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Canadian Intellectual Property Office

CA 3055006 C 2021/02/09

(11)(21) 3 055 006

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(22) Date de dépôt/Filing Date: 2012/06/21

(41) Mise à la disp. pub./Open to Public Insp.: 2012/12/27

(45) Date de délivrance/Issue Date: 2021/02/09

(62) Demande originale/Original Application: 2 875 484

(30) Priorité/Priority: 2011/06/24 (US61/501,034)

(51) Cl.Int./Int.Cl. *C12N 7/01* (2006.01),
A61K 39/145 (2006.01), *A61P 31/16* (2006.01),
A61P 37/04 (2006.01), *C07K 14/11* (2006.01),
C12N 15/44 (2006.01), *C12N 15/86* (2006.01),
C12N 7/00 (2006.01)

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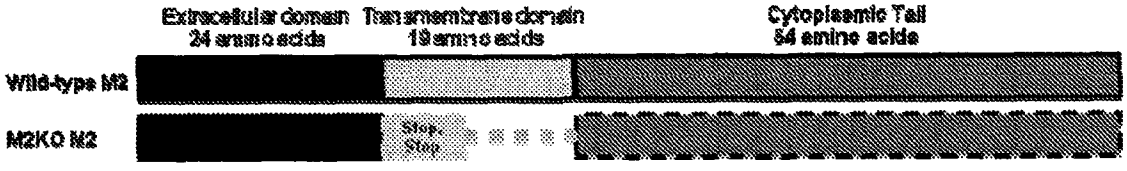
(54) Titre : MUTANTS DU VIRUS DE LA GRIPPE ET UTILISATIONS
(54) Title: INFLUENZA VIRUS MUTANTS AND USES THEREFOR



(57) Abrégé/Abstract:
Disclosed herein are compositions and methods related to mutant viruses, and in particular, mutant influenza viruses. The mutant viruses disclosed herein include a mutant M2 sequence, and are useful in immunogenic compositions, e.g., as vaccines. Also disclosed herein are methods, compositions and cells for propagating the viral mutants, and methods, devices and compositions related to vaccination.

ABSTRACT

Disclosed herein are compositions and methods related to mutant viruses, and in particular, mutant influenza viruses. The mutant viruses disclosed herein include a mutant M2 sequence, and are useful in immunogenic compositions, e.g., as vaccines. Also disclosed herein are methods, compositions and cells for propagating the viral mutants, and methods, devices and compositions related to vaccination.

INFLUENZA VIRUS MUTANTS AND USES THEREFOR

This application claims the benefit of U.S. Provisional Application No. 61/501,034, filed June 24, 2011.

BACKGROUND

[0001] Influenza is a leading cause of death among American adults. Each year, about 36,000 people die from influenza, and more than 200,000 people are hospitalized. Influenza is a highly contagious disease that is spread by coughing, sneezing and through direct physical contact with objects that carry the virus such as doorknobs and telephones. Symptoms of influenza include fever, extreme fatigue, headache, chills and body aches; about 50 percent of infected people have no symptoms but are still contagious. Immunization is 70-90 percent effective in preventing influenza in healthy people under the age of 65, as long as the antigenicities of the circulating virus strain match those of the vaccine.

[0002] Vaccination is the main method for preventing influenza, and both live attenuated and inactivated (killed) virus vaccines are currently available. Live virus vaccines, typically administered intranasally, activate all phases of the immune system and can stimulate an immune response to multiple viral antigens. Thus, the use of live viruses overcomes the problem of destruction of viral antigens that may occur during preparation of inactivated viral vaccines. In addition, the immunity produced by live virus vaccines is generally more durable, more effective, and more cross-reactive than that induced by inactivated vaccines, and live virus vaccines are less costly to produce than inactivated virus vaccines. However, the mutations in attenuated virus are often ill-defined, and reversion is a concern.

SUMMARY

[0003] In one aspect, the present disclosure provides a nucleic acid sequence comprising SEQ ID NO:1.

[0004] In one aspect, the present disclosure provides a nucleic acid sequence comprising SEQ ID NO:2.

[0005] In one aspect, the present disclosure provides a nucleic acid sequence comprising SEQ ID NO:3.

[0006] In one aspect, the present disclosure provides a composition comprising SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO: 3, operably linked to (i) a promoter, and (ii) a transcription termination sequence.

[0007] In one aspect, the present disclosure provides a recombinant influenza virus comprising a mutation in the M gene. In some embodiments, the recombinant influenza virus comprises SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO: 3. In some embodiments, the mutation in the M gene results in failure of the virus to express the M2 protein, or causes the virus to express a truncated M2 protein having the amino acid sequence of SEQ ID NO:4. In some embodiments, the mutation in the M gene does not revert to wild-type or to a non-wild-type sequence encoding a functional M2 protein for at least 10 passages in an *in vitro* host cell system. In some embodiments, the virus is an influenza A virus. In some embodiments, the virus is non-pathogenic in a mammal infected with the virus. In some embodiments, the *in vitro* cell system comprises Chinese Hamster Ovary cells. In some embodiments, the *in vitro* cell system comprises Vero cells.

[0008] In one aspect, the present disclosure provides a cell comprising the recombinant influenza virus of any one of claims 5-10. In some embodiments, the cell is *in vitro*. In some embodiments, the cell is *in vivo*.

[0009] In one aspect, the present disclosure provides a composition comprising: a recombinant influenza virus comprising a mutation in the M gene. In some embodiments, the composition comprises SEQ ID NO:1, SEQ ID NO: 2, or SEQ ID NO:3. In some embodiments, the mutation in the M gene results in failure of the virus to express the M2 protein, or causes the virus to express a truncated M2 protein having the amino acid sequence of SEQ ID NO:4. In some

embodiments, the virus is an influenza A virus. In some embodiments, the composition is non-pathogenic to a mammal administered the composition. In some embodiments, the composition elicits a detectable immune response in a mammal within about three weeks after administration of the composition to the mammal.

[0010] In one aspect, the present disclosure provides a vaccine comprising: a recombinant influenza virus comprising a mutation in the M gene. In some embodiments, the vaccine comprises SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO: 3. In some embodiments, the mutation in the M gene results in failure of the virus to express the M2 protein, or causes the virus to express a truncated M2 protein having the amino acid sequence of SEQ ID NO:4. In some embodiments, the virus is an influenza A virus. In some embodiments, the vaccine is non-pathogenic to a mammal administered the vaccine. In some embodiments, the vaccine elicits a detectable immune response in a mammal within about three weeks after administration of the vaccine to the mammal. In some embodiments, the vaccine comprises at least two different influenza viral strains in addition to the recombinant virus. In some embodiments, the vaccine comprises at least one influenza B virus or influenza B virus antigen. In some embodiments, the vaccine comprises at least one influenza C virus or influenza C virus antigen. In some embodiments, the vaccine comprises one or more viruses or viral antigens comprising human influenza A and pandemic influenza viruses from non-human species. In some embodiments, the vaccine comprises the human influenza A virus is selected from the group comprising H1N1, H2N2 and H3N2.

[0011] In one aspect, the present disclosure provides a method for propagating a recombinant influenza virus, comprising: contacting a host cell with a recombinant influenza virus SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO: 3; incubating the host cell for a sufficient time and under conditions suitable for viral replication, and isolating progeny virus particles.

[0012] In one aspect, the present disclosure provides a method of preparing a vaccine, comprising: placing a host cell in a bioreactor; contacting the host cell with a recombinant virus SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO: 3; incubating the host cell for a sufficient time and

under conditions suitable for viral propagation; isolating the progeny virus particles; and formulating the progeny virus particles for administration as a vaccine.

[0013] In one aspect, the present disclosure provides a method for immunizing a subject, comprising: administering a composition comprising a recombinant influenza virus comprising mutation in the M gene, wherein the mutation in the M gene results in failure of the virus to express the M2 protein, or causes the virus to express a truncated M2 protein having the amino acid sequence of SEQ ID NO:4.

[0014] In one aspect, the present disclosure provides a method for reducing the likelihood or severity of infection by influenza A virus in a subject comprising: administering a composition comprising a recombinant influenza virus comprising mutation in the M gene, wherein the mutation in the M gene results in failure of the virus to express the M2 protein, or causes the virus to express a truncated M2 protein having the amino acid sequence of SEQ ID NO:4. In some embodiments, the recombinant influenza virus comprises SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3. In some embodiments, the method comprises providing at least one booster dose of the composition, wherein the at least one booster dose is provided at three weeks after a first administration. In some embodiments, the method comprises administering the composition intranasally, intramuscularly or intracutaneously. In some embodiments, the method comprises administering is performed intracutaneously. In some embodiments, the method comprises administering is performed using a microneedle delivery device.

[0015] In one aspect, the present disclosure provides a method for intracutaneous administration of an immunogenic composition comprising: (a) providing a microneedle delivery device comprising (i) a puncture mechanism; (ii) an immunogenic composition layer comprising a plurality of microneedles capable of puncturing skin and allowing an immunogenic composition to be administered intracutaneously; and (b) depressing the puncture mechanism; wherein the immunogenic composition comprises a recombinant influenza virus comprising a mutation in the M gene, and wherein the mutation in the M gene results in failure of the virus to express the M2 protein, or causes the virus to express a truncated M2 protein having the amino

acid sequence of SEQ ID NO:4. In some embodiments, the recombinant influenza virus comprises SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3. In some embodiments, the microneedle array is initially positioned inside of a device housing, and upon actuation of a lever allows the microneedles to extend through the device bottom and insert into the skin thereby allowing infusion of the vaccine fluid into the skin.

[0016] In one aspect, the present disclosure provides a recombinant influenza virus comprising a mutation in the M gene, wherein the virus does not replicate in an unmodified host cell selected from the group consisting of a Chinese Hamster Ovary (CHO) cell, a Vero cell, a or Madin-Darby canine kidney cell. In some embodiments, the mutation in the M gene results in failure of the virus to express the M2 protein, or causes the virus to express a truncated M2 protein having the amino acid sequence of SEQ ID NO:4.

[0017] In one aspect, the present disclosure provides a recombinant cell comprising a nucleic acid encoding an influenza virus M2 ion channel gene, wherein the nucleic acid is expressed in the cell.

[0018] In one aspect, the present disclosure provides a recombinant cell comprising a 2,6-sialic acid receptor gene.

[0019] In one aspect, the present disclosure provides a recombinant cell comprising a cellular genome or an expression vector that expresses (i) a viral M2 ion channel gene, and (ii) a 2,6-sialic acid receptor gene. In some embodiments, the cell is a eukaryotic cell. In some embodiments, the eukaryotic cell is a Chinese Hamster Ovary cell or a Vero cell. In some embodiments, the recombinant cell further comprises a human influenza virus, wherein the virus does not express a functional M2 protein.

[0020] In one aspect, the present disclosure provides a method for producing recombinant influenza viral particles, comprising (A) infecting the cell of one of claims 47-52 with human influenza virus, wherein the cell either (i) constitutively expresses the functional M2 ion channel protein, or (ii) is induced after viral infection to express the functional M2 ion channel protein,

and wherein the virus successfully replicates only in the presence of the functional M2 ion channel proteins expressed by the cell; and (B) isolating the progeny virus particles. In some embodiments, the method further comprises formulating the isolated viral particles into a vaccine. In some embodiments, the virus comprises a human influenza virus, and wherein the virus does not express a functional M2 protein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIGURE 1 is a graphic depicting the role of M2 ion channel in an influenza virus life cycle, wherein (1) the influenza virus attaches to sialic acid receptors on a cell surface; (2) the virus is internalized into the cell; (3) the M2 ion channel is expressed on the viral surface; (4) the M2 ion channel opens to permit proton entry, leading to a release of viral RNA that enters the nucleus, is replicated and results in viral protein synthesis; and (5) the viral components are packaged into virions and released.

[0022] FIGURE 2 is a schematic diagram of wild-type and mutant M2 genes. The M2 gene of A/Puerto Rico/8/1934 (PR8) M segment was deleted by insertion of two stop codons downstream of the open reading frame of the M1 protein followed by deletion of 51 nucleotides in the transmembrane domain to inhibit expression of full-length M2 protein.

[0023] FIGURE 3 shows the nucleotide sequence of unprocessed M1 and M2.

[0024] FIGURE 4 is a chart showing the growth kinetics of M2KO(ΔTM) (upper panel) and wild-type PR8 (lower panel) viruses in normal MDCK cells and MDCK cells stably expressing M2 protein (M2CK). Cells were infected with viruses at multiplicity of infection of 10^{-5}. Virus titers in cell supernatants were determined. Wild-type PR8 grew to high titers in both cell types whereas M2KO(ΔTM) grew well only in M2CK cells and not at all in MDCK cells.

[0025] FIGURE 5 is a western blot showing that M2KO(ΔTM) virus produces viral antigens, but not M2, in normal cells. Cellular lysates were probed with PR8-infected mouse sera (panel A) or anti-M2 monoclonal antibody (panel B). Lane 1, Molecular weight marker; Lane 2,

MDCK cells infected with PR8; Lane 3 MDCK cells infected with M2KO(ΔTM); Lane 4, Uninfected MDCK cells.

[0026] FIGURE 6 is a chart showing the change in mouse body weight after inoculation with M2KO variants.

[0027] FIGURE 7A is a chart showing antibody response in mice inoculated with M2KO variants.

[0028] FIGURE 7B is a chart showing anti-PR8 IgG antibody titer in the serum of boosted mice 6 weeks post infection.

[0029] FIGURE 8 is a chart showing change in mouse body weight after influenza challenge, post-inoculation with M2KO variants.

[0030] FIGURE 9 is a chart showing mouse survival after influenza challenge, post-inoculation with M2KO variants.

[0031] FIGURE 10 is a chart showing the change in mouse body weight after inoculation with PR8 intranasally (IN), intradermally (ID) or intramuscularly (IM).

[0032] FIGURE 11A is a chart showing antibody titer in serum, collected at 2 weeks post-inoculation with PR8, from mouse with $1.8x10^1$ pfu (Lo) or $1.8x10^4$ pfu (Hi) concentration of virus. FIGURE 11B is a chart showing antibody titer in serum, collected at 7 weeks post-inoculation with PR8, from mouse with $1.8x10^1$ pfu (Lo) or $1.8x10^4$ pfu (Hi) concentration of vaccine.

[0033] FIGURE 12 is a chart showing mouse survival after influenza challenge, post-inoculation with PR8.

[0034] FIGURE 13 is a chart showing change in mouse body weight after influenza challenge, post-inoculation with PR8.

[0035] FIGURE 14 is a chart showing antibody titer in serum, collected from a mouse inoculated with $1.8x10^4$ pfu PR8 intradermally at 7 weeks post-inoculation.

[0036] FIGURE 15 is a chart showing the change in body weight of mice inoculated with $1.8x10^4$ pfu PR8 intradermally.

[0037] FIGURE 16 is a chart showing % survival post challenge for mice infected with a heterosubtypic virus.

[0038] FIGURE 17 is a chart showing ELISA titers of mice from different vaccination groups.

[0039] FIGURE 18 is a chart showing % survival of mice after homosubtypic virus infection.

[0040] FIGURE 19 is a chart showing % survival of mice after hetersubtypic virus challenge.

[0041] FIGURE 20 is a chart showing changes in body weight of inoculated ferrets. Ferrets were inoculated with 10^7 $TCID_{50}$ of M2KO(ΔTM) virus (panel A) or with 10^7 $TCID_{50}$ of A/Brisbane/10/2007 (H3N2) influenza A virus (panel B). Body weight was monitored for 3 days post inoculation.

[0042] FIGURE 21 is a chart showing changes in body temperature of inoculated ferrets. Ferrets were inoculated with 10^7 $TCID_{50}$ of M2KO(ΔTM) virus (panel A) or with 10^7 $TCID_{50}$ of A/Brisbane/10/2007 (H3N2) influenza A virus (panel B). Body temperature was monitored for 3 days post inoculation.

[0043] FIGURE 22 is a chart showing changes in body weight of ferrets after vaccination. Ferrets were inoculated with 10^7 $TCID_{50}$ of M2KO(ΔTM) virus [G1 and G3], with 10^7 $TCID_{50}$ of FM#6 virus [G2 and G4] or OPTI-MEM™ [G5]. Changes in body weight were monitored for 14 days following prime vaccination (panel A) and after receiving a booster vaccine (panel B).

[0044] FIGURE 23 is a chart showing changes in body weight of ferrets after challenge. Ferrets were challenged with 10^7 $TCID_{50}$ of A/Brisbane/10/2007 (H3N2) influenza A virus. Body weight was monitored for 14 days post inoculation.

[0045] FIGURE 24 is a chart showing changes in body temperature of ferrets after vaccination. Ferrets were inoculated with 10^7 $TCID_{50}$ of M2KO(ΔTM) virus [G1 and G3], with 10^7 $TCID_{50}$ of FM#6 virus [G2 and G4] or OPTI-MEM™ [G5]. Changes in body temperature were monitored for 14 days following prime vaccination (panel A) and after receiving a booster vaccine (panel B).

[0046] FIGURE 25 is a chart showing changes in body tempertature of ferrets after challenge. Ferrets were challenged with 10^7 $TCID_{50}$ of A/Brisbane/10/2007 (H3N2) influenza A virus. Body temperature was monitored for 14 days post inoculation.

[0047] FIGURE 26 is a chart showing changes in weight of ferrets after virus inoculation. Donor ferrets were inoculated on day 0 with either 10 7 $TCID_{50}$ of M2KO(ΔTM) virus (panel A) or with 10^7 TCIDso of A/Brisbane/10/2007 (H3N2) virus (panel B). 24 hours (Day 1) after inoculation donors were placed in a cage with direct contacts (DC) adjacent to a cage housing an aerosol contact (AC). Changes in body weight were monitored for 14 days following donor inoculation.

[0048] FIGURE 27 is a chart showing changes in body temperature of ferrets after virus inoculation. Donor ferrets were inoculated on day 0 with either 10^7 $TCID_{50}$ ofM2KO(ΔTM) virus (panel A) or with 10^7 $TCID_{50}$ of A/Brisbane/10/2007 (H3N2) virus (panel B). 24 hours (Day 1) after inoculation donors were placed in a cage with direct contacts (DC) adjacent to a cage housing an aerosol contact (AC). Changes in body temperature were monitored for 14 days following donor inoculation.

[0049] FIGURE 28 is a chart showing that M2KO(ΔTM) vaccine elicits humoral and mucosal responses. Panel A shows serum IgG and IgA titers following administration of PR8, M2KO(ΔTM), inactivated PR8 (IN, IM), or PBS. Panel B shows lung wash IgG and IgA titers following administration of PR8, M2KO(ΔTM), inactivated PR8 (IN, IM), or PBS.

[0050] FIGURE 29 is a chart showing that M2KO(ΔTM) vaccine protects mice from lethal homosubtypic and heterosubtypic viral challenge. Panel A shows mouse body weight change

following homologous PR8 (H1N1) challenge. Panel B shows mouse survival following heterologous Aichi (H3N2) challenge.

[0051] FIGURE 30 is a chart showing that M2KO(ΔTM) vaccine controls challenge virus replication in respiratory tract. Panel A shows viral titers following PR8 (H1N1) challenge. Panel B shows viral titers following Aichi (H3N2) challenge.

[0052] FIGURE 31 is a chart showing the kinetics of antibody response to M2KO(ΔTM) vaccine in sera.

[0053] FIGURE 32 is a chart showing the mucosal antibody response to M2KO(ΔTM) vaccine in sera and respiratory tract.

[0054] FIGURE 33 is a chart showing the kinetics of anti-HA IgG in mice in response to M2KO(ΔTM) vaccine.

[0055] FIGURE 34 is a chart showing that M2KO(ΔTM) vaccine induces immune responses similar to FluMist® and IVR-147. Panel A shows serum viral titers in animals administered FluMist® H3, M2KO(ΔTM) H3, IVR-147, and PBS. Panel B shows lung wash viral titers in animals administered FluMist® H3, M2KO(ΔTM) H3, IVR-147, and PBS. Panel C shows nasal turbinate viral titiers in animals administered FluMist® H3, M2KO(ΔTM) H3, IVR-147, and PBS.

[0056] FIGURE 35 is a chart showing that M2KO(ΔTM) vaccine protects against Aichi challenge. Panel A shows body weight loss following Aichi challenge in animals administered FluMist® H3, M2KO(ΔTM) H3, IVR-147, and PBS. Panel B shows the percent survival following Aichi challenge of animals administered FluMist® H3, M2KO(ΔTM) H3, IVR-147, and PBS.

[0057] FIGURE 36 is a chart showing that H5N1 M2KO(ΔTM) vaccine elicits IgG antibody titers against HA.

[0058] FIGURE 37 is a chart showing body weight following administration of M2KO(ΔTM) CA07, WT CA07, and FluMist® CA07 vaccines.

[0059] FIGURE 38 is a chart showing that M2KO(ΔTM) virus does not replicate in respiratory tract of mice.

[0060] FIGURE 39 is a chart showing that M2KO(ΔTM) vaccine displays rapid antibody kinetics.

[0061] FIGURE 40 is a chart showing that M2KO(ΔTM) vaccine protects against heterologous challenge with H3N2 virus, A/Aichi/2/1968.

[0062] FIGURE 41 is a chart showing that M2KO(ΔTM) vaccine primes for cellular responses that are recalled upon challenge.

[0063] FIGURE 42 is a chart showing that M2KO(ΔTM) virus generates mRNA levels similar to virus wild-type for M2.

[0064] FIGURE 43 is an agarose gel showing restriction digests of the pCMV-PR8-M2 expression plasmid. Lanes 1 & 5; 1 Kb DNA Ladder (Promega,Madison, WI, USA), Lane 2-4; Eco R1 digested pCMLV-PR8-M2: 0.375 μg (Lane 2), 0.75 μg (Lane 3), and 1.5 μg (Lane 4).

[0065] FIGURE 44 is a chart showing a sequence alignment of pCMV –PR8-M2 to the open reading frame of the influenza M2 gene.

[0066] FIGURE 45 is a chart showing M2KO(ΔTM) and FluMist® virus replication in the ferret respiratory tract.

[0067] FIGURE 46 is a chart showing M2KO(ΔTM) and FluMist® viral titers in nasal washes after intranasal challenge with A/Brisbane/10/2007 (H3N2) virus.

[0068] FIGURE 47 is a chart showing IgG titers in ferrets following vaccination with M2KO(ΔTM) and FluMist® prime group only.

[0069] FIGURE 48 is a chart showing IgG titers in ferrets following vaccination with M2KO(ΔTM) and FluMist,® prime-boost groups.

[0070] FIGURE 49 is a chart showing a summary of ELISA IgG titers in ferret sera from vaccination with M2KO(ΔTM) or FluMist® to post-challenge.

[0071] FIGURE 50 is a chart showing viral titers in nasal washes from ferrets in transmission study. M2KO(ΔTM) virus did not transmit (no virus detected), whereas the control Brisb/10 virus did transmit.**[0072]** FIGURE 51 is a chart showing IgG titers in subjects vaccinated with A/California, A/Perth, and B/Brisbane viruses intranasally (IN), intramuscularly (IM) and intradermally (ID FGN).

[0073] FIGURE 52 is a chart showing IgG titers in subjects administered a priming does or a priming and booster dose of A/Perth (H3N2) vaccine intramuscularly (IM) or intradermally (ID FGN).

[0074] FIGURE 53 is a chart showing viral titers in guinea pigs inoculated with FluLaval: A/California/7/2009 NYMC X-181, A/Victoria/210/2009 NYMC X-187 (an A/Perth/16/2009-like virus), and B/Brisbane/60/2008 by intramuscular (IM) and intradermal (ID) delivery at 0, 30, and 60 days post-inoculation.

[0075] FIGURE 54 is a chart showing the percent survival of H5N1 M2KO(ΔTM) vaccinated subjects challenged 5 months post-immunization with Vietnam/1203/2004 virus.

[0076] FIGURE 55 is a chart showing the percent survival of H5N1 M2KO(ΔTM) vaccinated subjects challenged 4 weeks post-immunization with Vietnam/1203/2004 virus.

DETAILED DESCRIPTION

I. Definitions

[0077] The following terms are used herein, the definitions of which are provided for guidance.

[0078] As used herein, the singular forms "a," "an," and "the" designate both the singular and the plural, unless expressly stated to designate the singular only.

[0079] The term "about" and the use of ranges in general, whether or not qualified by the term about, means that the number comprehended is not limited to the exact number set forth herein, and is intended to refer to ranges substantially within the quoted range while not departing from the scope of the invention. As used herein, "about" will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill in the art given the context in which it is used, "about" will mean up to plus or minus 10% of the particular term.

[0080] As used herein "subject" and "patient" are used interchangeably and refer to an animal, for example, a member of any vertebrate species. The methods and compositions of the presently disclosed subject matter are particularly useful for warm-blooded vertebrates including mammals and birds. Exemplary subjects may include mammals such as humans, as well as mammals and birds of importance due to being endangered, of economic importance (animals raised on farms for consumption by humans) and/or of social importance (animals kept as pets or in zoos) to humans. In some embodiments, the subject is a human. In some embodiments, the subject is not human.

[0081] As used herein, the terms "effective amount" or "therapeutically effective amount" or "pharmaceutically effective amount" refer to a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect, *e.g.*, an amount which results in the prevention of, disease, condition and/or symptom(s) thereof. In the context of therapeutic or prophylactic applications, the amount of a composition administered to the subject will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to the composition drugs. It will also depend on the degree, severity and type of disease or condition. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. In some embodiments, multiple doses are administered.

Additionally or alternatively, in some embodiments, multiple therapeutic compositions or compounds (*e.g.*, immunogenic compositions, such as vaccines) are administered.

[0082] As used herein, the terms "isolated" and/or "purified" refer to in vitro preparation, isolation and/or purification of a nucleic acid (*e.g.*, a vector or plasmid), polypeptide, virus or cell such that it is not associated with unwanted *in vivo* substances, or is substantially purified from unwanted *in vivo* substances with which it normally occurs. For example, in some embodiments, an isolated virus preparation is obtained by *in vitro* culture and propagation, and is substantially free from other infectious agents. As used herein, "substantially free" means below the level of detection for a particular compound, such as unwanted nucleic acids, proteins, cells, viruses, infectious agents, etc. using standard detection methods for that compound or agent.

[0083] As used herein the term "recombinant virus" refers to a virus that has been manipulated in vitro, *e.g.*, using recombinant nucleic acid techniques, to introduce changes to the viral genome and/or to introduce changes to the viral proteins. For example, in some embodiments, recombinant viruses may include both wild-type, endogenous, nucleic acid sequences and mutant and/or exogenous nucleic acid sequences. Additionally or alternatively, in some embodiments, recombinant viruses may include modified protein components, such as mutant or variant matrix, hemagglutinin, neuraminidase, nucleoprotein, non-structural and/or polymerase proteins.

[0084] As used herein the term "recombinant cell" or "modified cell" refer to a cell that has been manipulated *in vitro*, *e.g.*, using recombinant nucleic acid techniques, to introduce nucleic acid into the cell and/or to modify cellular nucleic acids. Examples of recombinant cells includes prokaryotic or eukaryotic cells carrying exogenous plasmids, expression vectors and the like, and/or cells which include modifications to their cellular nucleic acid (*e.g.*, substitutions, mutations, insertions, deletions, etc., into the cellular genome). An exemplary recombinant cell is one which has been manipulated *in vitro* to express an exogenous protein, such as a viral M2 protein.

[0085] As used herein the terms "mutant," "mutation," and "variant" are used interchangeably and refer to a nucleic acid or polypeptide sequence which differs from a wild-type sequences. In

some embodiments, mutant or variant sequences are naturally occurring. In other embodiments, mutant or variant sequence are recombinantly and/or chemically introduced. In some embodiments, nucleic acid mutations include modifications (*e.g.*, additions, deletions, substitutions) to RNA and/or DNA sequences. In some embodiments, modifications include chemical modification (*e.g.*, methylation) and may also include the substitution or addition of natural and/or non-natural nucleotides. Nucleic acid mutations may be silent mutations (*e.g.*, one or more nucleic acid changes which code for the same amino acid as the wild-type sequence) or may result in a change in the encoded amino acid, result in a stop codon, or may introduce splicing defects or splicing alterations. Nucleic acid mutations to coding sequences may also result in conservative or non-conservative amino acid changes.

[0086] As used herein, the term "vRNA" refers to the RNA comprising a viral genome, including segmented or non-segmented viral genomes, as well as positive and negative strand viral genomes. vRNA may be wholly endogenous and "wild-type" and/or may include recombinant and/or mutant sequences.

[0087] As used herein, the term "host cell" refers to a cell in which a pathogen, such as a virus, can replicate. In some embodiments, host cells are *in vitro*, cultured cells (*e.g.*, CHO cells, Vero cells, MDCK cells, etc.) Additionally or alternatively, in some embodiments, host cells are *in vivo* (*e.g.*, cells of an infected vertebrate, such as an avian or mammal). In some embodiments, the host cells may be modified, *e.g.*, to enhance viral production such as by enhancing viral infection of the host cell and/or by enhancing viral growth rate. By way of example, but not by way of limitation, exemplary host cell modifications include recombinant expression of 2-6-linked sialic acid receptors on the cell surface of the host cell, and/or recombinant expression of a protein in the host cells that has been rendered absent or ineffective in the pathogen or virus.

[0088] As used herein, the term "infected" refers to harboring a disease or pathogen, such as a virus. An infection can be intentional, such as by administration of a virus or pathogen (*e.g.*, by vaccination), or unintentional, such as by natural transfer of the pathogen from one organism to another, or from a contaminated surface to the organism.

[0089] As used herein, the term "attenuated," as used in conjunction with a virus, refers to a virus having reduced virulence or pathogenicity as compared to a non-attenuated counterpart, yet is still viable or live. Typically, attenuation renders an infectious agent, such as a virus, less harmful or virulent to an infected subject compared to a non-attenuated virus. This is in contrast to killed or completely inactivated virus.

[0090] As used herein, the term "type" and "strain" as used in conjunction with a virus are used interchangeably, and are used to generally refer to viruses having different characteristics. For example, influenza A virus is a different type of virus than influenza B virus. Likewise, influenza A H1N1 is a different type of virus than influenza A H2N1, H2N2 and H3N2. Additionally or alternatively, in some embodiments, different types of virus such as influenza A H2N1, H2N2 and H3N2 may be termed "subtypes."

[0091] As used herein, "M2KO" or "M2KO(ΔTM)" refers to SEQ ID NO:1, a virus comprising SEQ ID NO:1, or a vaccine comprising a virus comprising SEQ ID NO:1, depending on the context in which it is used. For example, in describing mutations of the M2 gene demonstrated herein, "M2KO" or "M2KO(ΔTM)" refers to SEQ ID NO:1. When describing the viral component of a vaccine, "M2KO" or "M2KO(ΔTM)" refers to a recombinant influenza virus which possesses internal 6 genes of PR8 (nucleoprotein (NP), polymerase genes (PA, PB1, PB2), non-structural (NS), matrix (M)), but which does not express functional M2 protein. When describing a vaccine, "M2KO" or "M2KO(ΔTM)" refers to a vaccine comprising the M2KO(ΔTM) recombinant virus.

[0092] As used herein , "M2KO(ΔTM) virus" encompasses a recombinant influenza virus which possesses internal 6 genes of PR8 (nucleoprotein (NP), polymerase genes (PA, PB1, PB2), non-structural (NS), matrix (M)), but which does not express functional M2 protein, alone or in combination with other viral components and/or genes encoding other viral components. In some embodiments, the M2KO(ΔTM) virus comprises genes of other influenza viruses. In some embodiments, the virus comprises the HA and NA genes of Influenza A/Brisbane/10/2007-like A/Uruguay/716/2007(H3N2). In some embodiments, the M2KO(ΔTM) virus comprises the HA

and NA genes of the A/Vietnam/1203/2004 (H5N1) virus. In some embodiments, the M2KO(ΔTM) virus comprises the HA and NA genes of the A/California/07/2009 (CA07) (H1N1pdm) virus.

II. Influenza A virus

A. General

[0093] Influenza is a leading cause of death among American adults. The causal agent of influenza are viruses of the family orthomyxoviridae including influenza A virus, influenza B virus and influenza C virus, with influenza A being the most common and most virulent in humans.

[0094] The influenza A virus is an enveloped, negative-strand RNA virus. The genome of influenza A virus is contained on eight single (non-paired) RNA strands the complements of which code for eleven proteins (HA, NA, NP, M1, M2, NS1, NEP, PA, PB1, PB1-F2, PB2). The total genome size is about 14,000 bases. The segmented nature of the genome allows for the exchange of entire genes between different viral strains during cellular cohabitation. The eight RNA segments are as follows. 1) HA encodes hemagglutinin (about 500 molecules of hemagglutinin are needed to make one virion); 2) NA encodes neuraminidase (about 100 molecules of neuraminidase are needed to make one virion); 3) NP encodes nucleoprotein; 4) M encodes two proteins (the M1 and the M2) by using different reading frames from the same RNA segment (about 3000 M1 molecules are needed to make one virion); 5) NS encodes two proteins (NS1 and NEP) by using different reading frames from the same RNA segment; 6) PA encodes an RNA polymerase; 7) PB1 encodes an RNA polymerase and PB1-F2 protein (induces apoptosis) by using different reading frames from the same RNA segment; 8) PB2 encodes an RNA polymerase.

[0095] There are several subtypes of influenza A, named according to an H number (for the type of hemagglutinin) and an N number (for the type of neuraminidase). Currently, there are 16 different H antigens known (H1 to H16) and nine different N antigens known (N1 to N9). Each

virus subtype has mutated into a variety of strains with differing pathogenic profiles; some pathogenic to one species but not others, some pathogenic to multiple species. Exemplary Influenza A virus subtypes that have been confirmed in humans, include, but are not limited to H1N1 which caused the "Spanish Flu" and the 2009 swine flu outbreak; H2N2 which caused the "Asian Flu" in the late 1950s; H3N2 which caused the Hong Kong Flu in the late 1960s; H5N1, considered a global influenza pandemic threat through its spread in the mid-2000s; H7N7; H1N2 which is currently endemic in humans and pigs; and H9N2, H7N2, H7N3, H5N2, H10N7.

[0096] Some influenza A variants are identified and named according to the known isolate to which they are most similar, and thus are presumed to share lineage (*e.g.*, Fujian flu virus-like); according to their typical host (example Human flu virus); according to their subtype (example H3N2); and according to their pathogenicity (example LP, Low Pathogenic). Thus, a flu from a virus similar to the isolate A/Fujian/411/2002(H3N2) can be called Fujian flu, human flu, and H3N2 flu.

[0097] In addition, influenza variants are sometimes named according to the species (host) the strain is endemic in or adapted to. The main variants named using this convention are: bird flu, human flu, swine influenza, equine influenza and canine influenza. Variants have also been named according to their pathogenicity in poultry, especially chickens, *e.g.*, Low Pathogenic Avian Influenza (LPAI) and Highly Pathogenic Avian Influenza (HPAI).

B. Life cycle and structure

[0098] The life cycle of influenza viruses generally involves attachment to cell surface receptors, entry into the cell and uncoating of the viral nucleic acid, followed by replication of the viral genes inside the cell. After the synthesis of new copies of viral proteins and genes, these components assemble into progeny virus particles, which then exit the cell. Different viral proteins play a role in each of these steps.

[0099] The influenza A particle is made up of a lipid envelope which encapsulates the viral core. The inner side of the envelope is lined by the matrix protein (M1), while the outer surface

is characterized by two types of glycoprotein spikes: hemagglutinin (HA) and neuraminidase (NA). M2, a transmembrane ion channel protein, is also part of the lipid envelope. *See e.g.*, **Figure 1**.

[0100] The HA protein, a trimeric type I membrane protein, is responsible for binding to sialyloligosaccharides (oligosaccharides containing terminal sialic acid linked to galactose) on host cell surface glycoproteins or glycolipids. This protein is also responsible for fusion between viral and host cell membranes, following virion internalization by endocytosis.

[0101] Neuraminidase (NA), a tetrameric type II membrane protein, is a sialidase that cleaves terminal sialic acid residues from the glycoconjugates of host cells and the HA and NA, and thus is recognized as receptor-destroying enzyme. This sialidase activity is necessary for efficient release of progeny virions from the host cell surface, as well as prevention of progeny aggregation due to the binding activity of viral HAs with other glycoproteins. Thus, the receptor-binding activity of the HA and the receptor-destroying activity of the NA likely act as counterbalances, allowing efficient replication of influenza.

[0102] The genome segments are packaged into the core of the viral particle. The RNP (RNA plus nucleoprotein, NP) is in helical form with three viral polymerase polypeptides associated with each segment.

[0103] The influenza virus life cycle begins with binding of the HA to sialic acid-containing receptors on the surface of the host cell, followed by receptor-mediated endocytosis. **Figure 1**. The low pH in late endosomes triggers a conformational shift in the HA, thereby exposing the N-terminus of the HA2 subunit (the so-called fusion peptide). The fusion peptide initiates the fusion of the viral and endosomal membrane, and the matrix protein (M1) and RNP complexes are released into the cytoplasm. RNPs consist of the nucleoprotein (NP), which encapsidates vRNA, and the viral polymerase complex, which is formed by the PA, PB1, and PB2 proteins. RNPs are transported into the nucleus, where transcription and replication take place. The RNA polymerase complex catalyzes three different reactions: (1) synthesis of an mRNA with a 5' cap and 3' polyA structure, (2) a full-length complementary RNA (cRNA), and (3) genomic vRNA

using the cDNA as a template. Newly synthesized vRNAs, NP, and polymerase proteins are then assembled into RNPs, exported from the nucleus, and transported to the plasma membrane, where budding of progeny virus particles occurs. The neuramimidase (NA) protein plays a role late in infection by removing sialic acid from sialyloligosaccharides, thus releasing newly assembled virions from the cell surface and preventing the self aggregation of virus particles. Although virus assembly involves protein-protein and protein-vRNA interactions, the nature of these interactions remains largely unknown.

C. Role of the M2 protein

[0104] As described above, spanning the viral membrane are three proteins: hemagglutinin (HA), neuramimidase (NA), and M2. The extracellular domains (ectodomains) of HA and NA are quite variable, while the ectodomain domain of M2 is essentially invariant among influenza A viruses. Without wishing to be bound by theory, in influenza A viruses, the M2 protein which possesses ion channel activity, is thought to function at an early state in the viral life cycle between host cell penetration and uncoating of viral RNA. Once virions have undergone endocytosis, the virion-associated M2 ion channel, a homotetrameric helix bundle, is believed to permit protons to flow from the endosome into the virion interior to disrupt acid-labile M1 protein-ribonucleoprotein complex (RNP) interactions, thereby promoting RNP release into the cytoplasm. In addition, among some influenza strains whose HAs are cleaved intracellularly (*e.g.*, A/fowl plagues/Rostock/34), the M2 ion channel is thought to raise the pH of the trans-Golgi network, preventing conformational changes in the HA due to conditions of low pH in this compartment. It was also shown that the M2 transmembrane domain itself can function as an ion channel. M2 protein ion channel activity is thought to be essential in the life cycle of influenza viruses, because amantadine hydrochloride, which blocks M2 ion channel activity, has been shown to inhibit viral replication. However, a requirement for this activity in the replication of influenza A viruses has not been directly demonstrated. The structure of the M2 protein is shown in **Figure 2**. The nucleic acid sequence of the M2 protein, along with the M1 sequence, is shown in **Figure 3**.

[0105] Although influenza B and C viruses are structurally and functionally similar to influenza A virus, there are some differences. For example, influenza B virus does not have an M2 protein with ion channel activity. Instead, the NB protein, a product of the NA gene, likely has ion channel activity and thus a similar function to the influenza A virus M2 protein. Similarly, influenza C virus does not have an M2 protein with ion channel activity. However, the CM1 protein of the influenza C virus is likely to have this activity.

III. M2 viral mutants

[0106] In one aspect, influenza A viruses harboring a mutant M2 vRNA sequence are disclosed. Typically, such mutants do not have M2 ion channel activity, exhibit attenuated growth properties *in vivo*, cannot produce infectious progeny and are non-pathogenic or show reduced pathogenesis in infected subjects. The mutant viruses are immunogenic, and when used as a vaccine, provide protection against infection with a counterpart wild-type and/or other pathogenic virus. Additionally, the M2 mutants disclosed herein are stable, and do not mutate to express a functional M2 polypeptide, regardless of the host cell used. Additionally or alternatively, in some embodiments, the M1 protein of these mutants is produced without detectable alteration to its function. In some embodiments, viruses harboring the mutant M2 nucleic acid sequences can not replicate in a host cell in which a corresponding wild-type virus could be propagated. By way of example, but not by way of limitation, in some embodiments, the wild-type virus can be grown, propagated and replicate in culturing MDCK cells, CHO cells and/or Vero cells, while the corresponding virus harboring a mutant M2 sequence cannot grow, replicate or be propagated in the same type of cells.

[0107] As noted above, in some embodiments, the M2 mutant virus is stable, and does not mutate or revert to wild-type or to a non-wild-type sequence encoding a functional M2 protein in a host cell. For example, in some embodiments, the M2 mutant virus is stable for 2 passages, 3 passages, 5 passages, 10 passages, 12 passages, 15 passages, 20 passages, 25 passages or more than 25 passages in a host cell. In some embodiments, the host cell is an unmodified host cell.

In other embodiments, the host cell is a modified host cell, such as a MDCK cell which expresses the M2 protein.

[0108] In some embodiments, the M2 mutants include one or more nucleic acid substitutions and/or deletions. In some embodiments, the mutations are localized in nucleic acids which code for one or more of the extracellular domain of the M2 protein, the transmembrane domain of the M2 proteins and/or the cytoplasmic tail of the M2 protein. Additionally or alternatively, in some embodiments, one or more nucleic acid mutations results in a splice variant, one or more stop codons and/or one or more amino acid deletions of the M2 peptide In some embodiments, viruses carrying the mutant M2 nucleic acid produce a non-functional M2 polypeptide. In some embodiments, viruses carrying the mutant M2 nucleic acid do not produce an M2 polypeptide. In some embodiments, viruses carrying the mutant M2 nucleic acid produce a truncated M2 polypeptide. In some embodiments, truncated M2 polypeptide has the amino acid sequence MSLLTEVETPIRNEWGCRCNGSSD.

[0109] Three exemplary, non-limiting M2 viral mutants (M2-1, M2-2 and M2-3) are provided below in Tables 1-3. In the tables, lower case letters correspond to the M2 sequence; upper case letters correspond to the M1 sequence; mutant sequence (*e.g.*, stop codons, splice defect) are in bold, underlined. Underlined (lower case) bases in the M2-2 mutant indicate the region deleted in the M2-1 and M2-3 mutants.

TABLE 1: M2-1 –(SEQ ID NO: 1) M2 ectodomain + 2 stop codons + TM deletion (PR8 M segment + 2 stops (786-791) without 792-842 (TM)); also known as "M2KOTMdel," "M2KOΔTM."
3'AGCAAAAGCAGGTAGATATTGAAAGatgagtcttctaaccgaggtcgaaacGTACGTACTCTCTA TCATCCCGTCAGGCCCCCTCAAAGCCGAGATCGCACAGAGACTTGAAGATGTCTTTG CAGGGAAGAACACCGATCTTGAGGTTCTCATGGAATGGCTAAAGACAAGACCAATC CTGTCACCTCTGACTAAGGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGT GAGCGAGGACTGCAGCGTAGACGCTTTGTCCAAAATGCCCTTAATGGGAACGGGGA TCCAAATAACATGGACAAAGCAGTTAAACTGTATAGGAAGCTCAAGAGGGAGATAA CATTCCATGGGGCCAAAGAAATCTCACTCAGTTATTCTGCTGGTGCACTTGCCAGTT GTATGGGCCTCATATACAACAGGATGGGGGCTGTGACCACTGAAGTGGCATTTGGC CTGGTATGTGCAACCTGTGAACAGATTGCTGACTCCCAGCATCGGTCTCATAGGCAA ATGGTGACAACAACCAATCCACTAATCAGACATGAGAACAGAATGGTTTTAGCCAG

CACTACAGCTAAGGCTATGGAGCAAATGGCTGGATCGAGTGAGCAAGCAGCAGAGG
CCATGGAGGTTGCTAGTCAGGCTAGACAAATGGTGCAAGCGATGAGAACCATTGGG
ACTCATCCTAGCTCCAGTGCTGGTCTGAAAAATGATCTTCTTGAAAATTTGCAGgcctat
cagaaacgaatgggggtgcagatgcaacggttcaagtgat**TAATAG**gatcgtctttttttcaaatgcatttaccgtcgctttaaatacgg
actgaaaggagggccttctacggaaggagtgccaaagtctatgagggaagaatatcgaaaggaacagcagagtgctgtggatgctgacg
atggtcattttgtcagcatagagctggagtaaAAAACTACCTTGTTTCTACT

[0110] The M2 polypeptide sequence produced from this mutant is as follows:

MSLLTEVETPIRNEWGCRCNGSSD. (SEQ ID NO: 4).

TABLE 2: M2-2 – SEQ ID NO: 2 M2 ectodomain + 2 stops + splice defect (PR8 M segment + 2 stops (786-791) +splice defect nt 52) (also known as "Splice def M2KO" or "Splice def")

3'AGCAAAAGCAGGTAGATATTGAAAGatgagtcttctaaccgaggtcgaaac**C**TACGTACTCTCTA
TCATCCCGTCAGGCCCCCTCAAAGCCGAGATCGCACAGAGACTTGAAGATGTCTTTG
CAGGGAAGAACACCGATCTTGAGGTTCTCATGGAATGGCTAAAGACAAGACCAATC
CTGTCACCTCTGACTAAGGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGT
GAGCGAGGACTGCAGCGTAGACGCTTTGTCCAAAATGCCCTTAATGGGAACGGGGA
TCCAAATAACATGGACAAAGCAGTTAAACTGTATAGGAAGCTCAAGAGGGAGATAA
CATTCCATGGGGCCAAAGAAATCTCACTCAGTTATTCTGCTGGTGCACTTGCCAGTT
GTATGGGCCTCATATACAACAGGATGGGGGCTGTGACCACTGAAGTGGCATTTGGC
CTGGTATGTGCAACCTGTGAACAGATTGCTGACTCCCAGCATCGGTCTCATAGGCAA
ATGGTGACAACAACCAATCCACTAATCAGACATGAGAACAGAATGGTTTTAGCCAG
CACTACAGCTAAGGCTATGGAGCAAATGGCTGGATCGAGTGAGCAAGCAGCAGAGG
CCATGGAGGTTGCTAGTCAGGCTAGACAAATGGTGCAAGCGATGAGAACCATTGGG
ACTCATCCTAGCTCCAGTGCTGGTCTGAAAAATGATCTTCTTGAAAATTTGCAGgcctat
cagaaacgaatgggggtgcagatgcaacggttcaagtgat**TAATAG**actattgccgcaaatatcattgggatcttgcacttgacattgt
ggattcttgatcgtctttttttcaaatgcatttaccgtcgctttaaatacggactgaaaggagggccttctacggaaggagtgccaaagtctatga
gggaagaatatcgaaaggaacagcagagtgctgtggatgctgacgatggtcattttgtcagcatagagctggagtaaAAAACTAC
CTTGTTTCTACT

[0111] No M2 polypeptide sequence is produced from this mutant.

TABLE 3: M2-3 – SEQ ID NO: 3 M2 ectodomain + 2 stops + splice defect + TM deletion (PR8 M segment + 2 stops (786-791) without 792-842 (TM)+splice defect nt 52) (also known as TMdel + Splice def M2KO)

3'AGCAAAAGCAGGTAGATATTGAAAGatgagtcttctaaccgaggtcgaaacCTACGTACTCTCTA
TCATCCCGTCAGGCCCCCTCAAAGCCGAGATCGCACAGAGACTTGAAGATGTCTTTG
CAGGGAAGAACACCGATCTTGAGGTTCTCATGGAATGGCTAAAGACAAGACCAATC
CTGTCACCTCTGACTAAGGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGT
GAGCGAGGACTGCAGCGTAGACGCTTTGTCCAAAATGCCCTTAATGGGAACGGGGA
TCCAAATAACATGGACAAAGCAGTTAAACTGTATAGGAAGCTCAAGAGGGAGATAA
CATTCCATGGGGCCAAAGAAATCTCACTCAGTTATTCTGCTGGTGCACTTGCCAGTT
GTATGGGCCTCATATACAACAGGATGGGGGCTGTGACCACTGAAGTGGCATTTGGC
CTGGTATGTGCAACCTGTGAACAGATTGCTGACTCCCAGCATCGGTCTCATAGGCAA
ATGGTGACAACAACCAATCCACTAATCAGACATGAGAACAGAATGGTTTTAGCCAG
CACTACAGCTAAGGCTATGGAGCAAATGGCTGGATCGAGTGAGCAAGCAGCAGAGG
CCATGGAGGTTGCTAGTCAGGCTAGACAAATGGTGCAAGCGATGAGAACCATTGGG
ACTCATCCTAGCTCCAGTGCTGGTCTGAAAAATGATCTTCTTGAAAATTTGCAGgcctat
cagaaacgaatgggggtgcagatgcaacggttcaagtgatTAATAGgatcgtctttttttcaaatgcatttaccgtcgctttaaatacgg
actgaaaggagggccttctacggaaggagtgccaaagtctatgagggaagaatatcgaaaggaacagcagagtgctgtggatgctgacg
atggtcattttgtcagcatagagctggagtaaAAAACTACCTTGTTTCTACT

[0112] No M2 polypeptide sequence is produced from this mutant.

[0113] Additionally or alternatively, in some embodiments, M2 mutations are introduced into the cytoplasmic tail. **Figure 2**. The M2 protein cytoplasmic tail is a mediator of infectious virus production. In some embodiments, truncations of the M2 cytoplasmic tail result in a decrease in infectious virus titers, a reduction in the amount of packaged viral RNA, a decrease in budding events, and a reduction in budding efficiency. It has been shown that the 5' sequence is more important than 3' sequence for genome packaging, and that a longer 5' sequence is better for genome packaging. In addition, studies have shown that nucleotide length is important, but the actual sequence is less so (random sequences are sufficient to generate viruses). Stable M2 cytoplasmic tail mutants have been challenging to develop, and the literature includes numerous examples of mutant reversion.

[0114] For example, Pekosz *et al* JVI, 2005; 79(6): 3595-3605, replaced two codons with stop codons at amino acid position 70, but the virus soon reverted. Another exemplary M2 cytoplasmic tail mutation is termed M2del11. In the M2del11 mutant, 11 amino acid residues are deleted from carboxyl end of cytoplasmic tail. This truncation is due to the introduction of

two stop codons, and a full length M2 polypeptide is not made. While this mutant is stable when passaged in M2 expressing MDCK cells (M2CK), it reverts to full length M2 during passaging in normal MDCK cells (J Virol. 2008 82(5):2486-92). Without wishing to be bound by theory, it is likely that reversion occurs with selective pressure in the MDCK cells.

[0115] Another M2 cytoplasmic tail mutant, M2Stop90ala78-81 did not reduce virus titer but ala70-77 did (JVI 2006; 80 (16) p8178-8189). Alanine-scanning experiments further indicated that amino acids at positions 74 to 79 of the M2 tail play a role in virion morphogenesis and affect viral infectivity. (J Virol. 2006 80(11):5233-40.)

[0116] Accordingly, presented herein are novel cytoplasmic mutants, with characteristics different than those described above. For example, in some embodiments, the cytoplasmic mutants are stable (do not revert to express a full-length M2 polypeptide) in MDCK cells. In some embodiments, the cytoplasmic mutants are stable for 2 passages, 3 passages, 5 passages, 10 passages, 15 passages, 20 passages, 25 passages or more than 25 passages in a host cell.

[0117] The wild-type M2 polypeptide is shown below in Table 4. For each of the sequences, the bold text indicates the transmembrane domain. The extracellular domain is first (left), followed by the transmembrane domain (center) and the cytoplasmic tail sequence (right).

TABLE 4: Wild-type M2 polypeptide and cytoplasmic tail mutants
Wild-type M2 polypeptide MSLLTEVETPIRNEWGCRCNGSSD**PLTIAANIIGILHLTLWIL**DRLFFKCIYRRFKYGLK GGPSTEGVPKSMREEYRKEQQSAVDADDGHFVSIELE
M2-4: M2del FG#1; delete M2's 44-54 aa (delete nucleotides 843-875; 11 aa) MSLLTEVETPIRNEWGCRCNGSSD**PLTIAANIIGILHLTLWIL**FKYGLKGGPSTEGVPKS MREEYRKEQQSAVDADDGHFVSIELE
M2-5: M2del FG#2; delete M2's 44-48 aa (delete nucleotides 843-857; 5 aa) MSLLTEVETPIRNEWGCRCNGSSD**PLTIAANIIGILHLTLWIL**KCIYRRFKYGLKGGPST EGVPKSMREEYRKEQQSAVDADDGHFVSIELE

M2-6: M2del FG#3; delete M2's 44 and 45 aa (delete nucleotides 843-848; 2 aa)

MSLLTEVETPIRNEWGCRCNGSSD**PLTIAANIIGILHLTLWIL**LFFKCIYRRFKYGLK
GGPSTEGVPKSMREEYRKEQQSAVDADDGHFVSIELE

[0118] M2-4 (M2del FG#1) was generated but was not passagable in normal MDCK cells, but may be passagable in a modified host cell (*e.g.*, a cell expressing a wild-type M2 polypeptide). M2-5 (M2del FG#2) and M2-6 (FG#3) were generated and passaged in normal MDCK cells. The nucleotide sequence of the M gene of these viruses are stable at least to passage 10 in MDCK cells. These mutants could be propagated and passaged in other cells as well (*e.g.*, cells that support influenza replication). It was also found that these mutants are not attenuated and are pathogenic.

[0119] As described in the Examples below, the M2 mutant viruses described herein do not replicate in the respiratory tract or disseminate to other organs in the ferret model and are not transmitted in the ferret model. Vaccines comprising M2 mutant elicit robust immune responses in mammals and protect mammals against influenza virus challenge. M2KO virus elicits both humoral and mucosal immune responses in mice, and protects mice from lethal homosubtypic and heterosubtypic challenge. Vaccines comprising M2 mutant virus as described herein provide effective protection against influenza challenge and have the advantage of being attenuated in mammalian hosts. These findings demonstrate that the M2 mutant viruses described herein are useful for vaccines against influenza.

IV. Cell-based virus production system

A. Producing "first generation" mutant viruses

[0120] Mutant virus, such as those carrying mutant M2 nucleic acid, can be generated by plasmid-based reverse genetics as described by Neumann *et al.*, *Generation of influenza A viruses entirely from clone cDNAs*, Proc. Natl. Acad. Sci. USA 96:9345-9350 (1999).

Briefly, eukaryotic host cells are transfected with one or more plasmids encoding the eight viral RNAs. Each viral RNA sequence is flanked by an RNA polymerase I promoter and an RNA polymerase I terminator. Notably, the viral RNA encoding the M2 protein includes the mutant M2 nucleic acid sequence. The host cell is additionally transfected with one or more expression plasmids encoding the viral proteins (*e.g.*, polymerases, nucleoproteins and structural proteins), including a wild-type M2 protein. Transfection of the host cell with the viral RNA plasmids results in the synthesis of all eight influenza viral RNAs, one of which harbors the mutant M2 sequence. The co-transfected viral polymerases and nucleoproteins assemble the viral RNAs into functional vRNPs that are replicated and transcribed, ultimately forming infectious influenza virus having a mutant M2 nucleic acid sequence, yet having a functional M2 polypeptide incorporated into the viral lipid envelope.

[0121] Alternative methods of producing a "first generation" mutant virus include a ribonucleoprotein (RNP) transfection system that allows the replacement of influenza virus genes with *in vitro* generated recombinant RNA molecules, as described by Enami and Palese, *High-efficiency formation of influenza virus transfectants,* J. Virol. 65(5):2711-2713.

[0122] The viral RNA is synthesized *in vitro* and the RNA transcripts are coated with viral nucleoprotein (NP) and polymerase proteins that act as biologically active RNPs in the transfected cell as demonstrated by Luytjes *et al.*, *Amplification, expression, and packaging of a foreign gene by influenza virus,* Cell 59:1107-1113.

[0123] The RNP transfection method can be divided into four steps: 1) Preparation of RNA: plasmid DNA coding for an influenza virus segment is transcribed into negative-sense RNA in an *in vitro* transcription reaction; 2) Encapsidation of the RNA: the transcribed RNA is then mixed with gradient purified NP and polymerase proteins isolated from disrupted influenza virus to form a biologically active RNP complex; 3) Transfection and rescue of the encapsidated RNA: the artificial ribonucleocapsid is transfected to the cells previously infected with a helper influenza virus that contains a different gene from the one being rescued; the helper virus will

amplify the transfected RNA; 4) Selection of transfected gene: because both the helper virus and the transfectant containing the rescued gene are in the culture supernatant, an appropriate selection system using antibodies is necessary to isolate the virus bearing the transfected gene.

[0124] The selection system allows for the generation of novel transfectant influenza viruses with specific biological and molecular characteristics. Antibody selection against a target surface protein can then be used for positive or negative selection.

[0125] For example, a transfectant or mutant virus that contains an M2 gene that does not express an M2 protein can be grown in a suitable mammalian cell line that has been modified to stably express the wild-type functional M2 protein. To prevent or inhibit replication of the helper virus expressing the wild-type M2 gene, and therefore the M2e protein at the membrane surface, antibodies against M2e can be used. Such antibodies are commercially available and would inhibit the replication of the helper virus and allow for the transfectant/mutant virus containing the mutant M2 to grow and be enriched in the supernatant. Inhibition of influenza virus replication by M2e antibodies has been described previously in *Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions,* J Virol 62:2762-2772 (1988) and Treanor *et al, Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice*, J. Virol. 64:1375-1377 (1990).

[0126] Additionally or alternatively, the same antibodies can be used to 'capture' the helper virus and allow for the enrichment of the transfectant. For example, the antibodies can be used to coat the bottom of a tissue culture dish or can be used in a column matrix to allow for enrichment for the transfectant in the supernatant or eluate.

[0127] The transfectant virus can be grown in M2 expressing cells in multi-well plates by limit dilution and then be identified and cloned, for example, by creating replica plates. For example, one-half of an aliquot of a given well of the multi-well plate containing the grown virus can be used to infect MDCK cells and the other half to infect MDCK cells that express M2 protein. Both the transfectant virus and helper virus will grow in MDCK cells that express M2 protein. However, only helper virus will grow in standard MDCK cells allowing for identifying the well

in the multi-well plate that contains the transfectant. The transfectant virus can be further plaque purified in the cells that express M2 protein.

B. Propagating viral mutants

[0128] In some embodiments, viral mutants described herein are maintained and passaged in host cells. By way of example, but not by way of limitation, exemplary host cells appropriate for growth of influenza viral mutants, such as influenza A viral mutants include any number of eukaryotic cells, including, but not limited to Madin-Darby canine kidney cells (MDCK cells), simian cells such as African green monkey cells (*e.g.*, Vero cells), CV-1 cells and rhesus monkey kidney cells (*e.g.*, LLcomk.2 cells), bovine cells (*e.g.*, MDBK cells), swine cells, ferret cells (*e.g.*, mink lung cells) BK-1 cells, rodent cells (*e.g.*, Chinese Hamster Ovary cells), human cells, *e.g.*, embryonic human retinal cells (*e.g.*, PER-C6®), 293T human embryonic kidney cells and avian cells including embryonic fibroblasts.

[0129] Additionally or alternatively, in some embodiments, the eukaryotic host cell is modified to enhance viral production, *e.g.*, by enhancing viral infection of the host cell and/or by enhancing viral growth rate. For example, in some embodiments, the host cell is modified to express, or to have increased expression, of 2,6-linked sialic acid on the cell surface, allowing for more efficient and effective infection of these cells by mutant or wild-type influenza A viruses. *See e.g.*, U.S. Patent Publication No. 2010-0021499, and U.S. Patent No. 7,176,021. Thus, in some illustrative embodiments, Chinese Hamster Ovary Cells (CHO cells) and/or Vero cells modified to express at least one copy of a 2,6-sialyltransferase gene (ST6GAL 1) are used. By way of example, but not by way of limitation, the *Homo sapiens* ST6 beta-galatosamide alpha-2,6-sialyltransferase gene sequence denoted by the accession number BC040009.1, is one example of a ST6Gal gene that can be integrated into and expressed by a CHO cell. One or more copies of a polynucleotide that encodes a functional ST6Gal I gene product can be engineered into a cell. That is, cells which have been stably transformed to express 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more than 12 copies of a ST6Gal I gene may be used. A single expression cassette may include one or more copies of

the ST6Gal I gene to be expressed, which is operably linked to regulatory elements, such as promoters, enhancers, and terminator and polyadenylation signal sequences, to facilitate the expression of the ST6Gal I gene or its copies. Alternatively, a single expression cassette may be engineered to express one copy of an ST6Gal I gene, and multiple expression cassettes integrated into a host cell genome. Accordingly, in some embodiments, at least one ST6Gal I gene is incorporated into the genome of a host cell, such that the cell expresses the ST6Gal I gene and its enzymatic protein product. Depending on the copy number, a single host cell may express many functional ST6Gal I gene proteins.

[0130] Suitable vectors for cloning, transfecting and producing stable, modified cell lines are well known in the art. One non-limiting example includes the pcDNA3.1 vectors (Invitrogen).

[0131] Additionally or alternatively, in some embodiments, the eukaryotic host cell is modified to produce a wild-type version of a mutant viral gene, thereby providing the gene to the virus in *trans*. For example, a viral strain harboring a mutant M2 protein may exhibit an enhanced growth rate (*e.g.*, greater viral production) when passaged in host cells producing the wild-type M2 protein. In some embodiments, the a viral strain harboring a mutant M2 protein may not grow or replicate in a cell which does not express a wild-type M2 gene. In addition, such host cells may slow or prevent viral reversion to a functional M2 sequence, because, for example, there is no selective pressure for reversion in such a host.

[0132] Method for producing both expression vectors and modified host cells are well known in the art. For example, an M2 expression vector can be made by positioning the M2 nucleic acid sequence (M2 ORF sequence; this is "wild-type" M2's start codon to stop codon (Table 5)) below in a eukaryotic expression vector.

Table 5: Wild-type M2 nucleic acid sequence
atgagtcttctaaccgaggtcgaaacgcctatcagaaacgaatgggggtgcagatgcaacggttcaagtgatcctctcactattgccgcaaa tatcattgggatcttgcacttgacattgtggattcttgatcgtctttttttcaaatgcatttaccgtcgctttaaatacggactgaaaggagggccttc tacggaaggagtgccaaagtctatgagggaagaatatcgaaaggaacagcagagtgctgtggatgctgacgatggtcattttgtcagcata gagctggagtaa

[0133] Host cells (*e.g.*, MDCK cells) can then be transfected by methods known in the art, *e.g.*, using commercially available reagents and kits, such as TransIT® LT1 (Mirus Bio, Madison, WI). By way of example, but not by way of limitation, cells can be selected and tested for M2 expression by cotransfection with a detectable marker or a selectable marker (*e.g.*, hygromycin-resistance) and/or by screening, for example, with indirect immunostaining using an M2 antibody. M2 expression can be determined by indirect immunostaining, flow cytometry or ELISA.

[0134] By way of example, but not by way of limitation, 293T human embryonic kidney cells and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and in minimal essential medium (MEM) containing 5% newborn calf serum, respectively. All cells were maintained at 37°C in 5% CO_2. Hygromycin-resistant MDCK cells stably expressing M2 protein from A/Puerto Rico/8/34 (H1N1) were established by cotransfection with plasmid pRHyg, containing the hygromycin resistance gene, and plasmid pCAGGS/M2, expressing the full-length M2 protein, at a ratio of 1:1. The stable MDCK cell clone (M2CK) expressing M2 was selected in medium containing 0.15 mg/mL of hygromycin (Roche, Mannheim, Germany) by screening with indirect immunostaining using an anti-M2 (14C2) monoclonal antibody (Iwatsuki *et al.*, JVI, 2006, vol.80, No.1, p.5233-5240). The M2CK cells were cultured in MEM supplemented with 10% fetal calf serum and 0.15 mg/mL of hygromycin. In M2CK cells, the expression levels and localization of M2 were similar to those in virus-infected cells (data not shown). M2 expressing Vero cells can be made in a similar fashion.

[0135] In some embodiments, cells and viral mutants are cultured and propagated by methods well known in the art. By way of example, but not by way of limitation, in some embodiments, host cells are grown in the presence of MEM supplemented with 10% fetal calf serum. Cells expressing M2 are infected at an MOI of 0.001 by washing with PBS followed by adsorbing

virus at 37°C. In some embodiments, viral growth media containing trypsin/TPCK is added and the cells are incubated for 2-3 days until cytopathic effect is observed.

[0136] Along these lines, disposable bioreactor systems have been developed for mammalian cells, with or without virus, whose benefits include faster facility setup and reduced risk of cross-contamination. The cells described herein, for instance, can be cultured in disposable bags such as those from Stedim, Bioeaze bags from SAFC Biosciences, HybridBagTM from Cellexus Biosytems, or single use bioreactors from HyClone or Celltainer from Lonza. Bioreactors can be 1 L, 10 L, 50 L, 250 L, 1000 L size formats. In some embodiments, the cells are maintained in suspension in optimized serum free medium, free of animal products. The system can be a fed-batch system where a culture can be expanded in a single bag from 1 L to 10 L for example, or a perfusion system that allows for the constant supply of nutrients while simultaneously avoiding the accumulation of potentially toxic by-products in the culture medium.

[0137] For long term storage, mutant virus can be stored as frozen stocks.

V. Vaccines and method of administration

A. Immunogenic compositions / vaccines

[0138] There are various different types of vaccines which can be made from the cell-based virus production system disclosed herein. The present disclosure includes, but is not limited to, the manufacture and production of live attenuated virus vaccines, inactivated virus vaccines, whole virus vaccines, split virus vaccines, virosomal virus vaccines, viral surface antigen vaccines and combinations thereof. Thus, there are numerous vaccines capable of producing a protective immune response specific for different influenza viruses where appropriate formulations of any of these vaccine types are capable of producing an immune response, *e.g.*, a systemic immune response. Live attenuated virus vaccines have the advantage of being also able to stimulate local mucosal immunity in the respiratory tract.

[0139] In some embodiments, vaccine antigens used in the compositions described herein are "direct" antigens, *i.e.* they are not administered as DNA, but are the antigens themselves. Such

vaccines may include a whole virus or only part of the virus, such as, but not limited to viral polysaccharides, whether they are alone or conjugated to carrier elements, such as carrier proteins, live attenuated whole microorganisms, inactivated microorganisms, recombinant peptides and proteins, glycoproteins, glycolipids, lipopeptides, synthetic peptides, or ruptured microorganisms in the case of vaccines referred to as "split" vaccines.

[0140] In some embodiments a complete virion vaccine is provided. A complete virion vaccine can be concentrated by ultrafiltration and then purified by zonal centrifugation or by chromatography. Typically, the virion is inactivated before or after purification using formalin or beta-propiolactone, for instance.

[0141] In some embodiments, a subunit vaccine is provided, which comprises purified glycoproteins. Such a vaccine may be prepared as follows: using viral suspensions fragmented by treatment with detergent, the surface antigens are purified, by ultracentrifugation for example. The subunit vaccines thus contain mainly HA protein, and also NA. The detergent used may be cationic detergent for example, such as hexadecyl trimethyl ammonium bromide, an anionic detergent such as ammonium deoxycholate; or a nonionic detergent such as that commercialized under the name TRITON X100. The hemagglutinin may also be isolated after treatment of the virions with a protease such as bromelin, then purified by standard methods.

[0142] In some embodiments, a split vaccine is provided, which comprises virions which have been subjected to treatment with agents that dissolve lipids. A split vaccine can be prepared as follows: an aqueous suspension of the purified virus obtained as above, inactivated or not, is treated, under stirring, by lipid solvents such as ethyl ether or chloroform, associated with detergents. The dissolution of the viral envelope lipids results in fragmentation of the viral particles. The aqueous phase is recuperated containing the split vaccine, constituted mainly of hemagglutinin and neuraminidase with their original lipid environment removed, and the core or its degradation products. Then the residual infectious particles are inactivated if this has not already been done.

[0143] In some embodiments, inactivated influenza virus vaccines are provided. In some embodiments, the inactivated vaccines are made by inactivating the virus using known methods, such as, but not limited to, formalin or ß-propiolactone treatment. Inactivated vaccine types that can be used in the invention can include whole-virus (WV) vaccines or subvirion (SV) (split) vaccines. The WV vaccine contains intact, inactivated virus, while the SV vaccine contains purified virus disrupted with detergents that solubilize the lipid-containing viral envelope, followed by chemical inactivation of residual virus.

[0144] Additionally or alternatively, in some embodiments, live attenuated influenza virus vaccines are provided. Such vaccines can be used for preventing or treating influenza virus infection, according to known method steps.

[0145] In some embodiments, attenuation is achieved in a single step by transfer of attenuated genes from an attenuated donor virus to an isolate or reassorted virus according to known methods (*see, e.g.*, Murphy, Infect. Dis. Clin. Pract. 2, 174 (1993)). In some embodiments, a virus is attenuated by mutation of one or more viral nucleic acid sequences, resulting in a mutant virus. For example, in some embodiments, the mutant viral nucleic acid sequence codes for a defective protein product. In some embodiments, the protein product has diminished function or no function. In other embodiments, no protein product is produced from the mutant viral nucleic acid.

[0146] The virus can thus be attenuated or inactivated, formulated and administered, according to known methods, as an immunogenic composition (*e.g.*, as a vaccine) to induce an immune response in an animal, *e.g.*, an avian and/or a mammal. Methods are well-known in the art for determining whether such attenuated or inactivated vaccines have maintained similar antigenicity to that of the clinical isolate or a high growth strain derived therefrom. Such known methods include the use of antisera or antibodies to eliminate viruses expressing antigenic determinants of the donor virus; chemical selection (*e.g.*, amantadine or rimantidine); HA and NA activity and inhibition; and DNA screening (such as probe hybridization or PCR) to confirm that donor genes encoding the antigenic determinants (*e.g.*, HA or NA genes) or other mutant sequences (*e.g.*,

M2) are not present in the attenuated viruses. *See, e.g.*, Robertson *et al.*, Giornale di Igiene e Medicina Preventiva, 29, 4 (1988); Kilbourne, Bull. M2 World Health Org., 41, 643 (1969); and Robertson *et al.*, Biologicals, 20, 213 (1992).

[0147] In some embodiments, the vaccine includes an attenuated influenza virus that lacks expression of a functional M2 protein. In some embodiments, the mutant virus replicates well in cells expressing M2 proteins, but in the corresponding wild-type cells, expresses viral proteins without generating infectious progeny virions.

[0148] Pharmaceutical compositions of the present invention, suitable for intradermal administration, inoculation or for parenteral or oral administration, comprise attenuated or inactivated influenza viruses, and may optionally further comprising sterile aqueous or non-aqueous solutions, suspensions, and emulsions. The compositions can further comprise auxiliary agents or excipients, as known in the art. *See, e.g.*, Berkow *et al.*, The Merck Manual, 15th edition, Merck and Co., Rahway, N.J. (1987); Goodman *et al.*, eds., Goodman and Gilman's The Pharmacological Basis of Therapeutics, Eighth Edition, Pergamon Press, Inc., Elmsford, N.Y. (1990); Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics, Third Edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, Md. (1987); and Katzung, ed., Basic and Clinical Pharmacology, Fifth Edition, Appleton and Lange, Norwalk, Conn. (1992).

[0149] In some embodiments, preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and/or emulsions, which may contain auxiliary agents or excipients known in the art. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers or occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable forms for suspending liposomes include emulsions, suspensions, solutions, syrups, and elixirs containing inert diluents commonly used in the art, such as purified water. Besides the inert diluents, such compositions can also

include adjuvants, wetting agents, emulsifying and suspending agents, or sweetening, flavoring, or perfuming agents.

[0150] When a composition of the present invention is used for administration to an individual, it can further comprise salts, buffers, adjuvants, or other substances which are desirable for improving the efficacy of the composition. For vaccines, adjuvants, substances that augment a specific immune response, can be used. Normally, the adjuvant and the composition are mixed prior to presentation to the immune system, or presented separately, but into the same site of the organism being immunized.

[0151] In some embodiments, the immunogenic compositions (*e.g.*, vaccines) disclosed herein include multiple, different types of virus or viral antigens, at least one of which includes a mutant M2 gene (*e.g.*, a virus comprising the M2KO(ΔTM) (SEQ ID NO:1) mutation), and/or a corresponding mutation in the M2 functional equivalent of that virus (*e.g.*, the NB protein of influenza B, or the CM1 protein of influenza C). In other embodiments, the immunogenic compositions include a single type of virus or viral antigen which includes a mutant M2 gene (*e.g.*, a virus comprising the M2KO(ΔTM) (SEQ ID NO:1) mutation) and/or a corresponding mutation in the M2 functional equivalent of that virus (*e.g.*, the NB protein of influenza B, or the CM1 protein of influenza C). For example, in some embodiments, the main constituent of an immunogenic compositions such as a vaccine composition includes one or more influenza viruses of type A, B or C, or any combination thereof or any combination of antigens from these viruses, wherein at least one virus includes a mutant M2 gene (*e.g.*, a virus comprising the M2KO(ΔTM) (SEQ ID NO:1) mutation) and/or a corresponding mutation in the M2 functional equivalent of that virus (*e.g.*, the NB protein of influenza B, or the CM1 protein of influenza C)For example, in some embodiments, at least two of the three types, at least two of different subtypes, at least two of the same type, at least two of the same subtype, or a different isolate(s) or reassortant(s) are provided in an immunogenic composition (*e.g.*, a vaccine). By way of example, but not by way of limitation, human influenza virus type A includes H1N1, H2N2 and H3N2 subtypes. In some embodiments, the immunogenic compositions (*e.g.*, vaccines) include a virus comprising a mutant M2 gene (*e.g.*, a virus comprising the M2KO(ΔTM) (SEQ ID NO:1)

mutation) and/or a corresponding mutation in the M2 functional equivalent of that virus (*e.g.*, the NB protein of influenza B, or the CM1 protein of influenza C) and about 0.1 to 200 μg, *e.g.*, 10 to 15 μg of hemagglutinin from each of the strains entering into the composition. Heterogeneity in a vaccine may be provided by mixing replicated influenza viruses for at least two influenza virus strains, such as from 2-50 strains, or any range or value therein. In some embodiments, influenza A or B virus strains having a modern antigenic composition are used. In addition, immunogenic compositions (*e.g.*, vaccines) can be provided for variations in a single strain of an influenza virus, using techniques known in the art.

[0152] In some embodiments, the vaccine comprises a virus comprising the M2KO(ΔTM) (SEQ ID NO:1) mutation together with other viral components and/or genes expressing other viral components. In some embodiments, the vaccine (*e.g.*, a virus comprising the M2KO(ΔTM) (SEQ ID NO:1) mutation) comprises genes from other viral strains, including but not limited to, for example, HA and NA genes from other viral strains. In some embodiments, the vaccine comprises HA and NA genes from human influenza virus type A subtypes H5N1, H1N1, H2N2 or H3N2. In some embodiments, the vaccine comprises HA and NA genes from, for example, PR8xBrisbane/10/2007, A/Vietnam/1203/2004, or A/California/07/2009 (CA07) viruses.

[0153] A pharmaceutical composition according to the present invention may further or additionally comprise at least one chemotherapeutic compound, *e.g.*, for gene therapy, an immunosuppressant, an anti-inflammatory agent or an immunostimulatory agent, or anti-viral agents including, but not limited to, gamma globulin, amantadine, guanidine, hydroxybenzimidazole, interferon-α, interferon-β, interferon-γ, tumor necrosis factor-α, thiosemicarbarzones, methisazone, rifampin, ribavirin, a pyrimidine analog, a purine analog, foscarnet, phosphonoacetic acid, acyclovir, dideoxynucleosides, a protease inhibitor, or ganciclovir.

[0154] The composition can also contain variable but small quantities of endotoxin-free formaldehyde, and preservatives, which have been found safe and not contributing to undesirable effects in the organism to which the composition of the invention is administered.

B. Administration

[0155] An immunogenic composition (*e.g.*, vaccine) as disclosed herein may be administered via any of the routes conventionally used or recommended for vaccines: parenteral route, mucosal route, and may be in various forms: injectable or sprayable liquid, formulation which has been freeze-dried or dried by atomization or air-dried, etc. Vaccines may be administered by means of a syringe or by means of a needle-free injector for intramuscular, subcutaneous or intradermal injection. Vaccines may also be administered by means of a nebulizer capable of delivering a dry powder or a liquid spray to the mucous membranes, whether they are nasal, pulmonary, vaginal or rectal.

[0156] A vaccine as disclosed herein may confer resistance to one or more influenza strains by either passive immunization or active immunization. In active immunization, an inactivated or attenuated live vaccine composition is administered prophylactically to a host (*e.g.*, a mammal), and the host's immune response to the administration protects against infection and/or disease. For passive immunization, the elicited antisera can be recovered and administered to a recipient suspected of having an infection caused by at least one influenza virus strain.

[0157] The present invention thus includes methods for preventing or attenuating a disease or disorder, *e.g.*, infection by at least one influenza virus strain. As used herein, a vaccine is said to prevent or attenuate a disease if its administration results either in the total or partial attenuation (*i.e.*, suppression) of a symptom or condition of the disease, or in the total or partial immunity of the individual to the disease.

[0158] At least one inactivated or attenuated influenza virus, or composition thereof, of the present invention may be administered by any means that achieve the intended purposes, using a pharmaceutical composition as previously described. For example, administration of such a composition may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, oral or transdermal routes. Parenteral administration can be by bolus injection or by gradual perfusion over time. In some embodiments, an immunogenic composition as disclosed herein is by intramuscular or subcutaneous application.

[0159] In some embodiments, a regimen for preventing, suppressing, or treating an influenza virus related pathology comprises administration of an effective amount of a vaccine composition as described herein, administered as a single treatment, or repeated as enhancing or booster dosages, over a period up to and including between one week and about 24 months, or any range or value therein. In some embodiments, an influenza vaccine as disclosed herein is administered annually.

[0160] According to the present invention, an "effective amount" of a vaccine composition is one that is sufficient to achieve a desired biological effect. It is understood that, in some embodiments, the effective dosage will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect wanted. The ranges of effective doses provided below are not intended to be limiting and represent exemplary dose ranges. Thus, in some embodiments, the dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art. The dosage of an attenuated virus vaccine for a mammalian (*e.g.*, human) adult can be from about 10^3-10^7 plaque forming units (PFU), or any range or value therein. The dose of inactivated vaccine can range from about 0.1 to 200, *e.g.*, 50 μg of hemagglutinin protein. However, the dosage should be a safe and effective amount as determined by conventional methods, using existing vaccines as a starting point.

C. Intracutaneous delivery

[0161] Live flu vaccines are traditionally delivered intranasally to mimic the natural route of infection and promote a similar immune response to that of natural virus infection. As an alternative, disclosed herein are intradermal delivery methods which involve the use of a novel microneedle device to capitalize on the immunological benefits of intradermal delivery. In some embodiments, an attenuated virus (*e.g.*, an M2 viral mutant) is used in a vaccine composition for intradermal administration. In some embodiments, an M2 viral mutant, which does not produce infectious progeny virus, is provided in a vaccine. Thus, any potential of reassortment with wild-type circulating influenza viruses is virtually eliminated.

[0162] In embodiments disclosed herein, intradermal delivery (intracutaneous) administers vaccine to the skin. In some embodiments, intradermal delivery is performed using a microneedle delivery device. As disclosed herein, intracutaneous delivery has numerous advantages. For example, the immunogenicity of the vaccine is enhanced by triggering the immunological potential of the skin immune system. The vaccine has direct access to the potent antigen-presenting dendritic cells of the skin, *i.e.*, epidermal Langerhans Cells and dermal dendritic cells. Skin cells produce proinflammatory signals which enhance the immune response to antigens introduced through the skin. Further, the skin immune system produces antigen-specific antibody and cellular immune responses. Intradermal delivery allows for vaccine dose sparing, *i.e.*, lower doses of antigen may be effective, in light of the above factors, when delivered intracutaneously.

[0163] And, because the vaccine is delivered to the skin through the device's microneedle array, the risk of unintended needle-sticks is reduced, and intracutaneous vaccine delivery via microneedle array is relatively painless compared to intramuscular injections with conventional needle and syringe.

[0164] Microneedle devices are known in the art, are known in the art, including, for example, those described in published U.S. patent applications 2012/0109066, 2011/0172645, 2011/0172639, 2011/0172638, 2011/0172637, and 2011/0172609. Microneedle devices may be made, for example, by fabrication from stainless steel sheets (*e.g.*, Trinity Brand Industries, Georgia; SS 304; 50 μm thick) by wet etching. In some embodiments, individual microneedles have a length of between about 500μm and 1000μm, *e.g.*, about 750μm, and a width of between about 100 μm to 500 μm, *e.g.*, about 200 μm. Vaccine can then be applied to the microneedles as a coating. By way of example, but not by way of limitation, a coating solution may include 1% (w/v) carboxymethyl cellulose sodium salt (low viscosity, USP grad; Carbo-Mer, San Diego CA), 0.5% (w/v) Lutrol F-68 NF (BASF, Mt. Olive, NJ) and the antigen (*e.g.*, soluble HA protein at 5 ng/ml; live, attenuated virus such as the M2 mutant virus described herein, etc.). To reach a higher vaccine concentration, the coating solution may be evaporated for 5 to 10 minutes at room temperature (~23°C). Coating may be performed by a dip coating process. The amount

of vaccine per row of microneedles can be determined by submerging the microneedles into 200 µl of phosphate-buffered saline (PBS) for 5 minutes and assaying for the antigen by methods known in the art.

[0165] In some embodiments, a microneedle device is used that is made mainly of polypropylene and stainless steel first-cut pieces that fit together with simple snap fits and heat seals. In some embodiments, the device is completely self-contained and includes the vaccine, a pump mechanism, an activation mechanism, and a microneedle unit. These components are hidden within a plastic cover. With the device, vaccine infusion is initiated by pressing an actuation button. Pressing the button simultaneously inserts the microneedles into the skin and initiates the pumping mechanism that exerts pressure on the primary drug container. When a spring mechanism exerts sufficient pressure on the vaccine reservoir, vaccine begins to flow through the microneedle array, and into the skin. In some embodiments, the delivery of the vaccine dose is completed within about 2 minutes after actuation of the device. After infusion is complete, the device is gently removed from the skin.

[0166] In some embodiments, a method for intracutaneous administration of an immunogenic composition (*e.g.*, a vaccine) is provided using a microneedle device. In some embodiments, the microneedle device comprises a puncture mechanism and an immunogenic composition layer comprising a plurality of microneedles capable of puncturing skin and allowing an immunogenic composition to be administered intracutaneously. In some embodiments, the method comprises depressing the puncture mechanism. In some embodiments, the immunogenic composition (*e.g.* vaccine) comprises a virus comprising a nucleic acid sequence encoding a mutant M2 protein that is expressed or a mutant M2 protein that is not expressed; wherein the expressed mutant M2 protein comprises, or consists of, the amino acid sequence of SEQ ID NO: 4. In some embodiments, the microneedle array is initially positioned inside of a device housing, and upon actuation of a lever allows the microneedles to extend through the device bottom and insert into the skin thereby allowing infusion of the vaccine fluid into the skin.

[0167] The delivery device described herein may be utilized to deliver any substance that may be desired. In one embodiment, the substance to be delivered is a drug, and the delivery device is a drug delivery device configured to deliver the drug to a subject. As used herein the term "drug" is intended to include any substance delivered to a subject for any therapeutic, preventative or medicinal purpose (*e.g.*, vaccines, pharmaceuticals, nutrients, nutraceuticals, etc.). In one such embodiment, the drug delivery device is a vaccine delivery device configured to deliver a dose of vaccine to a subject. In one embodiment, the delivery device is configured to deliver a flu vaccine. The embodiments discussed herein relate primarily to a device configured to deliver a substance transcutaneously. In some embodiments, the device may be configured to deliver a substance directly to an organ other than the skin.

EXAMPLES

[0168] While the following examples are demonstrated with influenza A, it is understood that the mutations and methods described herein are equally applicable to other viruses which express an M2, an M2-like protein or a protein with the same or similar function as the influenza A M2 protein.

Example 1: Generation of M2 viral mutants

[0169] M2 mutants were constructed as follows.

a) **M2-1**: M2 ectodomain + 2 stop codons + TM deletion (PR8 M segment + 2 stops (786-791) without 792-842 (TM))

[0170] Partial wild-type M genes from PR8 were amplified by PCR using oligo set 1 and oligo set 2 as shown below.

TABLE 6
Oligo Set 1
acacacCGTCTCTAGgatcgtctttttttcaaatgcatttacc

CACACACGTCTCCTATTAGTAGAAACAAGGTAGTTTTT
Oligo Set 2
acacacCGTCTCatcCTATTAatcacttgaaccgttgc
CACACACGTCTCCGGGAGCAAAAGCAGGTAG

[0171] The PCR products were then digested with BsmBI. An expression vector (pHH21) was also digested with BsmBI, and the digested PCR products were ligated into the vector using T4 DNA ligase. *E. coli* cells were transformed with the vector, and after appropriate incubation, vectors were isolated and purified by methods known in the art. The mutant M2 portion of the vector was characterized by nucleic acid sequencing.

b) **M2-2**: M2 ectodomain + 2 stops + splice defect (PR8 M segment + 2 stops (786-791) +splice defect nt 51)

[0172] Partial wild-type M genes from PR8 were amplified by PCR using the primer set shown below.

TABLE 7
PCR primers
5'acacacCGTCTCcCTACGTACTCTCTATCATCCCG
5'CACACACGTCTCCTATTAGTAGAAACAAGGTAGTTTTT

[0173] The PCR products were then digested with BsmBI. An expression vector (pHH21) was also digested with BsmBI. A double-stranded DNA fragment was then made by annealing the two nucleotides shown below.

TABLE 8

Annealing nucleotides
5'GGGAGCAAAAGCAGGTAGATATTGAAAGatgagtcttctaaccgaggtcgaaac
5'GTAGgtttcgacctcggttagaagactcatCTTTCAATATCTACCTGCTTTTGC

[0174] The digested vector, PCR product and double-stranded fragment were then ligated using T4 DNA ligase. *E. coli* cells were transformed with the vector, and after appropriate incubation, the vectors were isolated and purified by methods known in the art. The mutant M2 portion of the vector was characterized by nucleic acid sequencing.

c) **M2-3**: M2 ectodomain + 2 stops + splice defect + TM deletion (PR8 M segment + 2 stops (786-791) without 792-842 (TM)+splice defect nt 51)

[0175] The partial M2-1 mutant (M2 ectodomain + 2 stop codons + TM deletion (PR8 M segment + 2 stops (786-791) without 792-842 (TM)) was amplified from PR8 by PCR using the following primers:

TABLE 9
PCR primers
5'acacacCGTCTCcCTACGTACTCTCTATCATCCCG
5'CACACACGTCTCCTATTAGTAGAAACAAGGTAGTTTTT

[0176] The PCR products were then digested with BsmBI. An expression vector (pHH21) was also digested with BsmBI. A double-stranded DNA fragment was then made by annealing the two nucleotides shown below.

TABLE 10
Annealing nucleotides

5'GGGAGCAAAAGCAGGTAGATATTGAAAGatgagtcttctaaccgaggtcgaaac
5'GTAGgtttcgacctcggttagaagactcatCTTTCAATATCTACCTGCTTTTGC

[0177] The digested vector, PCR product and double-stranded fragment were then ligated using T4 DNA ligase. *E. coli* cells were transformed with the vector, and after appropriate incubation, the vectors were isolated and purified by methods known in the art. The mutant M2 portion of the vector was characterized by nucleic acid sequencing.

[0178] The sequence of each of the three M2 mutant constructs is provided in Tables 1-3.

Example 2: Generation and culturing of M2 mutant virus

[0179] This example demonstrates the culturing of the PR8 virus comprising the M2KO(ΔTM) (SEQ ID NO:1) mutation. Mutant viruses were generated as reported in Neumann *et al.*, *Generation of influenza A viruses entirely from clone cDNAs*, Proc. Natl. Acad. Sci. USA 96:9345-9350 (1999), with some modifications. Briefly, 293T cells were transfected with 17 plasmids: 8 PolI constructs for 8 RNA segments, one of which harbors the mutant M2 sequence, and 9 protein-expression constructs for 5 structural proteins as follows: NP (pCAGGS-WSN-NP0/14); M2 (pEP24c); PB1 (pcDNA774); PB2 (pcDNA762); and PA (pcDNA787) of A/Puerto Rico/8/34 (H1N1) virus.

[0180] The plasmids were mixed with transfection reagent (2 μL of Trans IT® LT-1 (Mirus, Madison, Wis.) per μg of DNA), incubated at room temperature for 15-30 minutes, and added to $1x10^6$ 293T cells. Forty-eight hours later, viruses in the supernatant were serially diluted and inoculated into M2CK cells. Two to four days after inoculation, viruses in supernatant of the last dilution well in which cells showing clear cytopathic effect (CPE) were inoculated into M2CK cells for the production of stock virus. The M genes of generated viruses were sequenced to confirm the gene and the presence of the intended mutations and to ensure that no unwanted mutations were present.

[0181] Mutant M2 viruses were grown and passaged as follows. M2CK host cells were grown in the presence of MEM supplemented with 10% fetal calf serum. Cells were infected at an MOI of 0.001 by washing with PBS followed by adsorbing virus at 37°C. Virus growth media containing trypsin/TPCK was added and the cells were incubated for 2-3 days until cytopathic effect was observed.

Example 3 M2KO Replication is Restricted in Normal Cells

[0182] Growth kinetics of the PR8 virus with the M2KO(ΔTM) (SEQ ID NO:1) mutation and wild-type PR8 were analyzed in both normal MDCK cells and MDCK cells stably expressing M2 protein (M2CK). Cells were infected with viruses at multiplicity of infection of 10^{-5}. Virus titers in cell supernatant were determined in MDCK or M2CK cells. Wild-type PR8 grew to high titers in both cell types whereas M2KO grew well only in M2CK cells and not at all in MDCK cells (**Figure 4**).

Example 4: M2KO Virus Produces Viral Antigens, But Not M2, in Normal Cells

[0183] This example demonstrates that the PR8 virus with the M2KO(ΔTM) (SEQ ID NO:1) mutation produces viral antigens, but not M2 protein, in normal cells. Viral protein expression was evaluated by infecting wild-type MDCK cells with wild-type PR8 or M2KO at a multiplicity of infection (MOI) of 0.5 in medium without trypsin to ensure that viruses complete only one life cycle. Viral proteins in the cell lysates were separated on a 4-12% SDS-PAGE gel and detected by Western blot using PR8 infected mouse sera (Panel A) or anti-M2 monoclonal antibody (14C2, Santa Cruz Biotechnology) (Panel B). **Figure 3A** shows that antisera against PR8 detects similar levels of protein expression for both PR8 and M2KO. When the lysates are probed with an anti-M2 monoclonal antibody (Panel B), M2 expression is detected only in PR8 infected cells, not M2KO. These results indicate that M2KO virus expresses all viral proteins, except M2 protein, to similar levels as PR8 virus (**Figure 5**)

Example 5: M2 mutants Are Attenuated In Vivo

[0184] An experiment was performed to demonstrate that M2 mutant viruses are attenuated *in vivo*. Six weeks old BALB/c, female mice (23 per group) were inoculated intranasally with one of the following mutants: **M2KO(yk)** as described in J. Virol (2009) 83:5947-5950; **M2-1** (TM del M2KO *aka* M2KO(ΔTM)) and **M2-2** (Splice def M2KO) (collectively termed "M2KO variants"). The mutant was administered at a dose of $1.2x10^4$ pfu per mouse. A control group of mice was given PBS. The mice were observed for 14 days after inoculation for any change in body weight and symptoms of infection. Additionally, after 3 days post-inoculation, virus titers were taken from the lungs and nasal turbinates (NT) from 3 mice in each group.

[0185] As shown in **Figure 6**, mice inoculated with the M2KO variants and PBS did not show any clinical symptoms of infection nor lose any body weight over the 14 day period. The change in body weight between the groups were comparable over the 14 day period. Additionally, no virus was detected in the titers that were gathered from the lungs and NT. Together, the lack of clinical symptoms, lack of loss of body weight and absence of virus indicate that the M2 mutant viruses are attenuated and not pathogenic in mice.

Example 6: M2 Mutants Induce Antibodies Against Influenza Virus and Protect Mice From Lethal Virus Challenge

[0186] Testing was also performed to determine antibody titers from the mice described in Example 5 above and their survival after being challenged with a lethal viral dose. Serum samples were taken 3 weeks after inoculation and anti-virus IgG antibody titers from the serum samples were determined by enzyme-linked immunosorbent assay (ELISA). The humoral response is shown in **Figure 7**, which shows that all three M2 mutants elevated anti-influenza virus antibodies higher than the control PBS group.

[0187] In addition, half of the mice within each of the groups were boosted 28 days after inoculation with same amount of M2 mutant virus. Serum was then collected 6 weeks after the first inoculation and IgG titers against the virus were determined. As shown in **Figure 7B**, mice

boosted by M2 mutant viruses had a higher level of anti-influenza virus antibodies than ones were not boosted.

[0188] 49 days after the first inoculation (3 weeks after the boost), the mice were challenged with a lethal dose of PR8 virus (40 mouse 50% lethal dose (MLD_{50})). As shown in **Figure 8** and **Figure 9**, all mice vaccinated with the M2KO variants survived the challenge and lost no weight. The control mice that were given only PBS, however, lost body weight and did not survive 8 days past the challenge date. On day 3 after the challenge, lungs and NT were obtained and virus titers determined in MDCK cells by plaque assay. As depicted in **Table 11**, lung virus titers in M2KO variants were at least one log lower than titers in naïve control PBS. And almost no viruses were detected in nasal turbinates in M2KO variants groups but more than 100,000 PFU/g were detected in the naïve control PBS group, indicating that the M2 mutant vaccines confer protection and limits the replication of the challenge virus.

TABLE 11: Virus Titer (log10 PFU/g) in Mouse Tissue After Challenge

	Lung	Nasal Turbinates
M2KO (yk)	6.1, 5.9, ND	1.7, ND, ND
M2KO (ΔTM)	5.8 ± 0.25	2.5, ND, ND
M2KO (splice def)	ND, ND, ND	ND, ND, ND
PBS	7.9 ± 0.27	5.3 ± 0.55

[0189] In another experiment, six weeks after immunization, the M2KO(ΔTM) groups were challenged with homosubtypic or heterosubtypic influenza viruses. Mice were challenged with Aichi (H3N2) virus and scored for survival for 14 days. Results for the heterotypic challenge are shown in **Figure 16**.

Example 7: Intradermal Vaccine Delivery

[0190] An experiment was performed to show that intradermal vaccine delivery / immunizing will protect a subject from influenza. BALB/c female, 6-7 weeks old mice (5 per group)

(Harland Laboratories) were inoculated either intranasally (IN), intramuscularly (IM) or intradmermally (ID) with PR8 virus ($3.5x10^7$pfu) at a concentration of $1.8x10^1$, $1.8x10^2$, $1.8x10^3$ or $1.8x10^4$ pfu (50 µl) per mouse. Control mice were also given PBS through the three different routes of administration. Body weight and survival were monitored for 14 days after inoculation. For the mouse experiments, allergy syringes with intradermal bevel needles were used.

[0191] Most vaccines are administered by intramuscular or subcutaneous injection using conventional needles and syringes. However, recent studies demonstrate that intradermal vaccine delivery achieves better immunogenicity than intramuscular or subcutaneous administration. Intradermal vaccination delivers antigen directly to the enriched skin immune system and has been shown to be effective for a range of vaccines, including rabies, hepatitis B and influenza. Intradermal delivery may also provide dose sparing, achieving the same immune response using less vaccine than required with intramuscular injection. The current state-of-the-art for intradermal delivery (using conventional needles and syringes) is the Mantoux technique, which requires extensive training, is difficult to perform, and often results in misdirected (subcutaneous) or incomplete administration. The lack of suitable delivery devices has hampered intradermal vaccination research and product development even though superior immune responses with this administration route have been documented.

[0192] As shown in **Table 12**, IN-inoculated mice succumbed to influenza infection at the higher doses of $1.8x10^3$ and $1.8x10^4$ pfu per mouse, with complete survival only at the lowest dose of $1.8x10^1$. However, IM- and ID-inoculated mice at all dosages survived. **Table 13** shows the median lethal dose for mice in the IN-inoculated group (MLD_{50}). **Figure 10** shows that IM- and ID-inoculated mice inoculated with $1.8x10^4$ pfu of the virus displayed no change in body weight, and shows the lack of survival for IN-inoculated mice inoculated with $1.8x10^4$ pfu of virus.

Table 12. Mice survival after PR8 inoculation	
Virus Dose	**Route of Administration**

(pfu)	IM	ID	IN
$1.8x10^1$	5/5	5/5	5/5
$1.8x10^2$	5/5	5/5	1/5
$1.8x10^3$	5/5	5/5	0/5
$1.8x10^4$	5/5	5/5	0/5
PBS	5/5	5/5	5/5

Table 13. Median lethal dose for mice (MLD_{50}).

Route	MLD_{50} (pfu[a]/mouse)
IN	76
IM	$> 1.8x10^4$
ID	$> 1.8x10^4$

[a]pfu: plaque forming unit.

[0193] Serum was collected at 2 weeks (**Figure 11A**) and 7 weeks (**Figure 11B**) after inoculation and evaluated for anti-PR8 IgG antibody as determined by an ELISA. "Hi" represents $1.8x10^4$ pfu inoculations, and "Lo" represents $1.8x10^1$ pfu. The responses of the IN-, IM- and ID-inoculated mice at both time periods are similar. At each time period, IN-inoculated mice presented the highest number of antibodies. Only IN-inoculated mice inoculated with $1.8x10^1$ pfu were identified (*i.e.*, "Lo"), because by this time, the IN-inoculated mice inoculated with higher doses had expired. IM- and ID-inoculated mice presented lower levels of antibodies than the IN-inoculated mice, although mice inoculated at the higher doses exhibited greater amounts of antibodies when compared with the control mice given only PBS. Additionally, over

time, the intradermal administration route produced more antibodies than the intramuscular route, as demonstrated by the higher titer levels shown in **Figure 11B**.

[0194] In another experiment, groups of IN-, IM- and ID-inoculated mice (5 mice per group, except for 4 mice in $1.8x10^3$ group) were challenged 8 week after vaccination. Specifically, $1.8x10^1$ IN-inoculated mice, $1.8x10^3$ IM-inoculated mice, $1.8x10^4$ IM-inoculated mice, $1.8x10^3$ ID-inoculated mice and $1.8x10^4$ ID-inoculated mice were challenged. Mice that lost more than 25% of their body weight were euthanized.

[0195] As shown in **Figure 12**, 100% of IM-inoculated mice at a dose of $1.8x10^3$ did not survive 8 days after the challenge. The survival rate of all ID-inoculated mice was between 40% and 60%. The survival rate of IM-inoculated mice at $1.8x10^4$, however, was 100%. **Figure 13** shows that the ID-inoculated and IM-inoculated ($1.8x10^4$) groups of mice had an initial average weight loss, but ended up with a relative low weight loss from the challenge date.

[0196] An evaluation of the ID-incoulated mice ($1.8x10^4$) showed that two mice (1 and 5 in **Figure 14** and **Figure 15**), elicited a better immune response than the other mice, and further did not develop symptoms of influenza infection (*e.g.*, body weight loss, rough fur, quietness, etc.). However, all mice in the IM-inoculated group ($1.8x10^4$) showed some symptoms and lost at least 10% in body weight.

Example 8: Stability of M2KO Variants

[0197] To test the stability of M2 gene of M2KO variants in wild-type cells, the M2KO variants were passaged in wild-type MDCK cells, which lacks M2 protein expression, along with M2CK cells which are M2 protein expressing MDCK cells. All M2KO variants were passageable in M2CK cells without any mutations until at least passage 10. Although, **M2-1** (TM del M2KO), **M2-2** (Splice def M2KO), and **M2-3** (TM del + Splice def M2KO) were not able to be passaged in wild-type MDCK cells (no cytopathic effect (CPE) is seen in wild-type MDCK cells), **M2KO(yk)** showed CPE even after 4^{th} passage in MDCK cells. M segment RNAs were extracted from **M2KO(yk)** passage 4 in wild-type MDCK and the cDNA were

sequenced. As shown in Table 14, two inserted stop codons of **M2KO(yk)** were edited and **M2KO(yk)** passage 4 in wild-type MDCK possessed full length M2 protein gene.

Table 14. Sequence around inserted 2 stop codons (nt 700-800 of M segment, stop codons at nt 786-791.)

Virus	Sequence
Original M2KO(yk)	3'CAACGGTTCAAGTGAT**TAATAA**ACTATTGCC
M2KO(yk) passage 2 in M2CK	3'CAACGGTTCAAGTGAT**TAATAA**ACTATTGCC
M2KO(yk) passage 4 in MDCK	3'CAACGGTTCAAGTGAT**TGGTGG**ACTGTTGCC

Example 9: M2KO Vaccinations

[0198] To demonstrate that the M2KO vaccine can stimulate an immune response similar to a natural influenza infection, a vaccine experiment was conducted. Natural influenza infection was represented by a low inoculum of PR8 virus and the standard inactivated flu vaccine was represented by inactivated PR8 virus (Charles River) delivered the standard intramuscular route and intranasally.

[0199] Six to seven week old BALB/c mice were immunized intranasally with live virus (10 pfu PR8), PR8 virus comprising M2KO(ΔTM) (10^4 pfu), or 1 μg inactivated PR8 virus, delivered both intranasally and intramuscularly. Mice given 10^4 infectious particles of M2KO(ΔTM) intranasally lost no weight and showed no signs of infection. Furthermore, the lungs of mice treated with M2KO(ΔTM) contained no detectable infectious particles three days post-inoculation. Sera was obtained from the immunized mice on day 21 and antibody titers against the hemagglutinin were determined by a standard ELISA assay. **Figure 17** shows that anti-HA IgG titers were highest in the live virus and M2KO(ΔTM) groups relative to the inactivated vaccine groups. Mucosal IgA antibody against influenza was detected in sera only in the live PR8 or M2KO vaccinated mice.

[0200] Six weeks after immunization, all groups were challenged with homosubtypic (PR8, H1N1) or heterosubtypic (Aichi, H3N2) influenza viruses. Both M2KO and inactivated vaccinations protected mice from homosubtypic virus infection (**Figure 18**). However, only M2KO vaccinated mice were protected from heterosubtypic virus challenge (**Figure 19**). The mice immunized with inactivated vaccine succumbed to infection similar to naïve mice.

Example 10 The M2KO(ΔTM) Virus Does not Replicate In The Respiratory Tract Or Other Organs

[0201] Summary – This example demonstrates that the M2KO(ΔTM) virus does not replicate in the respiratory tract or disseminate to other organs in the ferret model. The M2KO(ΔTM) virus was administered intranasally to 3 male ferrets at a dose level of $1x10^7$ $TCID_{50}$. As a control, second group of 3 male ferrets was administered A/Brisbane/10/2007 (H3N2) influenza A virus intranasally at a dose of $1x10^7$ $TCID_{50}$. Following virus inoculation, ferrets were observed until Day 3 post inoculation for mortality, with body weights, body temperatures and clinical signs measured daily. Necropsy was performed on all animals 3 days post inoculation. Organs were collected for histopathology and viral titers.

[0202] The control group receiving A/Brisbane/10/2007 (H3N2) exhibited a transient reduction in weight and an increase in body temperature 2 days after inoculation which was not observed in the M2KO(ΔTM) group. Activity levels were also reduced in the A/Brisbane/10/2007 group with sneezing observed on days 2-3 post infection. No changes in activity level or clinical signs associated with virus exposure were observed in the M2KO(ΔTM) group. Histopathological analysis revealed changes in the nasal turbinates in animals exposed to influenza A/Brisbane/10/2007 (H3N2) that were not seen in ferrets exposed to the M2KO(ΔTM) virus. Exposure to A/Brisbane/10/2007 resulted in atrophy of respiratory epithelium, infiltrates of neutrophils and edema in the nasal turbinates. No other organ was affected by the virus inoculation. Under the conditions of the experiment, the M2KO(ΔTM) virus did not induce clinical signs of infection or result in histological changes in the organs analyzed.

MATERIALS AND METHODS

[0203] A. Vaccine Material and Control Virus: The M2KO(ΔTM) virus is a recombinant virus which possesses internal 6 genes of PR8 (nucleoprotein (NP), polymerase genes (PA, PB1, PB2), non-structural (NS), matrix (M)), but which does not express functional M2 protein, as well as HA and NA genes of Influenza A/Brisbane/10/2007-like A/Uruguay/716/2007(H3N2). The A/Brisbane/10/2007 (H3N2) wild type virus served as the control virus and was supplied by IITRI. The viruses were kept frozen at -65°C until used.

[0204] B. Test Article and Positive Control Dose Formulation: The M2KO(ΔTM) virus dosing solution of $1x10^7$ $TCID_{50}$/mL per 316 μL was prepared by diluting 8 μL of 1x1010 $TCID_{50}$/mL into 2.528 mL PBS. The A/Brisbane/10/2007 (H3N2) at a titer of $1x10^7$ $TCID_{50}$/mL per 316 μL was used undiluted.

[0205] C. Animals and Animal Care: Eight male ferrets were purchased from Triple F Farms and six of the ferrets were placed on study. Animals were approximately 4 months of age at the time of study initiation. The animals were certified by the supplier to be healthy and free of antibodies to infectious diseases. Upon arrival the animals were single housed in suspended wire cages with slat bottoms, suspended over paper-lined waste pans. The animal room and cages had been cleaned and sanitized prior to animal receipt, in accordance with accepted animal care practices and relevant standard operating procedures. Certified Teklad Global Ferret Diet #2072 (Teklad Diets, Madison WI) and city of Chicago tap water were provided ad libitum and were refreshed at least once daily. Fluorescent lighting in the animal rooms was maintained on a 12-hr light/dark cycle. Animal room temperature and relative humidity were within respective protocol limits and ranged from 22.0 to 25.0°C and 33 to 56%, respectively, during the study.

[0206] D. Animal Quarantine and Randomization: The ferrets were held in quarantine for five days prior to randomization and observed daily. Based on daily observations indicating general good health of the animals the ferrets were released from quarantine for randomization and testing. Following quarantine, ferrets were weighed and assigned to treatment groups using a computerized randomization procedure based on body weights that produced similar group mean

values [ToxData® version 2.1.E.11 (PDS Pathology Data Systems, Inc., Basel, Switzerland)]. Within a group, all body weights were within 20% of their mean. Animals selected for the study receive a permanent identification number by ear tag and transponder and individual cage cards also identified the study animals by individual numbers and group. The identifying numbers assigned were unique within the study.

[0207] E. Experimental Design: All animal procedures were performed in an animal biosafety level–2 facility in accordance with the protocols approved by the animal care and use committee at IIT Research Institute. 6 male ferrets (Triple F Farms, Sayre PA), 4 months of age at the time of study initiation were utilized for the study. Prior to infection, ferrets were monitored for 3 days to measure body weight and establish baseline body temperatures. Temperature readings were recorded daily through a transponder (BioMedic data systems, Seaford, DE) implanted subcutaneously in each ferret. Blood was collected prior to study initiation via the jugular vein, and serum tested for influenza antibodies. Study animals free of influenza antibodies were randomized and divided into two groups (3 ferrets/group) as shown in **Table 15**. A group of 3 ferrets was anesthetized and inoculated intranasally with a single dose of 316 μL at $1x10^7$ $TCID_{50}$ of M2KO(ΔTM) virus. A control group (3 ferrets) was inoculated with 316 μL at $1x10^7$ $TCID_{50}$ of A/Brisbane/10/2007 (H3N2). Ferrets were observed daily to monitor body weight, body temperature and clinical symptoms. On Day 3 post-inoculation, ferrets (3 ferrets per group) were euthanized and necropsied. The following tissue samples were collected: nasal turbinates, trachea, lungs, kidneys, pancreas, olfactory bulbs, brains, livers, spleens, small and large intestines. One part of the collected samples was fixed with buffered neutral formalin for histological evaluation and the other part of the samples were stored at -65°C for virus titration.

Table 15: Immunization and sample collection schedule

Group	Dose	N	Organ collection (days post infection)
M2KO	$1x10^7$ $TCID_{50}$	3	3
Brisbane/10	$1x10^7$ $TCID_{50}$	3	3

[0208] F. Virus Inoculation: Ferrets were inoculated with either the M2KO(ΔTM) virus or wild type A/Brisbane/10/2007 (H3N2) influenza A virus. A vial of frozen stock was thawed and diluted to the appropriate concentration in phosphate buffered saline solution. Ferrets were anesthetized with ketamine/xylazine and the virus dose administered intranasally in a volume of 316 µL for the M2KO(ΔTM) virus and 316 µL for the A/Brisbane/10/2007 (H3N2) virus. To confirm the inoculation titer of the A/Brisbane/10/2007 (H3N2) virus, a $TCID_{50}$ assay was performed at IITRI on a portion of the prepared viral challenge solution. The viral titer assay was performed according to Illinois Institute of Technology Research Institute (IITRI) Standard Operating Procedures.

[0209] G. Moribundity/Mortality Observations: Following challenge, all animals were observed twice daily for mortality or evidence of moribundity. Animals were observed for 3 days post-challenge. Animals were euthanized by overdose with Sodium Pentobarbital 150 mg/kg, administered intravenously.

[0210] H. Body Weights and Body Weight Change: Body weights of animals were recorded upon receipt (random 10% sample), at randomization (Day -3 to 0), and daily after virus inoculation.

[0211] I. Clinical Observations: The change in temperature (in degrees Celsius) was determined daily for each ferret. Clinical signs of, inappetence, respiratory signs such as dyspnea, sneezing, coughing, and rhinorrhea and level of activity was assessed daily. A scoring system based on that described by Reuman, *et al.*, "Assessment of signs of influenza illness in

the ferret model," *J. Virol*, Methods 24:27-34 (1989), was used to assess the activity level as follows: 0, alert and playful; 1, alert but playful only when stimulated; 2, alert but not playful when stimulated; and 3, neither alert nor playful when stimulated. A relative inactivity index (RII) was calculated as the mean score per group of ferrets per observation (day) over the duration of the study.

[0212] J. Euthanasia: Study animals were euthanized by an intravenous dose of sodium pentobarbital 150 mg/kg. Death was confirmed by absence of observable heartbeat and respiration. Necropsies were performed on all study animals.

[0213] K. Necropsy: Nasal turbinates, trachea, lungs, kidneys, pancreas, olfactory bulbs, brain, liver, spleen, small and large intestines were harvested. One portion of each tissue was fixed in formalin and the other portion given to IITRI staff for freezing and storage. Tissue harvested for titers are: right nasal turbinates, upper 1/3 of trachea, right cranial lung lobe, right kidney, right arm of pancreas (near duodenum), right olfactory bulb, right brain, right lateral lobe of liver, right half of spleen (end of spleen seen on opening the abdominal cavity), small intestine and large intestine.

[0214] L. Histopathological analysis: Tissues were processed through to paraffin blocks, sectioned at approximately 5-microns thickness, and stained with hematoxylin and eosin (H & E).

[0215] M. Serum Collection: Pre-vaccination (Day -3) serum was collected from all ferrets. Ferrets were anesthetized with a ketamine (25 mg/kg) and xylazine (2mg/kg) mixture. A sample of blood (approximately 0.5-1.0 mL) was collected via the vena cava from each ferret and processed for serum. Blood was collected into Serum Gel Z/1.1 tubes (Sarstedt Inc. Newton, NC) and stored at room temperature for not more than 1 hour before collecting serum. Serum Gel Z/1.1 tubes were centrifuged at 10,000xg for 3 minutes and the serum collected. Individual pre-inoculation serum samples were collected and two aliquots made from each sample. One aliquot was tested prior to the initiation of the study to confirm ferrets are free of antibodies to influenza A viruses and one aliquot of the serum stored at -65°C.

[0216] N. Hemagglutination Inhibition (HI) Assay: Serum samples were treated with receptor-destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) to eliminate inhibitors of nonspecific hemagglutination. RDE was reconstituted per the manufacturer's instructions. Serum was diluted 1:3 in RDE and incubated 18-20 hours in a 37°C ± 2°C water bath. After the addition of an equal volume of 2.5% (v/v) sodium citrate, the samples were incubated in a 56 ± 2°C water bath for 30 ± 5 minutes. 0.85% NaCl was added to each sample to a final serum dilution of 1:10 after the RDE treatment. The diluted samples were then diluted into four two-fold dilutions (1:10 to 1:80) in duplicate in phosphate buffered saline (PBS) then incubated with 4 hemagglutinating units of A/Brisbane/10/2007 (H3N2) influenza A virus. After incubation, 0.5% chicken red blood cells were added to each sample and incubated. Presence or absence of hemagglutination was then scored.

[0217] O. Virus Titers: The concentration of infectious virus in the pre- and post-challenge virus inoculum samples was determined by $TCID_{50}$ in Madin-Darby Canine Kidney (MDCK) cells. Briefly, samples kept at -65°C were thawed and centrifuged to remove cellular debris. The resulting supernatant were diluted 10-fold in triplicate in 96-well microtiter plates in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) containing Pencillin/Streptomycin, 0.1% Gentamicin, 3% $NaCO_3$, 0.3% BSA fraction V (Sigma St. Louis, MO), 1% MEM vitamin solution (Sigma) and 1% L-glutamine (Mediatech, Manassas, VA, USA). After 10-fold serial dilutions were made, 100L was transferred into respective wells of a 96-well plate which contained a monolayer of MDCK cells. Plates were incubated at 37°C ± 2°C in 5 ± 2% CO_2 70% humidity. After 48 hours, the wells were observed for cytopathogenic effect (CPE). Supernatant from each well (50 µl) was transferred to a 96 well plate and the hemagglutination (HA) activity determined and recorded. The HA activity of the supernatant was assessed by HA assay with 0.5% packed turkey red blood cells (cRBCs). $TCID_{50}$ titers were calculated using the method of Reed LJ and Muench H, "A simple method for estimating 50% endpoints," *Am. J. Hygiene* 27: 493-497 (1938).

[0218] P. Data Analysis: Body weights and body weight gains (losses) and changes in body temperature were determined for each individual animal expressed as mean and standard deviations of the mean for each test group.

RESULTS

[0219] After inoculation with either the M2KO(ΔTM) virus or A/Brisbane/10/2007 (H3N2) influenza A virus, ferrets were monitored for survival and clinical signs of infection. Results are presented in **Table 16A and 16B**. All ferrets survived infection with M2KO(ΔTM) virus and A/Brisbane/10/2007 (H3N2). Ferrets inoculated with A/Brisbane/10/2007 presented respiratory signs (sneezing) on Day 2 and 3. The relative inactivity index of ferrets inoculated with A/Brisbane/10/2007 was 0.67; whereas ferrets inoculated with M2KO(ΔTM) showed no reduction activity level with a relative inactivity index of 0.0.

[0220] Changes in body weight and temperature after virus inoculation are shown in **Figure 20** and **Figure 21**. After inoculation with A/Brisbane/10/2007 (H3N2), a 2-3% loss of body weight was observed on Day 2 post inoculation in all animals. Minimal to zero weight loss was observed in ferrets inoculated with the M2KO(ΔTM) virus. One M2KO(ΔTM) inoculated ferret exhibited weight loss on Day 2 post inoculation of 1%. Elevated body temperatures of 40.3-40.7°C were observed in ferrets inoculated with A/Brisbane 10/2007 on Day 2 post inoculation. Body temperatures returned to normal range by Day 3. Body temperatures for M2KO(ΔTM) inoculated ferrets remained in normal range throughout the duration of the study. To determine if the M2KO(ΔTM) virus would replicate in the respiratory tract or other organs and induce pathology, tissues of ferrets were histologically examined on day 3 post inoculation and compared to those from ferrets inoculated with A/Brisbane/10/2007. In ferrets inoculated with A/Brisbane/10/2007, pathology was observed only in the nasal turbinates. Atrophy of respiratory epithelium, infiltrates of neutrophils and edema were observed in the nasal turbinates. No histopathological changes associated with viral infection were observed in ferrets inoculated with the M2KO(ΔTM) virus. The concentrations of pre- and post-challenge virus dosing solutions

were $10^{7.5}$ $TCID_{50}$/mL and $10^{7.75}$ $TCID_{50}$/mL, respectively, indicating good stability of the challenge material throughout administration.

Table 16A: Effect of virus inoculation on survival and clinical signs of infection in ferrets.

Group	N	Serum HI Titer[a]	Total number dead	Clinical signs[b]		
				Respiratory signs (observed day of onset)	Loss of Appetite	Lethargy (RII)[c]
M2KO	3	<10	0/3	0/3	0/3	0
Brisbane/10	3	<10	0/3	2/3 (2)	0/3	0.67

[a]Hemagglutination inhibition (HI) antibody titers to homologous virus in ferret serum prior to virus inoculation.
[b]Clinical signs were observed for 3 days after virus inoculation. Except for lethargy, findings for clinical signs are given as no. of ferrets with sign/total no. Respiratory signs included sneezing.
[c]Determined twice daily for 3 days of observation based on the scoring system and was calculated as the mean score per group of ferrets per observation (day) over the 3-day period. The relative inactivity index before inoculation was 0.

Table 16B: M2KO(ΔTM) Does Not Replicate in Ferret Respiratory Organs Harvested On Day 3

Virus Titer (log pfu/g)		
	Brisbane/10	M2KO(ΔTM)
Nasal Turbinates	5.43	0
Lung	0	0

CONCLUSION

[0221] This example shows that by Day 3 post inoculation, the M2KO(ΔTM) virus does not induce clinical signs of disease or histopathological changes associated with infection of wild type virus. This shows that the M2KO(ΔTM) virus of the present technology is useful for intranasal influenza vaccines.

Example 11: Immune Response and Protective Effects M2KO(ΔTM) Virus Relative To Other Vaccines

[0222] Summary – This example demonstrates the immune response elicited by the M2KO(ΔTM) vaccine and the protective effects of the vaccine in the ferret model. The M2KO(ΔTM) virus was administered intransally to 12 male ferrets at a dose level of $1x10^7$ $TCID_{50}$. As a control, a second group of 12 male ferrets was administered the FM#6 virus intranasally at a dose of $1x10^7$ $TCID_{50}$. A third group of ferrets was administered OPTI-MEM™ as a placebo control. A prime only or prime-boost vaccination regimen was utilized for each treatment group. Ferrets receiving the prime-boost vaccination regimen were administered the prime vaccine (Day 0) and the boost vaccination 28 days later (Day 28). Ferrets receiving only the prime vaccine were administered a single vaccination on the same day as the booster vaccine was given to the prime-boost ferrets (Day 28). Following each vaccination, ferrets were observed for 14 days post inoculation for mortality, with body weights, body temperatures and clinical signs measured daily. Nasal washes were collected from ferrets on days 1, 3, 5, 7 and 9 post-prime vaccination to look for viral shedding. Nasal washes and serum were collected weekly from all ferrets post-vaccination to evaluate antibody levels over time.

[0223] All animals were challenged intranasally on Day 56 with $1x10^7$ $TCID_{50}$ of A/Brisbane/10/2007 (H3N2). Following challenge, ferrets were monitored for 14 days post inoculation for mortality, with body weights, body temperatures and clinical signs measured daily. Nasal washes were collected on days 1, 3, 5, 7, 9 and 14 post challenge from ferrets in each group for viral titers. Additionally, serum was collected post-challenge (day 70) from

surviving ferrets for analysis. Necropsy was performed on 3 ferrets per group 3 days post challenge. Organs were collected for histopathology and viral titers.

[0224] No vaccine related adverse events were observed among the 5 groups. After challenge, the placebo control group exhibited an increase in body temperature 2 days after challenge and a reduction in weight. A reduction in weight was also observed in M2KO(ΔTM) and FM#6 vaccinated groups; however, the reduction was to less than that observed in the OPTI-MEM™ group. Activity levels were not reduced in any groups; however sneezing was observed in all groups after challenge. Histopathological analysis revealed an increase in severity of mixed cell infiltrates in the lung of vaccinated ferrets when compared to the lung infiltrates in the OPTI-MEM™ control group. In the nasal turbinates, animals receiving a prime or prime plus boost regimen of either M2KO(ΔTM) or FM#6 had lower severity of atrophy of respiratory epithelium when compared to the OPTI-MEM™ control group. Vaccination with the M2KO(ΔTM) virus appeared to provides similar protection against viral challenge as the FM#6 virus.

MATERIALS AND METHODS

[0225] A. Vaccine Material: The M2KO(ΔTM) virus is a recombinant virus which possesses internal 6 genes of PR8 (nucleoprotein (NP), polymerase genes (PA, PB1, PB2), non-structural (NS), matrix (M)), but which does not express functional M2 protein, as well as HA and NA genes of Influenza A/Brisbane/10/2007-like A/Uruguay/716/2007(H3N2). The FM#6 virus is clone #6 of the A/Uruguay/716/2007 (H3N2) influenza A virus from FluMist® (2009-2010 formula). The M2KO(ΔTM) virus and FM#6 virus were administered intranasally to the animals in a 316 μL dose of $1x10^7$ $TCID_{50}$ (50% Tissue Culture Infectious Doses).

[0226] B. Test Article and Positive Control Dose Formulation: The M2KO(ΔTM) virus dosing solution of $1x10^7$ $TCID_{50}$/mL per 316 μL was prepared by diluting 120 μL of $1x10^9$ $TCID_{50}$/mL into 3.680 mL PBS. The FM#6 virus at a titer of $1x10^7$ $TCID_{50}$/mL per 316 μL was prepared by diluting 120 μL of $1x10^9$ $TCID_{50}$/mL into 3.680 mL PBS.

[0227] C. Animals and Animal Care: Thirty-six male ferrets were purchased from Triple F Farms and 30 of the ferrets were placed on study. Animals were approximately 4 months of age at the time of study initiation. The animals were certified by the supplier to be healthy and free of antibodies to infectious diseases. Upon arrival the animals were single housed in suspended wire cages with slat bottoms, suspended over paper-lined waste pans. The animal room and cages had been cleaned and sanitized prior to animal receipt, in accordance with accepted animal care practices and relevant standard operating procedures. Certified Teklad Global Ferret Diet #2072 (Teklad Diets, Madison WI) and city of Chicago tap water were provided ad libitum and were refreshed at least three time per week. Fluorescent lighting in the animal rooms was maintained on a 12-hr light/dark cycle. Animal room temperature and relative humidity were within respective protocol limits and ranged from 20.0 to 25.0°C and 30 to 63%, respectively, during the study.

[0228] D. Animal Quarantine and Randomization: The ferrets were held in quarantine for seven days prior to randomization and observed daily. Based on daily observations indicating general good health of the animals the ferrets were released from quarantine for randomization and testing. Following quarantine, ferrets were weighed and assigned to treatment groups using a computerized randomization procedure based on body weights that produced similar group mean values [ToxData® version 2.1.E.11 (PDS Pathology Data Systems, Inc., Basel, Switzerland)]. Within a group, all body weights were within 20% of their mean. Animals selected for the study receive a permanent identification number by ear tag and transponder and individual cage cards also identified the study animals by individual numbers and group. The identifying numbers assigned were unique within the study.

[0229] E. Experimental Design: To assess the M2KO(ΔTM) vaccine efficacy, ferrets were immunized with M2KO(ΔTM) virus, cold adapted live attenuated virus (FM#6) or mock immunized by medium (OPTI-MEM™). The animals body weight, body temperature and clinical symptoms were monitored and immunological responses evaluated. 30 male ferrets (Triple F Farms, Sayre PA), 4 months of age at the time of study initiation were utilized for the study. All animal procedures were performed in an animal biosafety level–2 or biosafety level-3

facility in accordance with the protocols approved by the animal care and use committee at IIT Research Institute. Prior to inoculation, ferrets were monitored for 3 days to measure body weight and establish baseline body temperatures. Temperature readings were recorded daily through a transponder (BioMedic data systems, Seaford, DE) implanted subcutaneously in each ferret. Blood was collected prior to study initiation, and serum tested for influenza antibodies. Only ferrets with HAI (hemagglutination inhibition) titers 40 to A/Brisbane/10/2007 (H3N2) were considered seronegative and used in this study. Study animals were randomized and divided into 5 groups (6 ferrets/group) as shown in **Table 17**. Two groups (1 & 3) received the M2KO(ΔTM) virus and 2 groups (2 & 4) received the FM#6 virus. One group (5) was mock immunized with OPTI-MEM™ . Within each vaccine group, ferrets were divided into two vaccine regimens, six receiving a prime vaccination only (Prime only) and six receiving a prime vaccination followed by a booster vaccine 28 days after prime vaccination (Prime/Boost). Prime/Boost Groups: Ferrets were inoculated intranasally with a single dose of 316 μL of $1x10^7$ $TCID_{50}$ of M2KO(ΔTM) virus on days 0 and 28. Control groups were inoculated intranasally with 316 μL of $1x10^7$ $TCID_{50}$ (same dose as M2KO(ΔTM)) of FM#6 or mock inoculated with 316 μL of OPTI-MEM™ on days 0 and 28. Ferret body temperatures, weights, and clinical symptoms were monitored daily for 14 days post-inoculations. Nasal washes were collected from all ferrets, including OPTI-MEM™ control group, on days 1, 3, 5, 7, 9 and 14 post prime vaccination for virus titration in cells and on days 21 and 49 for antibody titration. Nasal wash samples were kept at -65°C. Blood was collected prior to inoculation (day -3 to -5) and days 7, 14, 21 35, 42, and 49 and serum kept at -65°C until measurement of antibody titer by ELISA and HI assay.

[0230] Prime only Groups: Ferrets were inoculated intranasally with a single dose of 316 μL of $1x10^7$ $TCID_{50}$ of M2KO(ΔTM) virus on day 28. Control groups were inoculated intranasally with 316 μL of $1x10^7$ $TCID_{50}$ (same dose as M2KO(ΔTM)) of FM#6 or mock inoculated with 316 μL of OPTI-MEM™ on day 28. Ferret body temperatures, weights, and clinical symptoms were monitored daily for 14 days post-inoculation. Nasal washes were collected from all ferrets on days 29, 31, 33, 35, 37, and 42 for virus titration in cells and on day 49 for antibody titration.

Nasal wash samples were kept at -65°C. Blood was collected prior to inoculation (day 23 to 25) and days 35, 42, and 49 and serum was kept at -65°C until measurement of antibody titer by ELISA and HAI assay. All ferrets were challenged with a dose of 316 µL of $1x10^7$ $TCID_{50}$ of wild-type A/Brisbane/10/2007 (H3N2) influenza virus on day 56, 4 weeks after the prime/boost vaccine was administered. Ferret body weight, body temperature and clinical symptoms were monitored for 14 days after challenge and nasal washes and organs collected. Nasal washes were collected from challenged ferrets on days 1, 3, 5, 7, 9, and 14 post-challenge (days 57, 59, 61, 63, 65, and 70) and the samples kept at -65°C for virus titration in cells. On Day 3 post-challenge (day 59), the animals (3 animals per group, total 15 animals) were euthanized and the following tissue samples collected: nasal turbinates, trachea, and lungs. One part of the collected samples was fixed with buffered neutral formalin for histological evaluation and the other part of the samples was stored at -65°C for virus titration. Blood was collected 14 days post-challenge (day 70) and all surviving animals were euthanized.

Table 17: Vaccination and sample collection schedule

Group	Vaccine Virus[1]	N	Vaccination (days)	Nasal Washes[2] (days)	Challenge (day)	Nasal Washes (days)	Organs[3] n=3 (day)	Serum collections
Prime only								
1	M2KO	6	28	29, 31, 33, 35, 37, 42, 49	56	57, 59, 61, 63, 65, 70	59	35, 42, 49, 70
2	FM#6	6	28	29, 31, 33, 35, 37, 42, 49	56	57, 59, 61, 63, 65, 70	59	35, 42, 49, 70
Prime/Boost								
3	M2KO	6	0, 28	1, 3, 5, 7, 9, 14, 21, 49	56	57, 59, 61, 63, 65, 70	59	7, 14, 21, 35, 42, 49, 70
4	FM#6	6	0, 28	1, 3, 5, 7, 9, 14, 21, 49	56	57, 59, 61, 63, 65, 70	59	7, 14, 21, 35, 42, 49, 70
5	Vehicle (Control)	6	0, 28	1, 3, 5, 7, 9, 14, 21, 49	56	57, 59, 61, 63, 65, 70	59	7, 14, 21, 35, 42, 49, 70

[1]Intranasally inoculated with a dose of $1x10^7$ $TCID_{50}$
[2]Nasal Washes only collected from animals after prime vaccination
[3]Organs (nasal turbinated, trachea and lung) collected from 3 ferrets per group for histology and viral titers.

[0231] F. Virus Inoculation: Ferrets were inoculated with either the M2KO(ΔTM) virus or FM#6 influenza A virus. A vial of frozen stock was thawed and diluted to the appropriate concentration in phosphate buffered saline solution. Ferrets were anesthetized with ketamine/xylazine and the virus dose administered intranasally in a volume of 316 µL for the M2KO(ΔTM) virus and 316 µL for the FM#6 virus. To confirm the inoculation titer of the M2KO(ΔTM) and FM#6 viruses, aliquots of the dosing solutions were collected prior to dosing (pre-dose) and after dosing (post-dose). The aliquots were stored at -65°C for virus titration.

[0232] G. Challenge Virus: Influenza A virus, strain A/Brisbane/10/2007, serotype H3N2 was used to challenge the ferrets. The virus was stored at approximately -65°C prior to use. The dose level of challenge virus used was prepared at $1x10^7$ $TCID_{50}$ in a volume of 316 µL. A quantitative viral infectivity assay, $TCID_{50}$ assay was performed at IITRI on a portion of the prepared viral challenge solution. The viral titer assay was performed according to IITRI Standard Operating Procedures.

[0233] H. Moribundity/Mortality Observations: Following challenge, all animals were observed twice daily for mortality or evidence of moribundity. Animals were observed for 14 days after vaccine inoculation and for 14 days after challenge.

[0234] I. Body Weights and Body Weight Change: Body weights were recorded within two days of receipt and at randomization. All study animals were weighed prior to inoculation, daily for 14 days following each vaccination and assessed daily for 14 days post challenge. Prior to inoculation, ferrets were monitored for 3-5 days to measure establish baseline body temperatures. Temperature readings were recorded daily for 14 days following each vaccination and recorded daily for 14 days post challenge through a transponder (BioMedic data systems, Seaford, DE) implanted subcutaneously in each ferret. The change in temperature (in degrees Celsius) was calculated at each time point for each animal.

[0235] J. Clinical Observations: The change in temperature (in degrees Celsius) was determined daily for each ferret. Clinical signs of, inappetence, respiratory signs such as dyspnea, sneezing, coughing, and rhinorrhea and level of activity was assessed daily. A scoring

system based on that described by Reuman, *et al.*, "Assessment of signs of influenza illness in the ferret model," *J. Virol*, Methods 24:27-34 (1989), was used to assess the activity level as follows: 0, alert and playful; 1, alert but playful only when stimulated; 2, alert but not playful when stimulated; and 3, neither alert nor playful when stimulated. A relative inactivity index (RII) was calculated as the mean score per group of ferrets per observation (day) over the duration of the study.

[0236] K. Survival Checks: Two survival checks were performed daily on all study animals throughout the study. Both survival checks occurred simultaneously with the clinical observations. The second check was performed later within the same day.

[0237] L. Nasal Washes: Ferrets were anesthetized with a ketamine (25 mg/kg) and xylazine (2mg/kg) mixture, and 0.5 ml of sterile PBS containing penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamicin (50 µg/ml) was injected into each nostril and collected in a specimen cup when expelled by the ferret. The nasal wash was collected into a cryovial and the recovered volume recorded.

[0238] M. Euthanasia: Study animals were euthanized by an intravenous dose of sodium pentobarbital 150 mg/kg. Death was confirmed by absence of observable heartbeat and respiration.

[0239] N. Necropsy: Necropsy was performed by Charles River Laboratories, Pathology Associates (PAI). The PAI team was comprised of a supervising pathologist and two prosectors. Nasal turbinates, trachea and lungs were harvested. One portion of each tissue was fixed in formalin and the other portion given to IITRI staff for freezing and storage. Tissue harvested for titers are: right nasal turbinates, upper 1/3 of trachea and right cranial lung lobe.

[0240] O. Histopathological analysis: Following each necropsy, tissues were transported to the PAI Chicago facility. Upon receipt, partial tissues from all 15 ferrets were processed through to paraffin blocks, sectioned at approximately 5-microns thickness, and stained with hematoxylin and eosin (H & E). All paraffin H & E slides were evaluated microscopically.

[0241] P. Serum Collection: Pre-vaccination serum (days -3 to -5 for groups 3, 4, and 5, and days 23 to 25 for groups 1 and 2) serum was collected from the ferrets. Post inoculation, serum was collected on days 7, 14, 21, 35, 42, 49, and 70 from groups 3, 4, and 5. Serum was collected on days 35, 42, 49, and 70 from groups 1 and 2. Ferrets were anesthetized with a ketamine (25 mg/kg) and xylazine (2mg/kg) mixture. A sample of blood (approximately 0.5-1.0 mL) was collected via the vena cava from each ferret and processed for serum. Blood was collected into Serum Gel Z/1.1 tubes (Sarstedt Inc. Newton, NC) and stored at room temperature for not more than 1 hour before collecting serum. Serum Gel Z/1.1 tubes were centrifuged at 10,000xg for 3 minutes and the serum collected.

[0242] Q. Hemagglutination Inhibition (HI) Assay: Serum samples were treated with receptor-destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) to eliminate inhibitors of nonspecific hemagglutination. RDE was reconstituted per the manufacturer's instructions. Serum was diluted 1:3 in RDE and incubated 18-20 hours in a 37°C ± 2°C water bath. After the addition of an equal volume of 2.5% (v/v) sodium citrate, the samples were incubated in a 56 ± 2°C water bath for 30 ± 5 minutes. 0.85% NaCl was added to each sample to a final serum dilution of 1:10 after the RDE treatment. The diluted samples were then diluted into four two-fold dilutions (1:10 to 1:80) in duplicate in phosphate buffered saline (PBS) then incubated with 4 hemagglutinating units of A/Brisbane/10/2007 (H3N2) influenza A virus. After incubation, 0.5% avian red blood cells were added to each sample and incubated for 30 ± 5 minutes. Presence or absence of hemagglutination was then scored.

[0243] R. Virus Titers: The concentration of infectious virus in the pre- and post-challenge virus inoculum samples was determined by $TCID_{50}$ assay in Madin-Darby Canine Kidney (MDCK) cells. Briefly, samples kept at -65°C were thawed and centrifuged to remove cellular debris. The resulting supernatant were diluted 10-fold in triplicate in 96-well microtiter plates in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) containing Pencillin/Streptomycin, 0.1% Gentamicin, 3% NaCO3, 0.3% BSA fraction V (Sigma St. Louis, MO), 1% MEM vitamin solution (Sigma) and 1% L-glutamine (Mediatech, Manassas, VA, USA). After 10-fold serial dilutions were made, 100 µL was transferred into respective wells of a

96-well plate which contained a monolayer of MDCK cells. Plates were incubated at 37°C ± 2°C in 5 ± 2% CO2 70% humidity. After 48 hours, the wells were observed for cytopathogenic effect (CPE). Supernatant from each well (50 µl) was transferred to a 96 well plate and the hemagglutination (HA) activity determined and recorded. The HA activity of the supernatant was assessed by HA assay with 0.5% packed turkey red blood cells (tRBCs). $TCID_{50}$ titers were calculated using the method of Reed LJ and Muench H, "A simple method for estimating 50% endpoints," *Am. J. Hygiene* 27: 493-497 (1938).

[0244] S. Data Analysis: Body weights and body weight gains (losses) and changes in body temperature were determined for each individual animal expressed as mean and standard deviations of the mean for each test group.

RESULTS

[0245] After intranasal vaccination with either the M2KO(ΔTM) virus or the FM#6 virus, ferrets were monitored daily for clinical signs of infection. Nasal washes were collected after prime vaccination to monitor viral shedding and serum collected to measure serum antibody titers. Results are presented in **Tables 18A, 18B, and 18C.**

Table 18A : Effect of vaccination on survival and clinical signs of infection in ferrets.

Group	Treatment	N	Serum HI Titer[a]	Total number dead	Clinical signs[b]		
					Respiratory signs (observed day of onset)	Loss of Appetite	Lethargy (RII)[c]
Prime							
1	M2KO	6	<10	0/6	2/6 (8)	0/6	0
2	FM#6	6	<10	0/6	0/6	0/6	0
3	M2KO	6	<10	0/6	0/6	0/6	0.07
4	FM#6	6	<10	0/6	0/6	0/6	0.29
5	Vehicle (Control)	6	<10	0/6	0/6	0/6	0
Boost							
3	M2KO	6	<10	0/6	0/6	0/6	0
4	FM#6	6	<10	0/6	2/6 (7)	0/6	0
5	Vehicle (Control)	6	<10	0/6	2/6 (4)	0/6	0

[a]Hemagglutination inhibition (HI) antibody titers to homologous virus in ferret serum prior to virus inoculation.
[b]Clinical signs were observed for 3 days after virus inoculation. Except for lethargy, findings for clinical signs are given as no. of ferrets with sign/total no. Respiratory signs included sneezing.
[c]Determined twice daily for 3 days of observation based on the scoring system and was calculated as the mean score per group of ferrets per observation (day) over the 3-day period. The relative inactivity index before inoculation was 0.

Table 18B: Virus Titers in Ferret Respiratory Organs After Challenge

	Nasal Turbinates (N=3, Log pfu/g)	Trachea (N=3, Log pfu/g)
M2KO(ΔTM) prime only	5.23 ± 0.24	**
FluMist® prime only	5.53 ± 0.82	2.52 ± 1.73
M2KO(ΔTM) prime-boost	6.16 ± 1.17	1.37 ± 1.06
FluMist® prime-boost	6.24 ± 1.31	3.30 ± 1.96
**Not Detected		

Table 18C: Mucosal IgA Responses in Ferret

α-HA ELISA IgA titers 14 days post-challenge:		
	Nasal Wash	Sera
M2KO(ΔTM) prime only	14	Not Tested
FluMist® prime only	29	Not Tested

[0246] All ferrets survived vaccination with M2KO(ΔTM) virus and FM#6 virus. After prime vaccination, two ferrets inoculated with M2KO(ΔTM) virus presented respiratory signs (sneezing) on Day 8. After boost vaccination, ferrets inoculated with the FM#6 virus presented respiratory signs (sneezing) 7 days post vaccination. Sneezing was also observed in the OPTI-MEM™ ferrets on day 4 post boost. After prime vaccination, the relative inactivity index of ferrets inoculated with M2KO(ΔTM) virus and FM#6 virus was 0.07 and 0.27, respectively. This reduction in activity was only observed in one group per virus after prime vaccination. After boost vaccination no reduction in activity level was observed. Changes in body weight and temperature after virus inoculation are shown in **Figure 22** and **Figure 23**. No weight loss was observed after vaccination; however, vaccination appeared to have an effect on weight gain. After vaccination, body weights of OPTI-MEM™ control ferrets increased 20% during the 14 day observation whereas body weight gain of the M2KO(ΔTM) or FM#6 vaccinated ferrets ranged from 6-15% after prime and 4-6% after boost. No increase in body temperature was observed in any groups after vaccination. Changes in body weight and temperature after challenge are shown in **Figure 24** and **Figure 25** and clinical signs summarized in **Table 19**.

Table 19: Effect of virus challenge on survival and clinical signs of infection in ferrets.

Group	Treatment	N	Total number dead	Clinical signs [a]		
				Respiratory signs (observed day of onset)	Loss of Appetite	Lethargy (RII)[b]
1	M2KO	6	0/6	5/6 (2)	0/6	0
2	FM#6	6	0/6	5/6 (1)	0/6	0
3	M2KO	6	0/6	3/6 (2)	0/6	0
4	FM#6	6	0/6	3/6 (2)	0/6	0
5	Vehicle (Control)	6	0/6	3/6 (3)	0/6	0

[a] Clinical signs were observed for 3 days after virus inoculation. Except for lethargy, findings for clinical signs are given as no. of ferrets with sign/total no. Respiratory signs included sneezing.
[b] Determined twice daily for 3 days of observation based on the scoring system and was calculated as the mean score per group of ferrets per observation (day) over the 3-day period. The relative inactivity index before inoculation was 0.

[0247] After challenge with A/Brisbne/10/2007 (H3N2), a 2-4% loss of body weight was observed on Day 2 post challenge in all animals. Throughout the 14 day observation period animal body weights remained below their initial weight. OPTI-MEM™ ferrets lost the most weight (8%). Weight loss among vaccinated ferrets was dependent on the vaccine regimen. Ferrets receiving the prime only regimen of M2KO(ΔTM) or FM#6 lost a maximum of 5% and 4% respectively. Ferrets receiving a booster lost a maximum of 3% for the FM#6 group and 2% for the M2KO(ΔTM) group. Elevated body temperatures post challenge were observed on Day 2 in OPTI-MEM™ ferrets and on Day 1 ferrets receiving the prime only regimens of

M2KO(ΔTM) or FM#6 (**Figure 25**). Body temperatures for ferrets receiving a booster remained within normal range.

[0248] To determine if the vaccination would prevent replication of challenge virus in the respiratory tract and reduce organ pathology tissues of challenged ferrets were histologically examined on day 3 post inoculation. Changes in the lungs of animals receiving the M2KO(ΔTM) prime only or prime/boost regimen were associated with increase in severity of mixed cell infiltrates in the lung when compared to the OPTI-MEM™ group. Minor differences in lung infiltrate incidences were observed between the M2KO(ΔTM) prime group and the M2KO(ΔTM) prime/boost group. An increase in the severity of mixed cell infiltrates in the lung was also seen in the FM#6 prime group and FM#6 prime/boost group when compared to the OPTI-MEM™ group. A slight increase in severity in lung mixed cell infiltrates was observed in the FM#6 prime/boost group over the FM#6 prime only group. In the nasal turbinates, animals receiving the prime or prime/boost of the M2KO(ΔTM) virus had lower severity of atrophy of respiratory epithelium when compared to the OPTI-MEM™ group. There were no differences in atrophy of the nasal turbinates when comparing prime versus prime/boost M2KO(ΔTM) groups. A slight increase in severity of atrophy of respiratory epithelium in animals receiving the FM#6 prime/boost regimen was observed versus animals FM#6 prime only regimen; the severity of atrophy of respiratory epithelium in all FM#6 animals was lower than that seen in the OPTI-MEM™ group. There was a decrease in incidence of neutrophilic infiltrates into the nasal cavity (lumen) in the M2KO(ΔTM) prime and prime/boost groups compared to the OPTI-MEM™ group. Neutrophilic luminal infiltrates in the M2KO(ΔTM) prime only group was interpreted as not different from the OPTI-MEM™ group. There was a slight increase in severity of luminal neutrophilic infiltrates in the FM#6 prime only and prime/boost groups when compared to the OPTI-MEM™ group. The concentrations of pre- and post-challenge virus dosing solutions were 10^7.83 $TCID_{50}$/mL and 10^7.25 $TCID_{50}$/mL, respectively, indicating good stability of the challenge material throughout administration.

[0249] **Figure 45** shows M2KO(ΔTM) and FluMist® virus replication in the ferret respiratory tract.

[0250] **Figure 46** shows M2KO(ΔTM) and FluMist® viral titers in nasal washes after intranasal challenge with A/Brisbane/10/2007 (H3N2) virus.

[0251] **Figure 47** shows IgG titers in ferrets following vaccination with M2KO(ΔTM) and FluMist,® prime group only.

[0252] **Figure 48** shows IgG titers in ferrets following vaccination with M2KO(ΔTM) and FluMist,® prime-boost groups.

[0253] **Figure 49** shows a summary of ELISA IgG titers in ferret sera from vaccination with M2KO(ΔTM) or FluMist® to post-challenge.

CONCLUSION

[0254] This example shows that intranasal administration of the M2KO(ΔTM) virus was not associated with any vaccine related adverse events (elevated body temperature, loss of weight or clinical signs). These results show that the M2KO(ΔTM) virus of the present technology is useful for use in an intranasal influenza vaccine.

Example 12: M2KO(ΔTM) Virus in Not Transmitted in the Ferret Model

[0255] Summary – This example demonstrates that the M2KO(ΔTM) virus is not transmitted in the ferret model. The M2KO(ΔTM) virus was administered intransally to 3 female ferrets at a dose level of $1x10^7$ $TCID_{50}$. As a control, a second group of 3 female ferrets was administered the A/Brisbane/10/2007 (H3N2) virus intranasally at a dose of $1x10^7$ $TCID_{50}$. Twenty four hours after inoculation, each donor ferret was introduced into a transmission chamber with two naive ferrets (a direct contact and aerosol contact). Following inoculation, ferrets were observed for 14 days post inoculation for mortality, with body weights, body temperatures and clinical signs measured daily. Nasal washes were collected from all inoculated donor ferrets on days 1, 3, 5, 7, 9 and from all contact (direct and aerosol) ferrets on days 2, 4, 6, 8, 10 to look for viral shedding. Nasal washes and serum were collected from all ferrets at the inoculation of the study (Day 14) to evaluate antibody levels. No clinical signs of infection were observed in the M2KO(ΔTM)

group; however, ferrets in the A/Brisbane/1 0/2007 (H3N2) group had weight loss, increased body temperatures and were sneezing. After inoculation with Brisbane/1 0, the donor ferrets exhibited an increase in body temperature 2 days after challenge and a reduction in weight. Activity levels were not reduced in any groups. Ferrets in direct contact with the donor ferrets showed progressive weight gain until day 4 post inoculation. A similar trend was observed in the aerosol contact ferrets beginning on day 6 post inoculation. The loss in body weight in the contact ferrets correlated with an increase in body temperature. Inoculation with the M2KO(ΔTM) virus does not elicit clinical signs of infection in inoculated animals. Spread to contact ferrets is unlikely.

MATERIALS AND METHODS

[0256] A. Vaccine Material: The M2KO(ΔTM) virus is a recombinant virus which possesses internal 6 genes of PR8 (nucleoprotein (NP), polymerase genes (PA, PB1, PB2), non-structural (NS), matrix (M)), but which does not express functional M2 protein, as well as HA and NA genes of Influenza A/Brisbane/10/2007-like A/Uruguay/716/2007(H3N2). M2KO(ΔTM) virus was administered intranasally to the animals in a 316 μL dose of 1xl07 $TCID_{50}$ (50% Tissue Culture Infectious Doses).

[0257] B. Test Article Dose Formulation: The M2KO(ΔTM) virus dosing solution of $1x10^7$ $TCID_{50}$/mL per 316 μL was prepared by diluting 45 of $1x10^9$ $TCID_{50}$/mL into 1.377 mL PBS.

[0258] C. Animals and Animal Care: 22 female ferrets were purchased from Triple F Farms and 18 of the ferrets were placed on study. Animals were approximately 4 months of age at the time of study initiation. The animals were certified by the supplier to be healthy and free of antibodies to infectious diseases. Upon arrival the animals were single housed in suspended wire cages with slat bottoms, suspended over paper-lined waste pans. The animal room and cages had been cleaned and sanitized prior to animal receipt, in accordance with accepted animal care practices and relevant standard operating procedures. Certified Teklad Global Ferret Diet #2072 (Teklad Diets, Madison WI) and city of Chicago tap water were provided ad libitum and were refreshed at least once daily. Fluorescent lighting in the animal rooms was maintained on a 12-hr

light/dark cycle. Animal room temperature and relative humidity were within respective protocol limits and ranged from 23.0 to 25.0°C and 36 to 50%, respectively, during the study.

[0259] D. Animal Quarantine and Randomization: The ferrets were held in quarantine for seven days prior to randomization and observed daily. Based on daily observations indicating general good health of the animals the ferrets were released from quarantine for randomization and testing. Following quarantine, ferrets were weighed and assigned to treatment groups using a computerized randomization procedure based on body weights that produced similar group mean values [ToxData® version 2.1.E.11 (PDS Pathology Data Systems, Inc., Basel, Switzerland)]. Within a group, all body weights were within 20% of their mean. Animals selected for the study receive a permanent identification number by ear tag and transponder and individual cage cards also identified the study animals by individual numbers and group. The identifying numbers assigned were unique within the study.

[0260] E. Experimental Design: To assess the transmissibility of the M2KO(ΔTM) virus, ferrets were inoculated with M2KO(ΔTM) virus or A/Brisbane/10/2007 (H3N2) virus. The animals body weight, body temperature, clinical symptoms and viral shedding were monitored and immunological responses evaluated. 18 female ferrets (Triple F Farms, Sayre PA), 4 months of age at the time of study initiation were utilized for the study. All animal procedures were performed in an animal biosafety level-2 or level 3 facility. Prior to inoculation, ferrets were monitored for 3 days to measure body weight and establish baseline body temperatures. Temperature readings were recorded daily through a transponder (BioMedic data systems, Seaford, DE) implanted subcutaneously in each ferret. Blood was collected prior to study initiation, and serum tested for influenza antibodies. Only ferrets with HI titers 40 to A/Brisbane/1 0/2007 (H3N2) virus were considered seronegative and used in this study. Study animals were randomized and divided into 2 groups (9 ferrets/group, 3/transmission chamber) as shown in **Table 20**. Ferrets in group 1 (Chambers A-C) were assigned to receive the M2KO(ΔTM) virus. Ferrets in group 2 (Chambers A-C) were assigned to receive the A/Brisbane/1 0/2007 (H3N2) virus. Within each group, ferrets were divided into inoculated donors or naive contacts.

Table 20: Study Design

Group	Chamber	Virus	N[1]	Inoculation (day)[2]	Donor Nasal Washes (days)	Contact Nasal Washes (days)	Serum collection
1	A	M2KO	3	0	1, 3, 5, 7, 9, 14	2, 4, 6, 8, 10, 14	14
1	B	M2KO	3	0	1, 3, 5, 7, 9, 14	2, 4, 6, 8, 10, 14	14
1	C	M2KO	3	0	1, 3, 5, 7, 9, 14	2, 4, 6, 8, 10, 14	14
2	A	Brisbane/ 10	3	0	1, 3, 5, 7, 9, 14	2, 4, 6, 8, 10, 14	14
2	B	Brisbane/ 10	3	0	1, 3, 5, 7, 9, 14	2, 4, 6, 8, 10, 14	14
2	C	Brisbane/ 10	3	0	1, 3, 5, 7, 9, 14	2, 4, 6, 8, 10, 14	14

[1]Each chamber consisted of three female ferrets: an infected donor ferret and 2 naïve contact ferrets (1 direct contact and 1 aerosol contact).
[2]Intranasally inoculated with a single dose of 316µl of $1x10^7$ $TCID_{50}$ of M2KO or $1x10^7$ $TCID_{50}$ of A/Brisbane/10/2007 (H3N2) virus.

[0261] Each group was housed in separate rooms, and individuals working with the animals followed a strict work flow pattern to prevent cross contamination between the two groups In each group, one donor ferret was inoculated intranasally with a single dose of 316 µL of $1x10^7$ $TCID_{50}$ of M2KO(ΔTM) (Group1) or $1x10^7$ $TCID_{50}$ of A/Brisbane/10/2007 (H3N2) virus (Group 2). Twenty-four hours post inoculation; each donor was placed in the same cage with 1 naive ferret (direct contact), dual housed within a wire cage. An additional ferret (aerosol contact) was placed in a separate adjacent wire cage (single housed) within the transmission chamber separated from the donor's cage by a distance of 10-12 cm. Ferret body temperatures, weights, and clinical symptoms were monitored daily for 14 days post-inoculation. Nasal washes were collected from all inoculated donor ferrets on days 1, 3, 5, 7, 9 and from all contact (direct and aerosol) ferrets on days 2, 4, 6, 8, 10 for virus titration in cells. Nasal washes were collected from all ferrets on day 14 for antibody titration. Nasal wash samples were kept at -65°C.

[0262] F. Transmission Chambers: Each transmission chamber was 2 cubic meters. A computerized air handling unit was used for HEPA filtration and to monitor and control environmental conditions within the transmission chambers. To provide directional airflow, HEPA-filtered air was supplied through an inlet port located at one end of the chamber, exited through an outlet port at the opposite end the chamber, HEPA filtered and exhausted into the room. Air exchange rate was 20 complete air changes per hour for each chamber, airflow was maintained as <0.1 m/sec. Chambers were maintained at a negative pressure of -0.15 inches of water. Ferrets were housed in wire cages with slat bottoms which were suspended over paper-lined waste pans. Ferrets were either dual housed in 32x24x14 cages or single housed in 24x24x14 wire cages which were placed inside each HEPA-filtered transmission chamber.

[0263] G. Virus Inoculation: Ferrets were inoculated with the M2KO(ΔTM) virus. A vial of frozen stock was thawed and diluted to the appropriate concentration in phosphate buffered saline solution. Ferrets were anesthetized with ketamine/xylazine and the virus dose administered intranasally in a volume of 316 μL for the M2KO(ΔTM). To confirm the inoculation titer of the M2KO(ΔTM) virus, aliquots of the dosing solutions were collected prior to dosing (pre-dose) and after dosing (post-dose). The aliquots were stored at 65°C for virus titration.

[0264] H. Challenge Virus: Influenza A virus, strain A/Brisbane/10/2007, serotype H3N2 was used to inoculate the control ferrets. The virus was stored at approximately -65°C prior to use. The dose level of challenge virus used was prepared at 1x1 07 $TCID_{50}$ in a volume of 316 μL. A quantitative viral infectivity assay, $TCID_{50}$ assay was performed at IITRI on a portion of the prepared viral challenge solution. The viral titer assay was performed according to IITRI Standard Operating Procedures.

[0265] I. Moribundity/Mortality Observations: Following challenge, all animals were observed twice daily for mortality or evidence of moribundity. Animals were observed for 14 days after vaccine inoculation and for 14 days after challenge.

[0266] J. Body Weights and Body Weight Change: Body weights were recorded within two days of receipt and at randomization. All study animals were weighed prior to inoculation, daily

for 14 days following each vaccination and assessed daily for 14 days post challenge. Prior to inoculation, ferrets were monitored for 3-5 days to measure establish baseline body temperatures. Temperature readings were recorded daily for 14 days following each vaccination and recorded daily for 14 days post challenge through a transponder (BioMedic data systems, Seaford, DE) implanted subcutaneously in each ferret. The change in temperature (in degrees Celsius) was calculated at each time point for each animal.

[0267] K. Clinical Observations: The change in temperature (in degrees Celsius) was determined daily for each ferret. Clinical signs of, inappetence, respiratory signs such as dyspnea, sneezing, coughing, and rhinorrhea and level of activity was assessed daily. A scoring system based on that described by Reuman, *et al.*, "Assessment of signs of influenza illness in the ferret model," *J. Virol*, Methods 24:27-34 (1989), was used to assess the activity level as follows: 0, alert and playful; 1, alert but playful only when stimulated; 2, alert but not playful when stimulated; and 3, neither alert nor playful when stimulated. A relative inactivity index (RII) was calculated as the mean score per group of ferrets per observation (day) over the duration of the study.

[0268] L. Survival Checks: Two survival checks were performed daily on all study animals throughout the study. Both survival checks occurred simultaneously with the clinical observations. The second check was performed later within the same day.

[0269] M. Nasal Washes: Ferrets were anesthetized with a ketamine (25 mg/kg) and xylazine (2mg/kg) mixture, and 0.5 ml of sterile PBS containing penicillin (100 U/ml), streptomycin (100 and gentamicin (50 was injected into each nostril and collected in a specimen cup when expelled by the ferret.

[0270] N. Euthanasia: Study animals were euthanized by an intravenous dose of sodium pentobarbital 150 mg/kg. Death was confirmed by absence of observable heartbeat and respiration. Necropsies were performed on all study animals.

[0271] O. Serum Collection: Pre-vaccination serum (days -3 to -5) and post inoculation serum (day 14) was collected from all ferrets. Ferrets were anesthetized with a ketamine (25 mg/kg) and xylazine (2mg/kg) mixture. A sample of blood (approximately 0.5-1.0 mL) was collected via the vena cava from each ferret and processed for serum. Blood was collected into Serum Gel Z/1.1 tubes (Sarstedt Inc. Newton, NC) and stored at room temperature for not more than 1 hour before collecting serum. Serum Gel Z/1.1 tubes were centrifuged at 10,000xg for 3 minutes and the serum collected. Individual pre-inoculation serum samples were collected and two aliquots made from each sample. One aliquot was tested prior to the initiation of the study to confirm ferrets are free of antibodies to influenza A viruses and one aliquot of the serum stored at -65°C.

[0272] P. Hemagglutination Inhibition (HI) Assay: Serum samples were treated with receptor-destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) to eliminate inhibitors of nonspecific hemagglutination. RDE was reconstituted per the manufacturer's instructions. Serum was diluted 1:3 in RDE and incubated 18-20 hours in a 37°C ± 2°C water bath. After the addition of an equal volume of 2.5% (v/v) sodium citrate, the samples were incubated in a 56± 2°C water bath for 30 ± 5 minutes. 0.85% NaCI was added to each sample to a final serum dilution of 1:10 after the RDE treatment. The diluted samples were then diluted into four two-fold dilutions (1:10 to 1:80) in duplicate in phosphate buffered saline (PBS) then incubated with 4 hemagglutinating units of A/Brisbane/10/2007 (H3N2) influenza A virus. After incubation, 0.5% avian red blood cells were added to each sample and incubated for 30 ± 5 minutes. Presence or absence of hemagglutination was then scored.

[0273] Q. Virus Titers: The concentration of infectious virus in the pre-and post-challenge virus inoculum samples was determined by $TCID_{50}$ assay in Madin-Darby Canine Kidney (MDCK) cells. Briefly, samples kept at -65°C were thawed and centrifuged to remove cellular debris. The resulting supernatant were diluted 10-fold in triplicate in 96-well microtiter plates in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) containing Pencillin/Streptomycin, 0.1% Gentamicin, 3% $NaCO_3$, 0.3% BSA fraction V (Sigma St. Louis, MO), 1% MEM vitamin solution (Sigma) and 1% L-glutamine (Mediatech, Manassas, VA, USA). After 10-fold serial dilutions were made, 1 OOflL was transferred into respective wells of

a 96-well plate which contained a monolayer of MDCK cells. Plates were incubated at 37°C ± 2°C in 5 ± 2% CO_2 70% humidity. After 48 hours, the wells were observed for cytopathogenic effect (CPE). Supernatant from each well (50 µl) was transferred to a 96 well plate and the hemagglutination (HA) activity determined and recorded. The HA activity of the supernatant was assessed by HA assay with 0.5% packed turkey red blood cells (tRBCs). $TCID_{50}$ titers were calculated using the method of Reed LJ and Muench H, "A simple method for estimating 50% endpoints," *Am. J. Hygiene* 27: 493-497 (1938).

[0274] R. Data Analysis: Body weights and body weight gains (losses) and changes in body temperature were determined for each individual animal expressed as mean and standard deviations of the mean for each test group.

RESULTS

[0275] After inoculation of donor ferrets with either the M2KO(ΔTM) virus or the A/Brisbane/1 0/2007 (H3N2) influenza A virus donor ferrets were introduced into transmission chambers containing naive contact ferrets. Ferrets were monitored daily for clinical signs of infection, nasal washes were collected to monitor viral shedding and serum collected to measure serum antibody titers. All ferrets survived inoculation with M2KO(ΔTM) virus and A/Brisbane/10/2007 (**Table 21**). No clinical signs of disease were observed in ferrets in the M2KO(ΔTM) group. Two of the three donor ferrets inoculated with A/Brisbane/10/2007 virus presented respiratory signs (sneezing) on Day 6 and 8. Direct contact ferrets in all chambers presented with sneezing on Day 8. No sneezing was observed in the aerosol contact ferrets. A reduction in activity level was not observed.

Table 21: Clinical signs in inoculated donor ferrets and contact ferrets.

Group	Treatment	N	Serum HI Titer[a]	Total number dead	Clinical signs[b]		
					Respiratory signs (observed day of onset)	Loss of Appetite	Lethargy (RI)[c]
M2KO							
Donors	M2KO	3	<10	0/3	0/3	0/3	0
Direct Contracts	M2KO	3	<10	0/3	0/3	0/3	0
Aerosol Contracts	M2KO	3	<10	0/3	0/3	0/3	0
Brisbane							
Donors	Brisbane/10	3	<10	0/3	2/3 (6,8)	0/3	0
Direct Contracts	Brisbane/10	3	<10	0/3	3/3 (8,8,8)	0/3	0
Aerosol Contracts	Brisbane/10	3	<10	0/3	0/3	0/3	0

[a]Hemagglutination inhibition (HI) antibody titers to homologous virus in ferret serum prior to virus inoculation.
[b]Clinical signs were observed for 14 days after virus inoculation. Except for lethargy, findings for clinical signs are given as no. of ferrets with sign/total no. Respiratory signs were sneezing, day of onset for each ferret in parentheses.
[c]Determined twice daily for 14 days of observation based on the scoring system and was calculated as the mean score per group of ferrets per observation (day) over the 14-day period. The relative inactivity index before inoculation was 0.

[0276] Changes in body weight and temperature after virus inoculation are shown in **Figure 26** and **Figure 27**. No significant weight loss was observed after inoculation with the M2KO(ΔTM) virus. The aerosol contacts averaged a 1% loss in weight on day; however, it is unlikely this due to exposure to virus. Body weights of ferrets in the M2KO(ΔTM) virus increased was 9% for donor ferrets and 10-11% for contact ferrets during the 14 day observation (**Figure 26A**). Body weight gain of the A/Brisbane/10/2007 was only 3% for donor ferrets and 6-8% for contact ferrets indicating a viral infection (**Figure 26B**). In the M2KO(ΔTM) group, body temperatures remained with in normal levels with the exception of Day 3 post infection (**Figure 27A**). Body temperatures were lower than normal for the aerosol contact ferrets. This was attributed to faulty or failing temperature transponders, temperatures were recorded within normal range throughout the rest of the study. Elevated body temperatures were observed on Day 2 in A/Brisbane/10/2007 donor ferrets and on Day 7 for aerosol contacts (**Figure 27B**). The concentrations of pre-and post-challenge virus dosing solution were $10^{7.50}$ $TCID_{50}$/mL and $10^{7.25}$ $TCID_{50}$/mL, respectively, indicating good stability of the challenge material throughout administration.

[0277] **Figure 50** shows viral titers in nasal washes from ferrets in a virus transmission study. The data shows that M2KO(ΔTM) virus does not transmit (no virus detected), whereas the control Brisb/10 virus is transmitted.

CONCLUSION

[0278] This example shows that ferrets inoculated with the A/Brisbane/10/2007 virus exhibited clinical signs of infection (sneezing, loss in body weight and a transient elevated body temperature), whereas ferrets inoculated with the M2KO(ΔTM) virus showed no clinical signs of disease. Therefore, inoculation of donor ferrets with the M2KO(ΔTM) did not appear to cause an infection or transmit virus via contact or via aerosol. These findings show that the M2KO(ΔTM) virus of the present technology is useful for intranasal influenza vaccines.

Example 13. M2KO(ΔTM) Virus Elicits Both Humoral and Mucosal Immune Responses in Mice.

[0279] This examples demonstrates that the M2KO(ΔTM) virus elicits both humoral and mucosal immune responses in mice. The immunogenicity of M2KO(ΔTM) was evaluated in mice and compared to the immune responses generated by other modes of vaccination. An immunogenicity study was performed containing the following groups as outline in **Table 22**: 1. M2KO(ΔTM) virus, 2. PR8 virus (10 pfu), live vaccine representative, 3. Inactivated PR8 virus (Charles River Laboratories, Wilmington, MA), 1 μg, intranasal (IN) 4. Inactivated PR8 virus, 1 μg, intramuscular (IM), or PBS only.

Table 22: Vaccine Groups in Immunogenicity Study

Immunogen	**Route of Delivery**	**Dose**	**Rationale**
M2KO(ΔTM) virus	Intranasal	$1x10^4$ pfu	Comprises M2KO(ΔTM) (SEQ ID NO:1) Mutation

PR8 virus	Intranasal	10 pfu	Represents the immune responses associated with a natural infection and/or live flu vaccine
Inactivated PR8, whole virus	Intranasal	1 μg	Demonstrates baseline response generated by killed flu virus delivered intranasally
Inactivated PR8, whole virus	Intramuscular	1 μg	Standard delivery route for traditional inactivated flu vaccine

[0280] To test the immunogenicity of M2KO(ΔTM) virus, mice were intranasally inoculated with $1.2x10^4$ pfu of M2KO(ΔTM), 10 pfu of wild-type PR8, 1 μg of inactivated whole PR8 (Charles River Laboratories, Wilmington, MA), or PBS as control, along with a group intramuscularly administered 1 μg of inactivated whole PR8. Three weeks after the immunization, serum and trachea-lung washes were collected from mice and anti-PR8 immunoglobulin G (IgG) and IgA levels were measured by enzyme linked immunosorbent assay (ELISA). Briefly, ELISA plates were coated by whole inactivated PR8, blocked by bovine serum albumin (BSA), and samples were applied. Mouse IgG and IgA antibodies were detected by horseradish peroxidase labeled anti-mouse IgG- and IgA- goat antibodies (KPL, Inc., Gaithersburg, MD) and SureBlue TMB (KPL, Inc.) substrate.

[0281] As expected, mice in the immunized groups showed significant elevation of anti-PR8 antibodies in serum and trachea-lung wash compare to the PBS only group (**Figure 28**). Anti-PR8 IgG levels in sera for M2KO(ΔTM) virus are higher than the inactivated PR8 groups and similar to live PR8 virus. More importantly anti-PR8 IgA antibodies were present only in the PR8 and M2KO(ΔTM) immunized mice in both sera and trachea-lung washes. These data suggest that M2KO(ΔTM) virus elicits significant humoral and mucosal immune response in mice.

Example 14 M2KO(ΔTM) Virus Protects Mice From Lethal Homosubtypic and Heterosubtypic Challenge.

[0282] This example demonstrates that the M2KO(ΔTM) virus protects mice from lethal homosubtypic and heterosubtypic challenge. The protective efficacy M2KO(ΔTM) virus was evaluated by challenging the immunized mice with lethal doses of the wild-type PR8 (H1N1; homosubtypic challenge) or mouse-adapted influenza A/Aichi/2/68 (Aichi; H3N2; heterosubtypic challenge) six weeks post-immunization. None of the mice immunized with either M2KO(ΔTM) or 10 pfu of PR8 and subsequently challenged by wild-type PR8 showed any clinical symptoms including weight loss (**Figure 29A**). In contrast, naive PBS mice died or were euthanized due to greater than 20% weight loss by day 5. Virus replication in the respiratory tracts of challenged mice was determined on day 3 post-challenge by $TCID_{50}$ assay in MDCK cells. As shown in **Figure 30A,** no virus was detected (limit of detection $10^{2.75}TCID_{50}$/organ) in the lungs of M2KO(ΔTM) or PR8 immunized mice indicating that M2KO(ΔTM) provided sterile immunity similar to PR8 infection. In contrast, challenge virus was recovered from the inactivated PR8 and PBS groups.

[0283] For heterosubtypic challenge, mice were challenged by Aichi (H3N2). M2KO(ΔTM) and wild-type PR8 immunized mice survived challenge whereas mice that received inactivated PR8 or PBS succumbed to infection (**Figure 29**). Virus titers in mouse respiratory tracts on day 3 post-challenge did not show significant reduction in M2KO(ΔTM) -vaccinated mice compared to mice in other groups (**Figure 30**). These results suggest that the cross-protection observed against Aichi challenge may in part be due to T-cell mediated immune responses induced by the M2KO(ΔTM) vaccine. Hemagglutination inhibition (HI) antibodies to Aichi were not detectable (less than 1:40) in post-challenge sera from challenged mice suggesting that protection was not mediated by neutralizing antibodies.

[0284] The M2KO(ΔTM) virus stimulates both humoral and cellular immune responses and confers protective immunity to animals against lethal homo- and hetero-subtypic challenge as summarized in **Table 23**.

Table 23 Protection After Homosubtypic (H1N1) and Heterosubtypic (H3N2) Influenza Challenge

Survival (%)		
Vaccine Group	PR8 (H1N1) Challenge	Aichi (H3N2) Chalenge
M2KO(ΔTM)	100%	100%
PR8	100%	100%
Inactivated PR8, IN	100%	0%
Inactivated PR8, IM	100%	20%
PBS	0%	0%

Example 15 M2KO(ΔTM) Vaccine Compared to Fluzone® and FluMist®

[0285] This example demonstrates the efficacy of the M2KO(ΔTM) virus compared to ive attenuated virus (FluMist®), Fluzone® inactivated flu vaccine. Mice were immunized with M2KO(ΔTM) virus, cold adapted live attenuated virus (FluMist®), Fluzone® inactivated flu vaccine or mock immunized by PBS. M2KO(ΔTM)-H3 virus was constructed by inserting the HA and NA coding sequences of Influenza A/Brisbane/10/2007-like, A/Uruguay/716/2007(H3N2) in to the M2KO(ΔTM) backbone (SEQ ID NO:1). FluMist®-H3, internal genes from the cold-adapted A/AA/6/60 backbone, containing the HA and NA genes of Influenza A/Brisbane/10/2007-like, A/Uruguay/716/2007(H3N2) was plaque purified from the 2009/2010 trivalent vaccine formulation. Fluzone® 2009/2010 formulation was used directly as the trivalent formulation.

[0286] Sera was obtained on days 7, 14, 21 post-immunization to compare the kinetics of antibody response by ELISA (**Figure 31**). M2KO(ΔTM)-H3 virus, a replication deficient virus, developed antibodies earlier than FluMist®-H3, a live flu virus vaccine that undergoes multi-

cycle replication in an attenuated manner. The inactivated vaccine Fluzone® had the highest antibody titers in sera as it is a concentrated presentation of antigen.

[0287] The presence of anti-HA mucosal antibody in sera, lung wash, and nasal turbinates was evaluated by ELISA. M2KO(ΔTM)-H3 and FluMist,® the two live flu vaccines, had higher IgA in the respiratory tract than the inactivated vaccine Fluzone® . **(Figure 32)**

Example 16: Comparison of Protection and Immunogenicity Elicited By Live Viruses.

[0288] Six-week-old female BALB/c mice, anesthetized with isoflurane, were infected intranasally on days 0 and 28 with 10^6 $TCID_{50}$/50µl of M2KO(ΔTM)-H3 (described above), FluMist® (2009-2010) (H3N2) IVR-147 (PR8xBrisbane/10/2007). IVR-147 is the wild-type version of the M2KO(ΔTM) virus; i.e. contains a functional M2 protein. Mock-infected control mice received 50 µl PBS instead of virus. Serum was collected weekly from all the mice and analyzed for the presence of anti-HA antibodies by ELISA. As shown in **Figure 33**, M2KO(ΔTM) virus and IVR-147 generated higher antibody levels with rapid kinetics compared to FluMist®.

[0289] Body weights of animals were monitored for 14 days after infection. Vaccinated mice did not lose any weight. On day 21 post-boost, 3 mice per group were euthanized and their trachea-lung washes, nasal washes, and sera were collected for antibody titer determinations **(Figure 34)**. M2KO(ΔTM) induced both humoral and mucosal antibodies to similar levels as FluMist® and IVR-147 in sera and respiratory tract.

[0290] Mice were intranasally challenged with $40MLD_{50}$ of A/Aichi/2/68 virus six weeks post-boost. Mice were observed for loss of body weight and survival for 14 days **(Figure 35)**. M2KO(ΔTM) protected mice from lethal Aichi challenge as indicated by less body weight loss (Panel A) and 100% survival (Panel B) in contrast to FluMist®. On day 3 post-challenge, 3 mice per group were euthanized and their lungs and nasal turbinates were collected for virus titer determinations **(Table 24)**. M2KO(ΔTM) controlled the challenge virus better than FluMist® as shown in **Table 24.**

Table 24. Challenge virus titers in respiratory tract.

	Lung (Log $TCID_{50}$/g)	Nasal Turbinate (Log $TCID_{50}$/g)
	Mean ± SD	Mean ± SD
M2KO H3	7.05 ± 0.14	4.37 ± 1.01
FluMist H3	7.32 ± 0.38	6.83 ± 1.50
IVR 147	7.08 ± 0.14	4.87 ± 0.14
PBS	7.95 ± 0.63	6.25 ± 0.29

Example 17: Generation of an M2KO(ΔTM) Vaccine Against Highly Pathogenic Avian H5n1 Influenza Virus

[0291] Summary: M2KO(ΔTM) is an influenza virus that lacks expression of a functional M2 protein. The M2 protein is crucial for initiation of influenza viral infection and for efficient viral RNA incorporation into progeny virions. M2KO(ΔTM) can enter cells and express viral proteins but cannot make infectious progeny viruses due to deletion of the M2 gene. M2KO(ΔTM) is produced in permissive M2 protein expressing cells but not in non-permissive wild-type cells. M2KO(ΔTM) elicits both mucosal and humoral immunity in mice and protects from both homo- and hetero-subtypic lethal challenge.

[0292] The H5N1 M2KO(ΔTM) virus contains the HA (avirulent) and NA genes of A/Vietnam/1203/2004 on the M2KO(ΔTM) backbone. By "M2KO(ΔTM) backbone" is meant the sequence of PR8 comprising the M2KO(ΔTM) (SEQ ID NO:1) mutation. The A/Vietnam/1203/2004 HA (avirulent) and NA sequences used are shown below.

>Avirulent VN1203 HA ORF + PR8 non-coding

AGCAAAAGCAGGGGAAAATAAAAACAACCAAAATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGT
GATCAGATTTGCATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTTAC
ACATGCCCAAGACATACTGGAAAAGAAACACAACGGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTAATTTTGAGAG
ATTGTAGCGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTG
GAGAAGGCCAATCCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTATTGAGCAG
AATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGTCATGAAGCCTCATTAGGGGTGAGCTCAG
CATGTCCATACCAGGGAAAGTCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATA
AAGAGGAGCTACAATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCGGCAGAGCA
GACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACACTAAACCAGAGATTGGTACCAAGAATAG
CTACTAGATCCAAAGTAAACGGGCAAAGTGGAAGGATGGAGTTCTTCTGGACAATTTTAAAGCCGAATGATGCAATCAAC
TTCGAGAGTAATGGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATTATGAAAAG
TGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTCTAGCATGCCATTCCACAATA
TACACCCTCTCACCATTGGGGAATGCCCCAAATATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGC
CCTCAAAGAGAGACTAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGGTTG
GTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAG
TCACCAATAAGGTCAACTCGATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA
AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTATAATGCTGAACTTCTGGTTCT
CATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGG
ATAATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATGGAAAGTGTAAGAAAT
GGAACGTATGACTACCCGCAGTATTCAGAAGAAGCGAGACTAAAAAGAGAGGAAATAAGTGGAGTAAAATTGGAATCAAT
AGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCGAGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCT
TATGGATGTGCTCCAATGGGTCGTTACAATGCAGAATTTGCATTTAAGATTAGAATTTCAGAGATATGAGGAAAAACACC
CTTGTTTCTACT

>VN1203 NA ORF + PR8 non-coding

AGCAAAAGCAGGGGTTTAAAATGAATCCAAATCAGAAGATAATAACCATCGGATCAATCTGTATGGTAACTGGAATAGTT
AGCTTAATGTTACAAATTGGGAACATGATCTCAATATGGGTCAGTCATTCAATTCACACAGGGAATCAACACCAATCTGA
ACCAATCAGCAATACTAATTTTCTTACTGAGAAAGCTGTGGCTTCAGTAAAATTAGCGGGCAATTCATCTCTTTGCCCCA
TTAACGGATGGGCTGTATACAGTAAGGACAACAGTATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAGAGCCG
TTCATCTCATGCTCCCACTTGGAATGCAGAACTTTCTTTTTGACTCAGGGAGCCTTGCTGAATGACAAGCACTCCAATGG
GACTGTCAAAGACAGAAGCCCTCACAGAACATTAATGAGTTGTCCTGTGGGTGAGGCTCCCTCCCCATATAACTCAAGGT
TTGAGTCTGTTGCTTGGTCAGCAAGTGCTTGCCATGATGGCACCAGTTGGTTGACGATTGGAATTTCTGGCCCAGACAAT
GGGGCTGTGGCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGAGTTGGAGGAACAACATACTGAGAACTCA
AGAGTCTGAATGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGACGGACCAAGTAATGGTCAGGCATCACATA
AGATCTTCAAAATGGAAAAAGGGAAAGTGGTTAAATCAGTCGAATTGGATGCTCCTAATTATCACTATGAGGAATGCTCC
TGTTATCCTAATGCCGGAGAAATCACATGTGTGTGCAGGGATAATTGGCATGGCTCAAATCGGCCATGGGTATCTTTCAA
TCAAAATTTGGAGTATCAAATAGGATATATATGCAGTGGAGTTTTCGGAGACAATCCACGCCCCAATGATGGAACAGGTA
GTTGTGGTCCGGTGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAATGGTGTCTGGATCGGG
AGAACCAAAAGCACTAATTCCAGGAGCGGCTTTGAAATGATTTGGGATCCAAATGGGTGGACTGAAACGGACAGTAGCTT
TTCAGTGAAACAAGATATCGTAGCAATAACTGATTGGTCAGGATATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAG
GACTAGATTGCATAAGACCTTGTTTCTGGGTTGAGTTGATCAGAGGGCGGCCCAAAGAGAGCACAATTTGGACTAGTGGG
AGCAGCATATCTTTTTGTGGTGTAAATAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCCGAGTTGCCATTCACCAT
TGACAAGTAGTCTGTTCAAAAAACTCCTTGTTTCTACT

[0293] Generation of H5N1 M2KO(ΔTM): The avirulent HA and NA of A/Vietnam/1203/2004 (H5N1) were chemically synthesized by GeneArt® Gene Synthesis based on the CDC sequences for each gene (CDC ID: 2004706280, Accession Numbers: EF541467 and EF541403). The sequences of the constructs were confirmed and sub-cloned into appropriate vectors to allow for the generation of seed virus using standard protocols.

[0294] M2KO(ΔTM) VN1203avHA,NA (H5N1 M2KO(ΔTM)) virus was amplified in M2CK cells (MDCK cells stably expressing the M2 protein), the supernatant clarified of cell debris and concentrated 100-fold by Centricon Plus-70 (Millipore). This virus was used as the immunogen in the mice study.

[0295] Mouse Study Design: Mice (7-8 weeks old, female BALB/c) were intranasally inoculated with H5N1 M2KO(ΔTM) (10^6 $TCID_{50}$/mouse), M2KO(ΔTM) CA07HA, NA (10^6 $TCID_{50}$/mouse) or VN1203 protein (1.5 μg) administered intramuscularly. Body weight and clinical symptoms were observed for 14 days post-inoculation. Sera was collected on days 7, 14, 21 post-inoculation. Mice were boosted on day 28 with a new prime only group initiated at the same time.

[0296] Boost immunization and 'prime only' groups: On day 28 the mice previously inoculated with H5N1 M2KO(ΔTM) were boosted with a second immunization of 10^6 pfu/mouse. At the same time the 'prime only' groups were given their first dose. Weight loss was followed for all groups following the day 28 inoculation. The mice that received a boost dose of M2KO(ΔTM) vaccine lost at most 5% of their body weight. The 'prime only' group lost up to 10% of their body weight.

Table 25. Vaccine groups in mice study

Group[1]	**Immunogen**	**Doses**	**Route of Administration**	**Challenge Virus**
1	H5N1 M2KO(ΔTM)	2	Intranasal	Challenged 5 months post-immunization with 20 MLD_{50} A/VN/1203/2004
2	H5N1 M2KO(ΔTM)	1	Intranasal	
3	H1N1pdm M2KO(ΔTM)	2	Intranasal	
4	H5 HA VN1203 protein	2	Intramuscular	
5	Naïve (OPTI-MEM™)	2	Intranasal	
6	H5N1 M2KO(ΔTM)	1	Intranasal	Challenged 4 weeks post-immunization with 20 MLD_{50} A/VN/1203/2004
7	Naïve (OPTI-MEM™)	1	Intranasal	
[1]5 mice/group for survival assessment after challenge				

[0297] H5N1 M2KO(ΔTM) elicits IgG antibody titers against HA: Sera was obtained from mice on day 7, 14, 21 post-inoculation and analyzed by ELISA for antibodies against the hemagglutinin. M2KO(ΔTM) generated at least 100 fold higher titers than H5 HA protein (**Figure 36**). Mice were boosted on day 28 and sera was obtained a week later (day 35). The

M2KO(ΔTM) titers were boosted 130 fold, whereas the HA protein only boosted 13 fold. The first week bleed at day 35 for the M2KO(ΔTM) prime only groups demonstrated high IgG titers as the first week of the prime-boost groups.

[0298] Mice were challenged with a lethal dose of Vietnam/1203/2004 virus (20 MLD_{50}). All H5N1 M2KO(ΔTM) vaccinated (prime only and prime-boost) mice survived (**Figures 54 and 55**). The high survival rate of mice challenged 5 months post-immunization suggests that the H5N1 M2KO(ΔTM) vaccine primes memory responses. Mice challenged 4 weeks post-immunization had received only one dose of vaccine, indicating that the M2KO(ΔTM) vaccine stimulates a strong immune response. H1N1pdm M2KO(ΔTM) immunized mice also survived H5N1 challenge after 5 months indicating that M2KO(ΔTM) primes cross-reactive immune responses that provide protection against heterologous challenge.

Example 18: H1N1pdm: FluMist® CA07 vs M2KO(ΔTM) CA07

[0299] The HA and NA cDNA clones of A/California/07/2009 (CA07) (H1N1pdm) were generated by standard molecular biology protocols. The sequences of the constructs were confirmed and sub-cloned into appropriate vectors to allow for the generation of seed M2KO(ΔTM) virus and M2WTCA07/PR8 virus using standard protocols. FluMist® CA07 (H1N1pdm) was plaque purified in MDCK cells from FluMist® 2011-2012 vaccine Lot# B11K1802. The A/California/07/2009 (CA07) HA and NA sequences used are shown below.

A/California/07/2009 (H1N1) HA in M2KOTMdel

AGCAAAAGCAGGGGAAAACAAAAGCAACAAAAATGAAGGCAATACTAGTAGTTCTGCTATATACATTTGCAACCGCAAAT
GCAGACACATTATGTATAGGTTATCATGCGAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGT
AACACACTCTGTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGG
GTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATT
GTGGAAACACCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCAATTGAG
CTCAGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGGCCCAATCATGACTCGAACAAAGGTGTAA
CGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCCA
AAGCTCAGCAAATCCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTAGTGC
TGACCAACAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGTCATCAAGATACAGCAAGAMGTTCAAGCCGG
AAATAGCAATAAGACCCAAAGTGAGGGATCRAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAGCCGGGAGACAAA
ATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGAGATATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTAT
CATTTCAGATACACCAGTCCACGATTGCAATACAACTTGTCAAACACCCAAGGGTGCTATAAACACCAGCCTCCCATTTC
AGAATATACATCCGATCACAATTGGAAAATGTCCAAAATATGTAAAAAGCACAAAATTGAGACTGGCCACAGGATTGAGG
AATATCCCGTCTATTCAATCTAGAGGCCTATTTGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGA
TGGATGGTACGGTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTG
ACGAGATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAATACACAGTTCACAGCAGTAGGTAAAGAGTTCAACCAC
CTGGAAAAAAGAATAGAGAATTTAAATAAAAAAGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTGTT
GGTTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTATATGAAAAGGTAAGAAGCCAGC
TAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAATTTTACCACAAATGCGATAACACGTGCATGGAAAGTGTC
AAAAATGGGACTTATGACTACCCAAAATACTCAGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGGGGTAAAGCTGGA
ATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAA
TCAGTTTCTGGATGTGCTCTAATGGGTCTCTACAGTGTAGAATATGTATTTAACATTAGGATTTCAGAAGCATGAGAAAA
AAACACCCTTGTTTCTACT

> A/California/07/2009 (H1N1) NA in M2KOTMdel

AGCAAAAGCAGGAGTTTAAAATGAATCCAAACCAAAAGATAATAACCATTGGTTCGGTCTGTATGACAATTGGAATGGCT
AACTTAATATTACAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGGGAATCAAAATCAGATTGA
AACATGCAATCAAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACT
TTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCTCTGCCCTGTTAGTGGATGGGCTATATAC
AGTAAAGACAACAGTGTAAGAATCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGCTCCCCCTT
GGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAATGACAAACATTCCAATGGAACCATTAAAGACAGGAGCC
CATATCGAACCCTAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTTGAGTCAGTCGCTTGGTCA
GCAAGTGCTTGTCATGATGGCATCAATTGGCTAACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAA
GTACAACGGCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAATGTGCATGTG
TAAATGGTTCTTGCTTTACTGTAATGACCGATGGACCAAGTAATGGACAGGCCTCATACAAGATCTTCAGAATAGAAAAG
GGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGA
AATCACATGTGTGTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACCAGAATCTGGAATATCAGA
TAGGATACATATGCAGTGGGATTTTCGGAGACAATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCT
AATGGAGCAAATGGAGTAAAAGGGTTTTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTC
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TAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCT
TGCTTCTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAGCAGCATATCCTTTTGTGG
TGTAAACAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTTACCATTGACAAGTAATTTGTTCAAA
AAACTCCTTGTTTCTACT

[0300] Mice (7-8 weeks old, female BALB/c) were intranasally inoculated with M2KO(ΔTM) CA07 (10^6 $TCID_{50}$/mouse), M2WT CA07 (10^6 $TCID_{50}$/mouse), FluMist® CA07 (10^6 $TCID_{50}$/mouse) or OPTI-MEM™ as naïve control. Body weight and clinical symptoms were observed for 14 days post-inoculation. **Figure 37** shows that M2KO(ΔTM) and FluMist® vaccinated mice did not lose weight whereas the virus that contains the WT M2 loses weight and succumbs to infection. These results demonstrate that deletion of the M2 gene attenuates the virus and that M2KO(ΔTM) is attenuated.

[0301] **Figure 38** M2KO(ΔTM), FluMist,® and M2 wild-type viral titers lungs and nasal terminates. Lungs and nasal turbinates were harvested on day 3 post-vaccination for titration of virus on cells. No virus was detected in either the lungs or the nasal turbinates in the M2KO(ΔTM) immunized mice. In contrast, FluMist® did have virus replication in both the lung and nasal turbinates although at lower levels than the wild-type virus.

[0302] **Figure 39** M2KO(ΔTM) and FluMist® titers in sera collected 7, 14, and 21 days post-inoculation and anti-HA IgG titers were determined by ELISA. M2KO(ΔTM) induced higher responses that were detected earlier than FluMist® responses. By day 21 peak antibody levels were reached by both viruses.

[0303] **Figure 40** shows the percent survival of mice challenged 12 weeks post-immunization with 40 MLD_{50} of heterologous virus, mouse-adapted influenza A/Aichi/2/1968 (H3N2). Body weight change and clinical symptoms were observed for 14 days after challenge. All the M2KO(ΔTM) (H1N1pdm HA ,NA) immunized mice were protected against the Aichi (H3N2) challenge whereas only 80% of the FluMist® (H1N1pdm HA ,NA) were protected. The surviving FluMist® mice lost close to 20% of their body weight whereas M2KO(ΔTM) mice lost ~10% of their body weight.

[0304] **Table 26** shows the virus titers in the lungs and nasal turbinates that were collected on day 3 post-challenge. M2KO(ΔTM) and FluMist® controlled challenge virus replication in the lungs and nasal turbinates to similar levels whereas naïve mice displayed virus titers that were a log higher in both the lung and the nasal turbinates.

[0305] Intracellular staining of cells in bronchoalveolar lavage (BAL). BAL was collected 3 days post-challenge and stained with surface markers for immunostaining by flow cytometry to detect CD8+CD4+, CD8+CD4-, CD8-CD4+, CD8-CD4- cell populations. Both CD4+ and CD8+ cell populations were greater in the vaccinated mice than the naïve mice indicating that M2KO(ΔTM) primed for a cellular response similar to FluMist®.. M2KO(ΔTM) vaccinated mice had greater CD8+CD4- cell population than FluMist® (49% vs 40%) (**Figure 41**).

Table 26. Virus titers in respiratory tract of mice.

	Lung (log pfu/g)	Nasal Turbinate (log pfu/g)
M2KO CA07	5.95 ± 0.59	5.61 ± 0.47
FluMist CA07	5.94 ± 0.46	3.88 ± 0.64
Naive	6.86 ± 0.06	6.52 ± 1.05

Example 19 M2KO(ΔTM) mRNA Expression Relative to FluMist® and Wild-Type Virus

[0306] In some embodiments, the M2KO(ΔTM) virus is produced in cells that stably provide M2 protein *in trans* resulting in a virus that has functional M2 protein in the viral membrane but does not encode M2 in its genome. Therefore, we hypothesize that the M2KO(ΔTM) virus behaves similar to wild-type virus in the initial infection and first round of replication in normal cells. We suggest that mRNA levels of viral antigens are similar to wild-type levels early in infection and stimulate a potent immune response sooner than attenuated replicating viral vaccines.

[0307] Human lung carcinoma (A549) cells were infected at a multiplicity of infection of 0.5 with M2KO(ΔTM), FluMist® and wild-type viruses. Unadsorbed virus was removed by washing five times with PBS. After addition of virus growth media, the infected cells were placed in the 35°C CO_2 incubator. No trypsin was added to the growth medium to ensure single-cycle

replication for all viruses. Cell monolayers were harvested and RNA extracted at 4, 9 and 22 hours post infection.

[0308] Total RNA (100ng) from control and infected A549 cells were used for quantitative RT-PCR analysis. cDNA was synthesized with oligo-dT primers and Superscript II reverse transcriptase(Invitrogen) and quantified by real-time quantitative PCR analysis using gene-specific primers for an early influenza gene, M1, and a late influenza gene, HA and cytokine IP-10 gene. Reactions were performed using SYBR Green reagent (Invitrogen, Carlsbad) according to the manufacturer's instructions. Reaction efficiency was calculated by using serial 10-fold dilutions of the housekeeping gene γ-actin and sample genes. Reactions were carried out on an ABI 7300 realtime PCR system (Applied Biosystems, Foster City, CA,USA) and the thermal profile used was Stage 1: 50°C for 30 min; Stage 2: 95°C for 15 min; Stage 3: 94°C for 15 sec, 55°C for 30 sec; and 72°C for 30 sec, repeated for 30 cycles. All quantitations (threshold cycle [CT] values) were normalized to that of the housekeeping gene to generate ΔCT, and the difference among the ΔCT value of the sample and that of the reference (wild-type sample) was calculated as -ΔΔCT. The relative level of mRNA expression was expressed as 2-ΔΔCT.

[0309] M2KO(ΔTM) virus HA mRNA expression was similar to wild-type M2 virus for H3 (**Table 27**), PR8 (**Table 28**) and H1N1pdm (**Figure 42**) at 4 hour post-infection. Cold-adapted FluMist® was less than wild-type and M2KO(ΔTM) in the early timepoints due to slower replication kinetics. When M1, an early timepoint gene, mRNA expression was tested, similar results were observed (**Table 27, Figure 42**). These results suggest that M2KO(ΔTM) generates similar levels of mRNA in the early infection cycle to produce de novo viral antigens that create a 'danger signal' similar to wild-type virus and induce a potent immune response.

Table 27. Relative mRNA expression of H3 HA genes.

4hr pi	2^-ΔΔCt (compare to IVR-147)		
	neat	1:10 dilution	1:100 dilution
IVR-147	1.0	1.0	1.0
M2KO	15.4	16.1	10.8
FluMist	2.3	2.1	1.4
Mock	0.0	0.0	N/A

22hr pi	2^-ΔΔCt (compare to IVR-147)		
	neat	1:10 dilution	1:100 dilution
IVR-147	1.0	1.0	1.0
M2KO	1.0	1.4	1.9
FluMist	1.8	3.1	1.5
Mock	0.0	0.0	N/A

Table 28. Relative mRNA expression of the PR8 HA and M1 genes.

		HA		M1	
	Sample	neat	1:10	neat	1:10
4 hr p.i.	PR8 WT	1.00	1.00	1.00	1.00
	PR8 M2KOTMdel	3.07	1.29	3.15	2.64
	Mock	0.02	0.30	0.15	0.32
9 hr p.i.	PR8 WT	1.00	1.00	1.00	1.00
	PR8 M2KOTMdel	2.05	3.27	4.04	5.11
	Mock	0.00	0.00	0.00	0.00

Example 20: Generation of M2 Vero Production Cells

[0310] The M2 gene of PR8 virus was cloned into expression vector pCMV-SC (Stratagene, La Jolla, CA) by standard molecular techniques to generate pCMV-PR8-M2. The plasmid was digested with EcoR1 to confirm the presence of the 300 bp M2 gene and the 4.5 Kb vector as shown in **Figure 43**. The sequence of the plasmid containing the M2 gene insert was confirmed as shown in **Figure 44**.

[0311] Generation of M2 Vero cells: The pCMV-PR8-M2 plasmid described earlier and containing a neomycin resistant gene, was transfected into Vero cells (ATCC CCL-81) by using the Trans IT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. Briefly, on the day before transfection, Vero cells were plated at $5x10^5$ cells/100-mm dish. On day 1, 10 μg of plasmid DNA was mixed with 20 μg of Trans IT-LT1 in 0.3 ml of OptiMEM (Invitrogen) and was incubated with these cells at 37°C in 5% CO_2 overnight. On day 2, the transfection mixture was replaced with a complete medium that is modified Eagle's medium (MEM) supplemented with 5% newborn calf serum. The medium also contained 1mg/ml of geneticin (Invitrogen), a broad spectrum antibiotic that is used to select mammalian cells expressing the neomycin protein. Resistant cells (Vero cells stably expressing M2 gene) began to grow in the selection medium, the medium was replaced with fresh selection medium and geneticin-resistant clones were isolated by limited dilution in TC-96 plates. The surface expression of the M2 protein was demonstrated by immunostaining using a M2 specific monoclonal antibody, 14C2 (Santa Cruz Biotechnology).

[0312] Infection of parental and modified M2 Vero cells with M2KO(ΔTM) virus: The ability of M2 Vero cells to serve as production cells for M2KO(ΔTM) virus was tested by infection with M2KO(ΔTM)-PR8 virus. Briefly, monolayers of M2 Vero and parent Vero cells were infected with ten-fold serial dilutions (10^{-1} to 10^{-6}) of M2KO(ΔTM)-PR8 virus using standard influenza infection procedures. The infected cells were incubated at 35°C and observed for cytopathic effect (CPE) daily. Only M2 Vero cells displayed CPE indicating virus growth. Supernatant was harvested on day 4 from the 10^{-3} well and virus titer was determined by $TCID_{50}$ assay on MDCK cells stably expressing M2 gene (M2CK). M2KO(ΔTM)-PR8 virus titer grown in M2 Vero cells was $10^{6.75}$ $TCID_{50}$/ml indicating that M2 Vero cells can serve as production cells for the manufacture of M2KO(ΔTM) vaccine.

Example 21: Intradermal Delivery of Influenza Vaccines

[0313] This example demonstrates the immunogenicity of the seasonal influenza vaccine, FluLaval (2011-2012 formulation), when administered intramuscularly (IM), intradermally (ID),

and using a subcutaneous microneedle device such as that described in published U.S. Patent Application 2011/0172609. Hairless guinea pigs were inoculated on day 0 and select groups were boosted on day 30. Sera was collected on days 0, 30 and 60 and analyzed by enzyme-linked immunosorbent assay (ELISA) for hemagglutinin-specific IgG responses.

[0314] Results are shown in Figures 51-53. The data shows qualitative absorbance of antibody levels to the three strains formulated in the seasonal influenza vaccine FluLaval: A/California/7/2009 NYMC X-181, A/Victoria/210/2009 NYMC X-187 (an A/Perth/16/2009-like virus), and B/Brisbane/60/2008. At day 30, IM and ID delivery produced identical IgG responses to all viral HA. The ID prime only groups displayed higher titers at day 60, suggesting that ID delivery induces long lasting immunity to all viral HA.

The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. A recombinant influenza virus having a mutant M gene comprising SEQ ID NO:2.

2. The recombinant influenza virus of claim 1, wherein mutation of the M gene results in failure of the virus to express the M2 protein.

3. The recombinant influenza virus of claim 1, wherein the mutant M gene does not revert to wild-type or to a non-wild-type sequence encoding a functional M2 protein for at least 10 passages in an *in vitro* host cell system, wherein the host cell is modified to produce a wild-type version of the mutant gene, thereby providing the gene product to the virus in *trans*.

4. The recombinant virus of claim 1, wherein the virus is an influenza A virus.

5. The recombinant virus of claim 1, wherein the virus is non-pathogenic in a mammal infected with the virus.

6. The recombinant virus of claim 3, wherein the *in vitro* cell system comprises Chinese Hamster Ovary cells or Vero cells.

7. A composition comprising: a recombinant influenza virus having a mutant M gene comprising SEQ ID NO:2 and an excipient.

8. The composition of claim 7, wherein mutation of the M gene results in failure of the virus to express the M2 protein.

9. The composition of claim 7, wherein the virus is an influenza A virus.

10. The composition of claim 7, wherein the composition is non-pathogenic to a mammal administered the composition.

11. The composition of claim 7, wherein the composition elicits a detectable immune response in a mammal within about three weeks after administration of the composition to the mammal.

12. A method for propagating a recombinant influenza virus, comprising: contacting a host cell with a recombinant influenza virus comprising SEQ ID NO:2; and incubating the host cell for a sufficient time and under conditions suitable for viral replication, wherein the host cell is modified to produce a wild-type version of the influenza M gene, thereby providing the gene product to the virus in *trans*.

13. The method of claim 12, further comprising isolating progeny virus particles.

14. The method of claim 13, further comprising formulating the virus particles into a vaccine.

15. The method of claim 12, wherein the virus fails to express the M2 protein.

16. The method of claim 12, wherein the virus is an influenza A virus.

17. The method of claim 12, wherein the virus is non-pathogenic to a mammal administered the virus.

18. The method of claim 12, wherein the virus elicits a detectable immune response in a mammal within about three weeks after administration of a composition comprising the virus to the mammal.

19. The method of claim 12, wherein the mutant M gene does not revert to wild-type or to a non-wild-type sequence encoding a functional M2 protein for at least 10 passages of the host cell.

20. The method of claim 12, wherein the host cell is a Chinese Hamster Ovary cell or a Vero cell.

FIGURE 1

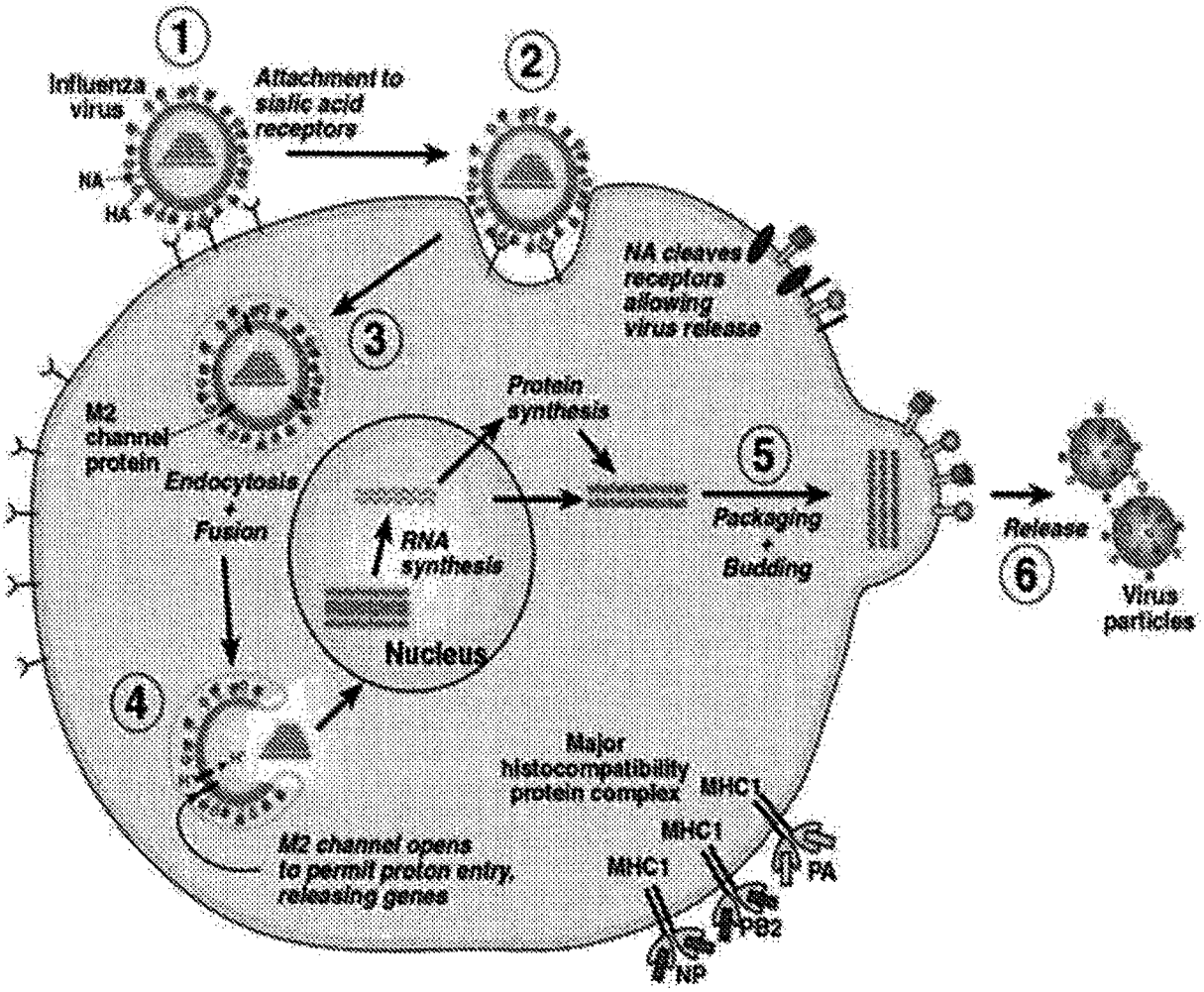

1
Influenza virus
NA
HA
Attachment to sialic acid receptors
2
3
M2 channel protein
Endocytosis + Fusion
NA cleaves receptors allowing virus release
Protein synthesis
RNA synthesis
Nucleus
5
Packaging + Budding
Release
6
Virus particles
4
M2 channel opens to permit proton entry, releasing genes
Major histocompatibility protein complex
MHC1
MHC1
MHC1
PA
PB2
NP

FIGURE 2

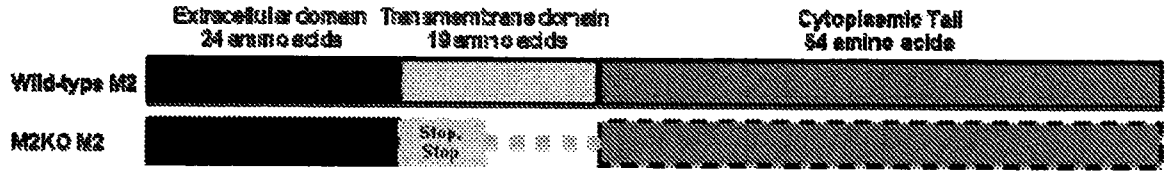

Extracellular domain
24 amino acids
Transmembrane domain
19 amino acids
Cytoplasmic Tail
54 amino acids
Wild-type M2
M2KO M2
Stop
Stop

FIGURE 3

"wild-type" M1/M2 nucleic acid sequence (3' to 5')

AGCAAAAGCAGGTAGATATTGAAAGatgagtcttctaaccgaggtcgaaacGTACGTACTCTCTATC
ATCCCGTCAGGCCCCCTCAAAGCCGAGATCGCACAGAGACTTGAAGATGTCTTTGCA
GGGAAGAACACCGATCTTGAGGTTCTCATGGAATGGCTAAAGACAAGACCAATCCT
GTCACCTCTGACTAAGGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGTGA
GCGAGGACTGCAGCGTAGACGCTTTGTCCAAAATGCCCTTAATGGGAACGGGGATC
CAAATAACATGGACAAAGCAGTTAAACTGTATAGGAAGCTCAAGAGGGAGATAACA
TTCCATGGGGCCAAAGAAATCTCACTCAGTTATTCTGCTGGTGCACTTGCCAGTTGT
ATGGGCCTCATATACAACAGGATGGGGGCTGTGACCACTGAAGTGGCATTTGGCCT
GGTATGTGCAACCTGTGAACAGATTGCTGACTCCCAGCATCGGTCTCATAGGCAAAT
GGTGACAACAACCAATCCACTAATCAGACATGAGAACAGAATGGTTTTAGCCAGCA
CTACAGCTAAGGCTATGGAGCAAATGGCTGGATCGAGTGAGCAAGCAGCAGAGGCC
ATGGAGGTTGCTAGTCAGGCTAGACAAATGGTGCAAGCGATGAGAACCATTGGGAC
TCATCCTAGCTCCAGTGCTGGTCTGAAAAATGATCTTCTTGAAAATTTGCAGgcctatcag
aaacgaatgggggtgcagatgcaacggttcaagtgatcctctcactattgccgcaaatatcattgggatcttgcacttgacattgtggattcttg
atcgtctttttttcaaatgcatttaccgtcgctttaaatacggactgaaaggagggccttctacggaaggagtgccaaagtctatgagggaaga
atatcgaaaggaacagcagagtgctgtggatgctgacgatggtcattttgtcagcatagagctggagtaaAAAACTACCTTGTT
TCTACT

FIGURE 4

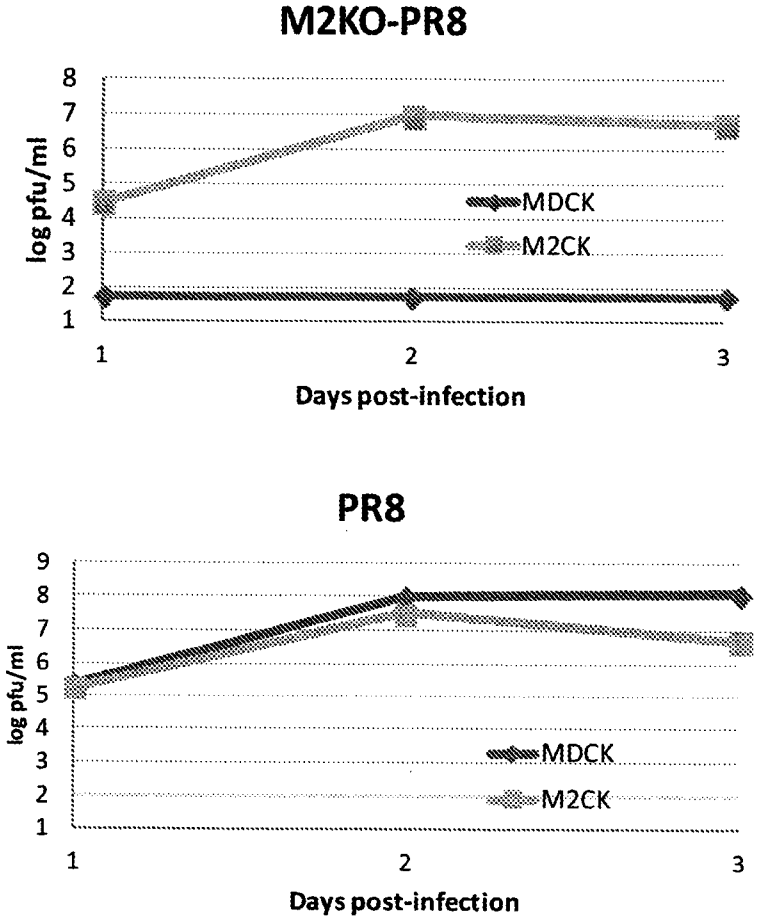
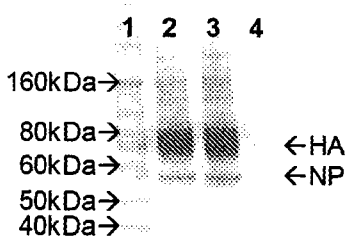

M2KO-PR8
log pfu/ml
8
7
6
5
4
3
2
1
MDCK
M2CK
1
2
3
Days post-infection
PR8
log pfu/ml
9
8
7
6
5
4
3
2
1
MDCK
M2CK
1
2
3
Days post-infection

FIGURE 5

A. Anti-PR8 polyclonal sera



B. Anti-M2 monoclonal antibody

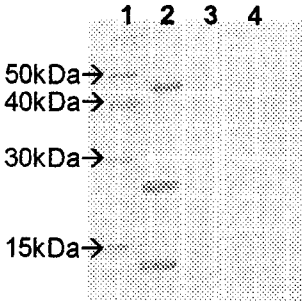


FIGURE 6

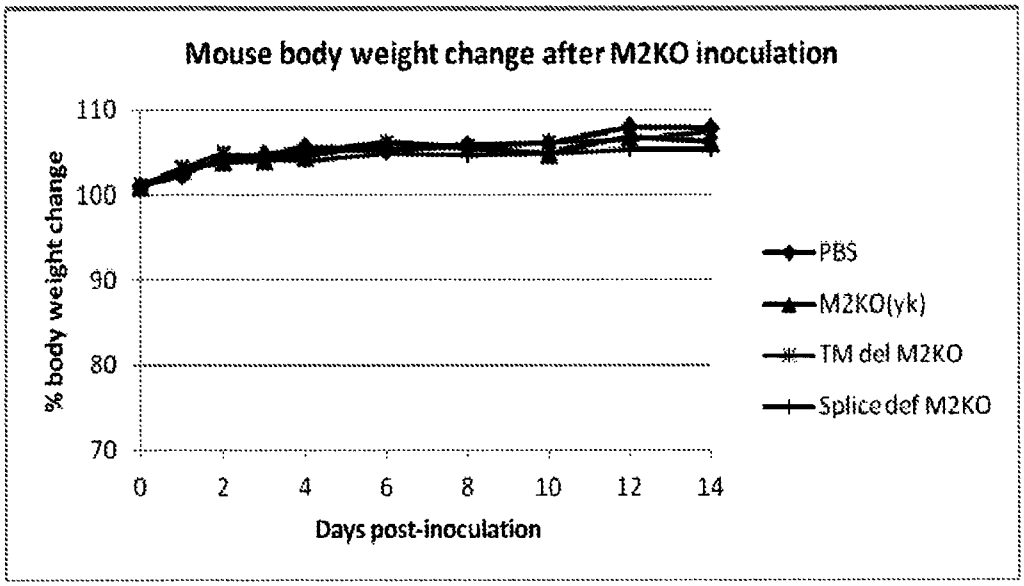
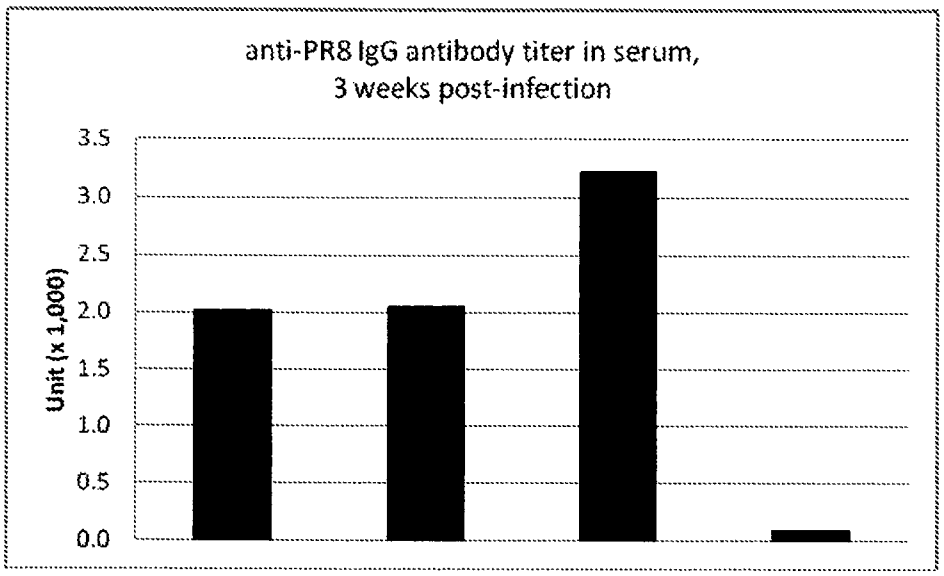

Mouse body weight change after M2KO inoculation
% body weight change
110
100
90
80
70
0
2
4
6
8
10
12
14
Days post-inoculation
PBS
M2KO(yk)
TM del M2KO
Splice def M2KO

FIGURE 7A



M2KO TM del Splice def PBS

FIGURE 7B

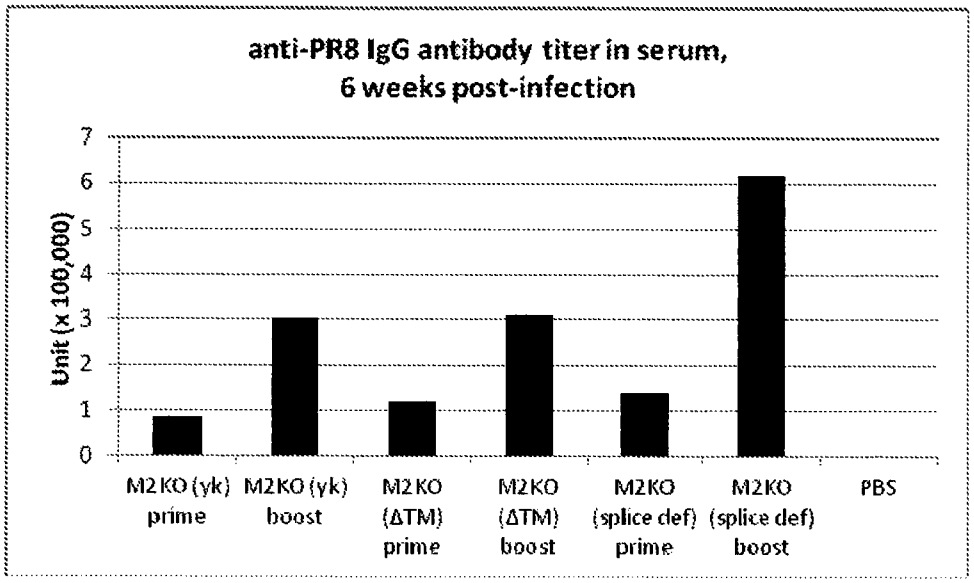


FIGURE 8

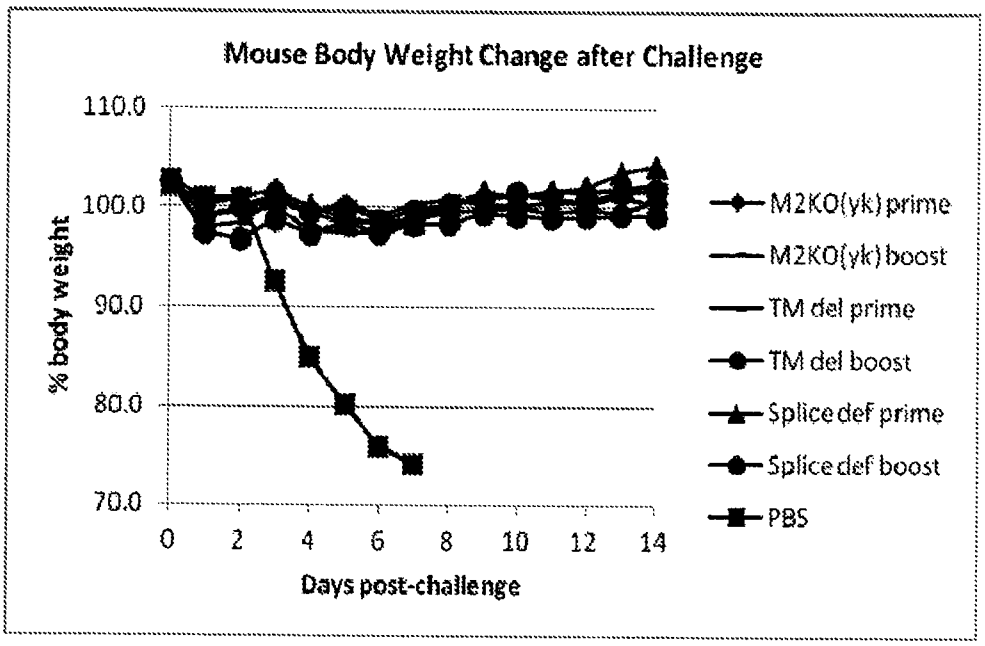
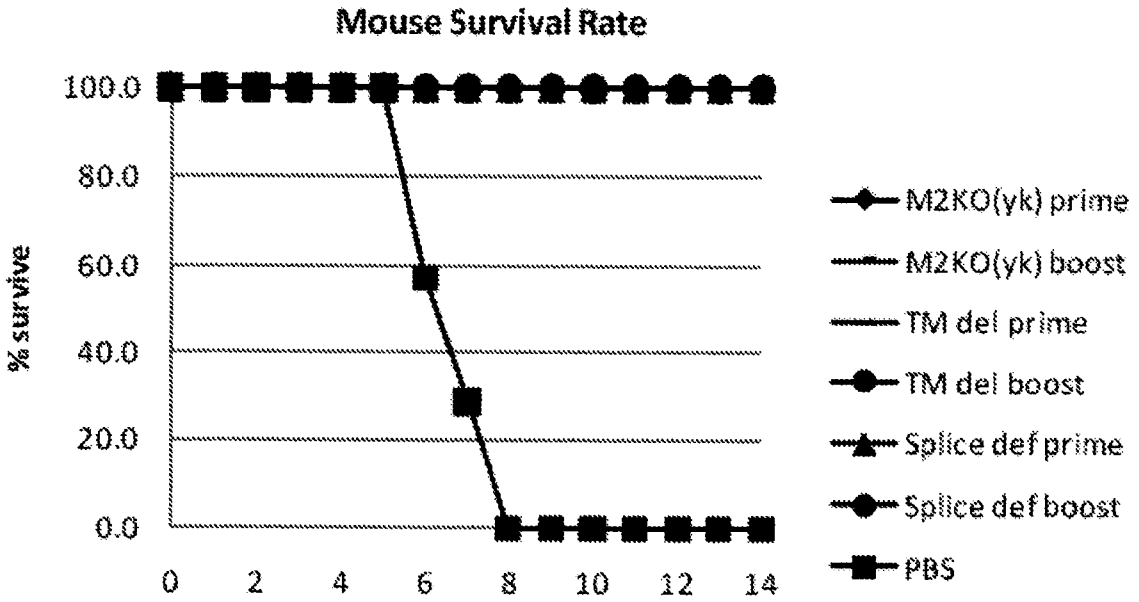

Mouse Body Weight Change after Challenge
110.0
100.0
90.0
80.0
70.0
% body weight
0
2
4
6
8
10
12
14
Days post-challenge
M2KO(yk) prime
M2KO(yk) boost
TM del prime
TM del boost
Splice def prime
Splice def boost
PBS

FIGURE 9



Days post-challenge

FIGURE 10

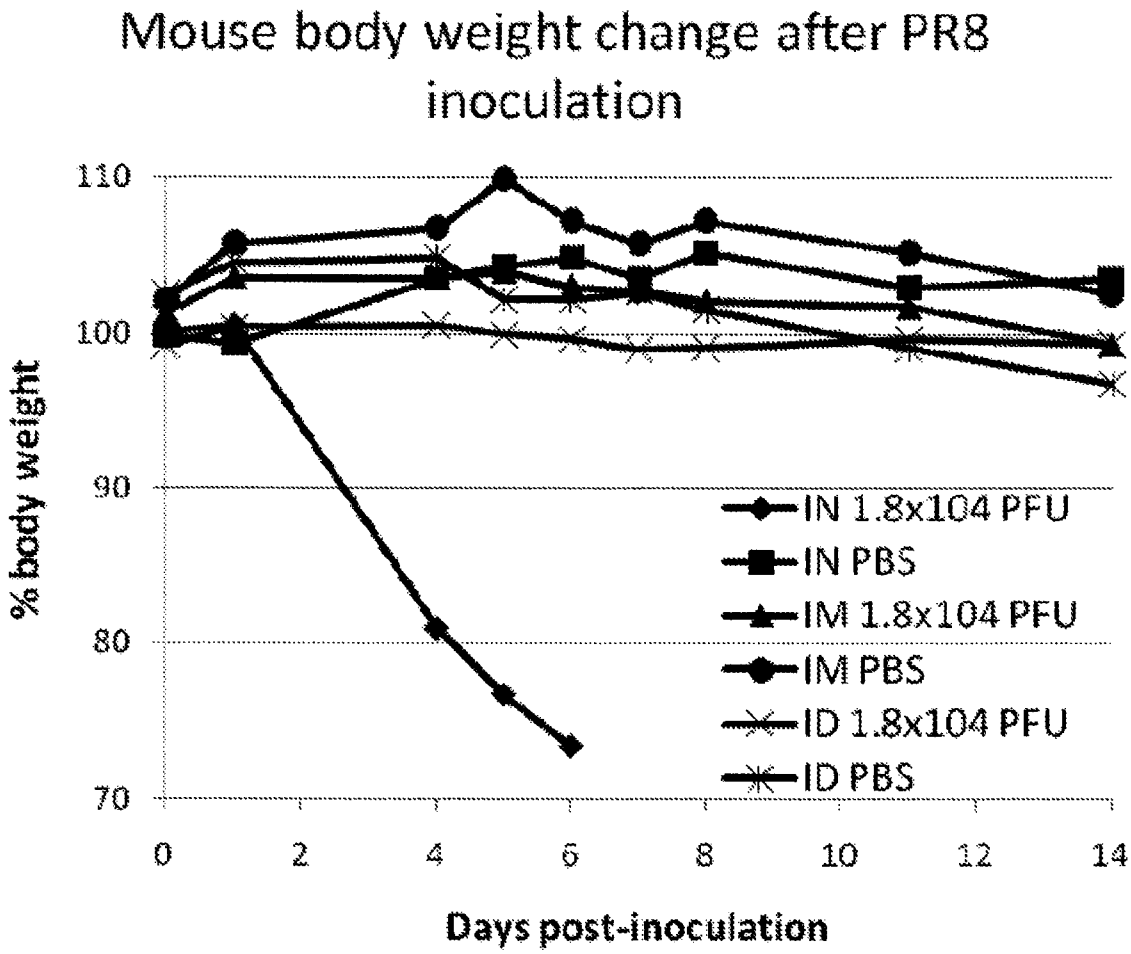

Mouse body weight change after PR8 inoculation
% body weight
110
100
90
80
70
0
2
4
6
8
10
12
14
Days post-inoculation
IN 1.8x104 PFU
IN PBS
IM 1.8x104 PFU
IM PBS
ID 1.8x104 PFU
ID PBS

FIGURE 11A

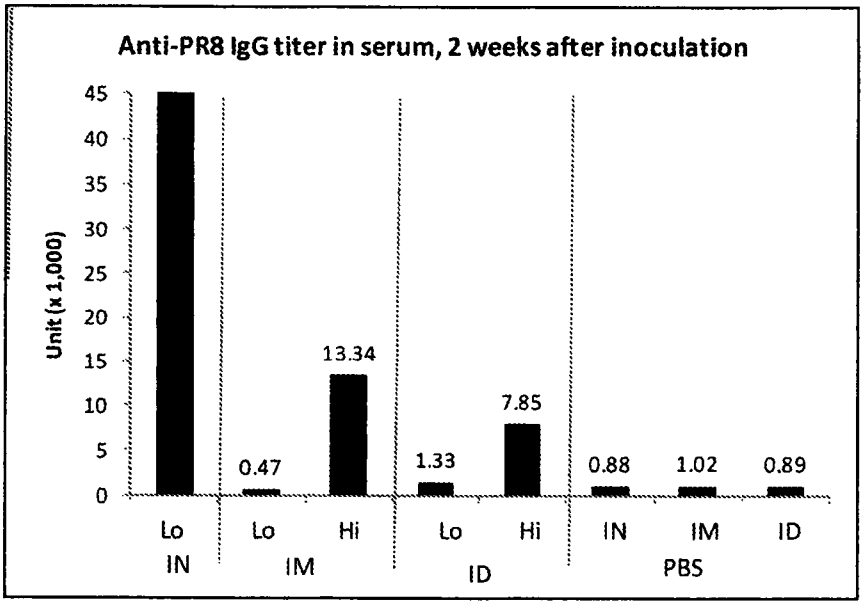


FIGURE 11B

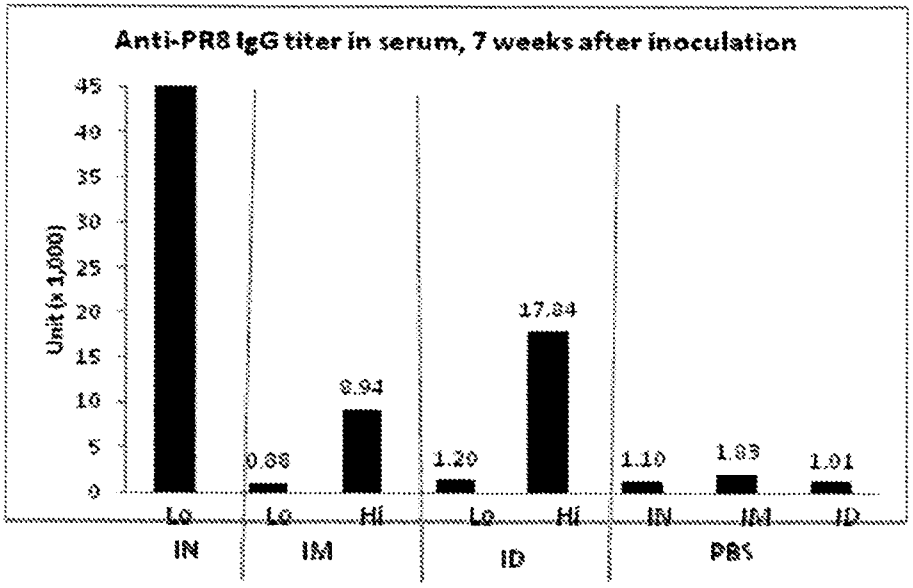


FIGURE 12

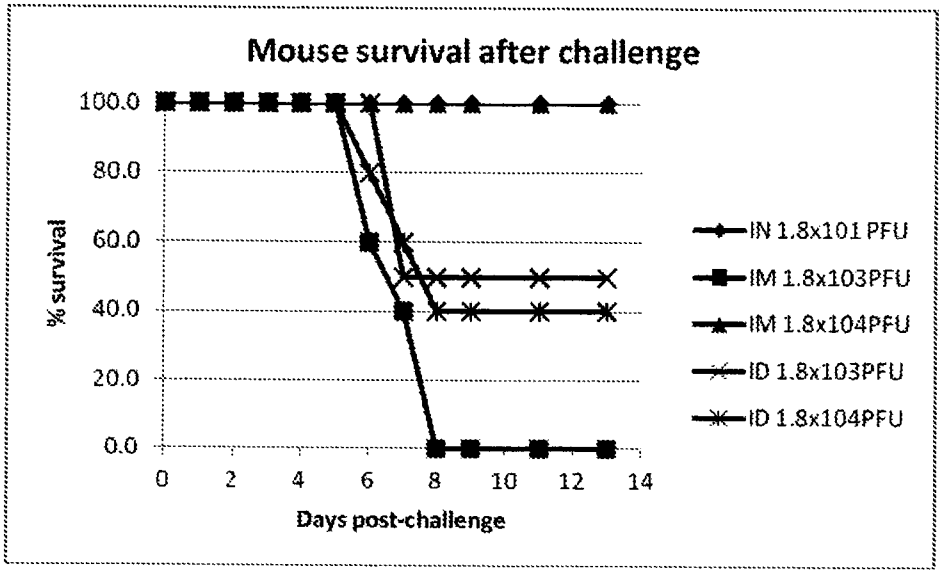

Mouse survival after challenge
100.0
80.0
60.0
40.0
20.0
0.0
% survival
0
2
4
6
8
10
12
14
Days post-challenge
IN 1.8x101 PFU
IM 1.8x103PFU
IM 1.8x104PFU
ID 1.8x103PFU
ID 1.8x104PFU

FIGURE 13

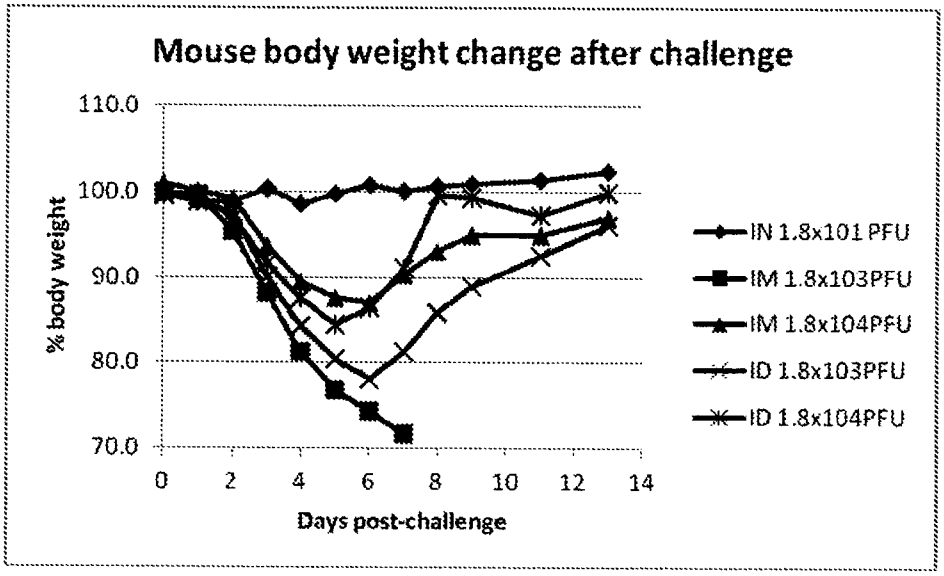

Mouse body weight change after challenge
110.0
100.0
90.0
80.0
70.0
% body weight
0
2
4
6
8
10
12
14
Days post-challenge
IN 1.8x101 PFU
IM 1.8x103PFU
IM 1.8x104PFU
ID 1.8x103PFU
ID 1.8x104PFU

FIGURE 14

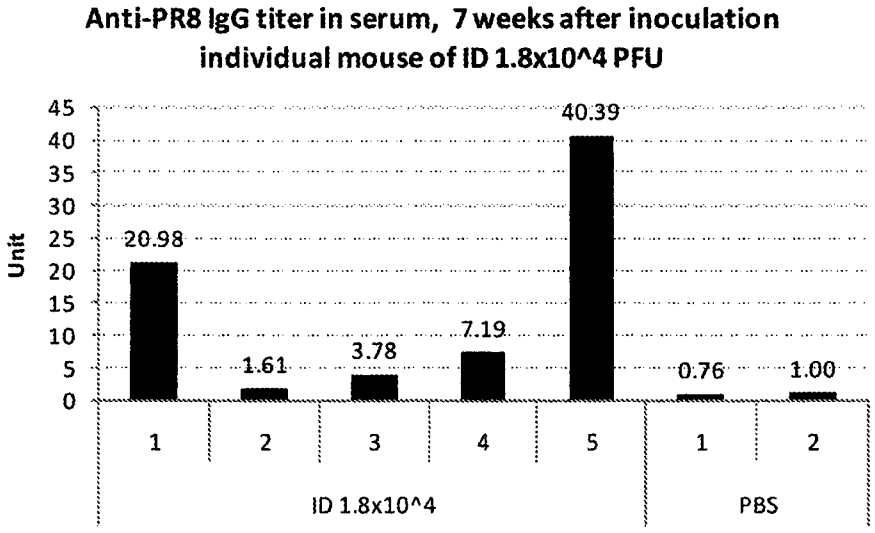

Anti-PR8 IgG titer in serum, 7 weeks after inoculation
individual mouse of ID 1.8x10^4 PFU
Unit
45
40
35
30
25
20
15
10
5
0
20.98
1.61
3.78
7.19
40.39
0.76
1.00
1
2
3
4
5
1
2
ID 1.8x10^4
PBS

FIGURE 15

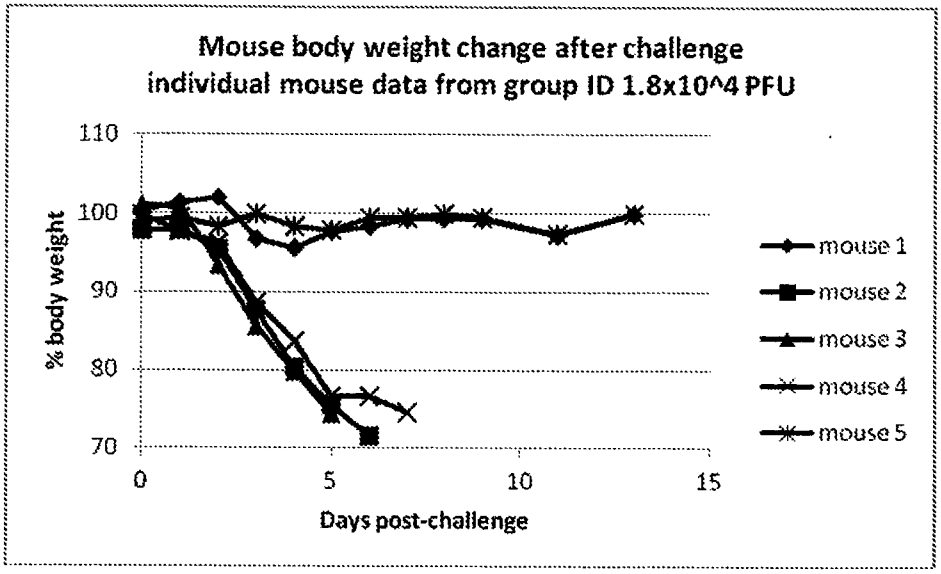

Mouse body weight change after challenge
individual mouse data from group ID 1.8x10^4 PFU
% body weight
110
100
90
80
70
0
5
10
15
Days post-challenge
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5

FIGURE 16

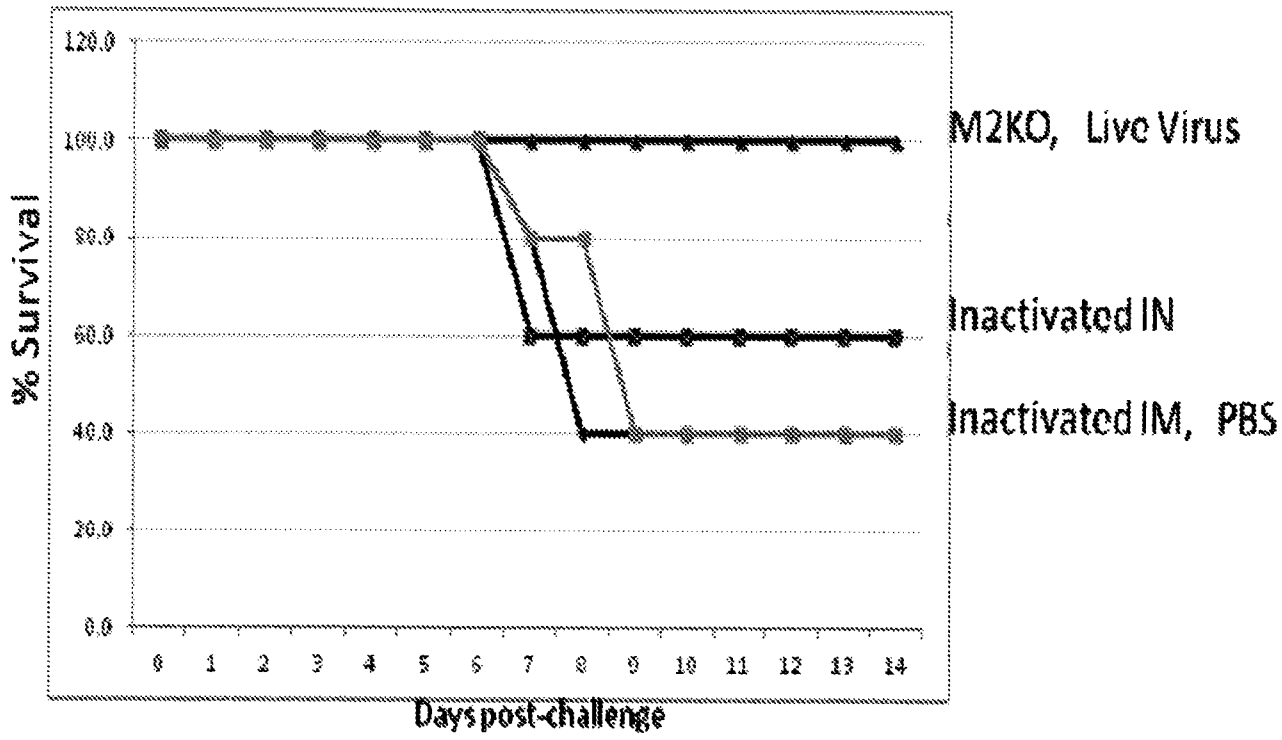

120.0
100.0
80.0
60.0
40.0
20.0
0.0
% Survival
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-challenge
M2KO, Live Virus
Inactivated IN
Inactivated IM, PBS

FIGURE 17

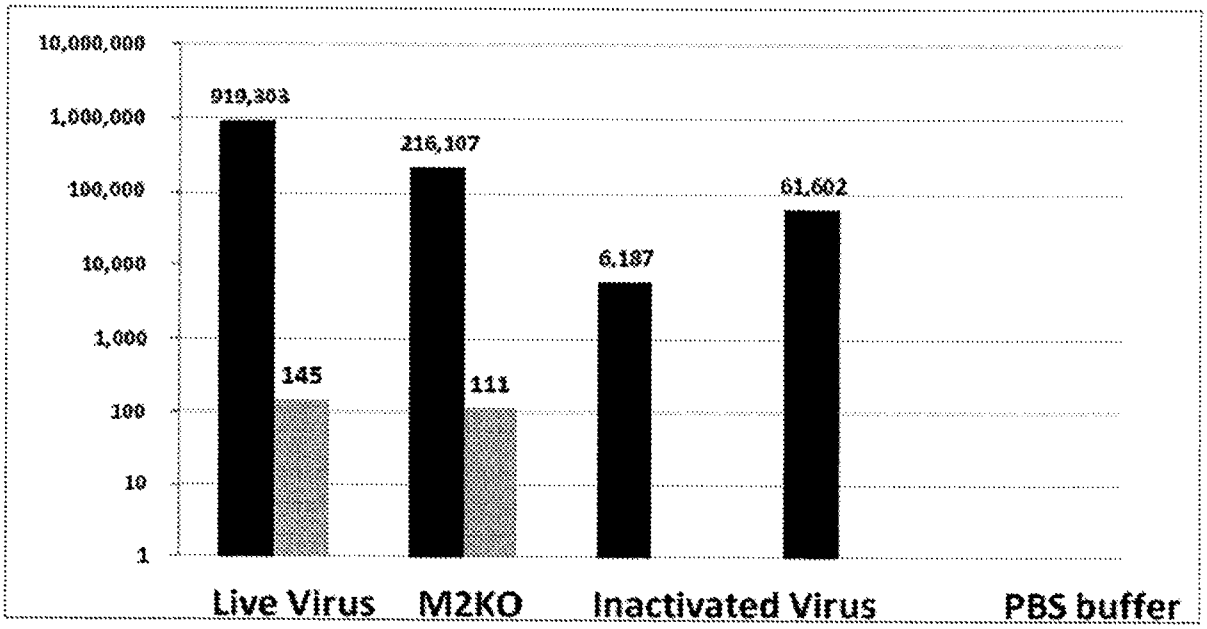

10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
919,303
145
216,107
111
6,187
61,602
Live Virus
M2KO
Inactivated Virus
PBS buffer
IgG
IgA

FIGURE 18

PR8 H1N1 challenge

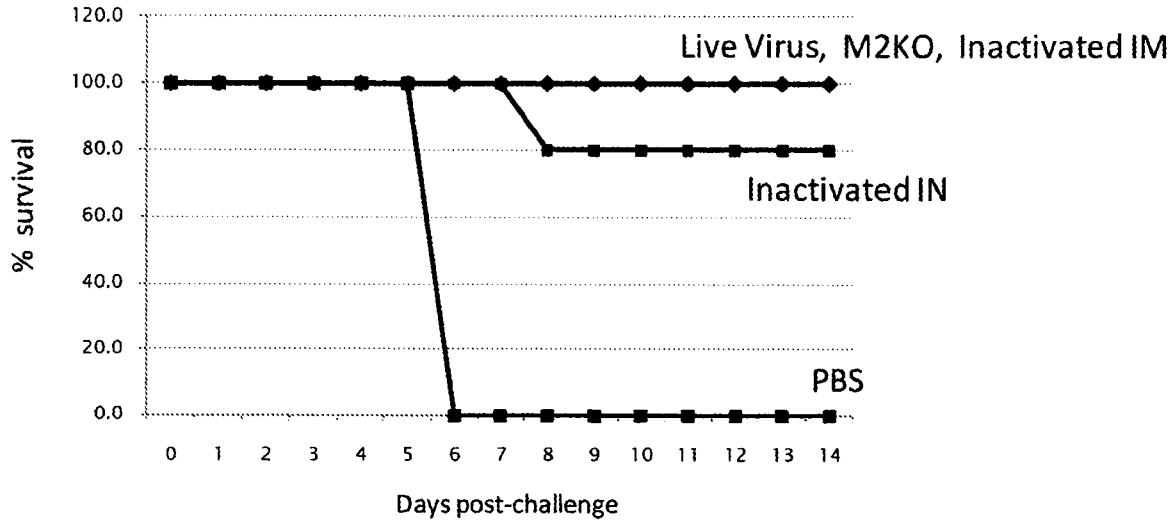


FIGURE 19

Aichi (H3N2) challenge

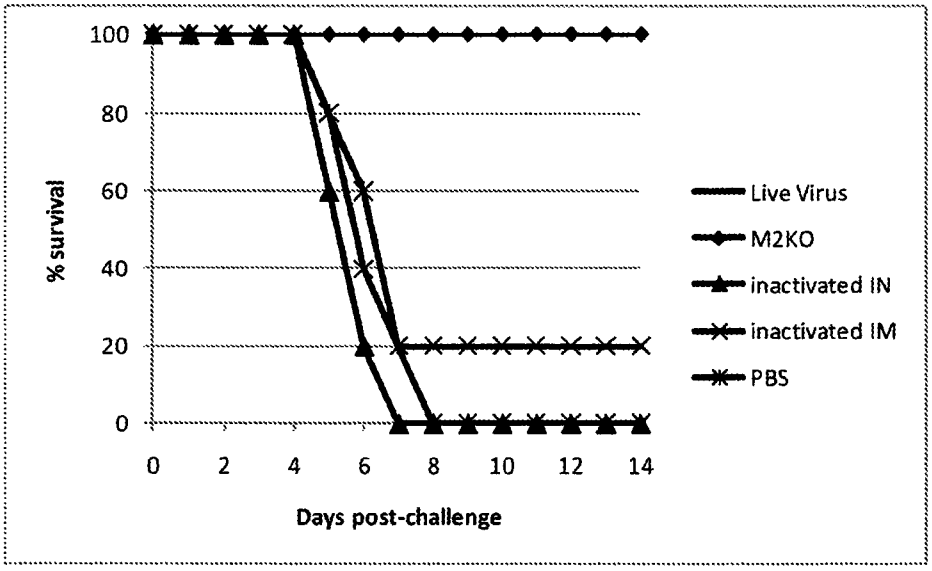
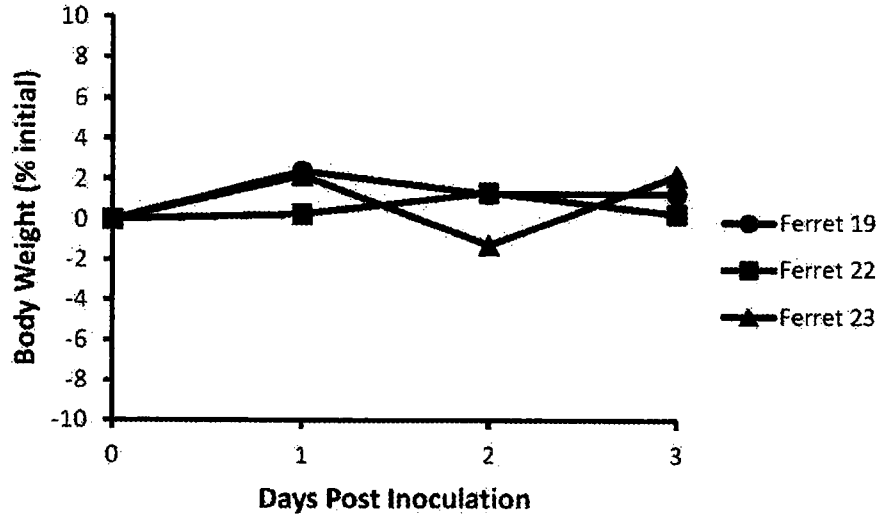


FIGURE 20

A.


Body Weight (% initial)
10
8
6
4
2
0
-2
-4
-6
-8
-10
0
1
2
3
Days Post Inoculation
Ferret 19
Ferret 22
Ferret 23

B.

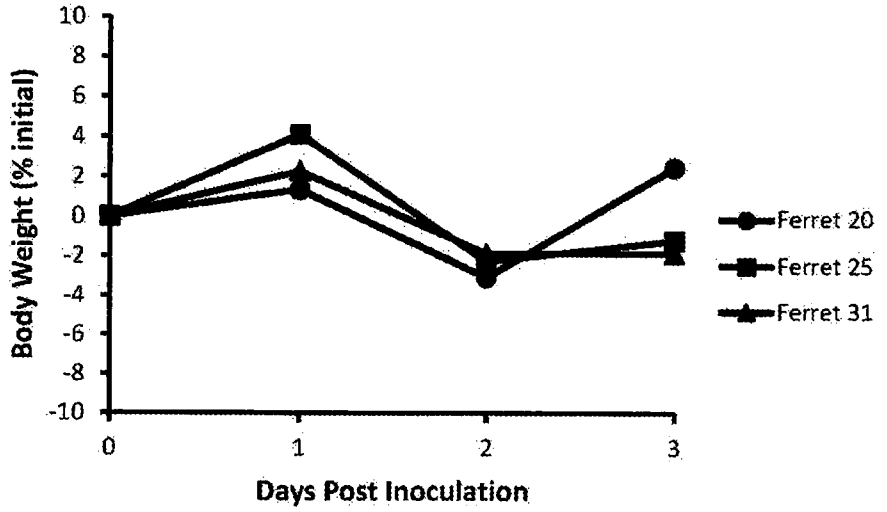
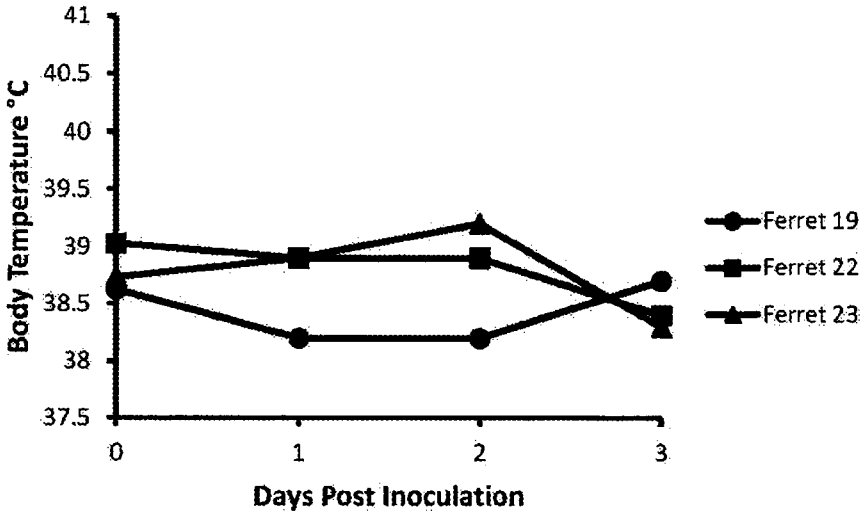

Body Weight (% initial)
10
8
6
4
2
0
-2
-4
-6
-8
-10
0
1
2
3
Days Post Inoculation
Ferret 20
Ferret 25
Ferret 31

FIGURE 21

A.


41
40.5
40
39.5
39
38.5
38
37.5
Body Temperature °C
0
1
2
3
Days Post Inoculation
Ferret 19
Ferret 22
Ferret 23

B.

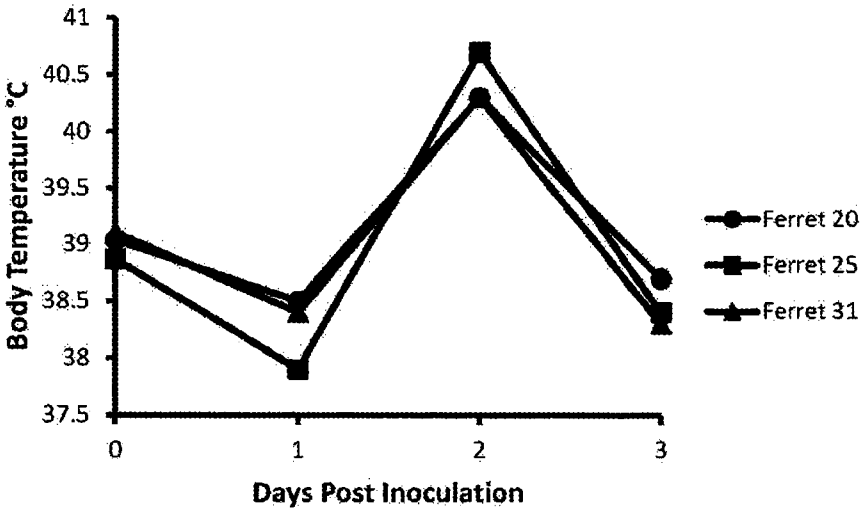
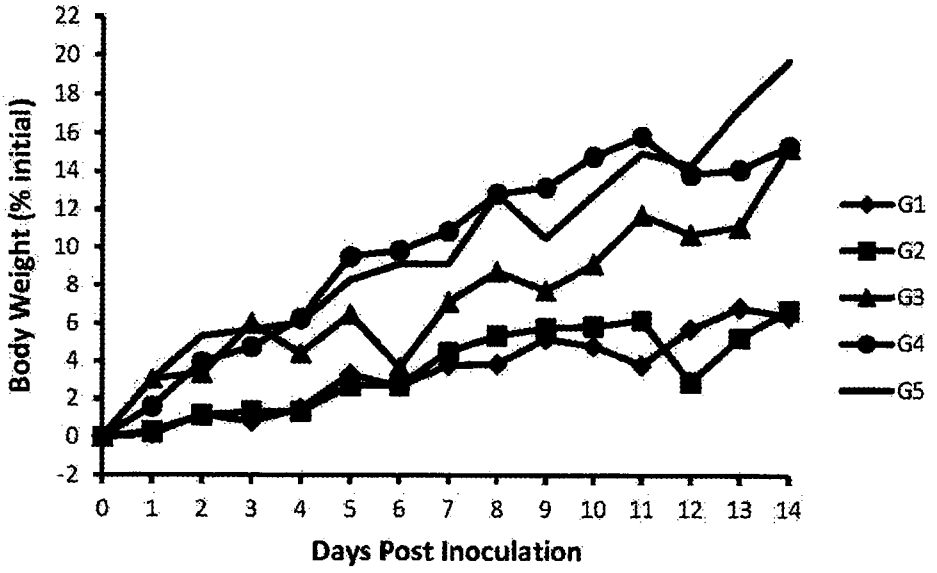

41
40.5
40
39.5
39
38.5
38
37.5
Body Temperature °C
0
1
2
3
Days Post Inoculation
Ferret 20
Ferret 25
Ferret 31

FIGURE 22

A.


Body Weight (% initial)
22
20
18
16
14
12
10
8
6
4
2
0
-2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
G1
G2
G3
G4
G5

B.

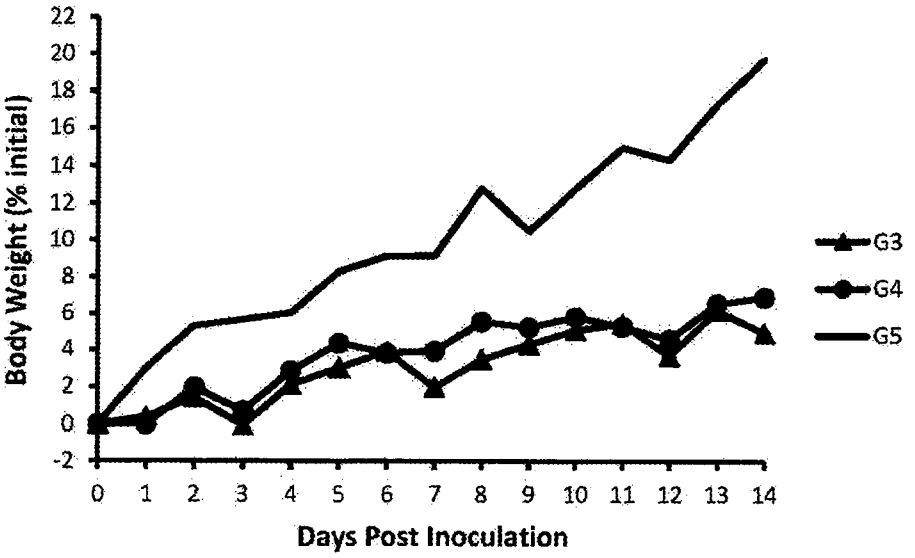

Body Weight (% initial)
22
20
18
16
14
12
10
8
6
4
2
0
-2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
G3
G4
G5

FIGURE 23

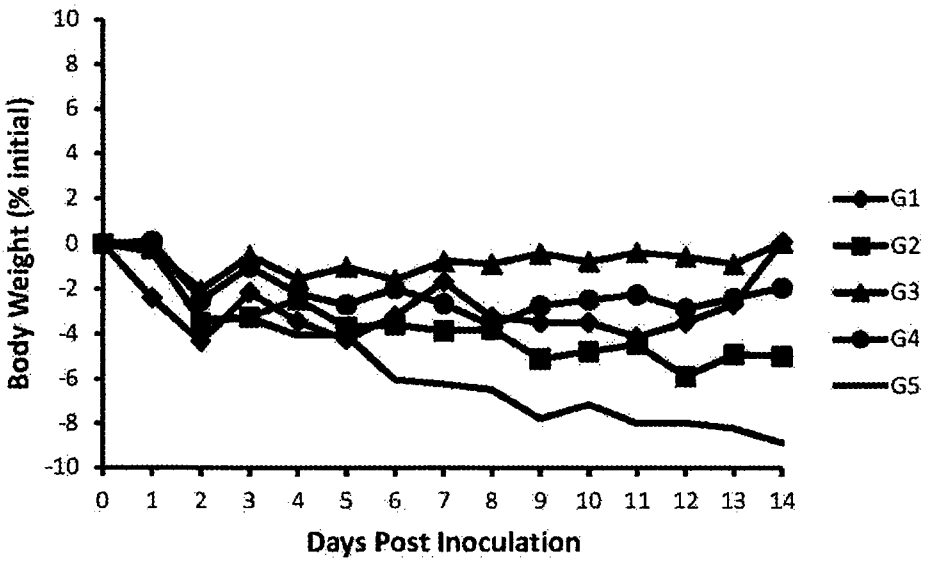
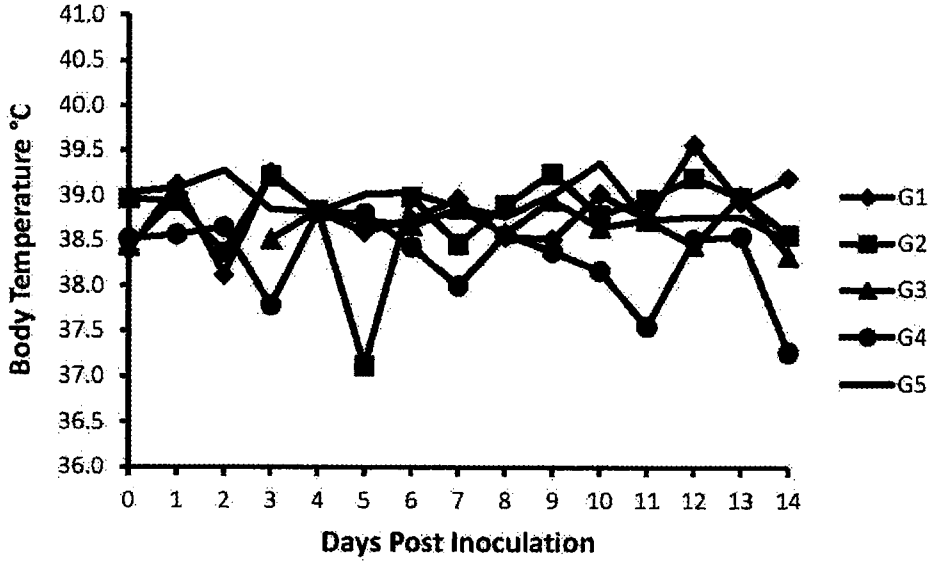

Body Weight (% initial)
10
8
6
4
2
0
-2
-4
-6
-8
-10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
G1
G2
G3
G4
G5

FIGURE 24

A.


41.0
40.5
40.0
39.5
39.0
38.5
38.0
37.5
37.0
36.5
36.0
Body Temperature °C
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
G1
G2
G3
G4
G5

B.

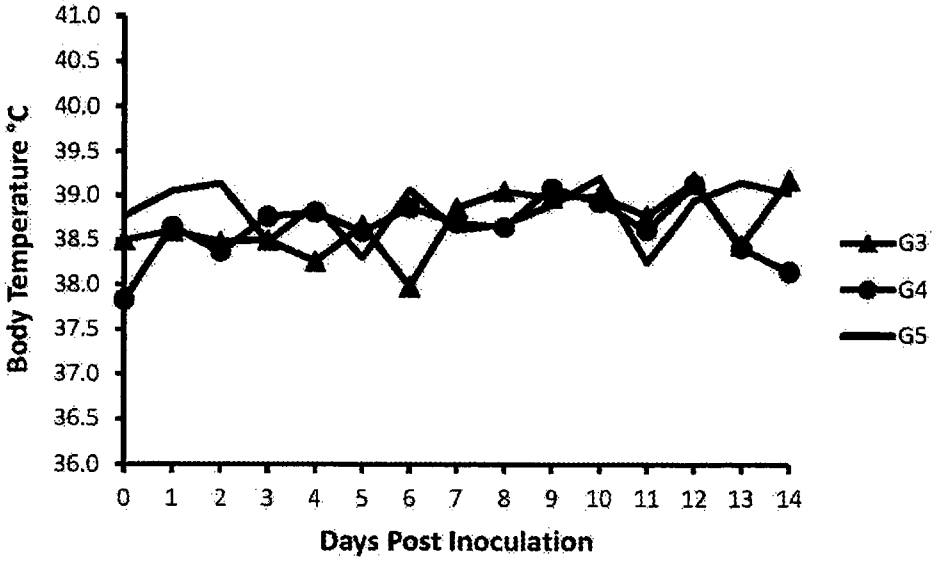

41.0
40.5
40.0
39.5
39.0
38.5
38.0
37.5
37.0
36.5
36.0
Body Temperature °C
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
G3
G4
G5

FIGURE 25

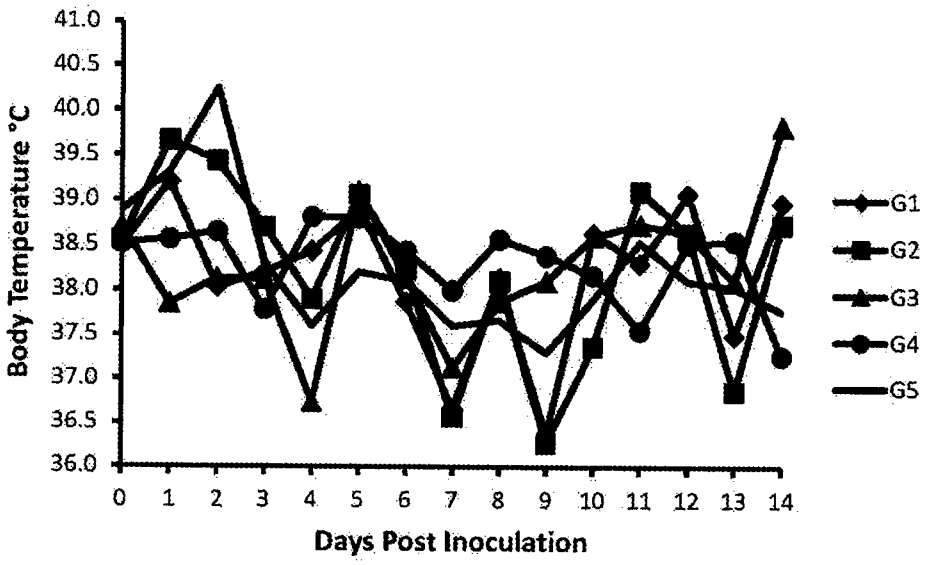
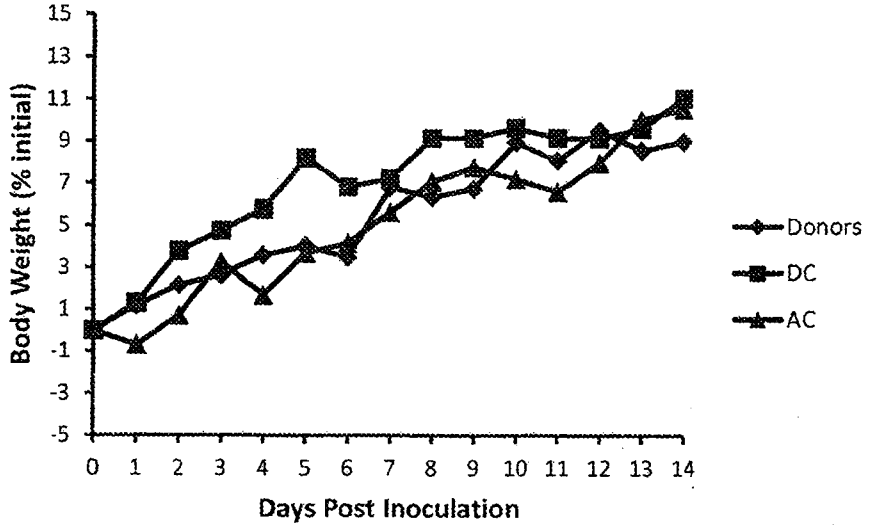

Body Temperature °C
41.0
40.5
40.0
39.5
39.0
38.5
38.0
37.5
37.0
36.5
36.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
G1
G2
G3
G4
G5

FIGURE 26

A.


Body Weight (% initial)
15
13
11
9
7
5
3
1
-1
-3
-5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
Donors
DC
AC

B.

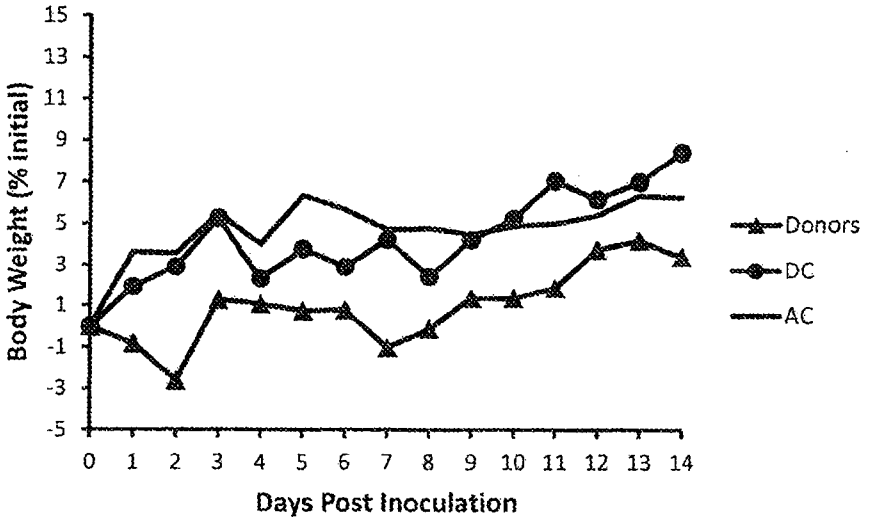
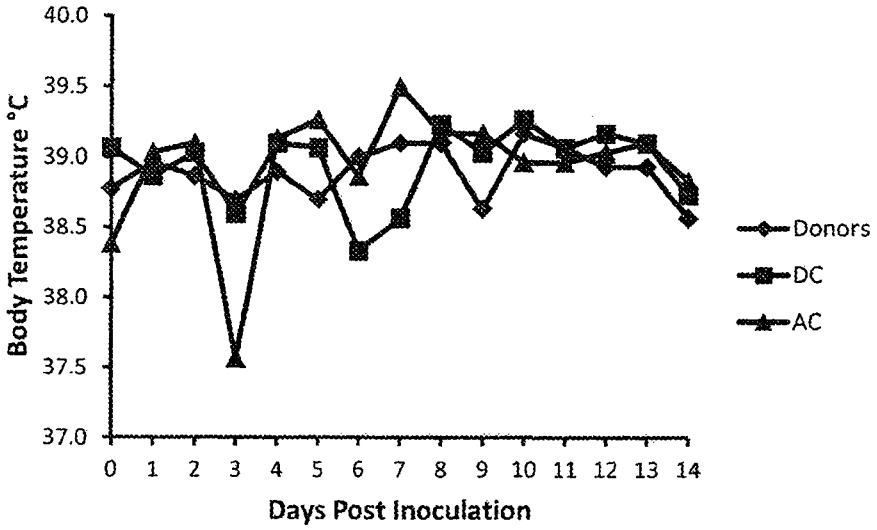

Body Weight (% initial)
15
13
11
9
7
5
3
1
-1
-3
-5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
Donors
DC
AC

FIGURE 27

A.


40.0
39.5
39.0
38.5
38.0
37.5
37.0
Body Temperature °C
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
Donors
DC
AC

B.

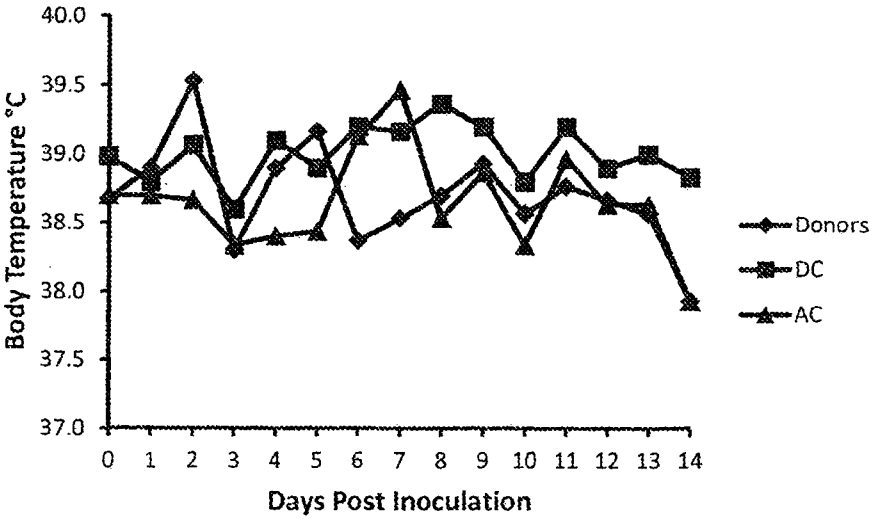
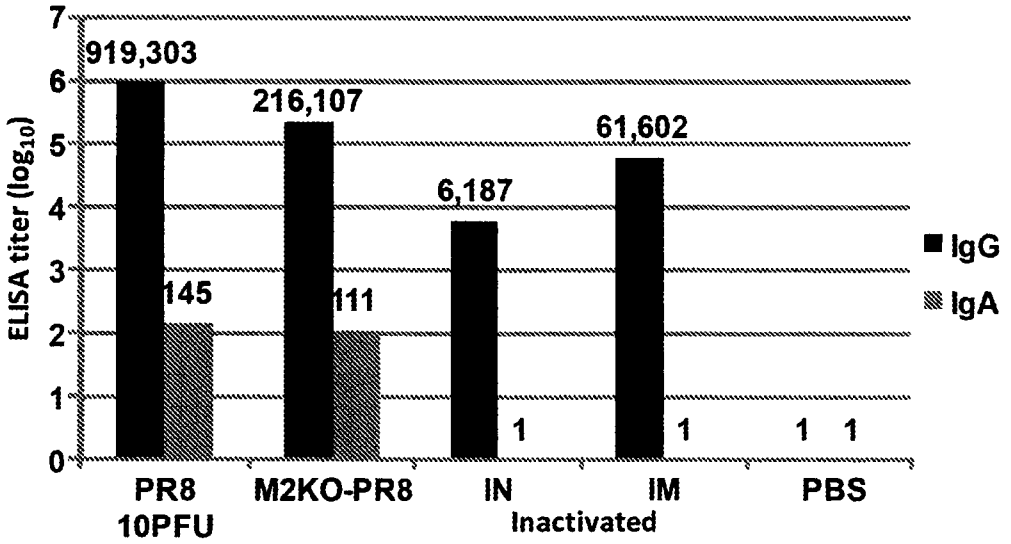

40.0
39.5
39.0
38.5
38.0
37.5
37.0
Body Temperature °C
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Inoculation
Donors
DC
AC

FIGURE 28

A. Sera



B. Lung Wash

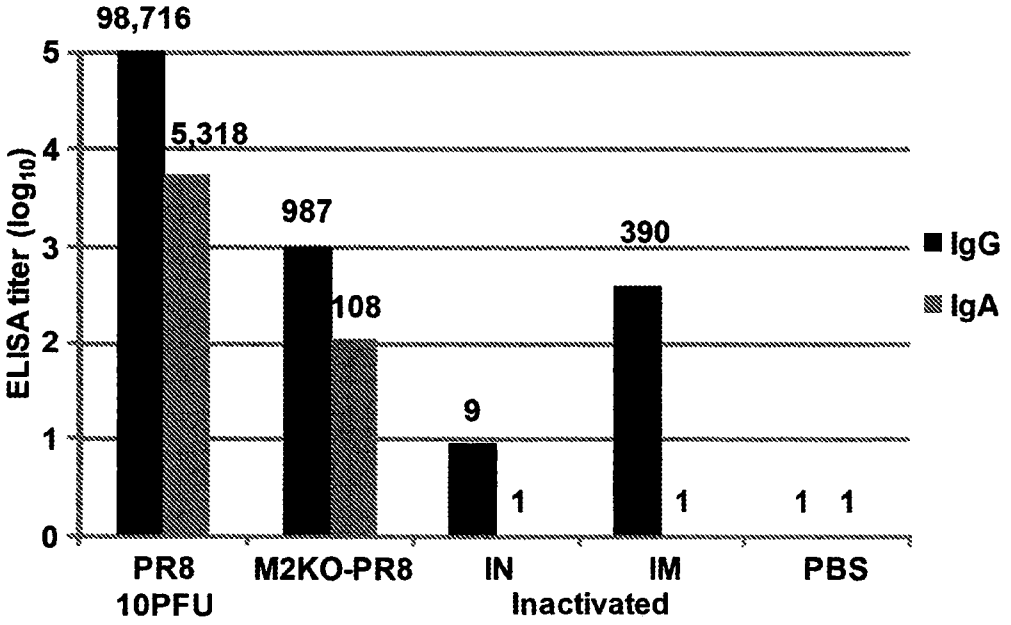
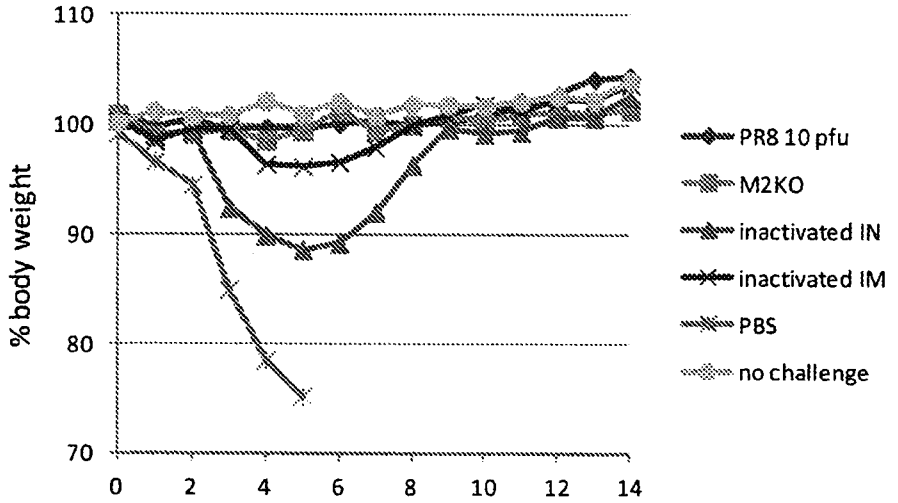


FIGURE 29

A. Mouse body weight change after homologous PR8 (H1N1) challenge.



B. Mouse survival after heterologous Aichi (H3N2) challenge.

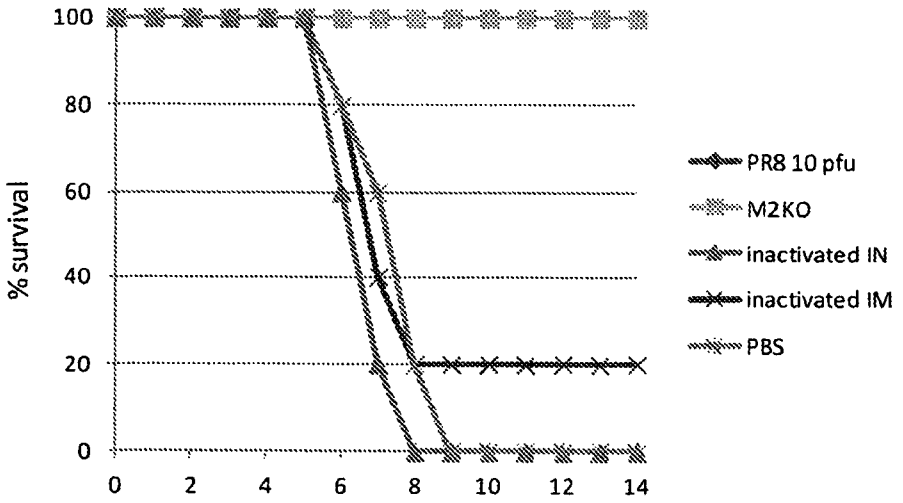
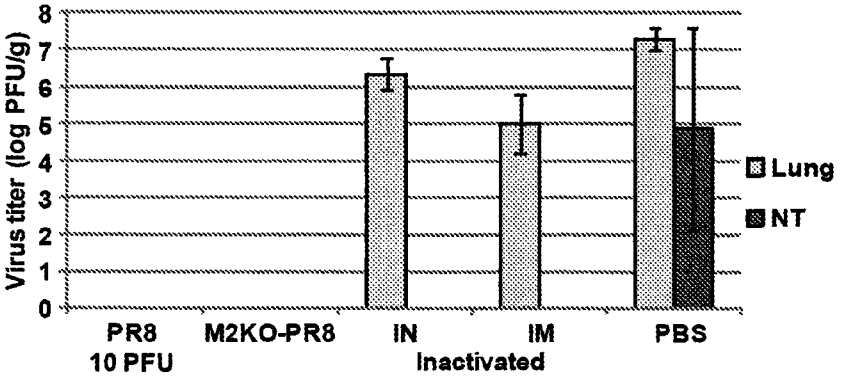
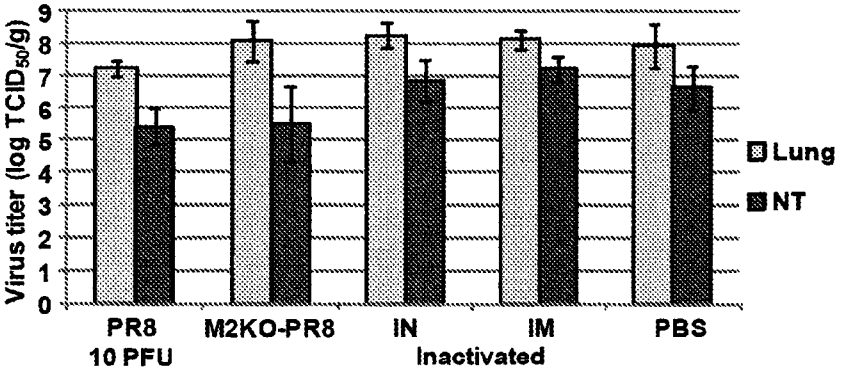


FIGURE 30

A. Virus titers after PR8 (H1N1) challenge



B. Virus titers after Aichi (H3N2) challenge



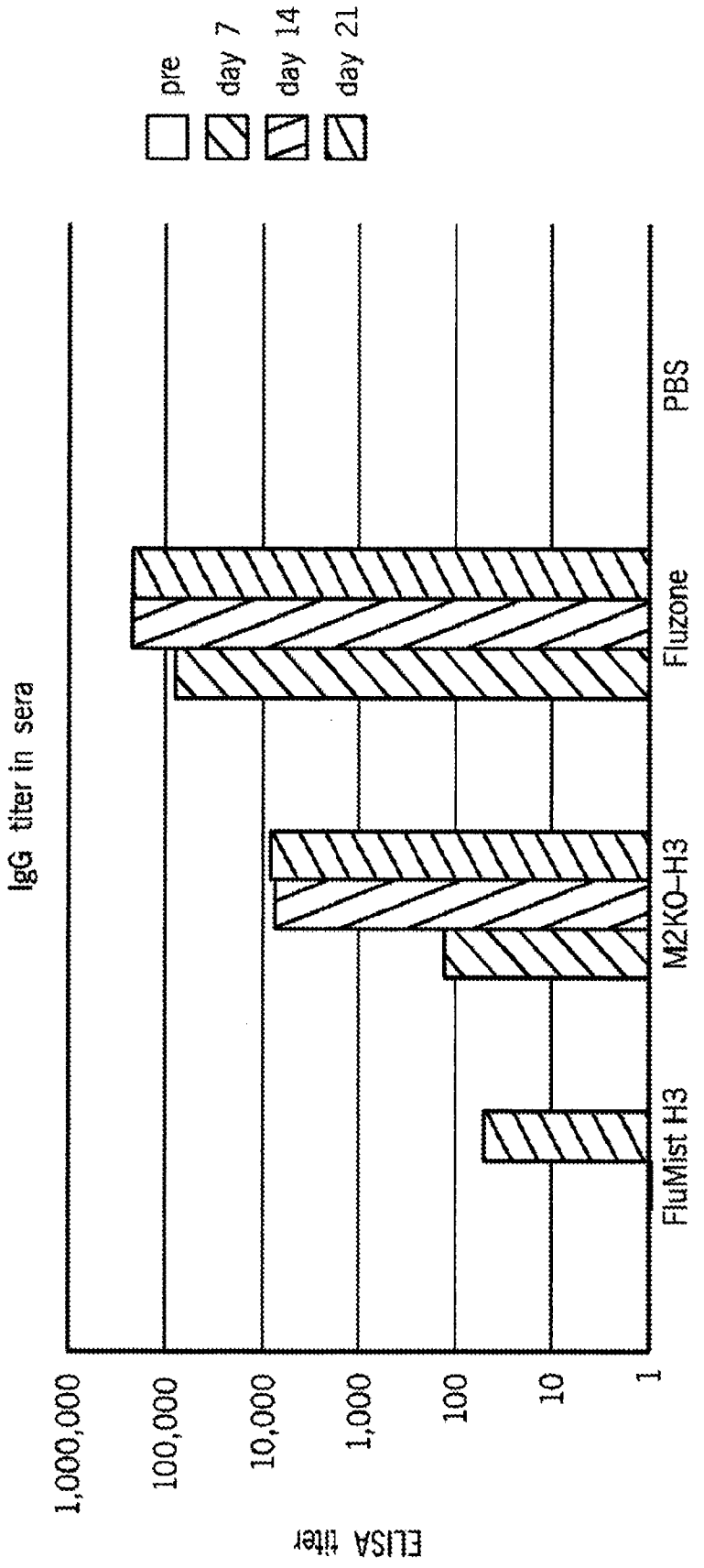

pre
day 7
day 14
day 21
IgG titer in sera
ELISA titer
1,000,000
100,000
10,000
1,000
100
10
1
PBS
Fluzone
M2KO-H3
FluMist H3

FIGURE 31

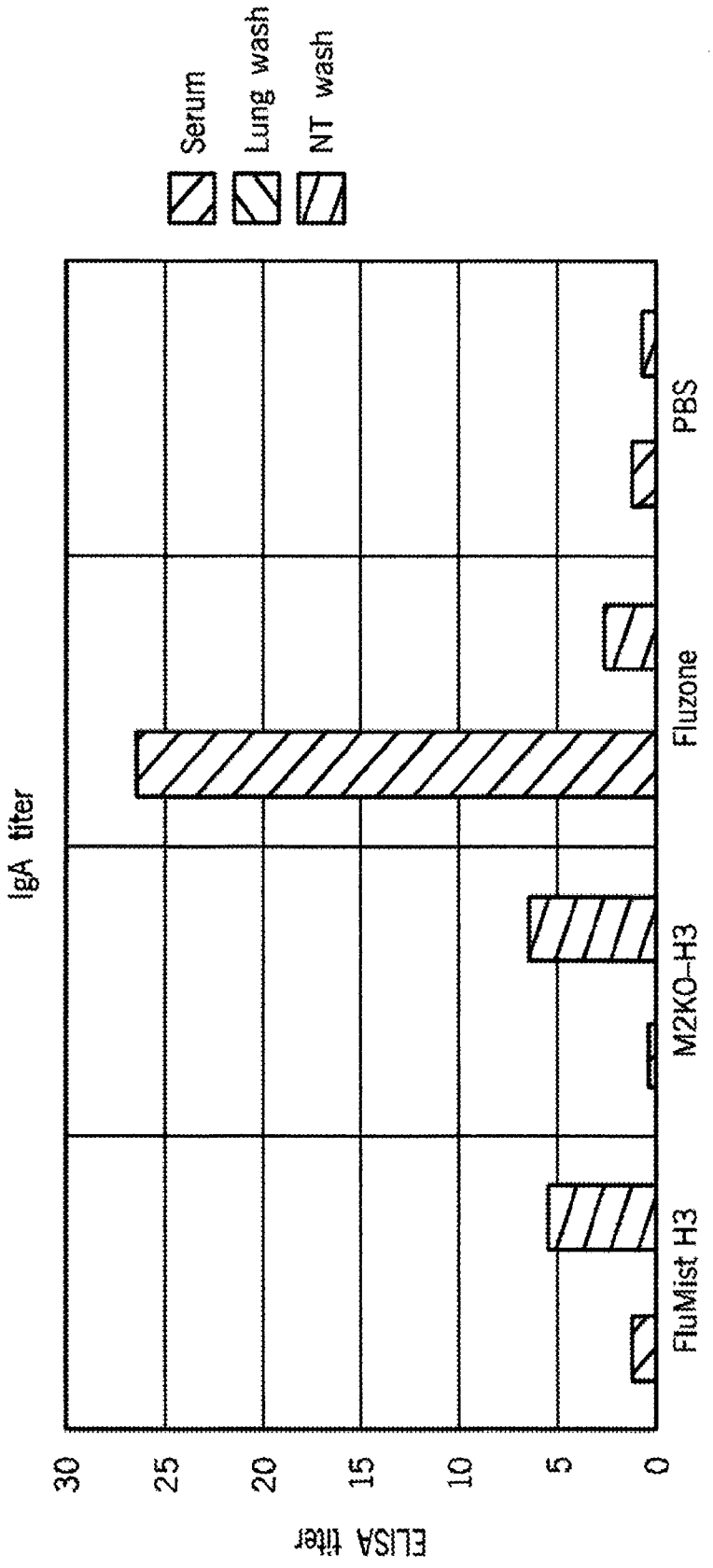

IgA titer
Serum
Lung wash
NT wash
ELISA titer
30
25
20
15
10
5
0
FluMist H3
M2KO-H3
Fluzone
PBS

FIGURE 32

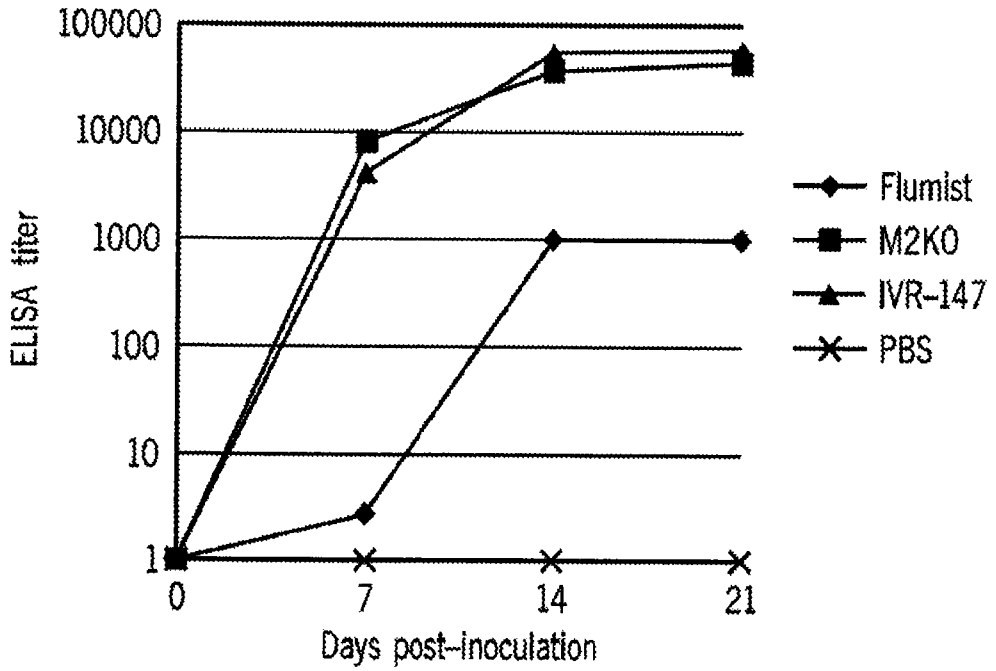

100000
10000
1000
100
10
1
ELISA titer
0
7
14
21
Days post-inoculation
Flumist
M2KO
IVR-147
PBS

FIGURE 33

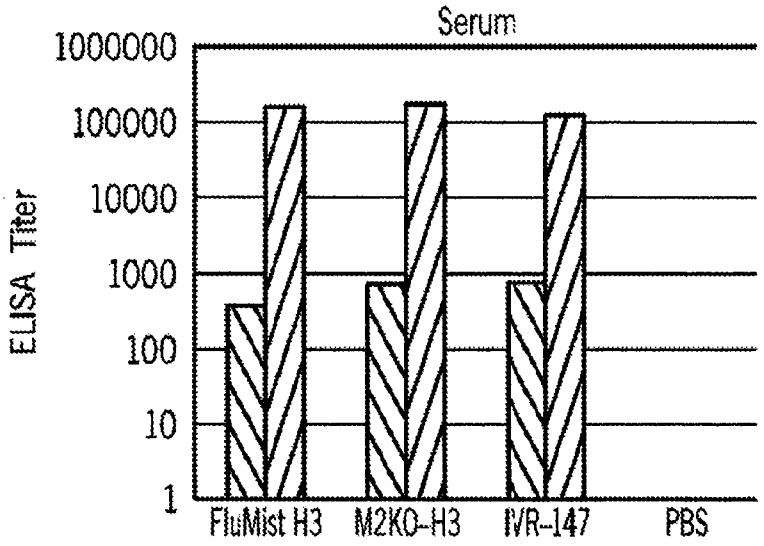


FIGURE 34A

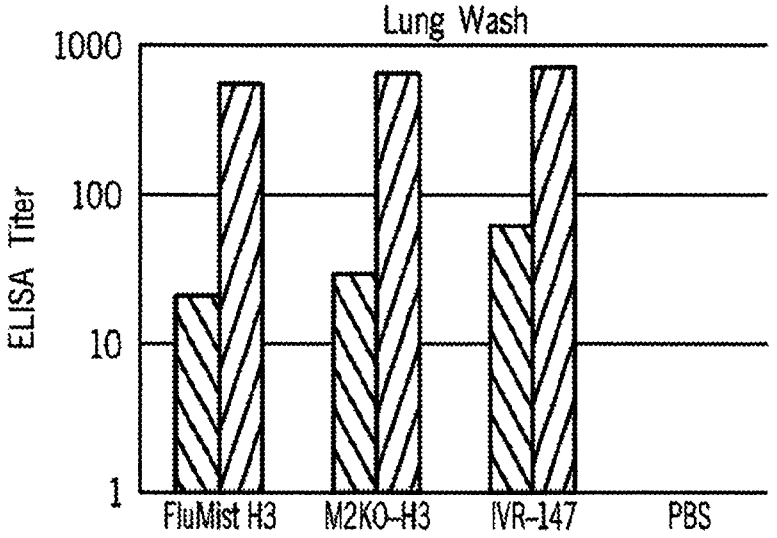


FIGURE 34B

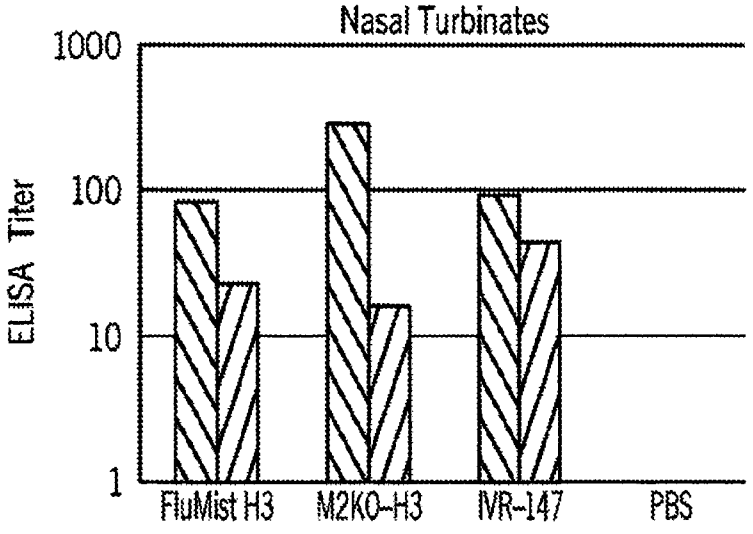


FIGURE 34C

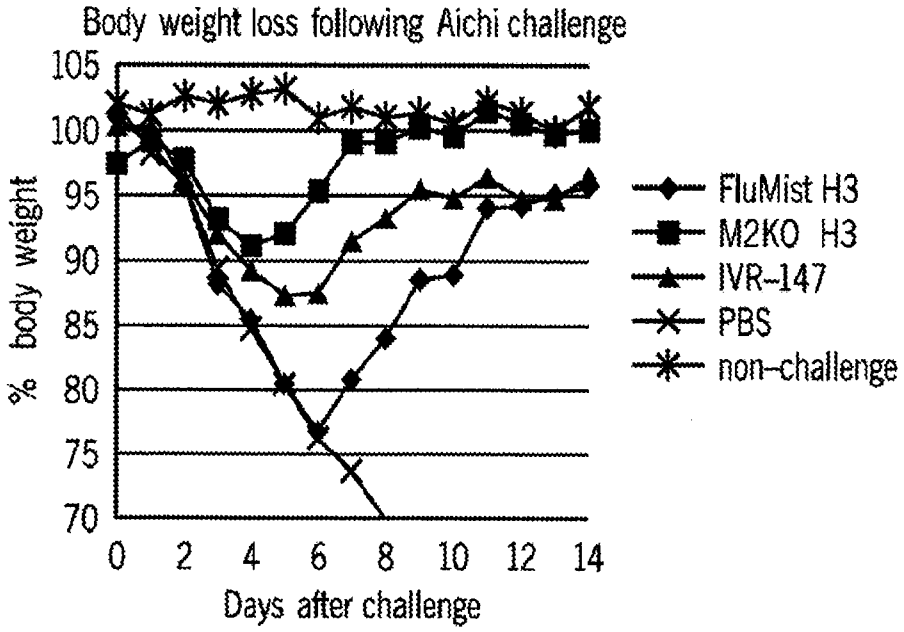


FIGURE 35A

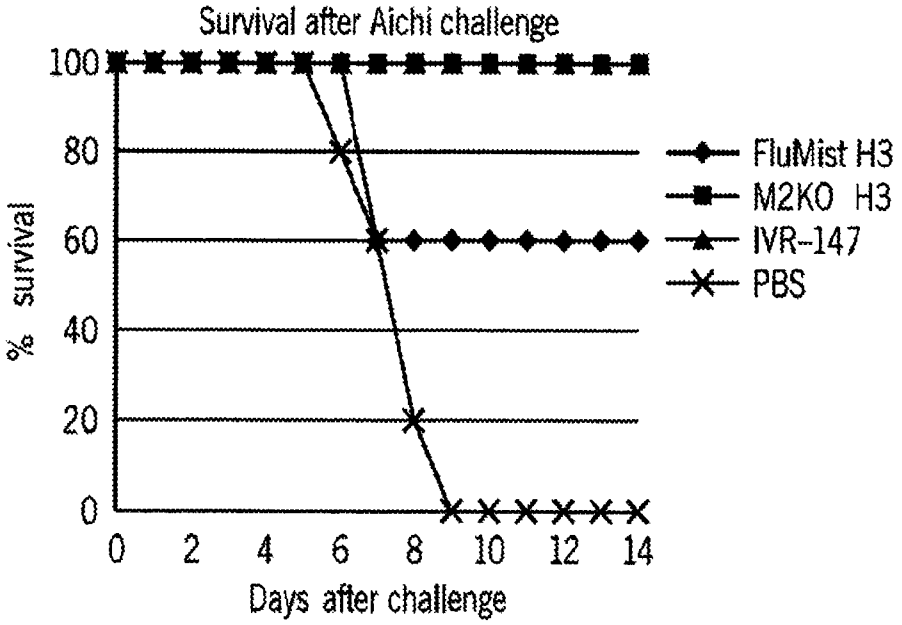
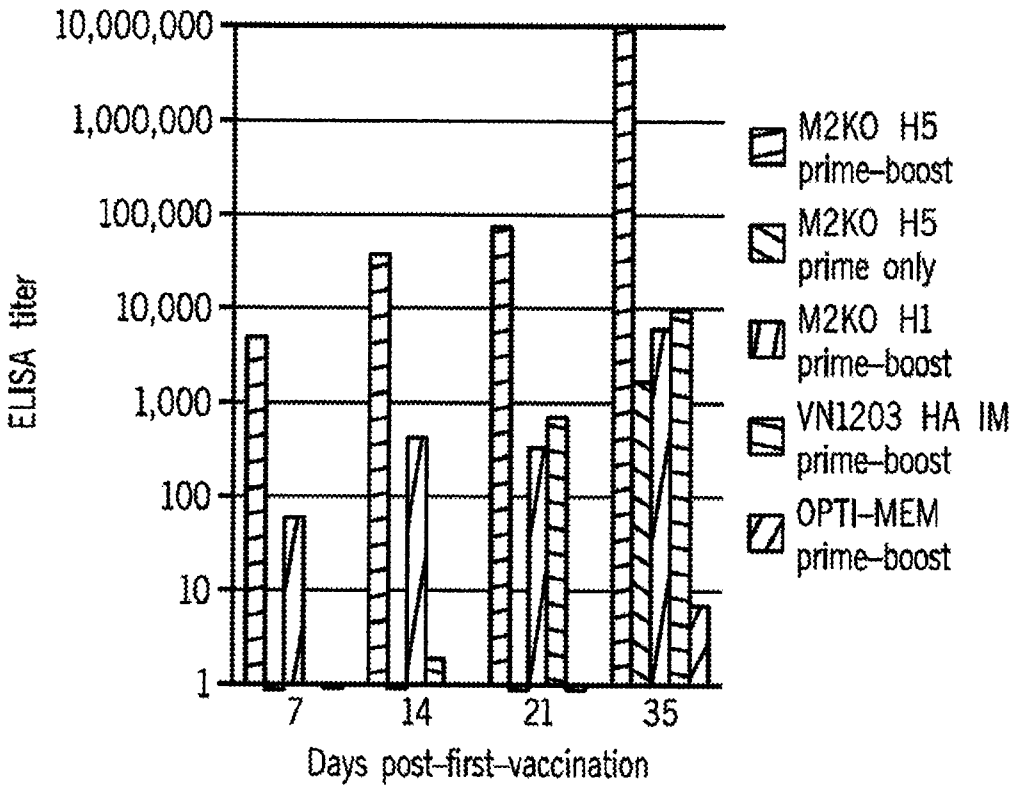


FIGURE 35B



Prime-boost: day 0 and 28
Prime: day 28

FIGURE 36

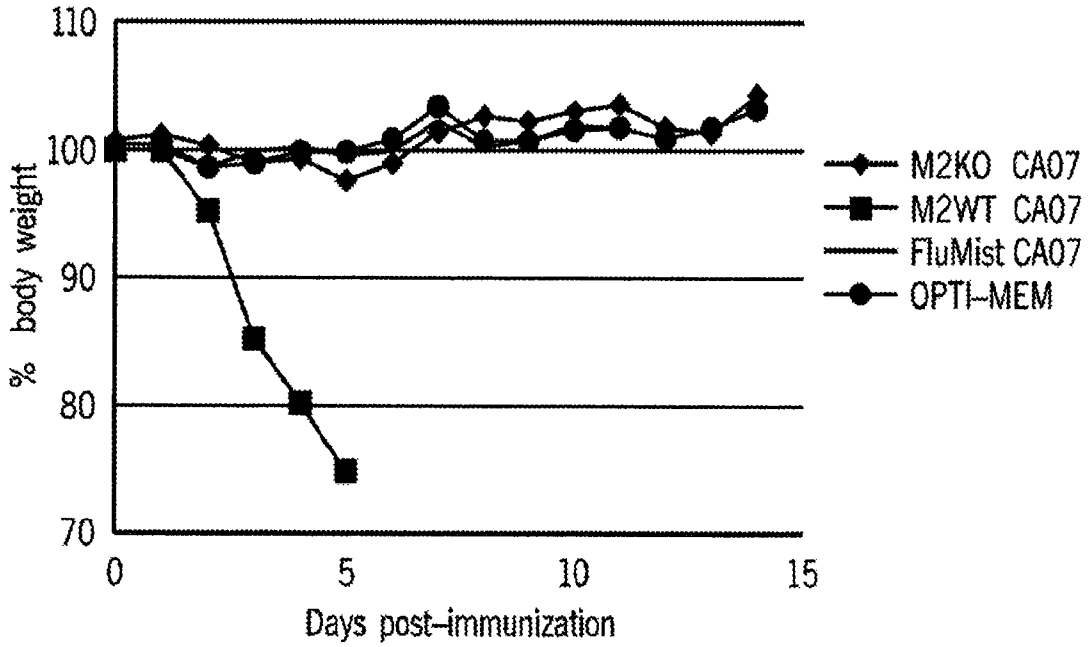
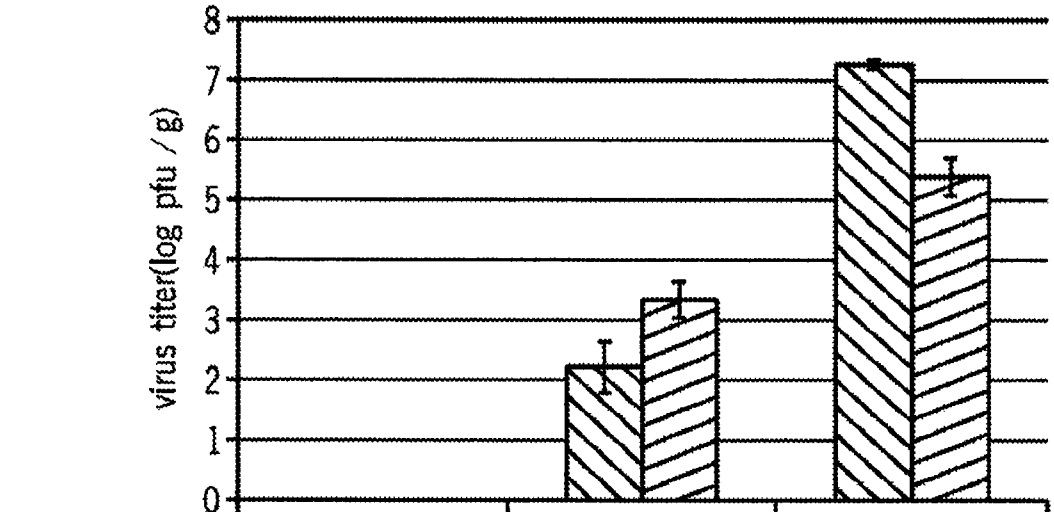

110
100
90
80
70
% body weight
0
5
10
15
Days post-immunization
M2KO CA07
M2WT CA07
FluMist CA07
OPTI-MEM

FIGURE 37



	M2KO CA07	FluMist CA07	M2WT CA07
Lung PFU /g	0.00	2.26	7.26
NT PFU /g	0.00	3.38	5.41

FIGURE 38

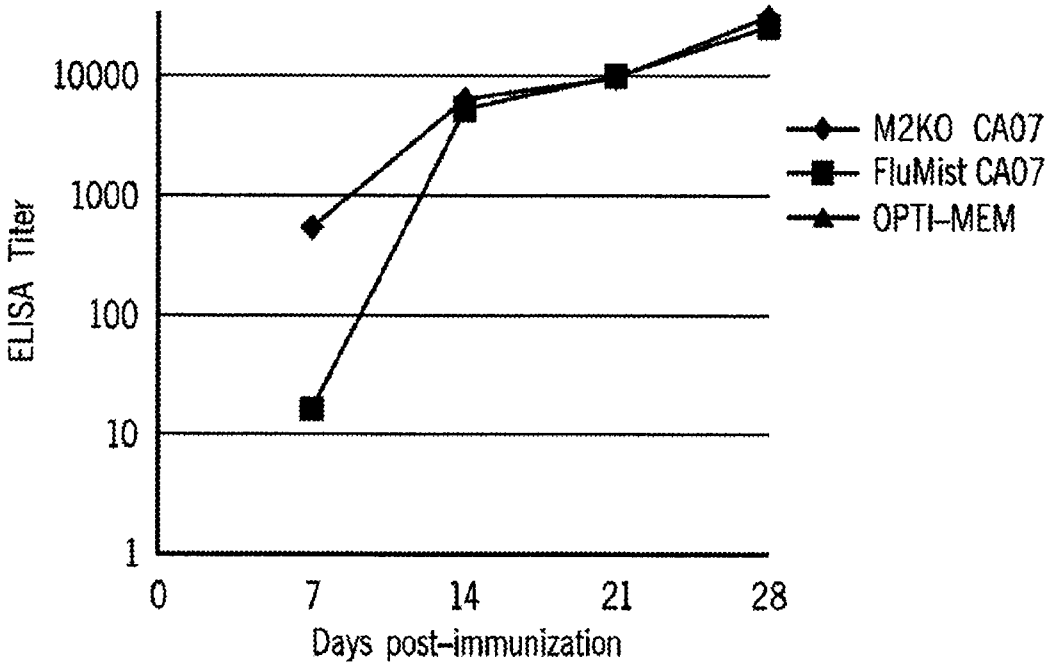

10000
1000
100
10
1
ELISA Titer
0
7
14
21
28
Days post-immunization
M2KO CA07
FluMist CA07
OPTI-MEM

FIGURE 39

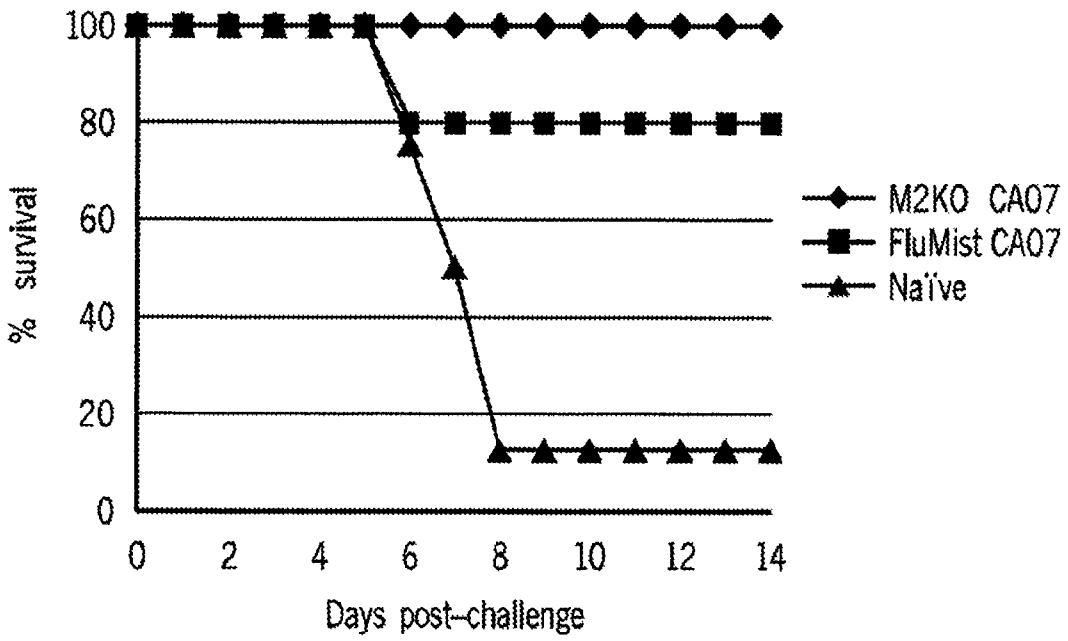

100
80
60
40
20
0
% survival
0
2
4
6
8
10
12
14
Days post-challenge
M2KO CA07
FluMist CA07
Naïve

FIGURE 40

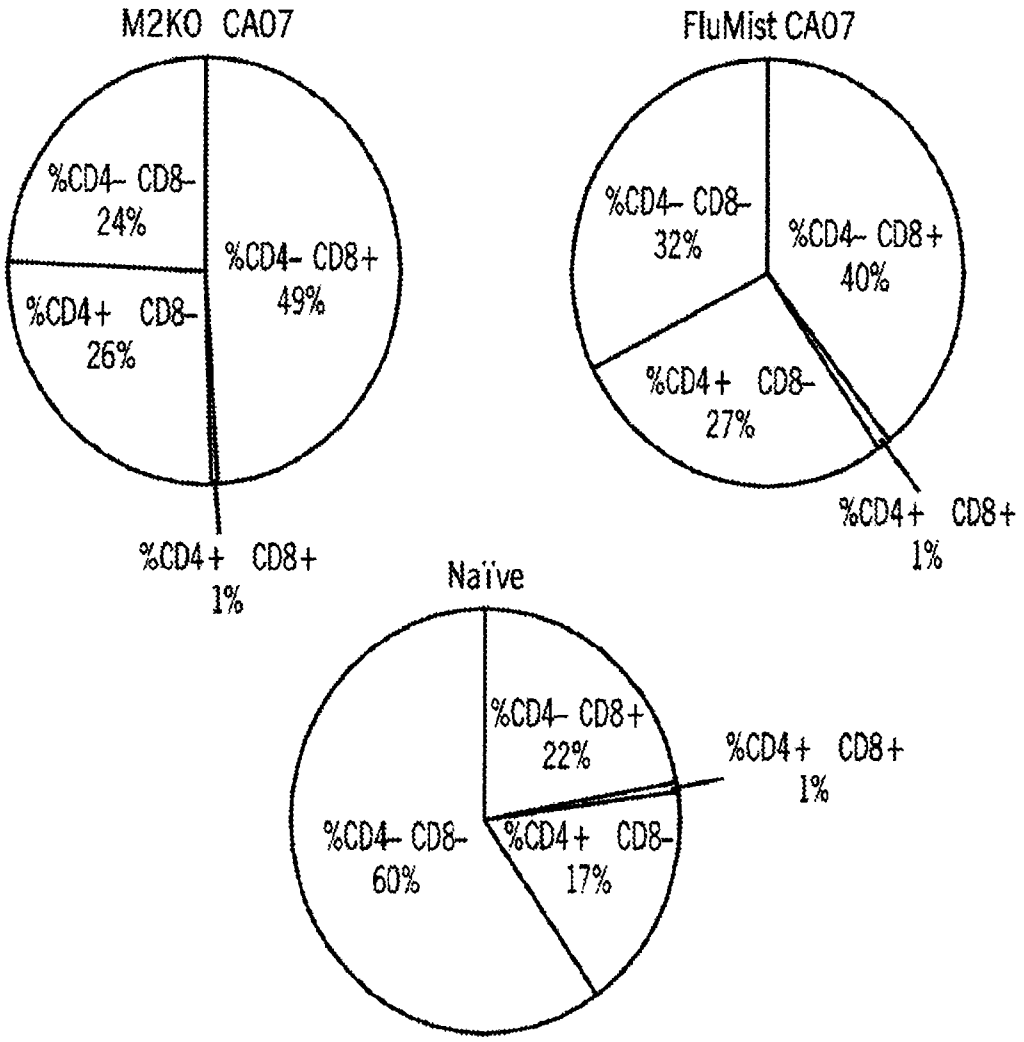

M2KO CA07
%CD4- CD8- 24%
%CD4- CD8+ 49%
%CD4+ CD8- 26%
%CD4+ CD8+ 1%
FluMist CA07
%CD4- CD8- 32%
%CD4- CD8+ 40%
%CD4+ CD8- 27%
%CD4+ CD8+ 1%
Naïve
%CD4- CD8+ 22%
%CD4+ CD8+ 1%
%CD4- CD8- 60%
%CD4+ CD8- 17%

FIGURE 41

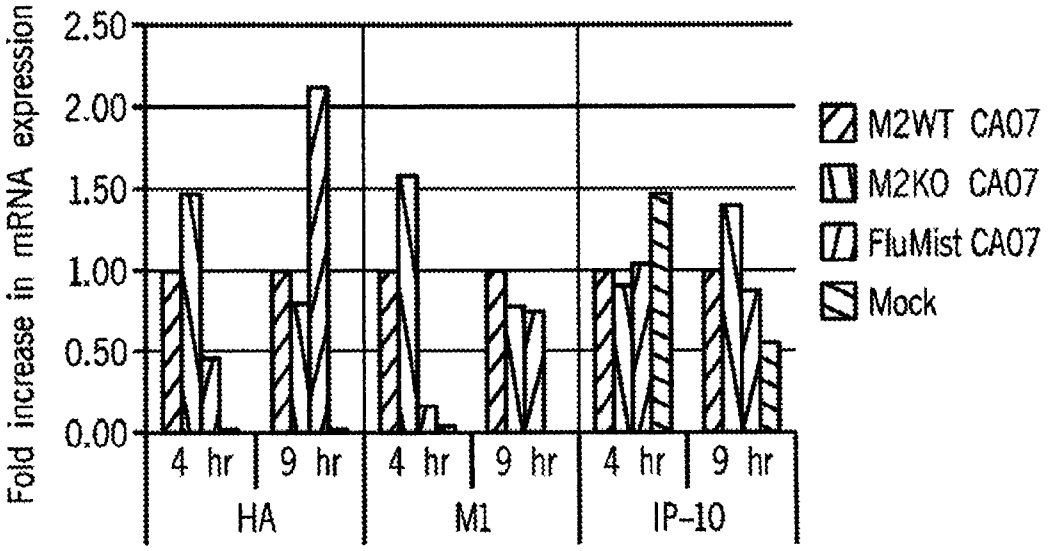

Fold increase in mRNA expression
2.50
2.00
1.50
1.00
0.50
0.00
4 hr
9 hr
4 hr
9 hr
4 hr
9 hr
HA
M1
IP-10
M2WT CA07
M2KO CA07
FluMist CA07
Mock

FIGURE 42

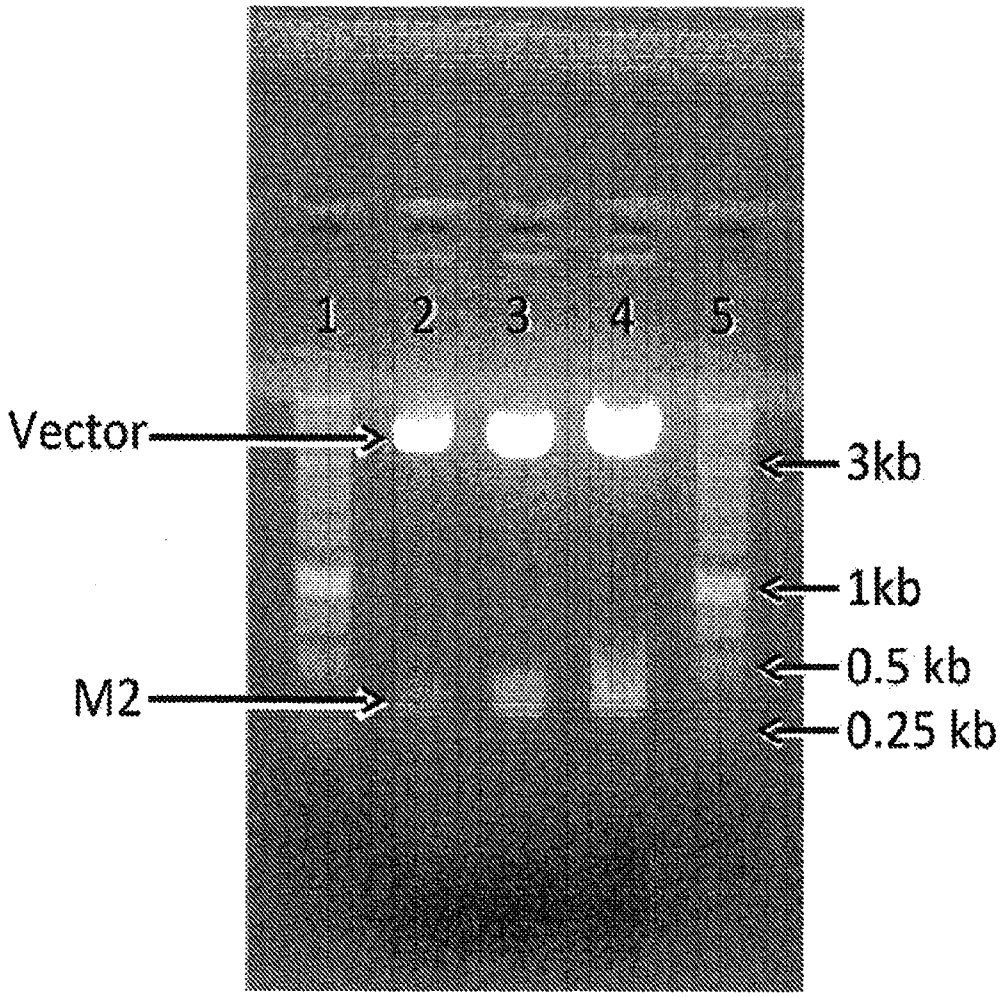


FIGURE 43

FIGURE 44A

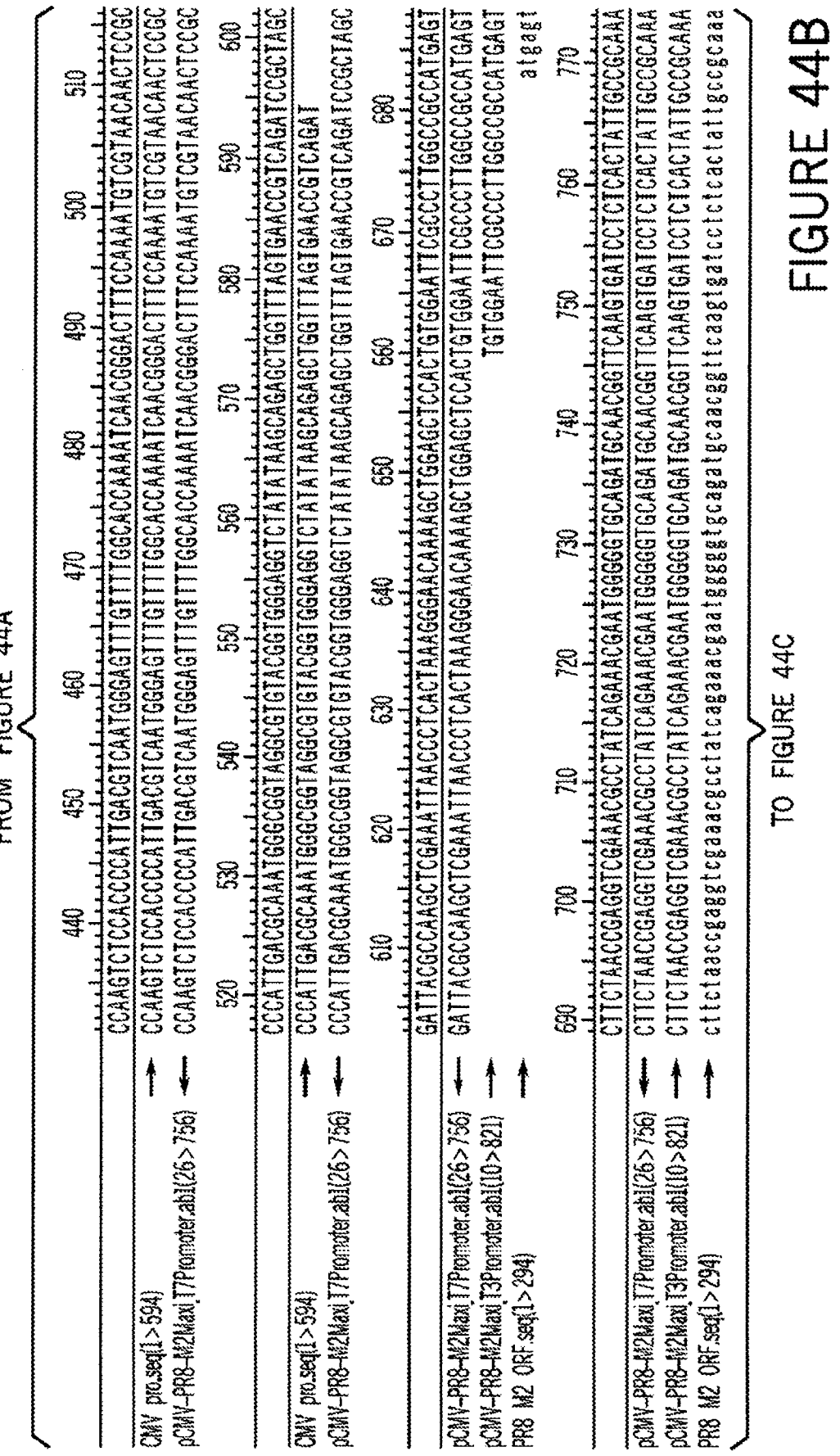
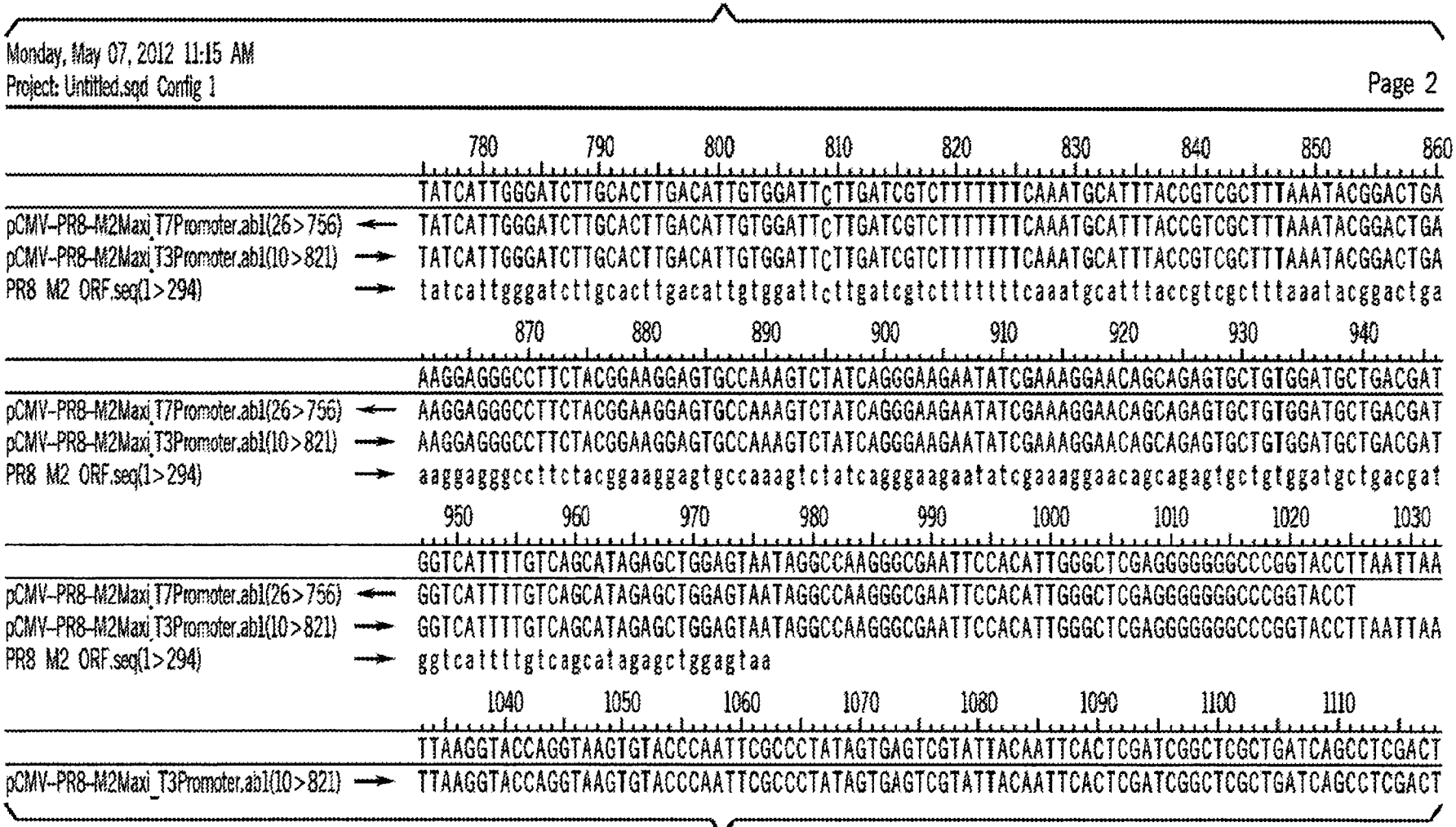


FIGURE 44B

FROM FIGURE 44B



TO FIGURE 44D

FIGURE 44C

FROM FIGURE 44C

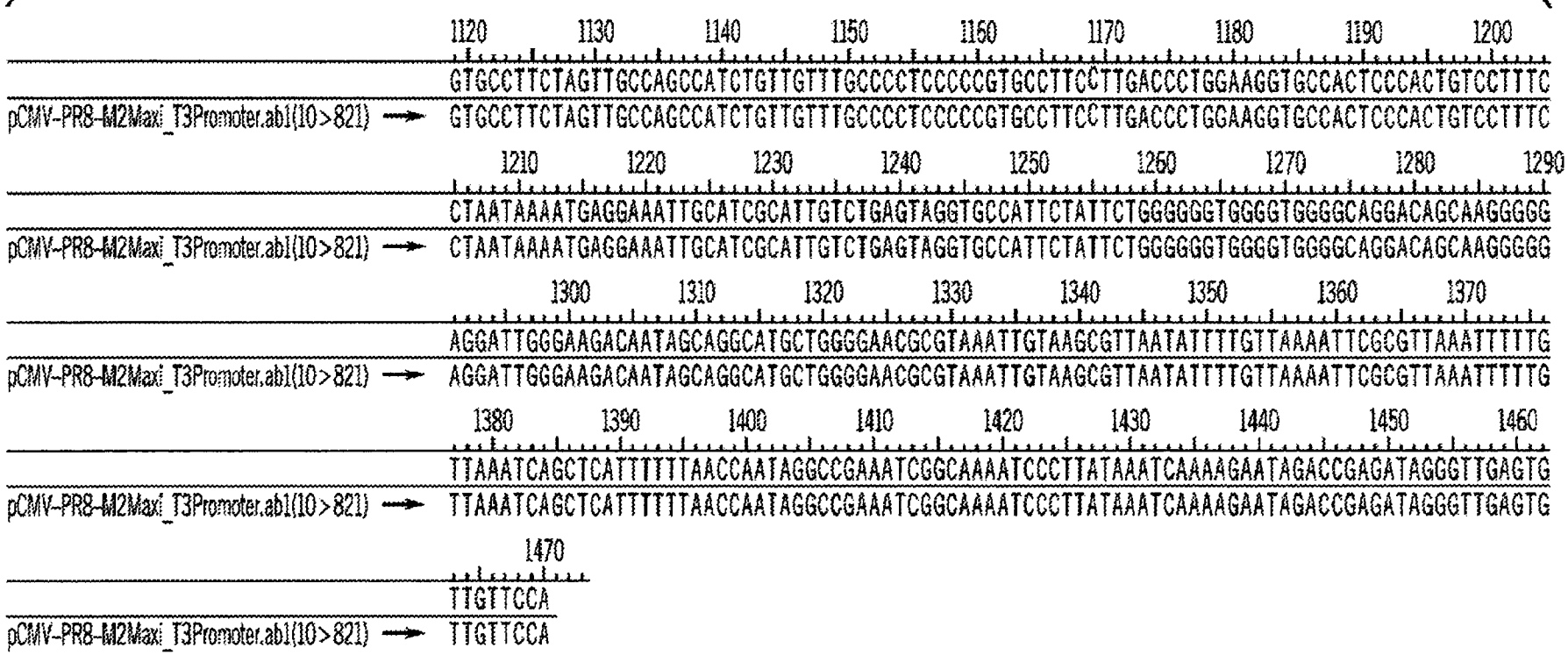


FIGURE 44D

FIGURE 45

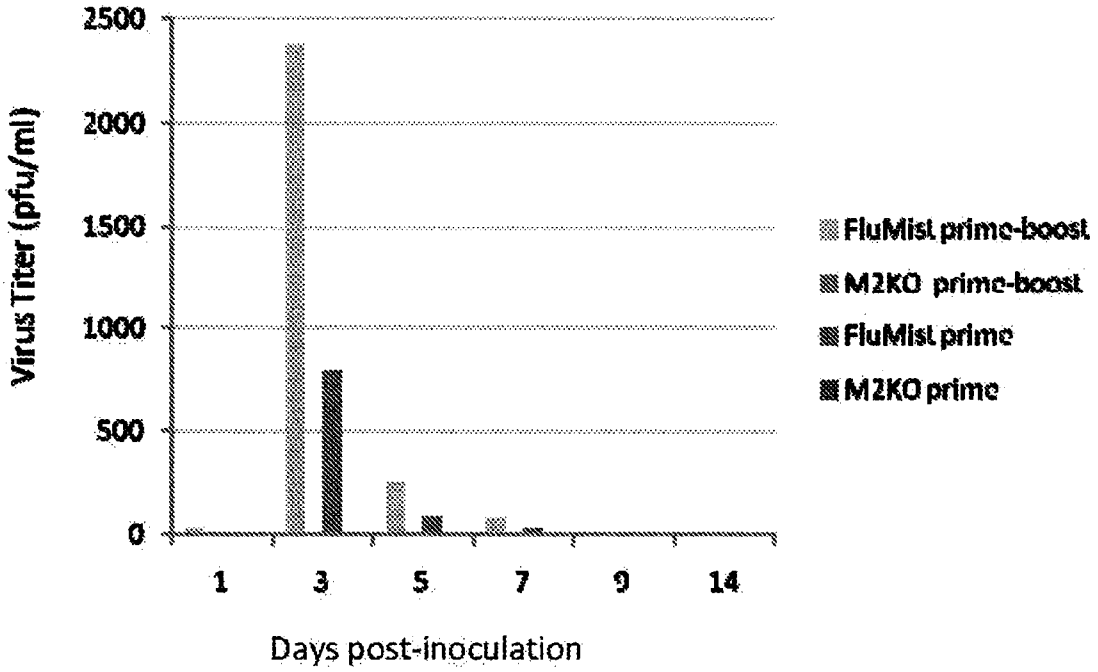

2500
2000
1500
1000
500
0
Virus Titer (pfu/ml)
1
3
5
7
9
14
Days post-inoculation
FluMist prime-boost
M2KO prime-boost
FluMist prime
M2KO prime

FIGURE 46

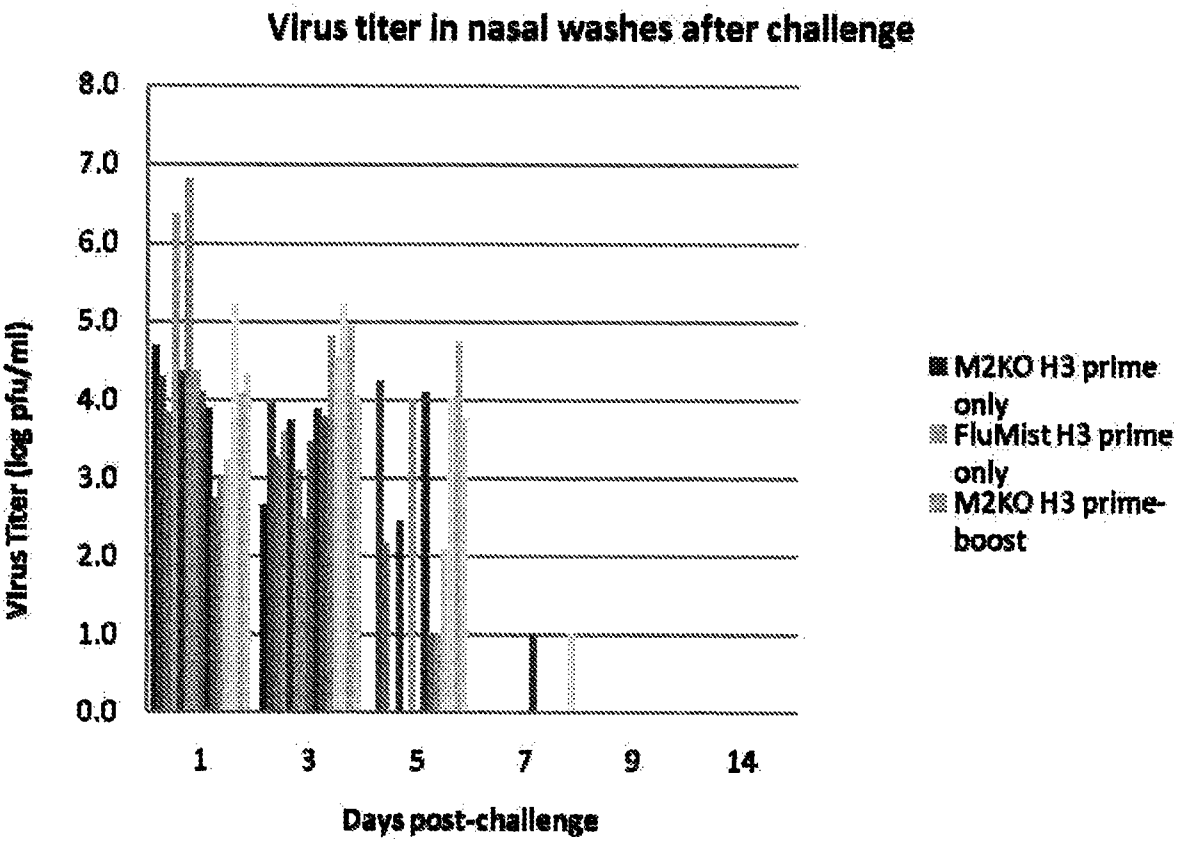

Virus titer in nasal washes after challenge
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Virus Titer (log pfu/ml)
1
3
5
7
9
14
Days post-challenge
M2KO H3 prime only
FluMist H3 prime only
M2KO H3 prime-boost

FIGURE 47

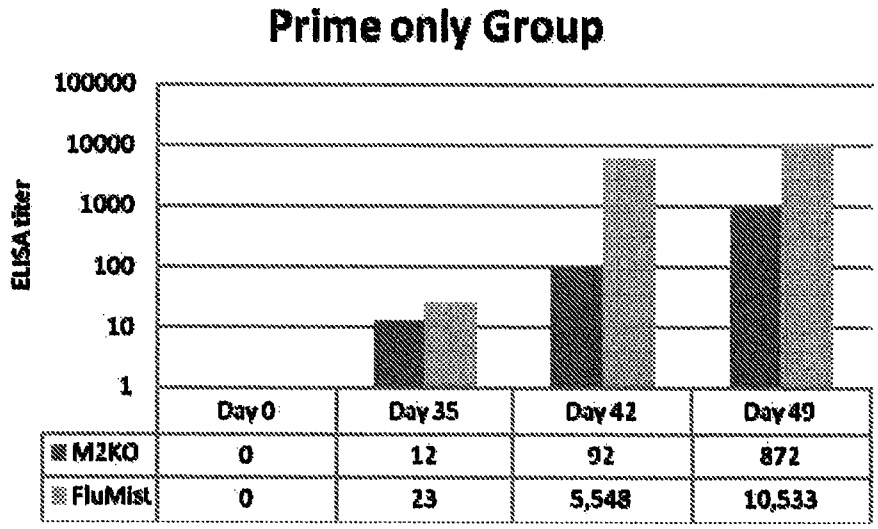

Prime only Group
ELISA titer
100000
10000
1000
100
10
1
Day 0
Day 35
Day 42
Day 49
M2KO
0
12
92
872
FluMist
0
23
5,548
10,533

FIGURE 48

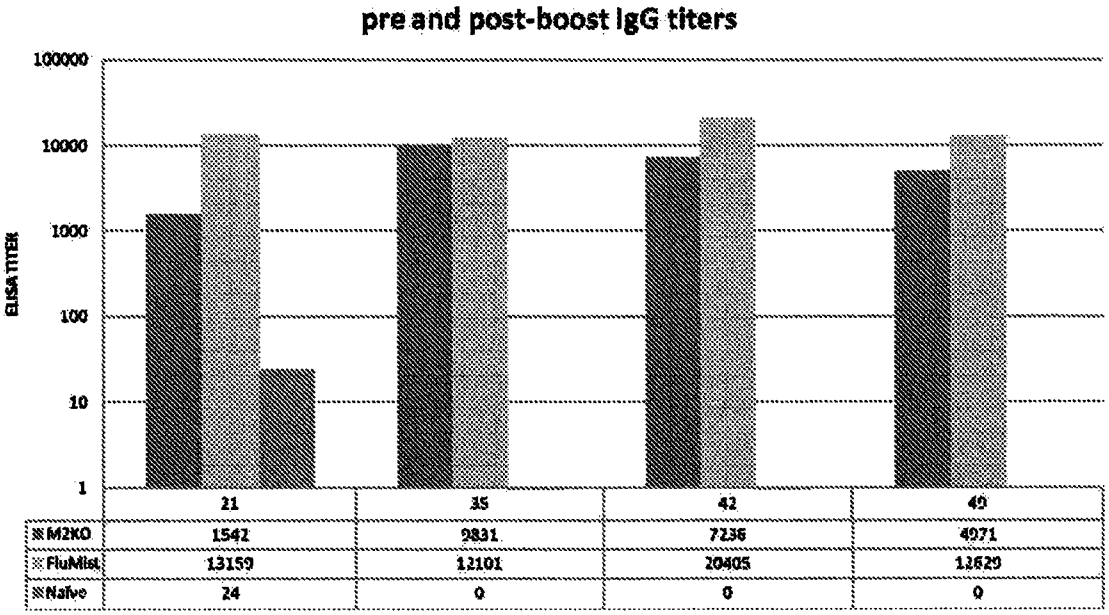

pre and post-boost IgG titers
ELISA TITER
100000
10000
1000
100
10
1
21
35
42
49
※M2KO
1542
9831
7236
4071
※FluMist
13159
12101
20405
12629
※Naive
24
0
0
0

FIGURE 49

BOOST

PRIME-BOOST	7	14	21	35	42	49	AFTER CHALLENGE day 70
M2KO	34	500	1,542	9,831	7,236	4,971	51,362
FluMist	55	8,989	13,159	12,101	20,405	12,629	64,725
Naïve	13	41	24	0	0	-	

PRIME ONLY	d.35	14 d.42 av	21 d.49 av	
M2KO	12	92	872	52,767
FluMist	23	5,548	10,533	56,240
Naïve				7,356

FIGURE 50

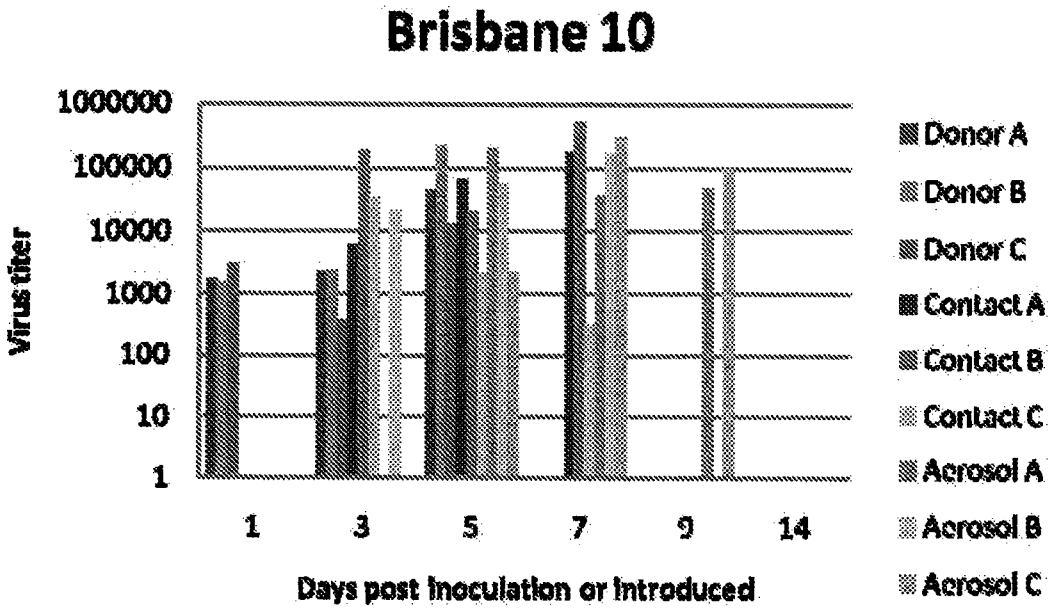

Brisbane 10
1000000
100000
10000
1000
100
10
1
Virus titer
1
3
5
7
9
14
Days post inoculation or introduced
Donor A
Donor B
Donor C
Contact A
Contact B
Contact C
Aerosol A
Aerosol B
Aerosol C

FIGURE 51

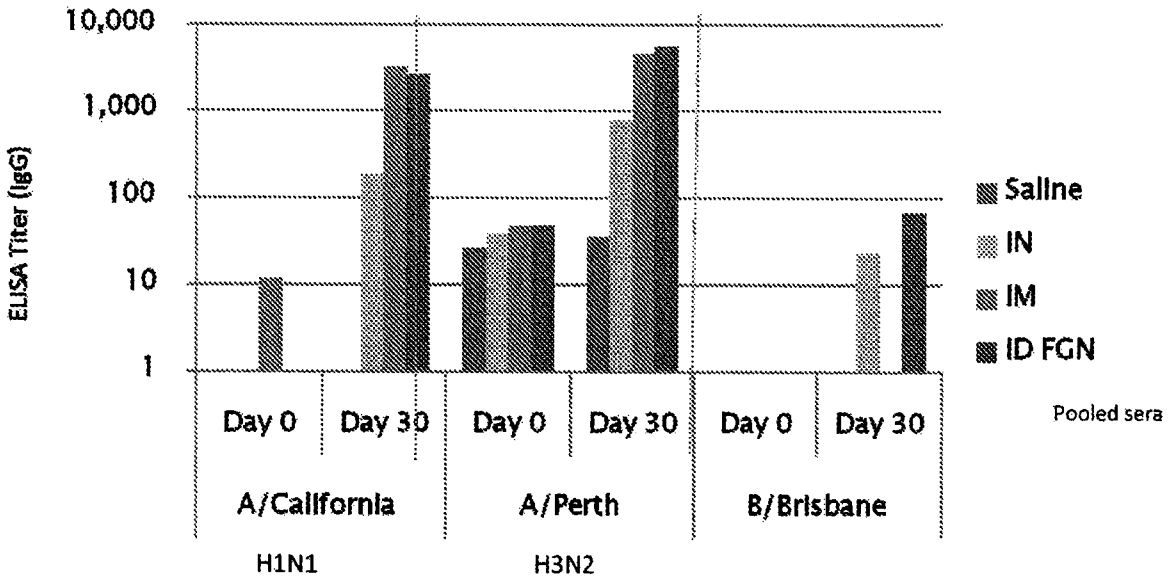

10,000
1,000
100
10
1
ELISA Titer (IgG)
Day 0
Day 30
Day 0
Day 30
Day 0
Day 30
A/California
A/Perth
B/Brisbane
H1N1
H3N2
Saline
IN
IM
ID FGN
Pooled sera

FIGURE 52

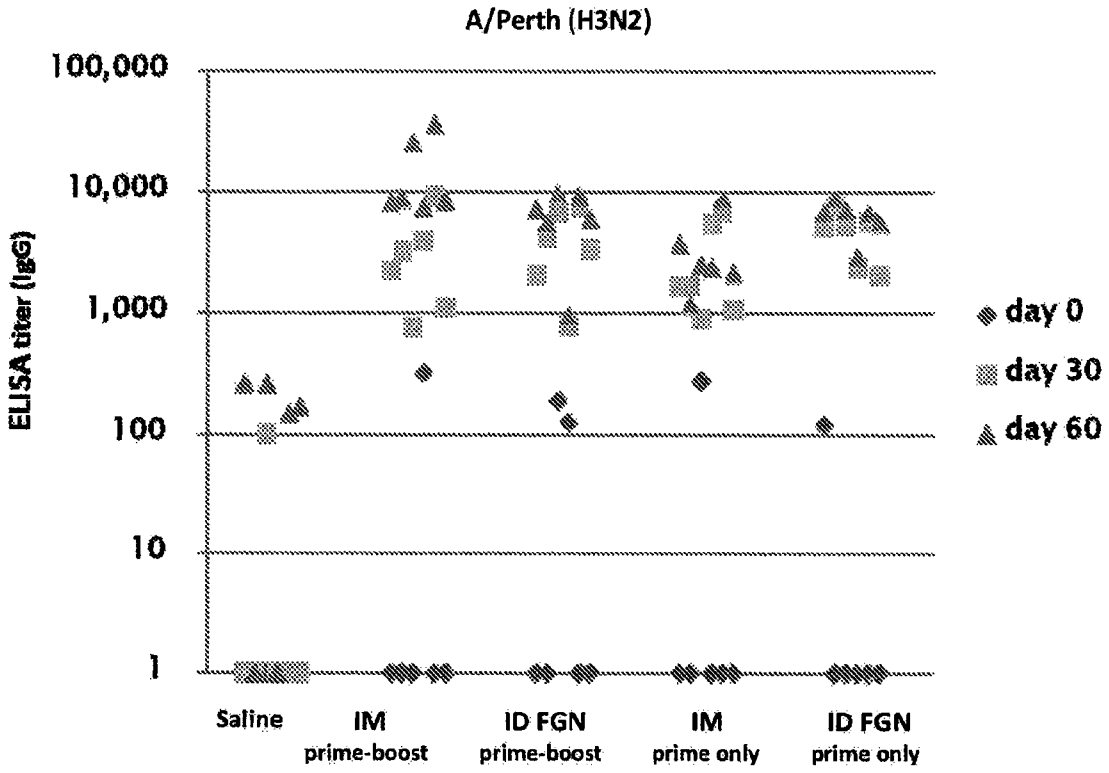

A/Perth (H3N2)
ELISA titer (IgG)
100,000
10,000
1,000
100
10
1
Saline
IM prime-boost
ID FGN prime-boost
IM prime only
ID FGN prime only
day 0
day 30
day 60

FIGURE 53

	Guinea Pig	day 0	day 30	day 60	
Saline	1	1	1	263	Av. 211 ± 59
	2	1	1	1	
	3	1	100	259	
	4	1	1	1	
	5	1	1	150	
	6	1	1	170	
IM (prime boost)	13	1	2,238	8,272	Av. 15,877 ± 12,333
	14	1	3,217	8,794	
	15	1	770	25,799	
	16	318	3,952	7,408	
	17	1	9,276	36,543	
	18	1	1,109	8,446	
ID FGN (prime boost)	19	1	2,072	7,220	Av. 6,391 ± 3,109
	20	1	4,254	5,545	
	21	188	6,765	9,589	
	22	124	792	941	
	23	1	7,402	9,015	
	24	1	3,397	6,034	
IM (prime only)	31	1	1,696	3,778	Av. 3,463 ± 2,704
	32	1	1,720	1,181	
	33	273	897	2,533	
	34	1	5,530	2,425	
	35	1	6,929	8,714	
	36	1	1,070	2,147	
ID FGN (prime only)	37	119	5,217	6,875	Av. 6,312 ± 1,966
	38	1	7,785	8,812	
	39	1	5,404	7,041	
	40	1	2,426	2,894	
	41	1	5,707	6,616	
	42	1	2,063	5,634	

FIGURE 54

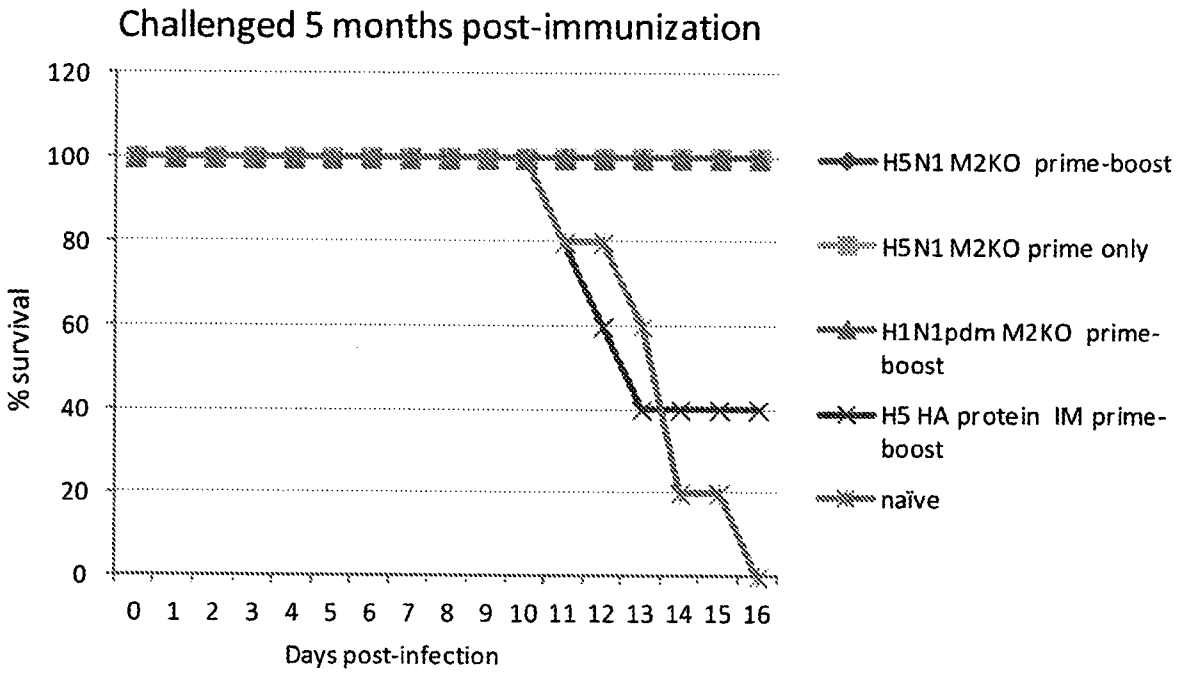
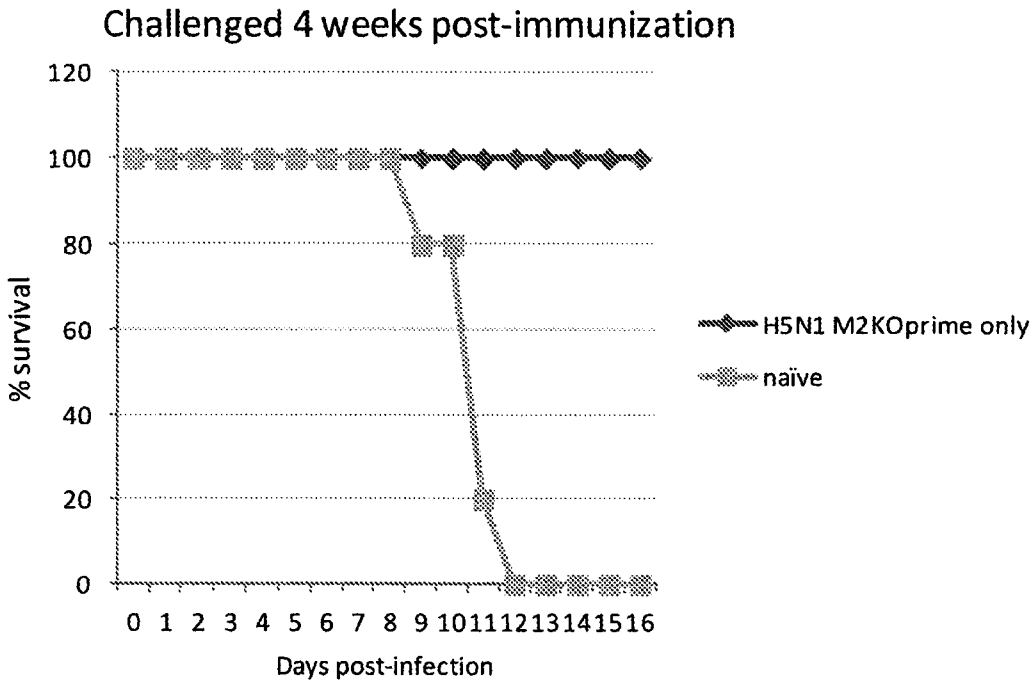
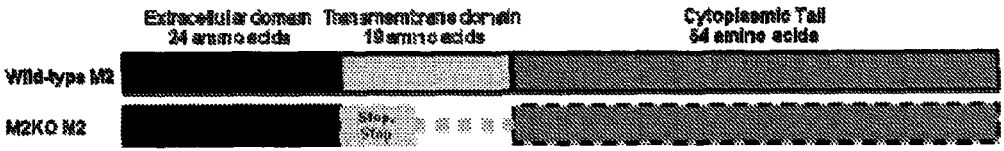

Challenged 5 months post-immunization
% survival
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Days post-infection
H5N1 M2KO prime-boost
H5N1 M2KO prime only
H1N1pdm M2KO prime-boost
H5 HA protein IM prime-boost
naïve

FIGURE 55


Challenged 4 weeks post-immunization
120
100
80
60
40
20
0
% survival
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Days post-infection
H5N1 M2KOprime only
naïve


Extracellular domain
24 amino acids
Transmembrane domain
19 amino acids
Cytoplasmic Tail
54 amino acids
Wild-type M2
M2KO M2
Stop
Stop