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(54) **Title:** METHODS AND COMPOSITIONS FOR THE TREATMENT AND PREVENTION OF VIRAL INFECTION

(57) **Abstract:** The instant invention provides nucleic acid molecules comprising a nucleic acid segment encoding a viral surface protein and a nucleic acid segment encoding a viral matrix protein for the immunization of treatment of viral infections. The invention also provides compositions and methods for treating or immunizing subjects having or at risk of having a viral infection.

METHODS AND COMPOSITIONS FOR THE TREATMENT AND PREVENTION OF VIRAL INFECTION

RELATED APPLICATIONS

This application claims the benefit of US Provisional Application No.: 60/786,747, filed March 27, 2006, the entire contents of which are incorporated herein by reference.

GOVERNMENT SUPPORT

Research supporting this application was carried out by the United States of America as represented by the Secretary, Department of Health and Human Services.

BACKGROUND OF THE INVENTION

The potential global threats posed by viral infections, e.g., pandemics, urgently demand new strategies for protection. Foremost in the minds of clinicians is the risk posed by rapidly mutating viruses against which vaccines rapidly become obsolete, e.g., influenza and HIV.

A vaccine is one of the most efficacious, safe, nontoxic and economical weapons to prevent disease and to control the spread of disease. Conventional vaccines are a form of immunoprophylaxis given before disease occurrence to afford immunoprotection by generating a strong host immunological memory against a specific antigen. The primary aim of vaccination is to activate the adaptive specific immune response, primarily to generate B and T lymphocytes against specific antigen(s) associated with the disease or the disease agent.

Certain viral diseases can currently be controlled for short periods of time, but efficacious and long-term prevention has not yet been obtained. For example, influenza is a contagious disease that is caused by the influenza virus. It attacks the respiratory tract in humans (nose, throat, and lungs). Influenza usually comes on suddenly and includes symptoms of, e.g., fever, headache, and dry cough. Most people who get influenza will recover in one to two weeks, but some people will develop life-

threatening complications (such as pneumonia) as a result of the flu. Millions of people in the United States--about 10% to 20% of U.S. residents--will get influenza each year. An average of about 36,000 people per year in the United States die from influenza, and 114,000 per year have to be admitted to the hospital as a result of influenza. Serious problems from influenza can happen at any age, but particularly in the elderly, e.g., 65 years and older, people with chronic medical conditions; and very young children are more likely to get complications, e.g., pneumonia, bronchitis, and sinus and ear infections from influenza.

Current vaccines made from inactivated or live attenuated viruses are in widespread use. Many researches believe that DNA or protein based vaccines hold much hope for improved vaccines. Moreover, this type of vaccine would eliminate many of the some problems with the current vaccines, e.g., apprehensions about using live attenuated viruses in pregnant women, yearly administrations of narrowly focused vaccines, etc. However, current DNA or protein-based vaccines are not as sensitive to the ongoing mutations of the viral strains as the inactivated viral vaccines and are not practical for use in immunizing subjects.

SUMMARY OF THE INVENTION

The instant invention is based on the finding that nucleic acid molecules containing a nucleic acid segment encoding a viral surface protein and a nucleic acid segment encoding a viral matrix protein are effective in the treatment and prevention of viral infection.

Accordingly, in one aspect, the instant invention provides a nucleic acid molecule comprising a nucleic acid segment encoding a viral surface protein and a nucleic acid segment encoding a viral matrix protein. This molecule can be used for the treatment and/or prevention of viral infection.

In a related embodiment, the viral surface protein is a protein from a Herpesviridae virus, a Poxviridae virus, a Flaviviridae virus, a Paramyxoviridae virus, a Rhabdoviridae virus, a Filoviridae virus, an Orthomyxoviridae virus, or a Retroviridae virus.

In another related embodiment, the matrix protein is a protein from a Herpesviridae virus, a Poxviridae virus, a Flaviviridae virus, a Paramyxoviridae virus,

a Rhabdoviridae virus, a Filoviridae virus, an Orthomyxoviridae virus, or a Retroviridae virus.

In one embodiment, the viral surface protein is a glycoprotein, e.g., an influenza glycoprotein, or immunogenic fragment thereof. For example, in certain embodiments, the influenza glycoprotein hemagglutinin, e.g., hemagglutinin subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16, or neuramidase, e.g., neuramidase subtype N1, N2, N3, N4, N5, N6, N7, N8 or N9. In one specific embodiment, the hemagglutinin is from subtype H5. In another specific embodiment the neuramidase is from subtype N1.

In another embodiment, the viral matrix protein is an influenza matrix protein, e.g., M1 or M2. In another embodiment, the viral matrix protein is a both M1 and M2.

In one embodiment, the glycoprotein is a HIV glycoprotein, e.g., gp120. In another embodiment, the viral matrix protein is a HIV matrix protein, e.g., MA.

In another aspect, the instant invention provides a nucleic acid molecule comprising a nucleic acid segment encoding an influenza hemagglutinin protein, or immunogenic fragments thereof, and a nucleic acid segment encoding the influenza M protein. In specific embodiments, the hemagglutinin is H3 and the M protein is M1 or M1 and M2.

In another aspect, the instant invention provides a nucleic acid molecule comprising a nucleic acid segment encoding the HIV gp120 protein and a nucleic acid segment encoding the HIV MA protein.

In another aspect, the instant invention provides a vector comprising one or more nucleic acid molecules described herein.

In another aspect, the instant invention provides a DNA vaccine comprising one or more nucleic acid molecules described herein.

In another aspect, the instant invention provides a method of vaccinating a subject against a virus comprising administering to the subject an effective amount of one or more nucleic acid molecules described herein.

In another aspect, the instant invention provides a method for inducing in a subject an immune response against influenza infection comprising administering to the subject an immunologically effective amount of a nucleic acid molecule comprising a segment encoding an influenza glycoprotein, or an immunogenic

fragment thereof, and a segment encoding an influenza matrix protein. In related embodiments, the influenza glycoprotein is hemagglutinin or neuramidase. In further related embodiments, the matrix protein is M1 or M2. In another related embodiment, the matrix proteins are M1 and M2.

In another aspect, the instant invention provides methods of vaccinating a subject against HIV comprising administering to the subject an effective amount of a nucleic acid molecule encoding a viral surface protein and a nucleic acid molecule encoding a viral matrix protein. In certain embodiments, the viral surface protein is an HIV glycoprotein, e.g., gp120. In another embodiment, the viral matrix protein is a HIV matrix protein, e.g., MA.

In another aspect, the instant invention provides methods of vaccinating a subject against HIV comprising, administering to the subject a nucleic acid molecule comprising a nucleic acid segment encoding an HIV gp120 protein and a nucleic acid segment encoding the HIV MA protein.

In another aspect, the instant invention provides kits comprising one or more of the nucleic acid molecules described herein instructions for use.

DESCRIPTION OF THE DRAWINGS

Figures 1A-E depict immunofluorescence staining of HA and M1 proteins in plasmids transfected MDCK cells. MDCK cells transfected with 0.5 μg of pHA (Figure 1B), pM1 (Figure 1C) or pHA/M1 double insertion plasmid (Figures 1A, D, E & F) were either stained directly with sheep anti-HA antibody (Figures 1B & D), or permeabilized with detergent first before stained with mouse anti-M1 antibody (Figures 1C & E), or with isotype control followed by secondary antibodies conjugated with fluorochromes (Figure 1A). Co-localization of HA and M1 is shown in Figure 1F.

Figures 2A-D depict virus-specific humoral responses of mice following DNA vaccination. Serum samples were collected at 2 weeks post 2nd booster and immediately before challenges. Mice immunized with heat-inactivated A/WSN/33 plus adjuvant (HI WSN) served as positive controls. Total IgG (Figure 2A) and its isotypes IgG1 (Figure 2B) and IgG2a (Figure 2C) ELISA and HA inhibition titers

(Figure 2D) were done with A/WSN/33 as antigen. The results are expressed as the geometric mean of 9 mice per group with standard errors.

Figures 3A-B depict antigen-specific IFN-γ responses of mice following DNA vaccination. Figure 3A: IFN-γ ELISPOT analysis. Spleen cells harvested at 2 weeks post 2nd DNA plasmid booster and right before challenges were re-stimulated with HA specific class-I peptides or M1 specific class-I peptide *in vitro* for 36 h, and then analyzed for IFN-γ specific ELISPOT. Data averaged on 3 individual mice per group is expressed as fold increases over vector vaccinated group, representing at least 2-3 repeated experiments. Figure 3B: Intracellular IFN-γ staining on splenocytes harvested on day 4 post A/WSN/33 challenges. The percentage of IFN-γ positive cells were gated on CD3+ and CD8α+ double positive population, and is presented as the average of 3 individual mice per group with standard errors.

Figures 4A-B depict HA or M1 epitope specific MHC class-I tetramer staining and cytolytic activity analysis by flow cytometry. Figure 4A: Splenocytes were harvested on day 4 post A/WSN/33 challenges, and then stained with recombinant mouse H-2L^d:Ig fusion protein (BD PharMingen) that was passively loaded with excessive H-2^d restricted HA or M1 class I peptides in vitro along with anti-CD3 FITC, anti-CD8α PE-Cy5 and anti-CD69 PE-Cy7 followed by PE labeled secondary antibodies (BD PharMingen). Stained cells were then analyzed on a BD LSR II by FACSDiva software (Beckman Dickson) using side scatter vs CD3+ FTIC gating strategy. Each dot plot was the representative of 3 individually analyzed mouse spleens per group with the average of the entire group shown. Figure 4B: Cytolytic activities of splenocytes at 2 weeks post 2nd DNA booster and right before challenges. CFSE labeled P815 target cells were incubated with effector cells for 5 h before staining with 7-AAD and the portions of CFSE and 7-AAD double positive P815 populations were resolved by a BD LSR II flow cytometer. The data is expressed as the average of 3 individual mice per group.

Figure 5 depicts body weight losses and survivals post wild type homologous and heterologous influenza virus challenges. Mice were challenged with lethal doses of wild type A/WSN/33 (H1N1) (A, 5 X 10^4 PFU/mouse; B, 5 X 10^6 PFU/mouse) or

A/Phil (H3N2) (C, 5 X 10^5 PFU/mouse) at 2 weeks post 2nd DNA booster, and body weights and survivals were monitored daily up to 2 weeks (n= 5 mice/group).

Figures 6A-B depict viral loads in the lungs of challenged mice. Mice were challenged with lethal doses of homologous A/WSN/33 (H1N1) or heterologous A/Phil (H3N2) influenza viruses, and lungs were harvested at day 4 post challenges. Viral loads in lung tissues were determined in vitro by MDCK cell based plaque assay. Figure 6A: Viral loads in the lungs of individual mice after homologous (upper) and heterologous (lower) challenges. Figure 6B depicts data combined according to DNA vaccination groups.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for generating, enhancing, or modulating a protective and/or therapeutic immune response to a virus in a subject. The methods comprise administering to a subject in need of therapeutic and/or preventative immunity one or more of the compositions described herein. The compositions include polynucleotides comprising a nucleic acid segment encoding a viral surface protein or an immunogenic fragment, variant, or derivative thereof, and a nucleic acid segment encoding a viral matrix protein. Upon administration of the composition according to this method, the nucleic acid molecule is expressed in the subject in a therapeutically or prophylactically effective amount.

The term "polynucleotide" is intended to encompass a singular nucleic acid or nucleic acid fragment as well as plural nucleic acids or nucleic acid fragments, and refers to an isolated molecule or construct, e.g., messenger RNA (mRNA), plasmid DNA (pDNA), DNA, or derivatives of pDNA (e.g., minicircles as described in (Darquet, A-M et al., Gene Therapy 4:1341-1349 (1997)). A polynucleotide may comprise a conventional phosphodiester bond or a non-conventional bond (e.g., an amide bond, such as found in peptide nucleic acids (PNA)).

The terms "nucleic acid" or "nucleic acid segment" refer to any one or more nucleic acid segments, e.g., DNA or RNA fragments, present in a polynucleotide or construct. A nucleic acid or nucleic acid segment may be provided in linear (e.g., mRNA) or circular (e.g., plasmid) form as well as double-stranded or single-stranded forms. DNA segments may include an entire gene, or a fragment thereof. In addition,

a DNA segment may comprise regulatory elements, e.g., promoters, enhancers, etc., that control the expression of the polypeptide encoded by the DNA segment. By "isolated" nucleic acid or polynucleotide is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, a recombinant polynucleotide contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the polynucleotides of the present invention. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically.

As used herein, the term "viral matrix protein" refers to a protein expressed naturally by a virus that makes up part of the matrix of the viral particle. Exemplary viral matrix proteins of the invention can be derived from an Orthomyxoviridae virus, a Retroviridae virus, a Herpesviridae virus, a Poxviridae virus, a Flaviviridae virus, a Paramyxoviridae virus, a Rhabdoviridae virus, or a Filoviridae virus. Exemplary viral proteins of the invention are influenza matrix proteins such as M1 or M2

As used herein, the term "viral surface protein" refers to a polypeptide or fragment thereof that is expressed naturally by a virus and is presented on the surrface of the viral particle. Exermplary viral surface proteins are glycoproteins, e.g., an influenza glycoprotein such as neuramidase or hemagglutinin or an HIV glycoprotein such as gp120.

As used herein, the term "plasmid" or "vector" refers to a construct made up of genetic material (i.e., nucleic acid molecules). Plasmids of the present invention may include genetic elements, e.g., promoters or regulatory elements, as described herein arranged such that an inserted coding sequence can be transcribed and translated in, for example, eukaryotic cells. Also, the plasmids include a sequence of two or more viral nucleic acid segments, e.g., a nucleic acid segment encoding a viral surface protein and a nucleic acid segment encoding a viral matrix protein. In certain embodiments described herein, a plasmid is a closed circular DNA molecule. In certain specific embodiments, the plasmids or vectors of the invention contain nucleic acid segments that encode a viral surface protein and a matrix protein. In specific

embodiments, these segments are under control of the same promoter, while in other embodiments these segments are under control of separate promoters.

Pharmaceutical compositions comprising the nucleic acid molecules encoding described herein, either alone or in combination, and a pharmaceutically acceptable carrier, are also provided by the present invention. As used herein, the phrase "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as those suitable for parenteral administration, such as, for example, by intramuscular, intraarticular (in the joints), intravenous, intradermal, intraperitoneal, and subcutaneous routes. Examples of such formulations include aqueous and non-aqueous, isotonic sterile injection solutions, which contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, slow-releasing bio-degradable polymers, solubilizers, thickening agents, stabilizers, and preservatives.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the vaccine dissolved in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the vaccine, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; (d) suitable emulsions; and (e) polysaccharide polymers such as chitians. The vaccine, alone or in combination with other suitable components, may also be made into aerosol formulations to be administered via inhalation, e.g., to the bronchial passageways. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

The concentration of the nucleic acid molecules of the invention in the pharmaceutical composition may vary depending on, for example, fluid volume or antigenicity, and in accordance with the particular mode of administration chosen.

As used herein, an "immune response" refers to the ability of a subject to elicit an immune reaction to a composition delivered to that subject. Examples of immune responses include an antibody response or a cellular, e.g., cytotoxic T-cell response. One or more compositions of the present invention may be used to prevent viral infection in subjects, e.g., as a prophylactic vaccine, to establish or enhance immunity to a virus in a healthy individual prior to exposure to a virus or contraction of viral disease, thus preventing the disease or reducing the severity of disease symptoms.

In one aspect the instant invention provides nucleic acid molecules, e.g., vectors, comprising a nucleic acid segment encoding a viral surface protein and a nucleic acid segment encoding a viral matrix protein that, for example, induce an immune response. The viral surface protein and viral matrix protein can be a protein from a Herpesviridae virus, a Poxviridae virus, a Flaviviridae virus, a Paramyxoviridae virus, a Rhabdoviridae virus, a Filoviridae virus, an Orthomyxoviridae virus, or a Retroviridae virus. In one embodiment, the viral surface protein and matrix protein are from the same genus, species and subtype, while in another embodiment, the viral surface protein and the viral matrix protein are from different genuses, species or subtypes.

In an exemplary embodiment, the viral surface protein and matrix protein are from influenza. For example, the viral surface protein can be a glycoprotein such as hemagglutinin, e.g., H1-H16, or neuraminidase, e.g., N1-N9, and the matrix protein can be, for example, M1 or M2 or both. In another exemplary embodiment, the viral surface protein and matrix protein are from HIV. For example, the viral surface protein is an HIV glycoprotein such as, for example, gp120, and the matrix protein can be, for example, MA. In a specific embodiment, the instant invention provides a DNA vaccine against influenza comprising a nucleic acid segment encoding influenza hemagglutinin protein (H1) and a nucleic acid segment encoding an influenza matrix protein. In other embodiments, the viral surface protein and matrix protein are from Ebola or Marburg. For example, the viral surface protein can be an Ebola or Marburg glycoprotein, e.g., GP1 or GP2, and the matrix protein can be an Ebola or Marburg matrix protein, e.g., VP24 or VP40. In certain embodiments, the viral surface protein and the matrix protein are from the same virus, or viral subtype. In other embodiments, the viral surface protein and the matrix protein are from different viruses or viral subtypes. In other embodiments, the viral surface protein and matrix protein are homologous to the exemplary proteins set forth above.

In related embodiments, the invention provides nucleic acid molecules comprising a segment encoding an internal viral proteins, e.g., nucleoprotein or polymerase, or homologs thereof, in addition to the viral surface and matrix proteins described herein.

Moreover, in at least one embodiment, the viral surface protein can be a fragment of the full length protein, e.g., an immunogenic fragment. Any fragment that

conveys sufficient immunogenic properties so as to cause an immunogenic reaction in the subject is contemplated for use in the methods and compositions of the invention.

As mentioned above, compositions of the present invention can be used both to prevent viral infection, i.e., to immunize against viral infection, and also to therapeutically treat viral infection. In individuals already exposed to a virus, e.g., influenza, or already suffering from disease, the present invention is used to further stimulate the immune system of the subject, thus reducing or eliminating the symptoms associated with that disease or disorder. As defined herein, "treatment " refers to the use of one or more compositions of the present invention to prevent, cure, retard, or reduce the severity of the infection symptoms in a subject, and/or result in no worsening of infection over a specified period of time in a subject which has already been exposed to a virus and is thus in need of therapy. The term "prevention" refers to the use of one or more compositions of the present invention to generate immunity in a subject which has not yet been exposed to a particular virus, thereby preventing or reducing disease symptoms if the subject is later exposed to the particular virus. The methods of the present invention therefore may be referred to as therapeutic vaccination or preventative or prophylactic vaccination. It is not required that any compositions of the present invention provide total immunity to a virus or totally cure or eliminate all viral infection symptoms.

As used herein, a "subject" refers to an individual for whom it is desirable to treat, i.e., to prevent, cure, retard, or reduce the severity of a viral infection or the symptoms thereof, and/or result in no worsening of the infection or symptoms thereof over a specified period of time. Subjects to treat and/or vaccinate include, for example, humans, apes, monkeys (e.g., owl, squirrel, cebus, rhesus, African green, patas, cynomolgus, and cercopithecus), orangutans, baboons, gibbons, and chimpanzees, dogs, cats, horses, donkeys, zebras, cows, cattle, pigs, sheep, goats, deer, rabbits, mice, ducks, geese, turkeys, chickens, and quail.

One or more compositions of the present invention can be utilized in a "prime boost" regimen. An example of a "prime boost" regimen may be found in Yang, Z. et al. J. Virol. 77:799-803 (2002). In these embodiments, one or more polynucleotide vaccine compositions of the present invention are delivered to a subject, thereby priming the immune response of the subject to a virus, and then a second immunogenic composition is utilized as a boost vaccination. One or more

compositions of the present invention are used to prime immunity, and then a second immunogenic composition, e.g., the same composition or another composition described herein.

The terms "priming" or "primary" and "boost" or "boosting" as used herein may refer to the initial and subsequent immunizations, respectively, i.e., in accordance with the definitions these terms normally have in immunology. However, in certain embodiments, e.g., where the priming component and boosting component are in a single formulation, initial and subsequent immunizations may not be necessary as both the "prime" and the "boost" compositions are administered simultaneously.

The instant invention also provides methods of treating or immunizing a subject by administering one or more compositions of the invention. In certain embodiments, one or more compositions of the present invention are delivered to a subject by methods described herein, thereby achieving an effective therapeutic and/or an effective preventative immune response. In certain embodiments, the methods of the invention further comprise the step of determining if a subject would benefit from treatment with a composition of the invention. More specifically, the compositions of the present invention may be administered to any tissue of a subject, including, but not limited to, muscle, skin, brain tissue, lung tissue, liver tissue, spleen tissue, bone marrow tissue, thymus tissue, heart tissue, e.g., myocardium, endocardium, and pericardium, lymph tissue, blood tissue, bone tissue, pancreas tissue, kidney tissue, gall bladder tissue, stomach tissue, intestinal tissue, testicular tissue, ovarian tissue, uterine tissue, vaginal tissue, rectal tissue, nervous system tissue, eye tissue, glandular tissue, tongue tissue, and connective tissue, e.g., cartilage.

Furthermore, the compositions of the present invention may be administered to any internal cavity of a subject, including, but not limited to, the lungs, the mouth, the nasal cavity, the stomach, the peritoneal cavity, the intestine, any heart chamber, veins, arteries, capillaries, lymphatic cavities, the uterine cavity, the vaginal cavity, the rectal cavity, joint cavities, ventricles in brain, spinal canal in spinal cord, the ocular cavities, the lumen of a duct of a salivary gland or a liver. Certain modes of administration to secretory organs of a gastrointestinal system using the salivary gland, liver and pancreas to release a desired polypeptide into the bloodstream are disclosed in U.S. Pat. Nos. 5,837,693 and 6,004,944, both of which are incorporated herein by reference in their entireties.

In certain embodiments, the compositions are administered to muscle, either skeletal muscle or cardiac muscle, or to lung tissue. Specific, but non-limiting modes of administration to lung tissue are disclosed in Wheeler, C. J., et al., Proc. Natl. Acad. Sci. USA 93:11454-11459 (1996).

According to the disclosed methods, compositions of the present invention can be administered by intramuscular (i.m.), subcutaneous (s.c.), or intrapulmonary routes. Other suitable routes of administration include, but are not limited to intratracheal, transdermal, intraocular, intranasal, inhalation, intracavity, intravenous (i.v.), intraductal (e.g., into the pancreas) and intraparenchymal (i.e., into any tissue) administration. Transdermal delivery includes, but not limited to intradermal (e.g., into the dermis or epidermis), transdermal (e.g., percutaneous) and transmucosal administration (i.e., into or through skin or mucosal tissue). Intracavity administration includes, but not limited to administration into oral, vaginal, rectal, nasal, peritoneal, or intestinal cavities as well as, intrathecal (i.e., into spinal canal), intraventricular (i.e., into the brain ventricles or the heart ventricles), inraatrial (i.e., into the heart atrium) and sub arachnoid (i.e., into the sub arachnoid spaces of the brain) administration.

Any mode of administration can be used so long as the mode results the generation of an immune response to a virus and/or the generation of a prophylactically or therapeutically effective immune response to a virus in a subject in need of such response. Administration means of the present invention include needle injection, catheter infusion, biolistic injectors, particle accelerators (e.g., "gene guns" or pneumatic "needleless" injectors) Med-E-Jet (Vahlsing, H., et al., J. Immunol. Methods 171:11-22 (1994)), Pigjet (Schrijver, R., et al., Vaccine 15: 1908-1916 (1997)), Biojector (Davis, H., et al., Vaccine 12: 1503-1509 (1994); Gramzinski, R., et al., Mol. Med. 4: 109-118 (1998)), AdvantaJet (Linmayer, I., et al., Diabetes Care 9:294-297 (1986)), Medi-jector (Martins, J., and Roedl, E. J. Occup. Med. 21:821-824 (1979)), gelfoam sponge depots, other commercially available depot materials (e.g., hydrogels), osmotic pumps (e.g., Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, topical skin creams, and decanting, use of polynucleotide coated suture (Qin, Y., et al., Life Sciences 65: 2193-2203 (1999)) or topical applications during surgery. Certain modes of administration are intramuscular needle-based injection and pulmonary application via catheter infusion. Energy-assisted plasmid delivery (EAPD) methods may also be employed to administer the

compositions of the invention. One such method involves the application of brief electrical pulses to injected tissues, a procedure commonly known as electroporation. See generally Mir, L. M. et al., Proc. Natl. Acad. Sci USA 96:4262-7 (1999); Hartikka, J. et al., Mol. Ther. 4:407-15 (2001); Mathiesen, I., Gene Ther. 6:508-14(1999); Rizzuto G. et al., Hum. Gen. Ther. 11:1891-900 (2000).

The nucleic acid molecules of the invention may also be delivered by a viral vector. The capacity of certain viral vectors to efficiently infect or enter cells, to integrate into a host cell genome and stably express viral genes, have led to the development and application of a number of different viral vector systems. Viral systems are currently being developed for use as vectors for ex vivo and in vivo gene transfer. For example, adenovirus, herpes-simplex virus, retrovirus and adeno-associated virus vectors are being evaluated currently for treatment of diseases such as cancer, cystic fibrosis, Gaucher disease, renal disease and arthritis U.S. Pat. No. 5,670,488).

In particular embodiments, an adenoviral (U.S. Pat. Nos. 6,383,795; 6,328,958 and 6,287,571, each specifically incorporated herein by reference); retroviral (U.S. Pat. Nos. 5,955,331; 5,888,502; and 5,830,725, each specifically incorporated herein by reference); Herpes-Simplex Viral (U.S. Pat. Nos. 5,879,934 and 5,851,826, each specifically incorporated herein by reference in its entirety); Adeno-associated virus (AAV); poxvirus (e.g., vaccinia virus); alpha virus (e.g., sindbis virus; Semliki forest virus); reovirus and influenza virus (type A or B or C); Chimeric poxviral/retroviral vectors; adenoviral/retroviral vectors and adenoviral/adeno-associated viral vectors, expression vectors are contemplated for the delivery of expression constructs.

Generally, the DNA vaccine administered may be in an amount of determined by a medical professional based on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgment of the practitioner and may be peculiar to each subject and antigen.

The vaccine for eliciting an immune response against one or more viruses, may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a

subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

Determining an effective amount of one or more compositions of the present invention depends upon a number of factors including, for example, the nucleic acid molecule being administered, the age and weight of the subject, the precise condition requiring treatment and its severity, and the route of administration. Based on the above factors, determining the precise amount, number of doses, and timing of doses are within the ordinary skill in the art and will be readily determined by the medical professional, e.g., a physician or veterinarian.

Compositions of the present invention may include various salts, excipients, delivery vehicles and/or auxiliary agents as are disclosed, e.g., in U.S. patent application Publication No. 2002/0019358, published Feb. 14, 2002. Furthermore, compositions of the present invention may include one or more transfection facilitating compounds that facilitate delivery of polynucleotides to the interior of a cell, and/or to a desired location within a cell.

As used herein, the terms "transfection facilitating compound," "transfection facilitating agent," and "transfection facilitating material" are synonymous, and may be used interchangeably. It should be noted that certain transfection facilitating compounds may also be "adjuvants" as described infra, i.e., in addition to facilitating delivery of polynucleotides to the interior of a cell, the compound acts to alter or increase the immune response to the antigen encoded by that polynucleotide. Examples of the transfection facilitating compounds include, but are not limited to inorganic materials such as calcium phosphate, alum (aluminum sulfate), and gold particles (e.g., "powder" type delivery vehicles); peptides that are, for example, cationic, intercell targeting (for selective delivery to certain cell types), intracell targeting (for nuclear localization or endosomal escape), and ampipathic (helix forming or pore forming); proteins that are, for example, basic (e.g., positively charged) such as histones, targeting (e.g., asialoprotein), viral (e.g., Sendai virus coat protein), and pore-forming; lipids that are, for example, cationic (e.g., DMRIE, DOSPA, DC-Chol), basic (e.g., steryl amine), neutral (e.g., cholesterol), anionic (e.g.,

phosphatidyl serine), and zwitterionic (e.g., DOPE, DOPC); and polymers such as dendrimers, star-polymers, "homogenous" poly-amino acids (e.g., poly-lysine, poly-arginine), "heterogeneous" poly-amino acids (e.g., mixtures of lysine & glycine), co-polymers, polylactide-co-glycolide (PLG), polyvinylpyrrolidinone (PVP), poloxamers (e.g. CRL 1005) and polyethylene glycol (PEG). A transfection facilitating material can be used alone or in combination with one or more other transfection facilitating materials. Two or more transfection facilitating materials can be combined by chemical bonding (e.g., covalent and ionic such as in lipidated polylysine, PEGylated polylysine) (Toncheva, et al., Biochim. Biophys. Acta 1380(3):354-368 (1988)), mechanical mixing (e.g., free moving materials in liquid or solid phase such as "polylysine+cationic lipids") (Gao and Huang, Biochemistry 35:1027-1036 (1996); Trubetskoy, et al., Biochem. Biophys. Acta 1131:311-313 (1992)), and aggregation (e.g., co-precipitation, gel forming such as in cationic lipids+poly-lactide, and polylysine+gelatin). Each of the references cited in this paragraph is incorporated herein by reference in its entirety.

One category of transfection facilitating materials is cationic lipids. Examples of cationic lipids are 5-carboxyspermylglycine dioctadecylamide (DOGS) and dipalmitoyl-phophatidylethanolamine-5-carboxy- spermylamide (DPPES). Cationic cholesterol derivatives are also useful, including {3β-[N-N',N'-dimethylamino)ethane]-carbomoyl}-cholesterol (DC-Chol). Dimethyldioctdecyl-ammonium bromide (DDAB), N-(3-aminopropyl)-N,N-(bis-(2-tetradecyloxyethyl))-N-methyl-ammonium bromide (PA-DEMO), N-(3-aminopropyl)-N,N-(bis-(2-dodecyloxyethyl))-N-meth-yl-ammonium bromide (PA-DELO), N,N,N-tris-(2-dodecyloxy)ethyl-N-(3-amino)p- ropyl-ammonium bromide (PA-TELO), and N1-(3-aminopropyl)((2-dodecyloxy)eth-yl)-N2-(2-dodecyloxy)ethyl-1-piperazinaminium bromide (GA-LOE-BP) can also be employed in the present invention.

Other hydrophobic and amphiphilic additives, such as, for example, sterols, fatty acids, gangliosides, glycolipids, lipopeptides, liposaccharides, neobees, niosomes, prostaglandins and sphingolipids, may also be included in compositions of the present invention. In such compositions, these additives may be included in an amount between about 0.1 mol % and about 99.9 mol % (relative to total lipid), about 1-50 mol %, or about 2-25 mol %.

Additional embodiments of the present invention are drawn to compositions comprising an auxiliary agent which is administered before, after, or concurrently with the polynucleotide. As used herein, an "auxiliary agent" is a substance included in a composition for its ability to enhance, relative to a composition which is identical except for the inclusion of the auxiliary agent, the entry of polynucleotides into subject cells in vivo, and/or the in vivo expression of polypeptides encoded by such polynucleotides. Certain auxiliary agents may, in addition to enhancing entry of polynucleotides into cells, enhance an immune response to an immunogen encoded by the polynucleotide. Auxiliary agents of the present invention include nonionic, anionic, cationic, or zwitterionic surfactants or detergents, with nonionic surfactants or detergents being preferred, chelators, DNase inhibitors, poloxamers, agents that aggregate or condense nucleic acids, emulsifying or solubilizing agents, wetting agents, gel-forming agents, and buffers.

Certain compositions of the present invention can further include one or more adjuvants before, after, or concurrently with the polynucleotide. The term "adjuvant" refers to any material having the ability to (1) alter or increase the immune response to a particular antigen or (2) increase or aid an effect of a pharmacological agent. It should be noted, with respect to polynucleotide vaccines, that an "adjuvant," can be a transfection facilitating material. Similarly, certain "transfection facilitating materials" described supra, may also be an "adjuvant." An adjuvant may be used with a composition comprising a polynucleotide of the present invention.

The ability of an adjuvant to increase the immune response to an antigen is typically manifested by a significant increase in immune-mediated protection. For example, an increase in humoral immunity is typically manifested by a significant increase in the titer of antibodies raised to the antigen, and an increase in T-cell activity is typically manifested in increased cell proliferation, or cellular cytotoxicity, or cytokine secretion. An adjuvant may also alter an immune response, for example, by shifting from one primary IgG subclass to another, or by changing a primarily humoral or Th_2 response into a primarily cellular or Th_1 response, or switching from primarily CD4+ T cell response to CD8+ T cell response, or by modifying the activities of antigen presenting cells.

Nucleic acid molecules of the present invention, e.g., plasmid DNA, mRNA, linear DNA or oligonucleotides, may be solubilized in any of various buffers. Suitable

buffers include, for example, phosphate buffered saline (PBS), normal saline, Tris buffer, and sodium phosphate (e.g., 150 mM sodium phosphate). Insoluble polynucleotides may be solubilized in a weak acid or weak base, and then diluted to the desired volume with a buffer. The pH of the buffer may be adjusted as appropriate. In addition, a pharmaceutically acceptable additive can be used to provide an appropriate osmolarity. Such additives are within the purview of one skilled in the art. For aqueous compositions used in vivo, sterile pyrogen-free water can be used. Such formulations will contain an effective amount of a polynucleotide together with a suitable amount of an aqueous solution in order to prepare pharmaceutically acceptable compositions suitable for administration to a human.

Compositions of the present invention can be formulated according to known methods. Suitable preparation methods are described, for example, in Remington's Pharmaceutical Sciences, 16th Edition, A. Osol, ed., Mack Publishing Co., Easton, Pa. (1980), and Remington's Pharmaceutical Sciences, 19th Edition, A. R. Gennaro, ed., Mack Publishing Co., Easton, Pa. (1995). Although the composition may be administered as an aqueous solution, it can also be formulated as an emulsion, gel, solution, suspension, lyophilized form, or any other form known in the art. In addition, the composition may contain pharmaceutically acceptable additives including, for example, diluents, binders, stabilizers, and preservatives.

Another embodiment of the present invention is a kit comprising the nucleic acid molecules of the present invention packaged in suitably sterilized containers, e.g., ampules, bottles, vials, and the like, either in multi-dose or in unit-dosage forms. The containers are preferably hermetically sealed after being filled with a vaccine/medicine preparation. Preferably, the vaccines/medicines are packaged in a container having a label affixed thereto, which label identifies the vaccine/medicine, and bears a notice in a form prescribed by a government regulatory agency such as the United States Food and Drug Administration reflecting approval of the vaccine/medicine under appropriate laws, dosage information, and the like. The label preferably contains information about the vaccine/medicine that is useful to a health care professional administering the vaccine to a subject. The kit also preferably contains printed informational materials relating to the administration of the vaccine/medicine, instructions, indications, and any necessary required warnings.

The kits of the invention may also contain a second agent for the treatment of a viral infection. The second agent can be a vaccine or can be an agent useful in managing the symptoms of viral infection.

Before administering the vaccines of this invention to humans, efficacy testing can be conducted using animals. In an example of efficacy testing, mice are vaccinated by intramuscular injection. After the initial vaccination or after optional booster vaccinations, the mice (and negative controls) are monitored for indications of vaccine-induced specific immune responses. Methods of measuring specific immune responses are described in the examples and are known in the art.

EXAMPLES

It should be appreciated that the invention should not be construed to be limited to the examples that are now described; rather, the invention should be construed to include any and all applications provided herein and all equivalent variations within the skill of the ordinary artisan.

Example 1

In this study, a new DNA vaccination that delivers M1, a gene that is highly conserved among different subtypes of influenza viruses, with HA into the same cells simultaneously is reported. In vaccinated mice, the HA/M1 double insertion plasmids not only induced HA specific neutralizing antibody responses as analyzed by ELISA and HA inhibition test, but also elicited HA and M1 specific CD8 T cell responses when evaluated by ELISPOT and intracellular staining for IFN-γ and class-I tetramer staining. In addition, the pHA/M1 double insertion plasmid immunization enhanced virus-specific CTL activities and reduced viral loads in the lungs of vaccinated mice. Finally, the mice immunized with the pHA/M1 double insertion plasmids not only survived the lethal challenges of wild type homologous A/WSN/33 (H1N1), but also showed improved cross-protection against wild type heterologous A/Philippines/2/82/X-79 (H3N2) compared to the groups that received pHA or pM1 alone or the mixture of both plasmids as vaccines.

Influenza A viruses of the Orthomyxoviridae family are a group of enveloped RNA viruses containing eight segmented genes encoding for more than 10 proteins. The typical clinical manifestation of an influenza A virus infection in humans is flu,

which is characterized as an acute and highly contagious respiratory disease, sometimes even lethal especially in immune underdeveloped or compromised populations such as infants and elderly people.

The initial infection occurs in host's upper respiratory tract when influenza A viruses attach to sialic acid on the surface of epithelial cells through one of the major viral surface glycoproteins, hemagglutinin (HA). Following quick endocytosis, influenza A viruses transcript viral RNAs inside the nucleus of host cells. Newly synthesized viral components are then transported to the cell cytoplasm membrane for assembling. Mature virions are released by removing sialic acid from the cell surface with the help of neuraminidase (NA), another major viral surface glycoprotein.

Traditional influenza vaccines have been focused on HA and NA, not only because of their availability, but also because of their excellent immunogenicity. For instance, anti-HA antibodies neutralize infectivity of influenza A viruses, while anti-NA antibodies prevent the release of virus particles. However, influenza A viruses undergo point mutations in HA and NA genes rapidly resulting in antigenic variations every year (antigenic drift). To cope with the emergence of these variant influenza virus strains, the World Health Organization (WHO), government health agencies and pharmaceutical industries work together to predict and re-evaluate candidate strains for influenza vaccine manufacture annually. But sometimes because of genetic reassortment between human virus and animal virus after a double infection in an animal host, a new strain of influenza A virus arises unexpectedly with marked changes in their HA or NA antigenicity (antigenic shift). This could be extremely lethal and may cause thousands or even millions of human lives, such as the 1918 Spainish A/H1N1, the 1957 Asian A/H2N2, and the 1968 Hong Kong A/H3N2 strains. The estimated death toll for the next potential pandemic has projected 89,000 to 207,000 lives in the US according to the model by the Center for Disease Control (CDC). The recent outbreaks of avian influenza (H5N1) in Southeast Asia even further deepened the anxiety globally. How we are going to be better prepared for the next pandemic will be on the top of our first priorities.

Recent strategies have been focused on some of the evolutionally conserved viral genes in influenza A viruses, such as nucleoprotein (NP) and the matrix gene (M) encoding a major structural M1 protein and a minor M2 protein in viral particles. Plasmids encoding NP or M1 genes have been shown to raise antigen-specific

cytotoxic T cell responses, and M2 peptide based vaccines were also able to provide protective neutralizing antibodies against influenza A viruses (Crowe S, Vaccine 2005, Ozaki, Viral Immunol 2005, Epstein S, Vaccine 2005, Neirynck S, Nat. Med. 1999).

Materials and Methods

Mice. Four-week-old female Balb/c (H-2^d) mice were purchased from National Cancer Institute (Frederick, MD), and were housed in sterile micro-isolator cages with water and feed *ad libitum*. All the animal experiments were conducted in a BSL-2 facility using ACUC approved protocols.

Viruses. Mouse-adapted influenza viruses A/WSN/33 (H1N1), and A/Philippines/2/82/X-79 (A/Phil, H3N2) were grown in the allantoic cavities of 9-day-old embryonated hen's eggs at 33^{0}C. Three days later, propagated viruses were harvested and stored at -70^{0}C until use. The infectivities of viruses were determined by plaque assay using Madin-Darby canine kidney (MDCK) cells, and were expressed as plaque forming unit (PFU) per milliliter.

Viral protein expression plasmids. Plasmids pBUDCE4.1-HA (pHA), pBUDCE4.1-M1 (pM1), and pBUDCE4.1-HA/M1 (pHA/M1) expressing influenza viral proteins HA, or M1 alone, or both were constructed using pBUDCE4.1 expression vector. The full-length HA cDNA copy of A/WSN/33 and M1 cDNA copy of A/PR/8/34 (a laboratory adapted vaccine internal gene donor strain) were included (Figure 1). Both HA and M1 genes were under the control of cytomegalovirus (CMV) immediate-early promoter in pHA and pM1, respectively, except in pHA/M1, the HA gene is driven by CMV promoter while the M1 gene is driven by human EF-1α promoter unless specified otherwise.

All plasmids were amplified in DH5α *E.coli* according to the manufacture's instructions and purified using endotoxin-free Maxiprep kit (Qiagen, Valencia, CA). The large-scale preparation of ultra pure and endotoxin free plasmids was provided by Elim Biopharmaceuticals Inc. (Hayward, CA). All the plasmids were confirmed by restriction enzyme digestions and sequencing analysis.

Immunofluorescence staining. MDCK cells grown on glass coverslips were transfected with the plasmids (pHA, pM1 or pHA/M1) using Lipofectamine 2000 (Invitrogen) and incubated in MEM with 2% FBS at 37^{0}C for 6 hr. The transfected MDCK cells were fixed with fresh prepared 4% formaldehyde in PBS at room temperature for 20 min followed by blocking with 3% nonfat powder milk in PBS for 1 h. The MDCK cells were then incubated with pooled monoclonal antibody to HA or M1, respectively at room temperature for 40 min as described before (Liu, virology 2002 for HA; JV 2002 for M1), and subsequently with donkey anti-mouse IgG conjugated with FITC for another 40 min. After thoroughly washing, the coverslips that contained transfected MDCK cells were mounted in 90% glycerol and 10% PBS in 3, 4, 5-trihydroxybenzoic acid *N*-propylester before visualized under an epifluorescent UV microscope.

Immunizations and Challenges. Mice were immunized three times in two-week intervals via subcutaneou (*s.c.*) route. Each mouse received a suboptimal dose (20 μg) of the following plasmids in 50 μl sterile PBS every time: (1) pHA, (2) pM1, (3) pHA/M1, or (4) the mixture of pHA and pM1 (20 μg/plasmid in total 50 μl PBS). A group of mice,vaccinated with 20 μg of pBUDCE4.1 empty vectors per mouse per time every two weeks were served as controls.

Two weeks after third immunization, mice were challenged with wild-type A/WSN/33 or wild-type A/Phil intranasally (*i.n.*) under light anesthesia of methoxyflurane. Mice were then monitored daily for BW changes and mortality for at least 14 days. For some experiments, the lungs of infected mice were harvested on day 4 post challenges and viral titers were determined by plaque assay as described above.

Virus-specific Antibody Response and HA Inhibition Assay. Serum samples were collected by tail-bleeding right before challenges and stored at -20^{0}C until use. Virus-specific antibodies in DNA immunized mice were analyzed by ELISA using sucrose gradient purified wild-type A/WSN/33 viruses as coating antigens (1 μg/well). After blocking with 5% nonfat milk powder in PBS containing 0.1% tween-20, the viruses-coated 96-well Immulon2 plates (ThermoLabSystems, Franklin, MA) were incubated with serially diluted serum samples at 37^{0}C for 2 h.

The plates were then washed thoroughly followed by incubation with secondary horse radish peroxidase labeled anti-mouse IgG, IgG1, and IgG2a (Southern Biotechnology). The color was developed by ABTS substrate (Kirkegaard & Perry) and read at 405 nm with an ELISA reader. The pooled hyper-immune sera from mice infected with a sublethal dose of wild-type A/WSN/33 were used as the standard. Antibody titers were expressed as the reciprocal of the end point dilution that yielded an OD value at least twice background. All samples were analyzed in duplicate.

HA inhibition assay was performed using chicken RBCs after sera were individually treated with receptor-destroying enzyme at 37^0C overnight followed by heat-inactivation at 56^0C for 30 min. HA inhibition titers were defined as the reciprocal of the highest dilution that yielded a complete inhibition of hemagglutination caused by 4 HA units of wild-type A/WSN/33.

Cytokine ELISPOT Analysis. Two weeks after 2nd booster immunization, single splenocyte suspension was prepared in RPMI1 1640 supplemented with 10% FBS, L-glutamine, nonessential amino acids, sodium pyruvate, HEPES buffer, β-mercaptoethanol, and penicillin/streptomycin. H-2^d restricted HA class I peptide (LYEKVKSQL) (Deng Y & Bennink J, JI 1997 158: 1507-1515; Oran A. & Robinson H, JI 2003 171: 1999-2005), and H-2^d restricted M1 peptide (KAVKLYRKLKRE) (Okuda K & Ihata A, Vaccine 2001 19: 3681-3691) were synthesized by the Core facility of Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, MD with more than 75% purity. Both peptide sequences were shared by wild type A/WSN/33 and A/PR8. Serial dilutions of splenocytes were added to anti-mouse IFN-γ (BD PharMingen, San Diego, CA) pre-coated 96-well plates, and were incubated with 25 μg/ml of H-2^d restricted HA or M1 class I peptides in the presence of 1 μg/ml anti-mouse CD28 (BD PharMingen). Because costimulatory anti-CD28 has been reported to enhance peptide-specific cytokine responses in ELISPOT assays (Oran A & Robinson H, JI , 2003, 171: 1999-2005), therefore a final concentration of 1μg/ml anti-mouse CD28 was included in all experiments unless other specified. After incubating at 37^0C, 5% CO2 for 36 h, the plates were extensively washed and then incubated with secondary biotin-labeled anti-mouse IFN-γ (BD PharMingen) at room temperature for anther 2 h. Following repeated washing, the plates were then treated with streptavidin-alkaline phosphatase

and developed with NBTS in the presence of 6% agarose. Spots were counted under a microscope and expressed as fold changes over background (vector pB immunized group without peptide stimulation *in vitro*).

MHC Class I Tetramer Stainings and Flow Cytometry. Recombinant soluble dimeric mouse H-2L^d:Ig fusion protein (BD PharMingen) was incubated with a 160-molar excess of H-2^d restricted HA or M1 class I peptides at 37^{0}C overnight. Peptide-loaded H-2L^d:Ig fusion protein was then used for immunofluorescent staining of mouse splenocytes along with FITC labeled anti-CD3, PE-Cy5 labeled anti-CD8α, and PE-Cy7 labeled anti-CD69 followed by PE labeled A85-1 monoclonal antibody (BD PharMingen). Splenocytes were also stimulated *in vitro* with heat-inactivated (HI) A/WSN/33 (500 HA units/ml) in the presence of anti-CD28 and GolgiPlug for 6 h, and then evaluated by intracellular staining using PE-labeled anti-IFN-γ antibodies (BD PharMingen). The stained splenocytes were analyzed on a BD LSR II with FACSDiva software (Becton Dickinson).

Cytolytic activity analysis. Mouse splenocytes were stimulated *in vitro* with HI A/WSN/33 (500 HA units/ml) pulsed synergistic mouse splenocytes in the presence of 2.5 μg/ml ConA for 5 days to generate effector cells (effector : stimulator = 10:1). Mouse P815 cells labeled with CFSE (Molecular Probes) were pulsed with HI A/WSN/33 and then incubated with effector cells at 37^{0}C for 5 h. After briefly stained with 7-AAD (Molecular Probes) in ice, the portion of CFSE and 7-AAD double positive P815 cells were resolved by a BD LSR II flow cytometer.

Statistical Analysis. Virus-specific antibody titers were expressed as geometric mean ± SEM. Survivals after lethal challenges of WT heterologous influenza viruses were assessed by Log-Rank analysis. A Student's *t*-test was used to compare pHA/M1 group with vector vaccinated control mice. A P value < 0.05 was considered statistically significant.

Results

Expression and co-localization of HA and M1 proteins in pHA/M1 transfected MDCK cells. The intracellular distributions of both HA and M1 proteins

in plasmid transfected MDCK cells were demonstrated by immunofluorescent staining with anti-HA or anti-M1 specific polyclonal antibodies (Figure 1). As seen in pHA transfected MDCK cells (Figure 1B), HA showed clear cell surface distribution by anti-HA polyclonal antibody staining, indicating that a biologically mature HA protein was expressed in vitro. Similar to pHA transfected cells, double insertion plasmid pHA/M1 transfected MDCK cells showed strong HA staining not only on cell surface but also in cytoplasm, suggesting a functional HA protein was produced in transfected cells (Figure 1D). In contrast to HA which is a cell surface glycoprotein, M1 is an internal protein that traffics from nucleus to cell surface for virion assembly and budding. Using anti-M1 polyclonal antibody, the intracellular distribution of M1 protein was observed mainly in the nucleus, and to a less extent, in the cytoplasm and membrane of either pM1 or pHA/M1 transfected cells after permeabilization with detergent (Figures 1C & E). After merging 1D & E, the co-localization of HA and M1 proteins in pHA/M1 transfected MDCK cells was seen clearly in the cytoplamic area, around nuclear membrane/Golgi apparatuses, and even on the cell surface as shown in Figure 1F, suggesting the association between M1 and HA proteins.

Virus-specific antibody responses induced by immunization with pHA/M1 double insertion plasmid. Previous studies by other groups have shown that HA or M1 based DNA vaccinations could induce protective immune responses in mice against the lethal challenges of influenza viruses of the same origin (Okuda K & Ihata A, Vaccine 2001 19:3681; Ozaki T & Okuda K, Viral Immunol 2005). In order to demonstrate the protective potential of pHA/M1 double insertion plasmid, we have administered Balb/c mice with suboptimal doses of either pHA or pM1 alone, or pHA/M1 double insertion, or the mixture of pHA and pM1 (pHA+pM1) in the current study. The empty vector and HI wild type A/WSN/33 (HI WSN) were used as negative and positive controls, respectively. As expected, virus-specific serum IgG responses were induced in all the experimental groups except the vector vaccinated negative controls (Figure 2). When comparing among all the DNA plasmids immunized groups, the increases were most marked for IgG2a and HA-specific neutralizing titers in the mice that received double insertion pHA/M1 plasmid vaccinations (Figures 2C & D).

Antigen-specific CD8+ T cell responses induced by pHA/M1 double insertion plasmid immunizations. One of the advantages of DNA vaccines is their ability to generate MHC class I-restricted CTL responses, which is crucial to effectively eliminate viruses and other intracellular pathogens. To assess antigen-specific CD8+ T cell responses induced by pHA/M1 double insertion plasmid DNA, a series of tests have been performed. As revealed by IFN-γ ELISPOT assay in Figure 3A, pHA/M1 double insertion plasmid DNA immunization induced strong IFN-γ producing T cell responses after restimulation with HA-specific, to a less extent, M1-specific MHC-class I peptides *in vitro*. This was confirmed by IFN-γ intracellular staining for CD3+ and CD8+ double positive T cells (Figure 3B).

Using tetramer staining, T cells specific for both HA and M1 class-I epitopes were readily detected in the spleens of all experimental mice on day 4 post infection including the vector vaccinated group. However, these HA and M1 class-I epitopes specific T cells were greatly enriched in the pHA/M1 double insertion plasmid vaccinated mice (HA/L^d, 6.2% and $M1/L^d$, 3.9% in pHA/M1 group vs HA/L^d, 3.0% and $M1/L^d$, 1.5% in vector group, respectively) (Figure 4A). Compared to other immunization groups, a greater portion of these HA or M1 class-I epitopes specific CD8+ T cells was already activated in the pHA/M1 double insertion plasmid vaccinated mice as revealed by staining for CD69, an early activation marker for T cells (Figure 4A).

In parallel with these enhanced antigen-specific CD8+ T cell responses, the pHA/M1 double insertion plasmid vaccination also improved their virus-specific cytolytic activities as shown in Figure 4B.

Protections induced by immunizations with pHA/M1 double insertion DNA plasmid. To determine the immunization conferred by the pHA/M1 double insertion plasmid immunization, mice were challenged with two lethal doses of wild type homologous A/WSN/33 viruses. The vector immunized controls showed signs of severe infection and progressive body weight loss at both lethal doses of homologous challenges (Figures 5A&B). The pHA/M1 double insertion plasmid group showed no signs of body weight loss after a homologous challenge with 5×10^4 PFU/mouse, but had approximate 20% body weight loss at a 100-fold higher dose of wild type A/WSN/33 challenge, which was quickly recovered after day 6 post-infection. In

contrast to the rest of groups yielded either partial (< 30%) or none protections, pHA/M1 double insertion plasmid vaccinated mice had 100% survivals at both lethal doses of homologous challenges.

In addition to its protection against influenza viruses of the same origins, the potential of pHA/M1 double insertion plasmid DNA to provide cross-protections against heterologous challenges has also been determined. While all the groups showed similar body weight loss during the initial week post-infection with wild type heterologous influenza virus A/Phil (H3N2) at 5 X 10^5 PFU/mouse, only pHA/M1 double insertion plasmid vaccinated group had more than 80% survival later (Figure 5C).

The pHA/M1 double insertion plasmid DNA vaccination also led to significant reductions of viral loads in the lungs on day 4 post-infection of wild type homologous A/WSN/33 (Figure 6A) or heterologous A/Phil (Figure 6B) when compared to vector immunized controls (Figure 6). The pHA immunized group also had reduced viral loads in lungs after a homologous challenge, but showed no differences from the vector vaccinated mice after a heterologous challenge. This indicated that the enhanced neutralizing responses by pHA/M1 double insertion DNA plasmid were likely due to co-expression of M1 with HA antigens. However, surprisingly pM1 alone immunized group had even higher lung titers than vector treated controls after both homologous and heterologous virus challenges.

Discussion

Currently the licensed influenza vaccines consist of two types: inactivated and live-attenuated. Compared to inactivated vaccines, live-attenuated vaccines may be more cross-protective, but also have greater risks to revert to a new pathogenic strain (Stech J & Garn H, Nat Med 2005 11:683). The improved DNA vaccine technology discussed herein, offers advantages over both inactivated and live-attenuated vaccines. Depending on antigens expressed and routes/means of administration, both neutralizing antibodies and cytotoxic T cell responses can be induced, the latter of which is crucial for efficient clearance of intracellular viruses and bacteria (Donnelly J & Liu M, JI 2005 175:633). Unlike live-attenuated vaccines with potential revertants, DNA vaccines have broader recipients and can be applied to immune compromised populations. Furthermore, in contrast to traditional vaccines

which development and manufacture are very time-consuming, DNA vaccines offer great flexibility and time efficiency in terms of the emergence of a live-threatening strain (Forde G, Nat Biotech 2005 23;1059).

One of the main goals of this study is to develop cross-protective vaccines against influenza A viruses. M1 is highly conserved among different subtypes of influenza A viruses and has been shown to be an ideal target for generating virus-specific CD8+ T cell responses (Okuda K & Ihata A, Vaccine 2001 19:3681; Ozaki T & Okuda K, Viral Immunol 2005). Human memory CD8+ T cells specific for M1 epitope have be been found to persist after acute infection with characteristics of both immature and mature phenotypes and expressing certain chemokine receptors (Hoji, A, Immunol 2005). In the current study, vaccination with a single plasmid DNA expressing both HA and M1 proteins simultaneously not only showed improved immune responses than individual plasmids or their mixtures, but also protected mice from the lethal challenges of both homologous and heterologous viruses. By expressing both HA and M1 proteins together through a single plasmid, it is assured to deliver both antigens into the same cells simultaneously. Transfections by mixtures of two or more plasmids may cause reduced expression efficiencies due to limited uptake capability of target cells and possible toxicity of co-transfected plasmids (data not shown). In addition, newly expressed mature M1 protein may also assemble virus-like particles along with co-expressed HA, hence might further enhance both antigens' uptake and process by professional antigen presenting cells. In the present study, we had failed to isolate virus-like particles directly in pHA/M1 transfected MDCK or vero cells *in vitro* (data not shown). This could be because the expression system we used was relatively weak to generate a large quantity of virus-like particles for electron microscopy analysis. However, the ability of forming virus-like particles by M1 gene *in vitro* has been demonstrated before in some overexpressing systems using either vaccinia virus or baculovirus recombinants (Gomez-Puertas P & Albo C, JV 2000 74:11538–11547; Latham T & Galarza J, JV 2001 75:6154). Recently Galarza *et al.* have reported that virus-like particles containing M1 and HA isolated from Sf9 insect cells were protective against homologous virus challenges with or without IL-12 as adjuvant (Galarza J, Virol Immunol 2005). Despite the success by purified virus-like particles formed by M1 and HA, this approach has the risks of potential contamination

from its insect expressing system, whereas the expressing system in our study is safe for human use.

During the natural course of influenza virus infection, viruses replicate rapidly in lungs and reach a maximal level within 3-5 days of infection, whereas virus-specific CD8+ T cells begin to traffick into lungs in the meantime. Following the continuous infiltration of virus specific CD8+ T cells into lungs, virus titers drop quickly and infectious virus particles will not be detected by the time when pulmonary CD8+ T cells peak on day 9-12 post infection (Eichelberger M & Doherty P, J Exp Med 1991 174:875; Turner S & Doherty P, JI 2001 167:2753; Wiley J & Harmsen A, JI 2001 167:3293; Lawrence C & Braciale T, JI 2005 174:5332). It has been suggested that, the delayed kinetics of virus specific CD8+ T cells relative to virus replications in infected lungs indicates the early CD8+ T cell response is crucial to contain virus replication (Lawrence C & Braciale T, JI 2005 174:5332). Although pHA or pM1 DNA alone protected mice from a low dose of homologous challenge, the pHA/M1 double insertion plasmid is more effective as indicated by higher level of HA-specific neutralizing antibodies raised, and more HA and M1 specific CD8+ T cells stimulated. As demonstrated by tetramer and intracellular IFN-γstainings, more HA and M1 specific CD8+ T cells were activated in pHA/M1 double insertion plasmid DNA vaccinated mice on day 4 post wild type challenges. While the viral titers in the lungs of these HA/M1 double insertion plasmid vaccinated mice were lower than vector vaccinated control groups at the same time This is in agreement with the observations published by other groups that, the clearance of viruses in lungs begins on day 5-7 post infection, which correlates with the early migration of virus-specific CD8+ T cells before the maximal pulmonary accumulation of effector CD8+ T cells by day 9-12 post infection (Bennink J & Doherty P, Immunol 1978 35:503; Lawrence C & Braciale T, JI 2005 174:5332; Price G & Moskophidis D, JV 2005 79;8545). The improved humoral and cellular immune responses by pHA/M1 double insertion plasmid are due to the co-expression of both HA and M1 by the same target cells, since vaccination with the mixture of pHA and pM1 had no better effects than pHA/M1 double insertion. In addition to forming virus-like particles by M1, on the other hand, the stability of membrane-bound M1 can also be enhanced by co-expressed HA through the interaction with the cytoplasmic tail and transmembrane domain of HA (Ali et al. Virology 2000, Barman et al., Virus Res 2001). The stable

presentation of both antigens on the cell surface may be beneficial for prolonged CD8+ T cell priming. Co-expression of M1 and HA also leads to co-localization of both proteins in Golgi region and the exocytic transport of glycoproteins (Barman et al., Virus Res 2001). Considering M1 is the essential force for nuclear transport and virion assembling, co-expression and co-localization of M1 with HA might be more efficiently to present mature HA apically than cells expressing HA alone. For instance, M1 may play an important role in support of nonpalmitoylated H3 HA mediated membrane fusion (Chen B & Lamb J, JV 2005 79: 13673) the implication of which remains to be explored.

In addition, it was observed that the pM1 alone immunized group had even higher lung titers than vector treated controls consistently after both homologous and heterologous virus challenges, even though the vaccination by pM1 had moderate protection against the challenge of a lower dose A/WSN/33 in the present study. This phenomenon seems inconsistent with the physiological behaviors of M1, which has been reported to inhibit transcriptase activity of ribonucleoprotein cores from virions, thus may downregulate endogenous viral RNA replication (Ye Z & Wagner R, JV 1989 63:3586; Perez D & Donis R, Virology 1998 249:52). One possibility is that M1 might be also involved in virus escape mechanisms since it is indispensable for virion assembly and budding.

The instant experiments provide a novel strategy of DNA vaccination against viral infection. Specifically, the experiments provide exemplary methods and compositions for the vaccination of subjects against viruses, e.g., influenza A virus by expressing both HA of A/WSN/33 (H1N1) and M1of A/PR/8/34 (H1N1) through a single plasmid. The data indicates that vaccination by pHA/M1 double insertion plasmid not only enhanced virus-specific neutralizing antibody titers, but also augmented virus-specific CD8+ T cell responses. More importantly, pHA/M1 double insertion DNA vaccination reduced the viral loads in lungs and protected mice from lethal H1N1 and H3N2 influenza A virus challenges. Ongoing studies are planned to extend the current findings to life threatening influenza A virus strains such as H5N1 and other avian influenza viruses by replacing the corresponding HA genes into the plasmid. The strategy suggested in the current study may provide an alternative other than traditional approaches to combat pandemic influenza virus outbreaks.

Incorporation by Reference

The contents of all references, patents, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A nucleic acid molecule comprising a nucleic acid segment encoding a viral surface protein and a nucleic acid segment encoding a viral matrix protein.

2. The nucleic acid molecule of claim 1, wherein the viral surface protein is a protein from a virus selected from the group consisting of an Orthomyxoviridae virus, a Retroviridae virus, a Herpesviridae virus, a Poxviridae virus, a Flaviviridae virus, a Paramyxoviridae virus, a Rhabdoviridae virus, and a Filoviridae virus.

3. The nucleic acid molecule of claim 1, wherein the matrix protein is a protein from a virus selected from the group consisting of an Orthomyxoviridae virus, a Retroviridae virus, a Herpesviridae virus, a Poxviridae virus, a Flaviviridae virus, a Paramyxoviridae virus, a Rhabdoviridae virus, and a Filoviridae virus.

4. The nucleic acid molecule of claim 1, wherein the viral surface protein is a glycoprotein.

5. The nucleic acid molecule of claim 2, wherein the glycoprotein is an influenza glycoprotein.

6. The nucleic acid molecule of claim 5, wherein the glycoprotein is hemagglutinin, or an immunogenic fragment thereof.

7. The nucleic acid molecule of claim 6, wherein the hemagglutinin is selected from the group consisting of hemagglutinin subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16.

8. The nucleic acid molecule of claim 7, wherein the hemagglutinin is from subtype H5.

9. The nucleic acid molecule of claim 5, wherein the glycoprotein is neuramidase or an immunogenic fragment thereof.

10. The nucleic acid molecule of claim 9, wherein the neuramidase is selected from the group consisting of neuramidase type N1, N2, N3, N4, N5, N6, N7, N8 and N9.

11. The nucleic acid molecule of claim 1, wherein the viral matrix protein is an influenza matrix protein.

12. The nucleic acid molecule of claim 11, wherein the influenza matrix protein is M1 or M2.

13. The nucleic acid molecule of claim 4, wherein the glycoprotein is a HIV glycoprotein.

14. The nucleic acid molecule of claim 13, wherein the glycoprotein is gp120.

15. The nucleic acid molecule of claim 1, wherein the viral matrix protein is a HIV matrix protein.

16. The nucleic acid molecule of claim 15, wherein the HIV matrix protein is MA.

17. A nucleic acid molecule comprising a nucleic acid segment encoding an influenza hemagglutinin protein and a nucleic acid segment encoding the influenza M protein.

18. The nucleic acid molecule of claim 17, wherein the hemagglutinin is H3 and the M protein is M1.

19. A nucleic acid molecule comprising a nucleic acid segment encoding the HIV gp120 protein and a nucleic acid segment encoding the HIV MA protein.

20. A vector comprising the nucleic acid molecule of any one of claims 1-19.

21. A DNA vaccine comprising the nucleic acid molecule of any one or more of claims 1-20.

22. A method of vaccinating a subject against a virus comprising:
administering to the subject an effective amount of a nucleic acid molecule of any one or more of claims 1-20.

23. A method for inducing in a subject an immune response against influenza infection comprising administering to the subject an immunologically effective amount a nucleic acid molecule comprising a segment encoding a influenza glycoprotein and a segment encoding an influenza matrix protein.

24. The method of claim 23, wherein the influenza glycoprotein is hemagglutinin or neuramidase.

25. The method of claim 23, wherein the matrix protein is M1 or M2.

26. A method of vaccinating a subject against HIV comprising:
administering to the subject an effective amount of a nucleic acid molecule encoding a viral surface protein and a nucleic acid molecule encoding a viral matrix protein.

27. The method of claim 26, wherein the viral surface protein is an HIV glycoprotein.

28. The method of claim 27, wherein the glycoprotein is gp120.

29. The method of claim 26, wherein the viral matrix protein is a HIV matrix protein.

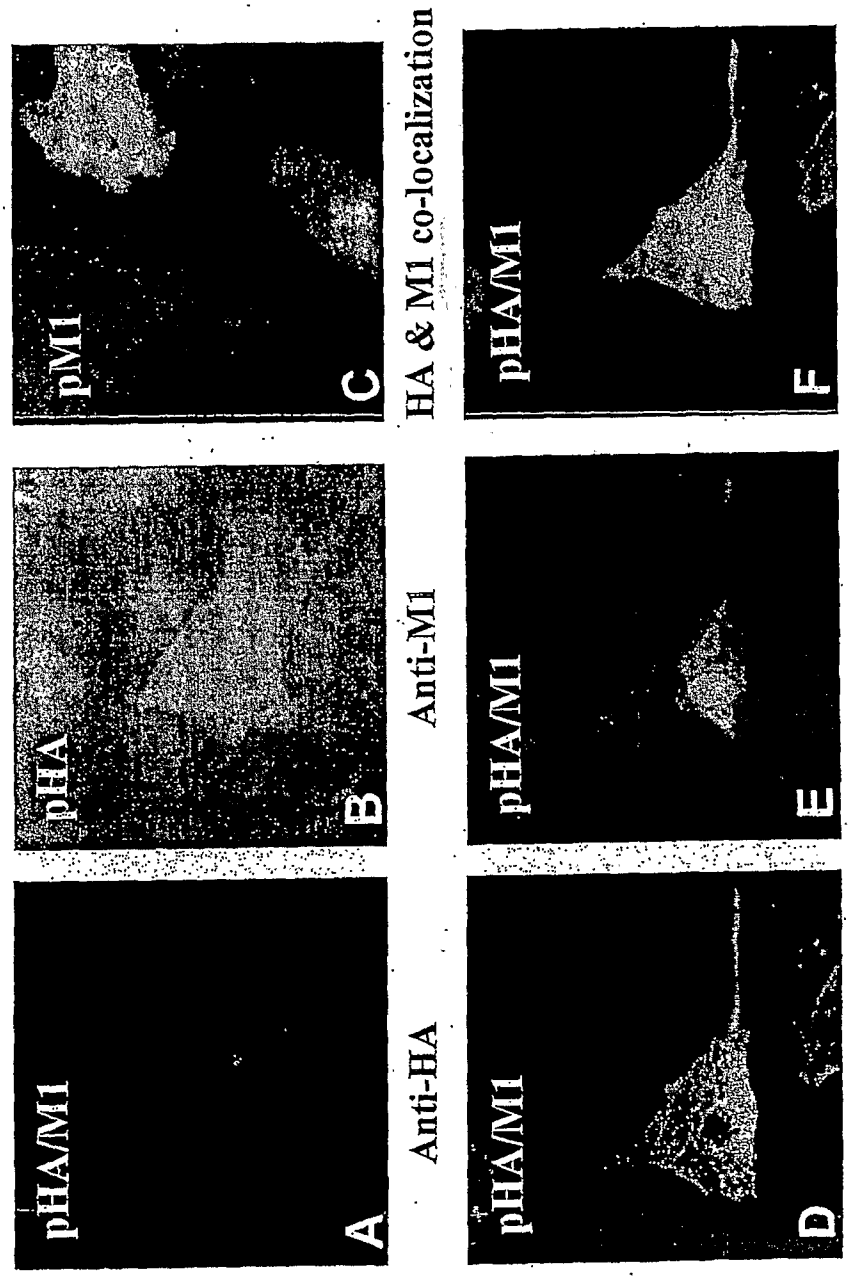
30. The method claim 29, wherein the HIV matrix protein is MA.

31. A method of vaccinating a subject against HIV comprising:

administering to the subject a nucleic acid molecule comprising a nucleic acid molecule comprising a nucleic acid segment encoding an HIV gp120 protein and a nucleic acid segment encoding the HIV MA protein.

32. A kit comprising the nucleic acid molecule of any one of claims 1-20 and instructions for use.

Figure 1



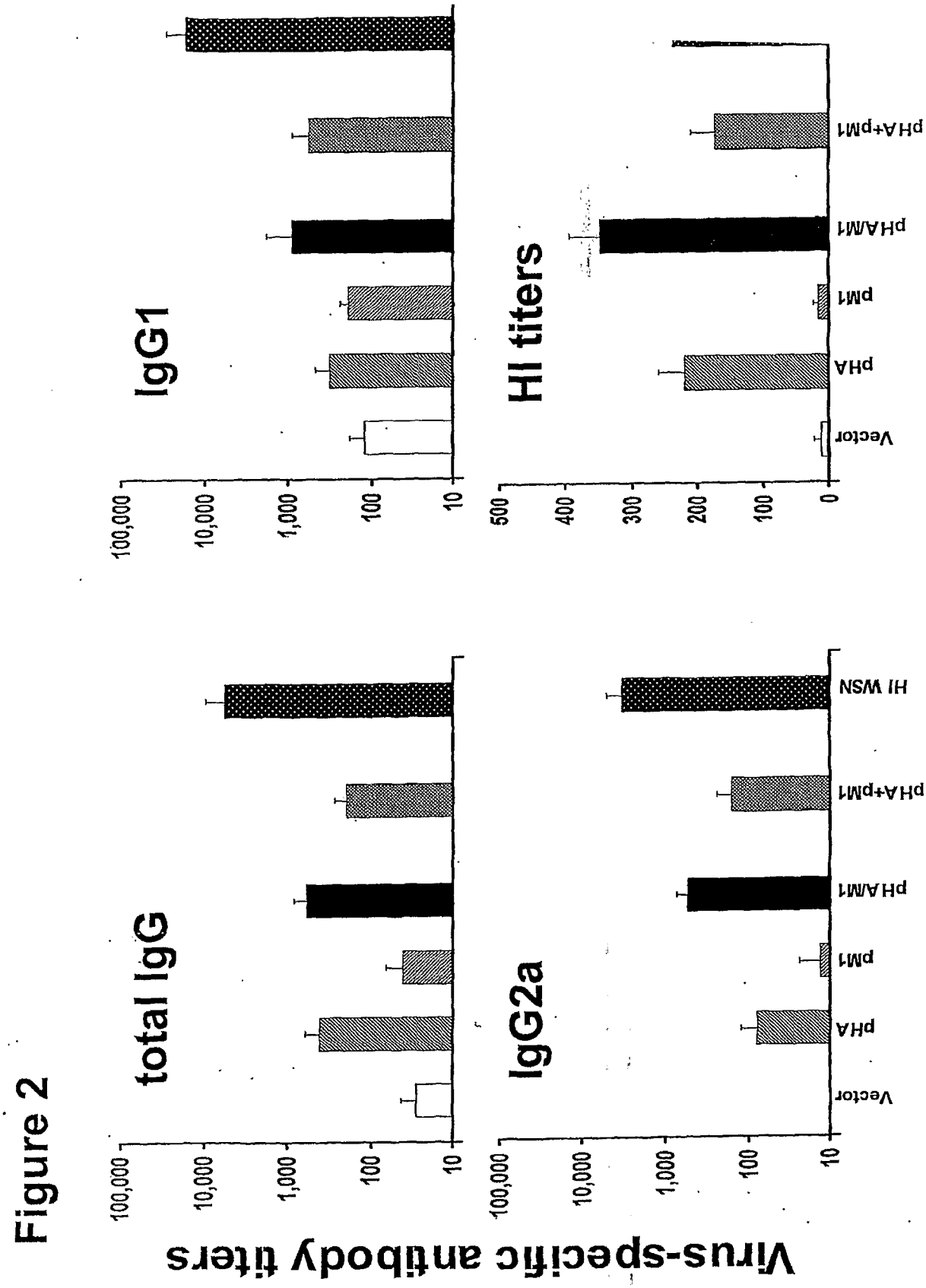
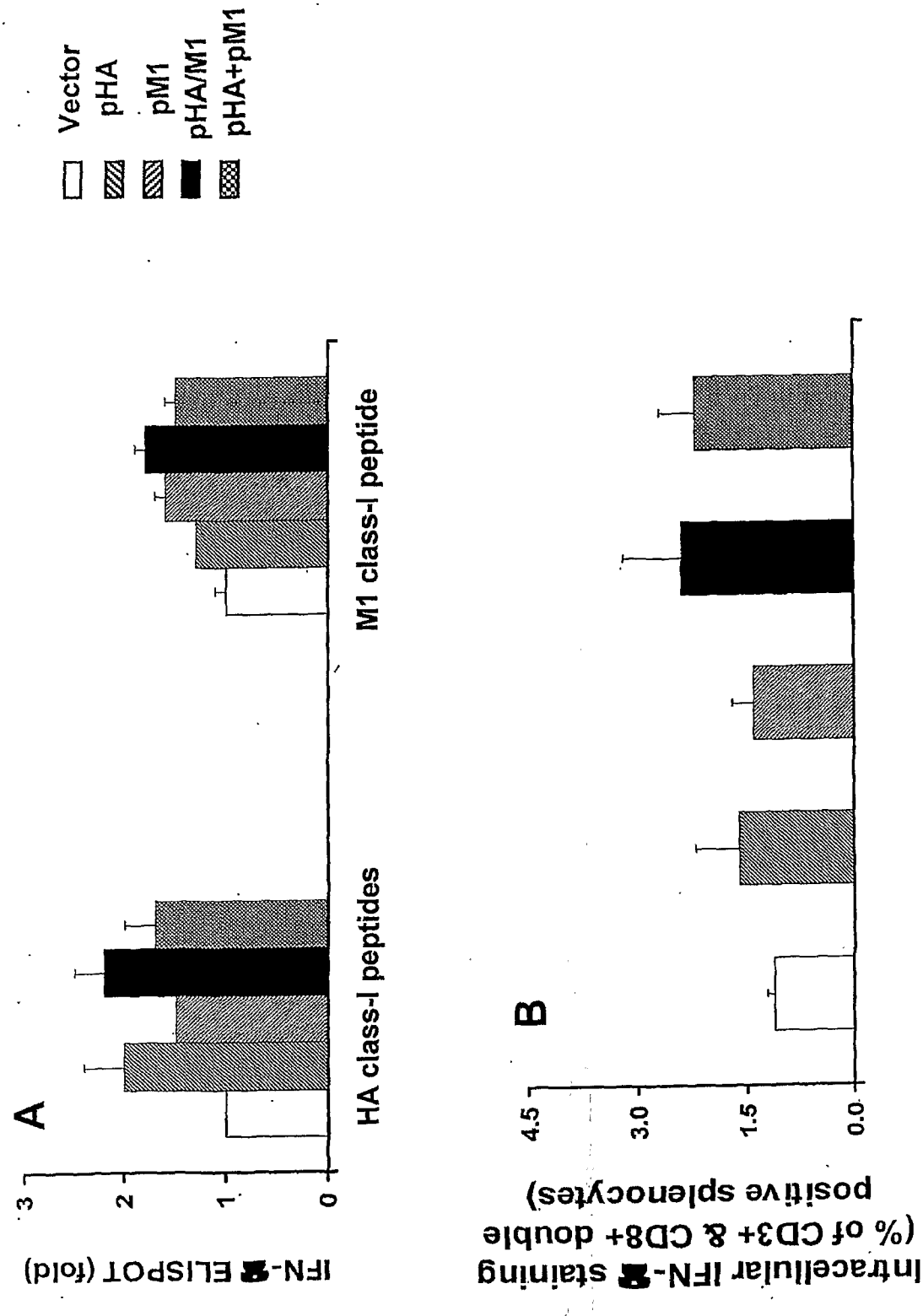


Figure 2

Figure 3



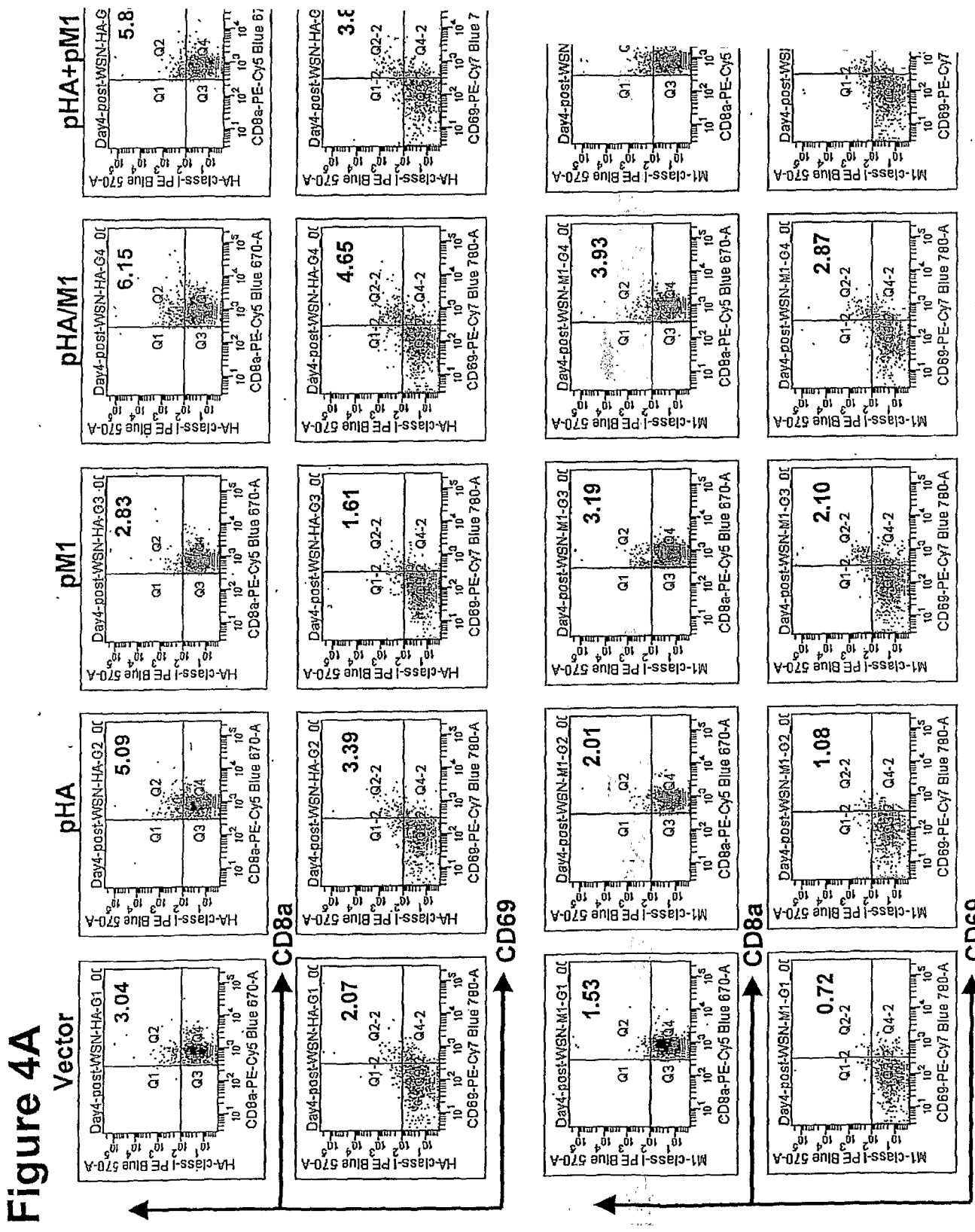


Figure 4A
Vector
pHA
pM1
pHA/M1
pHA+pM1
CD8a
CD69
HA class-I tetramer
M1 class-I tetramer

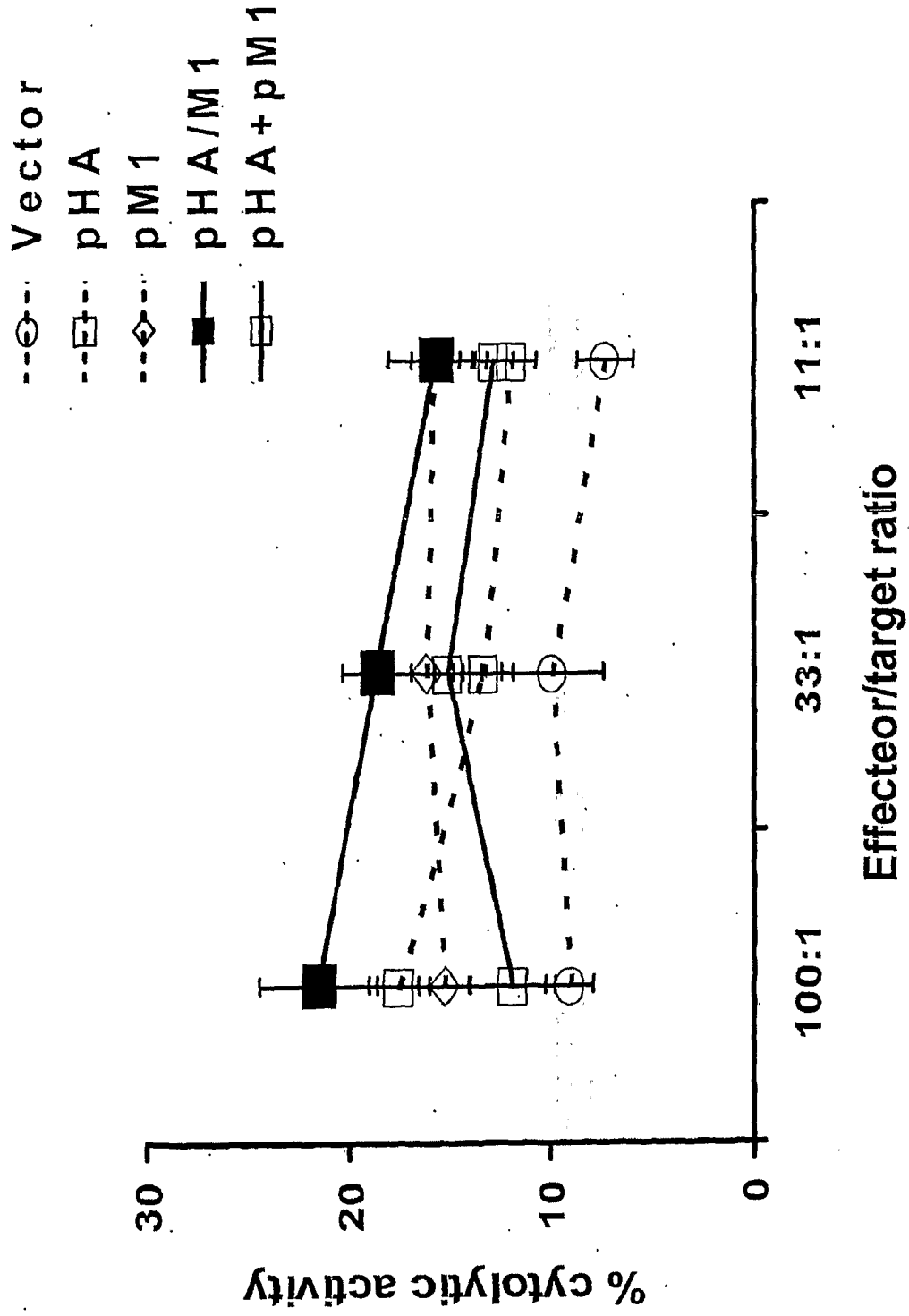
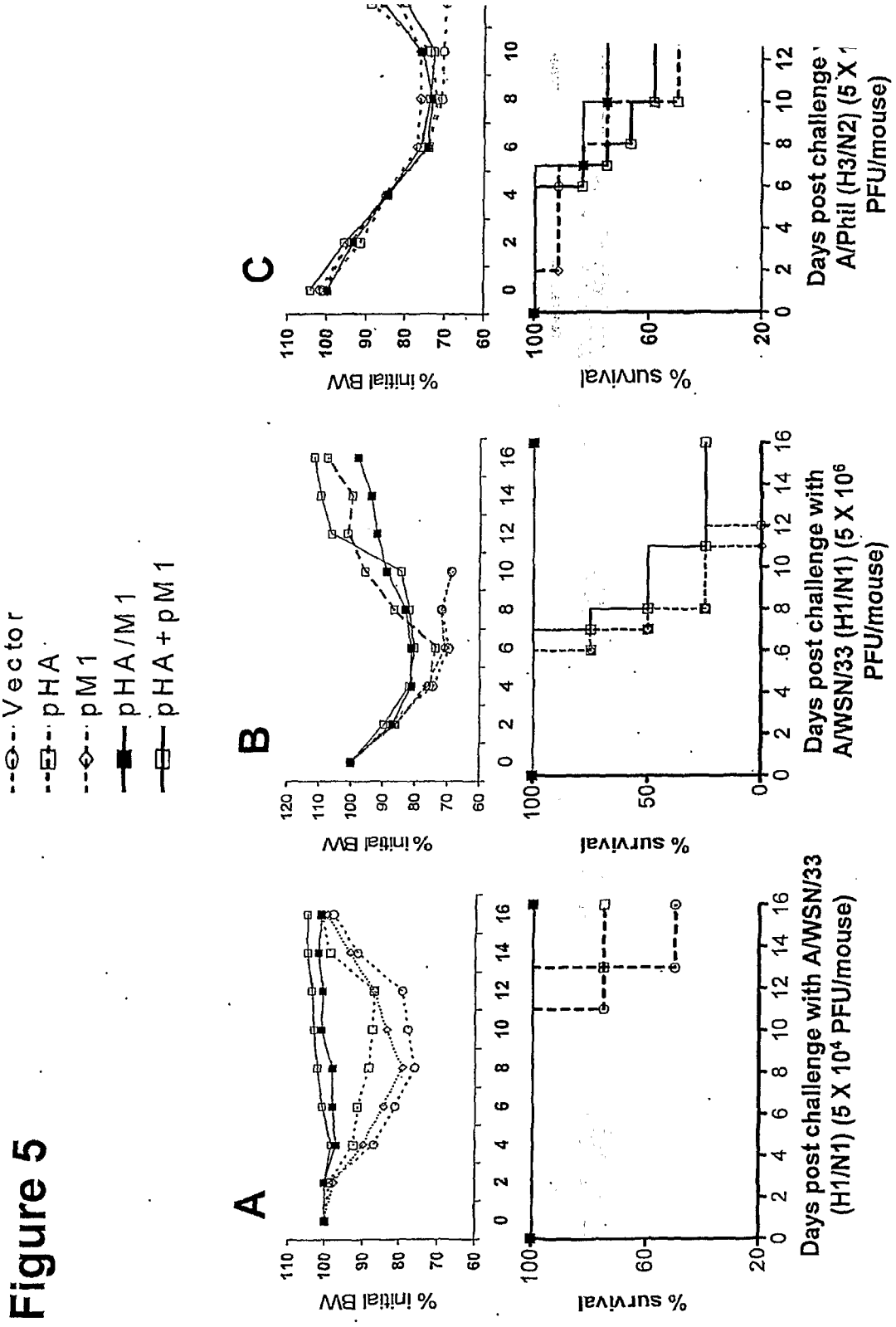


Figure 4B

Figure 5



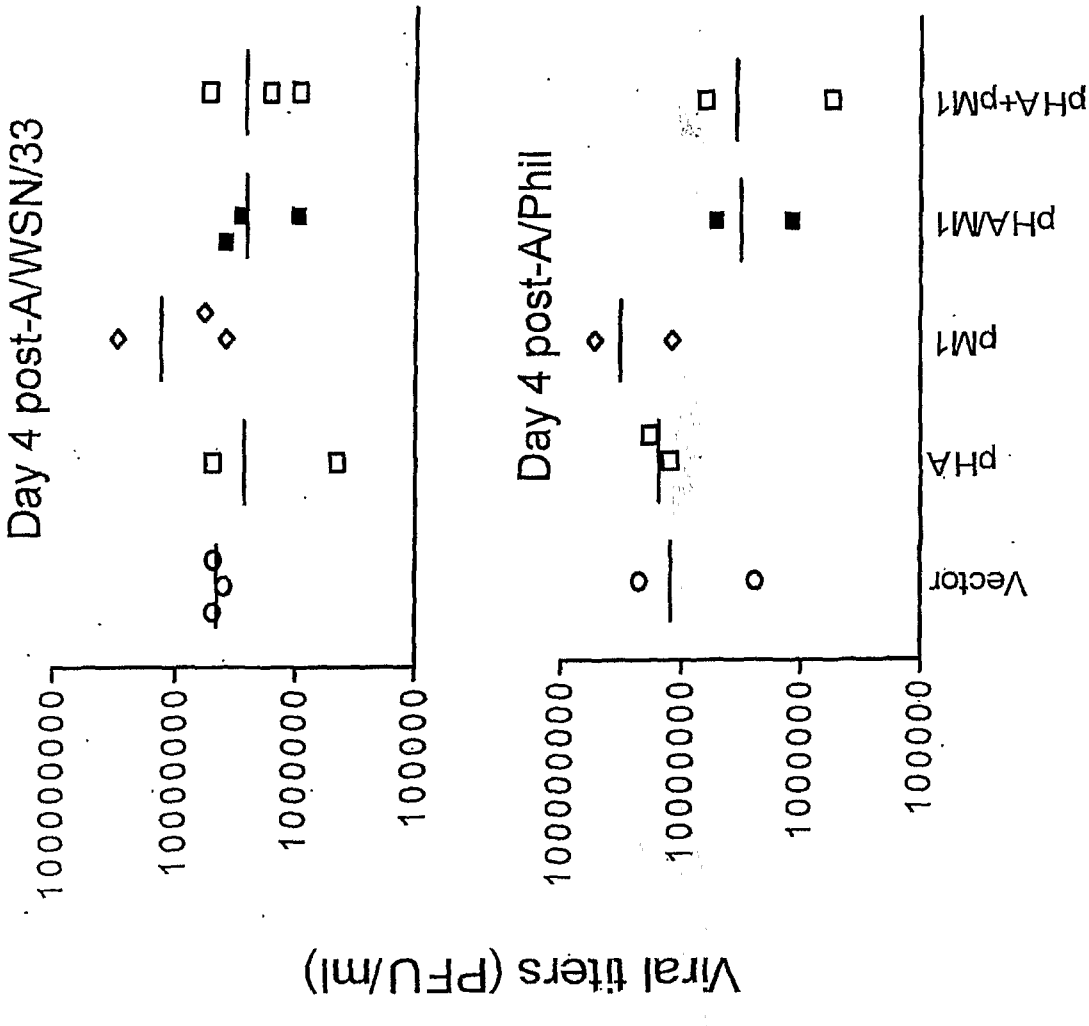

Day 4 post-A/WSN/33
Day 4 post-A/Phil
Viral titers (PFU/ml)
100000000
10000000
1000000
100000
Vector
pHA
pM1
pHAM1
pHA+pM1

Figure 6A

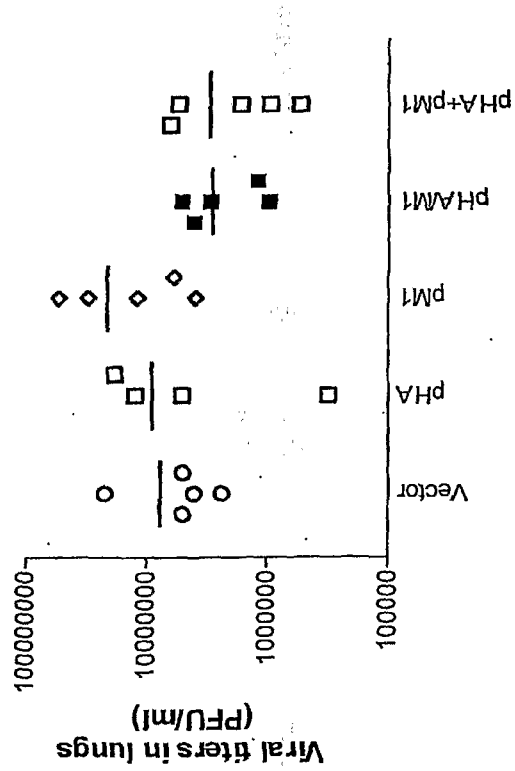

Day4 post-challenge
Viral titers in lungs (PFU/ml)
100000000
10000000
1000000
100000
Vector
pHA
pM1
pHAM1
pHA+pM1

Figure 6B