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(54) Title: NS1 TRUNCATED VIRUS FOR THE DEVELOPMENT OF CANINE INFLUENZA VACCINES

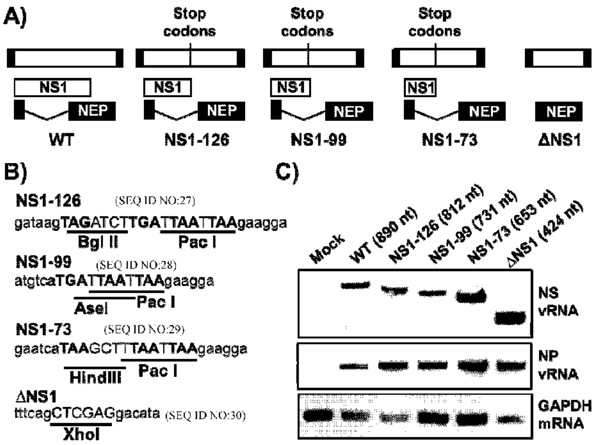

Figure 1A – Figure 1C

(57) **Abstract**: The present invention relates to compositions and methods for the treatment and prevention of canine influenza virus (CIV) and CIV-related pathology. The present invention is based on the development of mutant CIV, having one or more mutations in segment 8, which induces a CIV-specific immune response in a subject.

TITLE OF THE INVENTION
NS1 TRUNCATED VIRUS FOR THE DEVELOPMENT OF CANINE INFLUENZA VACCINES

CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 62/207,576 filed on August 20, 2015, the contents of which are incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

Influenza A viruses (IAVs) have a broad host range and mainly exist in the wild aquatic fowl reservoir (de Jong et al., 2007, J Virol, 81:4315-4322, Taubenberger et al., 2010, Cell host & microbe, 7:440-451, Webster et al., 1992, Microbiological reviews, 56:152-179, Yoon et al., 2014, Current topics in microbiology and immunology, 385:359-375). IAV has gained the capacity to cross the species barrier to infect and cause disease in new hosts (Parrish et al., 2005, Annual review of microbiology, 59:553-586, Parrish et al., 2015, J Virol, 89:2990-2994). Two IAV strains emerged in dogs in the past 16 years, first the equine-origin H3N8 CIV in the USA around 1999 (Crawford et al., 2005, Science, 310:482-485), and then the avian-origin CIV H3N2 in China around 2005 (Song et al., 2008, Emerging infectious diseases, 14:741-746). Serological evidence demonstrated that CIV H3N8 has been circulating in dogs in the United States (US) since 1999 (Hayward et al., 2010, J Virol, 84:12636-12645), the virus was first isolated in 2004 from Florida racing greyhounds exhibiting signs of respiratory disease (Crawford et al., 2005, Science, 310:482-485). In turn, H3N2 CIV has been circulating in Asia after 2005, and was imported to North America in February 2015. Therefore, CIVs represent new threats to canine health in the US, and potentially worldwide (Crawford et al., 2005, Science, 310:482-485, Holt et al., 2010, Journal of the American Veterinary Medical Association, 237:71-73, Pecoraro et al., 2013, Journal of veterinary diagnostic investigation, 25:402-406, Yoon et al., 2005, Emerging infectious diseases, 11:1974-1976). Vaccination remains the best prophylactic option against IAV infection (Nogales et al., 2014, J Virol, 88:10525-10540, Pica et al., 2013, Annual review of medicine, 64:189-202), but only inactivated influenza vaccines (IIV) against H3N8 and H3N2 are commercially available. IIVs require the production of large amounts of virus and do not induce significant cellular responses, which are important for generating long-term memory against subsequent influenza infections (Belshe et al., 2007, The New England journal of medicine, 356:685-696, Belshe et al., 2000, The Journal of infectious diseases, 181:1133-1137, Centers et al., 2010, MMWR, 59(16):485-486, Rimmelzwaan et al., 2007, Current opinion in biotechnology, 18:529-536). In contrast, live attenuated influenza vaccines (LAIVs) induce stronger innate and adaptive cellular and humoral immunity (Belshe et al., 2007, The New England journal of medicine, 356:685-696, Cox et al., 2015, J Virol, 89(6):3421-3426). In addition, LAIVs have the advantage of intranasal administration, avoiding possible muscle soreness and potential iatrogenic practices associated with the intramuscular administration of IIV.

An important public health concern is the ability of IAV to cause occasional pandemics when novel viruses are introduced into humans (Smith et al., 2009, Nature, 459:1122-1125). Dogs are susceptible to IAVs, and could become “mixing vessels” species for the generation of novel IAVs with pandemic potential to humans. Moreover, natural and experimental infections of dogs with human viruses have been reported (Dundon et al., 2009, Emerging infectious diseases, 16:2019-2021, Song et al., 2015, The Journal of general virology, 96:254-258), and reassortants between canine and human influenza viruses could result in the emergence of new viruses in humans.

In 2006, the American Veterinary Medical Association (AVMA) called for the urgent development of an effective vaccine against CIV. A vaccine made from inactivated virus have been developed that is administered subcutaneously as two doses to reduce the severity of the CIV disease and to reduce the incidence of CIV infection in naive dogs (Nobivac, Merck). However, to date, no LAIV for CIV infections has been developed. Thus there is a need in the art for improved vaccines for CIV. The present invention satisfies this unmet need.

SUMAMRY OF THE INVENTION

In one aspect, the present invention provides an immunological composition comprising a canine influenza virus (CIV), wherein the CIV comprises one or more mutations in segment 8 of the viral genome. In one embodiment, the segment 8 comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4

In one embodiment, the CIV comprises one or more mutations in segment 8, which encodes a truncation mutant of NS1. In one embodiment, the truncation mutant of NS1 is selected from the group consisting of NS1-126, NS1-99, and NS1-73. in one embodiment, the truncation mutant of NS1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7. In one embodiment, the CIV comprises one or more mutations in segment 8 such that NS1 is not expressed.

In one embodiment, the CIV is derived from H3N8 subtype of influenza A virus. In one embodiment the composition is used for the treatment or prevention of canine influenza in a subject.

In one aspect, the present invention provides a method for treating or preventing canine influenza in a subject. The method comprises administering to the subject an immunological composition comprising a canine influenza virus (CIV), wherein the CIV comprises one or more mutations in segment 8 of the viral genome.

In one embodiment, the segment 8 comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4

In one embodiment, the CIV comprises one or more mutations in segment 8, which encodes a truncation mutant of NS1. In one embodiment, the truncation mutant of NS1 is selected from the group consisting of NS1-126, NS1-99, and NS1-73. in one embodiment, the truncation mutant of NS1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7. In one embodiment, the CIV comprises one or more mutations in segment 8 such that NS1 is not expressed.

In one embodiment, the CIV is derived from H3N8 subtype of influenza A virus.

In one embodiment, the subject does not have canine influenza, and wherein the method induces immunity against one or more of: influenza A virus subtype H3N8 and influenza A virus subtype H3N2. In one embodiment, the subject is infected with at least one or more of: influenza A virus subtype H3N8 and influenza A virus subtype H3N2; and wherein the method induces a therapeutic immune response.

In one embodiment, the immunological composition is administered intranasally, intratracheally, orally, intradermally, intramuscularly, intraperitoneally, intravenously, or subcutaneously. In one embodiment, the subject is a dog.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of preferred embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1, comprising Figure 1A through Figure 1C depicts the results of experiments demonstrating the generation of CIV with truncated NS1 proteins. (Figure 1A and Figure 1B) Schematic representation of the WT and truncated NS1 H3N8 CIVs. C-terminal truncations were introduced to the NS1 protein, resulting in four constructs encoding the first 126, 99, or 73 amino acids or a deletion (ΔNS1) of the viral NS1 protein. The nuclear export protein (NEP) open reading frame was not altered (Figure 1A). The viral NS non-coding regions, NCR (black boxes) and the stop codons are indicated. Deletions, stop codons and/or restriction enzymes were introduced in each construct (Figure 1B). NS1 sequence (lowercase), stop codons (bold, uppercase) and restriction sites (underlined, uppercase) are indicated. (Figure 1C) RT-PCR of the WT and truncated NS H3N8 CIVs: MDCK cells were infected (MOI 3) with the indicated H3N8 CIVs. At 20 hours post-infection total RNA was extracted and the presence of vRNAs encoding NS and NP as well as cellular mRNA encoding GAPDH was assessed by RT-PCR. Expected nucleotide (nt) sizes are indicated. The sizes of molecular markers (bp) are indicated to the left.

Figure 2, comprising Figure 2A through Figure 2C depicts the results of experiments demonstrating the characterization of CIV WT and NS1-truncated viruses. (Figure 2A and Figure 2B) Multicycle growth kinetics of CIV WT and NS1-truncated viruses. MDCK cells were infected with the indicated viruses (MOI of 0.001) and incubated at 37°C (Figure 2A) or 33°C (Figure 2B). Supernatants were collected at 24, 48, 72, and 96 hours post-infection (h.p.i.) and viral titers were determined by immunofocus assay (FFU/ml). Data is expressed as the means of triplicate wells. Dotted black lines indicate limit of detection (20 FFU/ml). $P<0.05$: *

WT versus ΔNS1, ** WT versus NS1-126 and NS1-99 and *** WT versus NS1-73, using a Student's t test. (Figure 2C) Plaque assays: Plaque sizes of WT and NS1-truncated H3N8 CIVs in MDCK cells at 33°C were evaluated at 3 days post-infection.

Figure 3, comprising Figure 3A through Figure 3E, depicts the results of experiments evaluating the activation of the IFN-β promoter by CIV WT and NS1-truncated viruses. (Figure 3A) Schematic representation of the IFN-β induction bioassays: MDCK cells constitutively expressing GFP-CAT and firefly luciferase (FFluc) reporter genes under the control of the IFNβ promoter (MDCK IFNβ GFP-CAT/FFluc) were infected with WT H3N8 CIV or NS1 mutants (MOI 3). At 12 h p.i. IFNβ promoter activation was determined by assessing GFP (fluorescence microscope) and FFluc (luciferase assay) expressions. Supernatants of the same MDCK infected cells were collected and, after UV virus inactivation, used to treat fresh MDCK cells for 24 hours prior to infection with rNDV-GFP. GFP expression from rNDV-GFP-infected cells was determined at 14 h p.i. using fluorescent microscopy. (Figure 3B) Indirect immunofluorescence: WT and NS1-truncated H3N8 CIV infections of MDCK IFNβ GFP-CAT/FFluc cells were evaluated at 12 h p.i. using an anti-NP antibody. DAPI was used for nuclear staining. (Figure 3C and Figure 3D) Activation of the IFNβ promoter: At 12 hours post-infection of the MDCK IFNβ GFP-CAT/FFluc cells, activation of the IFNβ promoter was determined by assessing GFP expression (Figure 3C) and FFluc activity. (Figure 3D) Inhibition of NDV infection: Supernatants of previously infected MDCK cells were collected, UV-inactivated and used to treat fresh MDCK cells. After 24 hours of incubation, cells were infected with the IFN-sensitive rNDV-GFP (MOI 3). At 14 h p.i., rNDV-GFP-infected cells were quantified. (Figure 3E). Scale bars, 200 μM (Figure 3B and Figure 3C). Data shown in Figure 3D and Figure 3E represents the means and SDs of triplicate wells. *, $P<0.05$ (WT versus NS1-126, WT versus NS1-99, WT versus NS1-73 or WT versus ΔNS1) using Student's t test.

Figure 4 depicts the results of experiments evaluating the attenuation of NS1-truncated CIV: 5- to-7-week-old C57BL/6 female mice (N=6) were infected i.n. with $1x10^5$ PFU of WT CIV or NS1 mutants (NS1-126, -99, -73 and ΔNS1). Three mice were euthanized at days 2 and 4 post-infection and lungs were harvested for virus titrations using an immunofocus assay (FFU/ml). The dotted black line indicates the limit of detection (200 FFU/ml). ND, virus not detected. * (black lines for D2 and grey lines for D4) indicate P values < 0.05 using a Student's t test from Microsoft Excel.

Figure 5, comprising Figure 5A and Figure 5B depicts the results of experiments evaluating the immunogenicity and protection efficacy of NS1 mutant H3N8 CIVs. Female 5- to-7-week-old C57BL/6 mice were vaccinated with the H3N8 CIV IIV (Nobivac; 100 μl i.m), or with $1x10^3$ PFU of WT or mutant H3N8 CIVs (ΔNS1, NS1-73, NS1-99 and NS1-126, i.n.); or mock vaccinated with PBS. (Figure 5A) Induction of humoral responses: At 14 days post-vaccination, mice were bled and sera were collected and evaluated by ELISA for IgG antibodies against total WT H3N8 CIV proteins using cell extracts of MDCK-infected cells. Mock-infected cell extracts were used to evaluate the specificity of the antibody response. OD, optical density. Data represent the means +/- SDs of the results for 4 individual mice. (Figure 5B) Protection efficacy: Two weeks post-vaccination, mice were challenged with $1x10^5$ PFU of CIV WT H3N8. To evaluate virus replication, mice were euthanized at days 2 (N=3) and 4 (N=3) post-infection with WT H3N8 CIV, lungs were harvested and viral titers determined by immunofocus assay (FFU/ml). The dotted black line indicates the limit of detection (200 FFU/ml). ND, virus not detected. Data represent the means +/- SDs. *, $P<0.05$ (PBS vs. ΔNS1 or PBS vs. Nobivac) using a Student's t test.

Figure 6, comprising Figure 6A through Figure 6B, depicts the results of experiments of ex *vivo* infection of canine tracheal explants with A/canine/New York/2009 H3N8 WT or mutant (ΔNS1, NS1-73, NS1-99 and NS1-126) viruses. (Figure 6A) Histological features of dog tracheas infected with 200 PFU of H3N8 CIVs (WT or mutants) or mock-infected with infection media. Lesions are shown in sections stained with haematoxylin and eosin (H&E). (Figure 6B) Infected cells were detected by immunohistochemical staining of the NP viral protein. Positive cells are stained in brown. Black horizontal bars represent 20μm. (Figure 6C) Graphical representation of bead clearance assays in infected and control explants. Lines represent the average time to clear the beads in three independent experiments. Error bars represent SEM. (Figure 6D) Growth kinetics of H3N8 CIVs (WT and mutants) in canine tracheal explants. Vertical bars represent average from three independent experiments. Error bars represent SEM.

Figure 7 depicts the results of experiments evaluating the induction of humoral responses by NS1-truncated CIV vaccination. Female 6- to-8-week-old

C57BL/6 mice were immunized intranasally with the H3N8 CIV inactivated vaccine (Nobivac; 100uµ intramuscular), or with 1x10^3 PFU of H3N8 CIV wild-type (WT) or NS1-truncated viruses (ΔNS1, NS1-73, NS1-99 and NS1-126); or mock vaccinated with PBS. At 14 days post-infection, mice were bled and the sera were collected and evaluated by ELISA for IgG antibodies against total influenza virus protein using cell extracts of MDCK cells infected with CIV H3N2 wild-type (A/Ca/IL/41915/2015). Mock-infected cell extracts were used to evaluate the specificity of the antibody response. Data represent the means +/- SDs of the results for 4 individual mice.

Figure 8 depicts the results of experiments evaluating the protection efficacy of CIV ΔNS1 against CIV H3N2: Female 6- to-8-week-old C57BL/6 mice (n=6) were immunized intranasally with the H3N8 CIV inactivated vaccine (Nobivac; 100µl intramuscular), or with 1x10^3 PFU of CIV wild-type (WT) or ΔNS1 or mock vaccinated with PBS. Two weeks post-vaccination, mice were challenged with 1x10^5 PFU of CIV H3N2 wild-type (A/Ca/IL/41915/2015). To evaluate viral lung replication, mice were sacrificed at days 3 (n=3) post-challenge and lungs were harvested, homogenized, and used to quantify viral titers by immunofocus assay (FFU/ml) using an anti-NP MAb (HB-65). Dotted black lines indicate limit of detection (200 FFU/ml). Data represent the means +/- SDs.

Figure 9 depicts the nucleotide sequence of modified segment 8, wherein the segment is modified such that it does not express NS1 (ΔNS1), derived from H3N8 CIV (Influenza A/canine/NY/dog23/2009).

Figure 10, comprising Figure 10A and Figure 10B, depict the nucleotide sequence of modified segment 8 (Figure 10A), and the encoded amino acid sequence (Figure 10B) of the NS1-126 mutant, derived from H3N8 CIV (Influenza A/canine/NY/dog23/2009).

Figure 11, comprising Figure 11A and Figure 11B, depict the nucleotide sequence of modified segment 8 (Figure 11A), and the encoded amino acid sequence (Figure 11B) of the NS1-99 mutant, derived from H3N8 CIV (Influenza A/canine/NY/dog23/2009).

Figure 12, comprising Figure 12A and Figure 12B, depict the nucleotide sequence of modified segment 8 (Figure 12A), and the encoded amino acid sequence (Figure 12B) of the NS1-73 mutant, derived from H3N8 CIV (Influenza A/canine/NY/dog23/2009).

DETAILED DESCRIPTION

The present invention relates to compositions and methods for the treatment and prevention of canine influenza virus (CIV) and CIV-related pathology. The present invention is based in part upon the discovery that mutations in segment 8 of the CIV genome, resulting in the truncation of NS1 or the lack of NS1 expression, induces a CIV-specific immune response and can be used to protect against CIV infection. In certain embodiments, the NS1-mutated CIV described herein is a live-attenuated canine influenza vaccine (LACIV).

In certain embodiments, the present invention provides a composition for the treatment and prevention of canine influenza virus (CIV) and CIV-related pathology. In one embodiment, the composition comprises a mutant CIV having one or more mutations in segment 8 of the viral genome. For example, in one embodiment, the mutant CIV encodes mutant NS1. In certain embodiments, mutant NS1 is a truncation mutant, selected from NS1-126, NS1-99, or NS1-73. In one embodiment, the mutant CIV comprises one or more mutations in segment 8 that results in the lack of expression of NS1 (denoted herein as delNS1 or ΔNS1).

In certain embodiments, the present invention provides a method for treating or preventing CIV and CIV-related pathology, comprising administering a composition comprising a mutant CIV described herein. In certain embodiments, the method comprises intranasal delivery of the mutant CIV.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

As used herein, each of the following terms has the meaning associated with it in this section.

The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

“About” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of

±20%, ±10%, ±5%, ±1%, or ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

The term “antibody,” as used herein, refers to an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and $F(ab)_2$, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

The term “antigen” or “Ag” as used herein is defined as a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an “antigen” as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a “gene” at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological sample.

As used herein, the term “autologous” is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

As used herein, by “combination therapy” is meant that a first agent is administered in conjunction with another agent. “In conjunction with” refers to administration of one treatment modality in addition to another treatment modality. As such, “in conjunction with” refers to administration of one treatment modality before, during, or after delivery of the other treatment modality to the individual. Such combinations are considered to be part of a single treatment regimen or regime.

As used herein, the term “concurrent administration” means that the administration of the first therapy and that of a second therapy in a combination therapy overlap with each other.

A “disease” is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal’s health continues to deteriorate. In contrast, a “disorder” in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal’s state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal’s state of health.

An “effective amount” as used herein, means an amount which provides a therapeutic or prophylactic benefit.

The term “expression” as used herein is defined as the transcription and/or translation of a particular nucleotide sequence driven by its promoter.

“Expression vector” refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

“Homologous” refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared X 100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

The term “immunoglobulin” or “Ig,” as used herein, is defined as a class of proteins, which function as antibodies. Antibodies expressed by B cells are sometimes referred to as the BCR (B cell receptor) or antigen receptor. The five members included in this class of proteins are IgA, IgG, IgM, IgD, and IgE. IgA is the primary antibody that is present in body secretions, such as saliva, tears, breast milk, gastrointestinal secretions and mucus secretions of the respiratory and genitourinary tracts. IgG is the most common circulating antibody. IgM is the main immunoglobulin produced in the primary immune response in most subjects. It is the most efficient immunoglobulin in agglutination, complement fixation, and other antibody responses, and is important in defense against bacteria and viruses. IgD is the immunoglobulin that has no known antibody function, but may serve as an antigen receptor. IgE is the immunoglobulin that mediates immediate hypersensitivity by causing release of mediators from mast cells and basophils upon exposure to allergen.

As used herein, the term “immune response” includes T-cell mediated and/or B-cell mediated immune responses. Exemplary immune responses include T cell responses, e.g., cytokine production and cellular cytotoxicity, and B cell responses, e.g., antibody production. In addition, the term immune response includes immune responses that are indirectly affected by T cell activation, e.g., antibody production (humoral responses) and activation of cytokine responsive cells, e.g., macrophages. Immune cells involved in the immune response include lymphocytes, such as B cells and T cells (CD4+, CD8+, Th1 and Th2 cells); antigen presenting cells (e.g., professional antigen presenting cells such as dendritic cells, macrophages, B lymphocytes, Langerhans cells, and non-professional antigen presenting cells such as keratinocytes, endothelial cells, astrocytes, fibroblasts, oligodendrocytes); natural killer cells; myeloid cells, such as macrophages, eosinophils, mast cells, basophils, and granulocytes.

“Isolated” means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not “isolated,” but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

"Parenteral" administration of an immunogenic composition includes, e.g., subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether in vitro or in situ, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

The term "simultaneous administration," as used herein, means that a first therapy and second therapy in a combination therapy are administered with a time separation of no more than about 15 minutes, such as no more than about any of 10, 5, or 1 minutes. When the first and second therapies are administered simultaneously, the first and second therapies may be contained in the same composition (e.g., a composition comprising both a first and second therapy) or in separate compositions (e.g., a first therapy in one composition and a second therapy is contained in another composition).

By the term "specifically binds," as used herein with respect to an antibody, is meant an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A," the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

The term “normal temperature” or “normal body temperature” as used herein refers to the temperature of a healthy subject. For example, in certain instances the “normal body temperature” in a human subject is in the range of about 36°C to about 38°C. In certain instances, in a canine subject, “normal body temperature” is in the range of about 38°C to about 39.5°C.

The tem “elevated temperature” or “elevated body temperature” as used herein refers to a temperature in a subject that is greater than the “normal body temperature” of a subject of a given organism. In certain instances “elevated body temperature” may be indicative of a fever, infection, or other illness. In certain instances, elevated body temperature in a human subject is greater than about 37°C. In certain instances, elevated body temperature in a canine subject is greater than about 38.5°C.

The term “therapeutic” as used herein means a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, remission, or eradication of a disease state.

The term “therapeutically effective amount” refers to the amount of the subject compound that will elicit the biological or medical response of a tissue, system, or subject that is being sought by the researcher, veterinarian, medical doctor or other clinician. The term “therapeutically effective amount” includes that amount of a compound that, when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the signs or symptoms of the disorder or disease being treated. The therapeutically effective amount will vary depending on the compound, the disease and its severity and the age, weight, etc., of the subject to be treated.

To “treat” a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject.

The term “transfected” or “transformed” or “transduced” as used herein refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A “transfected” or “transformed” or “transduced” cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

The present invention provides immunological compositions and methods useful for the inhibition, prevention and treatment of canine influenza and canine influenza related diseases and disorders. In one embodiment, the immunological composition comprises a live-attenuated virus (LAV).

In one embodiment, the present invention provides a mutant form of a canine influenza virus. For example, it is demonstrated herein that mutations in segment 8 of the CIV genome, resulting the expression of truncated NS1 or the lack of NS1 expression, provides antigen-specific immune responses and protection against CIV. In one embodiment, the mutant CIV provides at least the same antigen-specific immune responses and protection against CIV compared to wildtype CIV. In certain embodiments, the mutant CIV provides greater antigen-specific immune responses and protection against CIV as compared to inactivated CIV.

In general, wild-type influenza viruses contain a segmented genome with 8 segments as described in Table 1 below:

Table 1:

Segment	Gene Product
1	PB2 (Polymerase (basic) protein 2)
2	PB1 (Polymerase (basic) protein 1)
3	PA (Polymerase (acidic) protein)
4	HA (Hemagglutinin)
5	NP (Nucleoprotein)

6	NA (Neuraminidase)
7	M1 (Matrix protein 1) and M2 (Matrix protein 2)
8	NS1 (non-structural protein 1) and NEP/NS2 (non-structural protein 2)

In certain embodiments, the present invention provides an immunological composition comprising segment 8, wherein segment 8 comprises one or more mutations. For example, in certain embodiments, the immunological composition comprises an LAV, comprising one or more mutations in segment 8.

The present invention also provides methods of preventing, inhibiting, and treating CIV and CIV-related diseases and disorders. In one embodiment, the methods of the invention induce immunity against CIV by generating an immune response directed to CIV. In one embodiment, the methods of the invention induce production of CIV-specific antibodies. In one embodiment, the methods of the invention prevent CIV-related pathology. In one embodiment, the methods of the invention comprise administering an immunological composition comprising a mutant CIV, wherein the mutant CIV comprises one or more mutations in segment 8, to a subject in need thereof. In one embodiment, the methods comprise administering an immunological composition to a subject in need thereof, thereby inducing immunity to CIV.

Compositions

The present invention provides immunological compositions that when administered to a subject in need thereof, elicit an immune response directed against canine influenza virus (CIV). In some embodiments, the composition includes polypeptides, nucleotides, vectors, or vaccines. Further, when the compositions are administered to a subject, they elicit an immune response that serves to protect the inoculated subject against canine influenza. As exemplified herein, the composition can be obtained in large quantities for use as a vaccine.

In one embodiment, the present invention provides compositions that are useful as immunomodulatory agents, for example, in stimulating immune responses and in preventing canine influenza and canine influenza-related pathology. In certain embodiments, the composition comprises a live-attenuated virus (LAV).

The immunological compositions can be used as immunostimulatory agents to induce the production of CIV-specific antibodies and protect against canine influenza and canine influenza-related pathology. In one embodiment, the composition of the invention comprises a mutant CIV, wherein the mutant CIV comprises one or more mutations in the viral genome. For example, in one embodiment, the mutant CIV comprises one or more mutations in segment 8 of the viral genome. In one embodiment, the one or more mutations in segment 8 of the viral genome encode a mutant NS1 protein. For example, in one embodiment, the one or more mutations in segment 8 of the viral genome encode a truncation mutant of NS1, where NS1 is truncated at its C-terminus. In one embodiment, the one or more mutations in segment 8 of the viral genome results in the lack of NS1 protein expression. In one embodiment, the mutant segment 8 of the mutant CIV still encodes wildtype NEP/NS2 protein.

In one embodiment, the LACIV is based upon the genome of Influenza A/canine/NY/dog23/2009 H3N8. Wildtype nucleic acid sequences for each segment of Influenza A/canine/NY/dog23/2009 H3N8 and wildtype amino acid sequences for the encoded proteins are summarized in Table 2 below:

Table 2:

Wildtype sequences for Influenza A/canine/NY/dog23/2009 H3N8	
Segments	Gene Products
Segment 1 (SEQ ID NO: 9)	PB2 (SEQ ID NO: 10)
Segment 2 (SEQ ID NO: 11)	PB1 (SEQ ID NO: 12)
Segment 3 (SEQ ID NO: 13)	PA (SEQ ID NO: 14)
Segment 4 (SEQ ID NO: 15)	HA (SEQ ID NO: 16)
Segment 5 (SEQ ID NO: 17)	NP (SEQ ID NO: 18)
Segment 6 (SEQ ID NO: 19)	NA (SEQ ID NO: 20)

Segment 7 (SEQ ID NO: 21)	M1 (SEQ ID NO: 22)	M2 (SEQ ID NO: 23)
Segment 8 (SEQ ID NO: 24)	NS1 (SEQ ID NO: 25)	NEP/NS2 (SEQ ID NO: 26)

In one embodiment, the composition comprises one or more mutations in the nucleic acid sequences of segment 8, encoding NS1 and NEP/NS2 proteins. In one embodiment, certain embodiments, the composition encodes mutant NS1. In one embodiment, the composition encodes a truncation mutant of NS1. In one embodiment, the composition comprises a mutation in segment 8 that results in the lack of expression of NS1. In one embodiment, the composition encodes wildtype NEP/NS2.

In some embodiments, the invention provides a composition comprising one or more mutations in segment 8. For example, in one embodiment, the composition comprises segment 8 having one or more mutation which results in the production of a truncation mutant of NS1, where the truncation mutant of NS1 is truncated at its C-terminus, as compared to wildtype NS1. For example, in one embodiment, the mutant NS1 comprises the amino acid sequence of SEQ ID NO: 25, except having one or more amino acids deleted from the C-terminus. In one embodiment, the mutant NS1comprises amino acids 1-126 of NS1, referred to herein as NS1-126. For example, in one embodiment, NS1-126 comprises the amino acid sequence of SEQ ID NO: 5. In one embodiment, the mutant NS1 comprises amino acids 1-99 of NS1, referred to herein as NS1-99. For example, in one embodiment, NS1-99 comprises the amino acid sequence of SEQ ID NO: 6. In one embodiment, the mutant NS1 comprises amino acids 1-73 of NS1, referred to herein as NS1-73. For example, in one embodiment, NS1-73 comprises the amino acid sequence of SEQ ID NO: 7.

In one embodiment, the composition comprises a nucleic acid sequence encoding a mutant NS1 having an amino acid sequence of SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7. In one embodiment, the composition comprises a nucleic acid sequence encoding a mutant NS1 that is substantially homologous to SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7. For example, in certain embodiments, the composition comprises a nucleic acid sequence that encodes a mutant NS1 that is at least 50% homologous, at least 60% homologous, at least 70% homologous, at least 80% homologous, at least 90% homologous, at least 95% homologous, at least 98% homologous, at least 99% homologous, or at least 99.5% homologous to SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7.

In one embodiment, the composition comprises a mutant segment 8 comprising the nucleotide sequence of SEQ ID NO: 2. In one embodiment, the composition comprises nucleotide sequence that is substantially homologous to SEQ ID NO: 2. For example, in certain embodiments, the composition comprises a nucleotide sequence that is at least 50% homologous, at least 60% homologous, at least 70% homologous, at least 80% homologous, at least 90% homologous, at least 95% homologous, at least 98% homologous, at least 99% homologous, or at least 99.5% homologous to SEQ ID NO: 2.

In one embodiment, the composition comprises a mutant segment 8 comprising the nucleotide sequence of SEQ ID NO: 3. In one embodiment, the composition comprises nucleotide sequence that is substantially homologous to SEQ ID NO: 3. For example, in certain embodiments, the composition comprises a nucleotide sequence that is at least 50% homologous, at least 60% homologous, at least 70% homologous, at least 80% homologous, at least 90% homologous, at least 95% homologous, at least 98% homologous, at least 99% homologous, or at least 99.5% homologous to SEQ ID NO: 3.

In one embodiment, the composition comprises a mutant segment 8 comprising the nucleotide sequence of SEQ ID NO: 4. In one embodiment, the composition comprises nucleotide sequence that is substantially homologous to SEQ ID NO: 4. For example, in certain embodiments, the composition comprises a nucleotide sequence that is at least 50% homologous, at least 60% homologous, at least 70% homologous, at least 80% homologous, at least 90% homologous, at least 95% homologous, at least 98% homologous, at least 99% homologous, or at least 99.5% homologous to SEQ ID NO: 4.

In one embodiment, the composition comprises segment 8 having one or more mutation which results in the deletion of NS1, where NS1 is not expressed by segment 8, referred to herein as delNS1 or ΔNS1.

In one embodiment, the composition comprises a mutant segment 8 comprising the nucleotide sequence of SEQ ID NO: 1. In one embodiment, the composition comprises nucleotide sequence that is substantially homologous to SEQ

ID NO: 1. For example, in certain embodiments, the composition comprises a nucleotide sequence that is at least 50% homologous, at least 60% homologous, at least 70% homologous, at least 80% homologous, at least 90% homologous, at least 95% homologous, at least 98% homologous, at least 99% homologous, or at least 99.5% homologous to SEQ ID NO: 1.

In one embodiment, the composition comprises segment 8 having one or more mutation which results in mutant NS1 or lack of NS1 expression, but still expresses wildtype NEP/NS2. In one embodiment, the composition comprises a nucleic acid sequence encoding a NEP/NS2 having an amino acid sequence of SEQ ID NO: 8. In one embodiment, the composition comprises a nucleic acid sequence encoding a NEP/NS2 that is substantially homologous to SEQ ID NO: 8. For example, in certain embodiments, the composition comprises a nucleic acid sequence that encodes a NEP/NS2 that is at least 50% homologous, at least 60% homologous, at least 70% homologous, at least 80% homologous, at least 90% homologous, at least 95% homologous, at least 98% homologous, at least 99% homologous, or at least 99.5% homologous to SEQ ID NO: 8.

In certain embodiments, the composition comprises one or more mutations in the nucleic acid sequences of segment 8, while comprising wildtype nucleic acid sequences for the rest of the segmented genome. For example, in one embodiment, the composition comprises one or more mutations in segment 8 and comprises wildtype segment 1, segment 2, segment 3, segment 4, segment 5, segment 6, and segment 7.

In certain embodiments, the composition comprises one or more mutations in segment 8, in combination with one or more mutations in one or more other segments of the viral genome.

For example, in one embodiment, the composition further comprises one or more mutations in segment 1 and/or segment 2. In one embodiment, the composition comprises a mutation in segment 1 and/or segment 2, encoding a point mutation in PB2 and/or PB1 that render the CIV temperature sensitive. An exemplary point mutations of PB2 is N265S. Exemplary point mutations of PB1 include a K391E point mutation, a E581G point mutation, and a A661T point mutation, as described in PCT Patent Application PCT/US2016/______, filed on August 19, 2016, claiming priority to U.S. Provisional Patent Application No. 62/207,571, each of which applications are incorporated by reference in their entirety.

For example, in one embodiment, the composition further comprises one or more mutations in segment 4. In one embodiment, the composition comprises a deletion mutant of segment 4, such that HA is not expressed, as described in PCT Patent Application PCT/US2016/______, filed on August 19, 2016, claiming priority to U.S. Provisional Patent Application No. 62/207,579, each of which applications are incorporated by reference in their entirety.

In certain embodiments, the composition comprises a mutated segment 8, as described herein, in combination with one or more nucleotide sequences encoding another antigen. For example, in certain embodiments, the composition comprises a mutated segment 8, as described herein, in combination with one or more nucleotide sequences encoding one or more antigens of another virus or strain. For example, in certain aspects, the H3N8 NS1 mutants described herein can be used as a master donor virus (MDV). For example, an MDV comprising an H3N8 modified segment 8 described herein, can be modified to comprise one or more nucleotide sequences encoding one or more of PB2, PB1, PA, NP, HA, NA, M1, M2, NS1, or NEP/NS2 from another influenza strain. As such a composition comprising an H3N8 modified segment 8 described herein can provide protection against a different strain, when the composition expresses an antigen of the different strain. For example, in one embodiment, a composition comprises the backbone of a NS1 mutant H3N8 described herein, further comprising one or more nucleotide sequences encoding one or more of PB2, PB1, PA, NP, HA, NA, M1, M2, NS1, or NEP/NS2 from another influenza strain. In one embodiment, the composition comprises the backbone of a NS1 mutant H3N8 described herein, further comprising one or more nucleotide sequences encoding one or more of HA or NA of a different influenza strain, including but not limited to H3N2 CIV. For example, the composition comprising the backbone of a NS1 mutant H3N8 described herein, may be modified to express one or more viral proteins of a newly emergent strain, thereby providing protection against the newly emergent strain.

In certain embodiments, the composition comprises a polynucleotide encoding a truncation mutant of NS1. In one embodiment, the composition comprises a polynucleotide that results in the lack of NS1 expression. The polynucleotide can be RNA or DNA. In one embodiment, the composition comprises a DNA vaccine.

The nucleic acid sequences include both the DNA sequence that is transcribed into RNA and the RNA sequence that is translated into a polypeptide.

According to other embodiments, the polynucleotides of the invention are inferred from the amino acid sequence of the polypeptides of the invention. As is known in the art several alternative polynucleotides are possible due to redundant codons, while retaining the biological activity of the translated polypeptides.

Further, the invention encompasses an isolated nucleic acid comprising a nucleotide sequence having substantial homology to a nucleotide sequence of an isolated nucleic acid encoding a polypeptide disclosed herein. Preferably, the nucleotide sequence of an isolated nucleic acid encoding a polypeptide of the invention is “substantially homologous,” that is, is about 60% homologous, more preferably about 70% homologous, even more preferably about 80% homologous, more preferably about 90% homologous, even more preferably, about 95% homologous, and even more preferably about 99% homologous to a nucleotide sequence of an isolated nucleic acid encoding a polypeptide of the invention.

It is to be understood explicitly that the scope of the present invention encompasses homologs, analogs, variants, fragments, derivatives and salts, including shorter and longer polypeptides and polynucleotides, as well as polypeptide and polynucleotide analogs with one or more amino acid or nucleic acid substitution, as well as amino acid or nucleic acid derivatives, non-natural amino or nucleic acids and synthetic amino or nucleic acids as are known in the art, with the stipulation that these modifications must preserve the immunologic activity of the original molecule. Specifically any active fragments of the active polypeptides as well as extensions, conjugates and mixtures are included and are disclosed herein according to the principles of the present invention.

The invention should be construed to include any and all isolated nucleic acids which are homologous to the nucleic acids described and referenced herein, provided these homologous nucleic acids encode polypeptides having the biological activity of the polypeptides disclosed herein.

The skilled artisan would understand that the nucleic acids of the invention encompass a RNA or a DNA sequence encoding a polypeptide of the invention, and any modified forms thereof, including chemical modifications of the DNA or RNA which render the nucleotide sequence more stable when it is cell free or when it is associated with a cell. Chemical modifications of nucleotides may also be used to enhance the efficiency with which a nucleotide sequence is taken up by a cell or the efficiency with which it is expressed in a cell. Any and all combinations of modifications of the nucleotide sequences are contemplated in the present invention.

Further, any number of procedures may be used for the generation of mutant, derivative or variant forms of a protein of the invention using recombinant DNA methodology well known in the art such as, for example, that described in Sambrook et al. (2012, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York).. Procedures for the introduction of amino acid changes in a polypeptide or polypeptide by altering the DNA sequence encoding the polypeptide are well known in the art and are also described in these, and other, treatises.

According to yet another embodiment, composition of the invention, comprising the nucleic acid sequences or combination of nucleic acid sequences of the present invention, is capable of generating a CIV-specific immune response. In another embodiment, the composition of the invention, comprising the nucleic acid sequences or combination of nucleic acid sequences of the present invention, is capable of generating CIV-specific antibodies. In certain embodiments, the composition is able to protect against CIV, including H3N8 CIV and H3N2 CIV.

In one embodiment, the composition of the invention comprises a polypeptide, or a fragment of a polypeptide, a homolog, a variant, a derivative or a salt of a polypeptide having the sequence of any one or more of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or SEQ ID NO: 8.

The invention should also be construed to include any form of a polypeptide having substantial homology to the polypeptides disclosed herein. Preferably, a polypeptide which is “substantially homologous” is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous, more preferably about 90% homologous, even more preferably, about 95% homologous, and even more preferably about 99% homologous to amino acid sequence of the polypeptides disclosed herein.

According to yet another embodiment, composition of the invention, comprising the polypeptide or combination of polypeptides of the present invention, is capable of generating a CIV-specific immune response. In another embodiment, the composition of the invention, comprising the polypeptide or combination of polypeptides of the present invention, is capable of generating CIV-specific antibodies. In certain embodiments, the composition is able to protect against CIV, including H3N8 CIV and H3N2 CIV.

The present invention should also be construed to encompass "mutants," "derivatives," and "variants" of the polypeptides of the invention (or of the DNA encoding the same) which mutants, derivatives and variants are polypeptides which are altered in one or more amino acids (or, when referring to the nucleotide sequence encoding the same, are altered in one or more base pairs) such that the resulting polypeptide (or DNA) is not identical to the sequences recited herein, but has the same biological property as the polypeptides disclosed herein.

Mutated virus and LAV

The invention relates in part to the generation, selection and identification of mutant CIV that generate a CIV-specific immune response, and the use of such viruses in vaccine and pharmaceutical formulations. In certain embodiments, the mutant CIV is a live-attenuated CIV (LACIV).

As described herein, in certain embodiments the mutant CIV comprises one or more mutations in segment 8, the results in either the lack of NS1 expression or the expression of truncation mutants of NS1. The mutant CIV induces CIV-specific immune responses and antibody production, and is thus able to protect against CIV and CIV-related pathology.

Any mutant virus or strain which has at least one mutation can be selected and used in accordance with the invention. In one embodiment, naturally occurring mutants or variants, or spontaneous mutants can be selected that include at least one mutation in segment 8, as described elsewhere herein. In another embodiment, mutant viruses can be generated by exposing the virus to mutagens, such as ultraviolet irradiation or chemical mutagens, or by multiple passages and/or passage in non-permissive hosts. Screening in a differential growth system can be used to select for those mutants having at least one mutation in segment 8, as described elsewhere herein. For viruses with segmented genomes, the attenuated phenotype can be transferred to another strain having a desired antigen by reassortment, (i.e., by coinfection of the attenuated virus and the desired strain, and selection for reassortants displaying both phenotypes).

In another embodiment, mutations can be engineered into an influenza virus, including, but not limited to H3N8 CIV or H3N2 CIV using "reverse genetics" approaches. In this way, natural or other mutations which confer the attenuated phenotype can be engineered into vaccine strains. For example, deletions, insertions, or substitutions of the coding region of segment 8, encoding NS1 can be engineered. Deletions, substitutions or insertions in the non-coding region of segment 8 are also contemplated. To this end, mutations in the signals responsible for the transcription, replication, polyadenylation and/or packaging of segment 8 can be engineered. In certain embodiments, the mutation comprises the insertion of a stop signal, which terminates translation of the NS1 protein, thereby producing a truncated NS1 mutant.

In certain instances, the reverse genetics technique involves the preparation of synthetic recombinant viral RNAs that contain the non-coding regions of the negative strand virus RNA which are essential for the recognition by viral polymerases and for packaging signals necessary to generate a mature virion. The recombinant RNAs are synthesized from a recombinant DNA template and reconstituted in vitro with purified viral polymerase complex to form recombinant ribonucleoproteins (RNPs) which can be used to transfect cells. In some instances, a more efficient transfection is achieved if the viral polymerase proteins are present during transcription of the synthetic RNAs either in vitro or in vivo. The synthetic recombinant RNPs can be rescued into infectious virus particles. The foregoing techniques are described in U.S. Pat. No. 5,166,057 issued Nov. 24, 1992; in U.S. Pat. No. 5,854,037 issued Dec. 29, 1998; in European Patent Publication EP 0702085A1, published Feb. 20, 1996; in U.S. patent application Ser. No. 09/152,845; in International Patent Publications PCT WO97/12032 published Apr. 3, 1997; WO96/34625 published Nov. 7, 1996; in European Patent Publication EP-A780475; WO 99/02657 published Jan. 21, 1999;. WO 98/53078 published Nov. 26, 1998; WO 98/02530 published Jan. 22, 1998; WO 99/15672 published Apr. 1, 1999; WO 98/13501 published Apr. 2, 1998; WO 97/06270 published Feb. 20, 1997; and EPO 780 47SA1 published Jun. 25, 1997, each of which is incorporated by reference herein in its entirety.

Attenuated viruses generated by the reverse genetics approach can be used in the vaccine and pharmaceutical formulations described herein. Reverse genetics techniques can also be used to engineer additional mutations to other viral genes important for vaccine production—i.e., the epitopes of useful vaccine strain variants can be engineered into the attenuated virus. Alternatively, completely foreign epitopes, including antigens derived from other viral or non-viral pathogens can be engineered into the attenuated strain.

In an alternate embodiment, a combination of reverse genetics techniques and reassortant techniques can be used to engineer attenuated viruses having the desired epitopes. For example, an attenuated virus (generated by natural selection, mutagenesis or by reverse genetics techniques) and a strain carrying the desired vaccine epitope (generated by natural selection, mutagenesis or by reverse genetics techniques) can be co-infected in hosts that permit reassortment of the segmented genomes. Reassortants that display both the attenuated phenotype and the desired epitope can then be selected.

The attenuated virus of the present invention can itself be used as the active ingredient in vaccine or pharmaceutical formulations. In certain embodiments, the attenuated virus can be used as the vector or "backbone" of recombinantly produced vaccines. To this end, the "reverse genetics" technique can be used to engineer mutations or introduce foreign epitopes into the attenuated virus, which would serve as the "parental" strain. In this way, vaccines can be designed for immunization against strain variants, or in the alternative, against completely different infectious agents or disease antigens.

For example, in one embodiment, the immunological composition of the invention comprises a mutant virus, engineered to express one or more epitopes or antigens of CIV along with epitopes or antigens of another pathogen. For example, the virus can be engineered to express neutralizing epitopes of other preselected strains. Alternatively, epitopes of other viruses can be built into the mutant virus. Alternatively, epitopes of non-viral infectious pathogens (e.g., parasites, bacteria, fungi) can be engineered into the virus.

In one embodiment, the viruses selected for use in the invention is capable of inducing a robust anti-CIV response in the host—a feature which contributes to the generation of a strong immune response when used as a vaccine, and which has other biological consequences that make the viruses useful as pharmaceutical agents for the prevention and/or treatment of other viral infections, or other diseases.

The mutant viruses, which induce a CIV -specific immune response in hosts, may also be used in pharmaceutical formulations for the prophylaxis or treatment of other influenza infections, or influenza-related pathology. In this regard, the tropism of the virus can be altered to target the virus to a desired target organ, tissue or cells in vivo or ex vivo. Using this approach, the CIV-specific immune response can be induced locally, at the target site, thus avoiding or minimizing the side effects of systemic treatments. To this end, the attenuated virus can be engineered to express a ligand specific for a receptor of the target organ, tissue or cells.

Vaccine

In certain aspects, the immunological composition is useful as a vaccine, where the immunological composition induces an immune response to the antigen in a cell, tissue or mammal. Preferably, the vaccine induces a protective immune response in the mammal. As used herein, an "immunological composition" may comprise, by way of examples, a mutant virus, a live-attenuated virus (LAV), an antigen (e.g., a polypeptide), a nucleic acid encoding an antigen (e.g., an antigen expression vector), or a cell expressing or presenting an antigen or cellular component. In particular embodiments the immunological composition comprises or encodes all or part of any polypeptide antigen described herein, or an immunologically functional equivalent thereof. In other embodiments, the immunological composition is in a mixture that comprises an additional immunostimulatory agent or nucleic acids encoding such an agent. Immunostimulatory agents include but are not limited to an additional antigen, an immunomodulator, an antigen presenting cell or an adjuvant. In other embodiments, one or more of the additional agent(s) is covalently bonded to the antigen or an immunostimulatory agent, in any combination. In certain embodiments, the antigenic composition is conjugated to or comprises an HLA anchor motif amino acids.

In the context of the present invention, the term "vaccine" refers to a substance that induces anti-CIV immunity or suppresses CIV upon inoculation into an animal.

The invention encompasses vaccine formulations comprising a mutant virus, wherein the mutant virus is a canine influenza virus (CIV). In one embodiment, the vaccine comprises a mutant CIV comprising one or more mutations in segment 8, and a suitable excipient. The virus used in the vaccine formulation may be selected from naturally occurring mutants or variants, mutagenized viruses or genetically engineered viruses. Mutant strains of CIV can also be generated via reassortment techniques, or by using a combination of the reverse genetics approach and reassortment techniques. Naturally occurring variants include viruses isolated from nature as well as spontaneous occurring variants generated during virus propagation. The mutant virus can itself be used as the active ingredient in the vaccine formulation. Alternatively, the mutant virus can be used as the vector or “backbone” of recombinantly produced vaccines. To this end, recombinant techniques such as reverse genetics (or, for segmented viruses, combinations of the reverse genetics and reassortment techniques) may be used to engineer mutations or introduce foreign antigens into the attenuated virus used in the vaccine formulation. In this way, vaccines can be designed for immunization against strain variants, or in the alternative, against completely different infectious agents or disease antigens.

In one embodiment, the vaccine formulation comprises a plurality of mutant CIV. For example, in one embodiment, the vaccine formulation may comprise one or more of the mutant CIV, described herein, in combination with other mutant CIV that induce an anti-CIV immune response. For example, in one embodiment, the vaccine formulation comprises a live-attenuated CIV having one or more mutations in segment 1 and/or segment 2. In one embodiment, the vaccine formulation comprises a mutant single-cycle infectious CIV comprising a deletion mutant in segment 4, resulting in the lack of HA expression.

In one embodiment, the present invention comprises a method of generating a mutant virus, comprising contacting a host cell with a polynucleotide comprising the nucleic acid sequences of segment 8, having one or more mutations, described elsewhere herein.

Propagation of the virus in culture is known to persons in the art. Briefly, the virus is grown in the media compositions in which the host cell is commonly cultured. Suitable host cells for the replication of CIV include, e.g., Vero cells, BHK cells, MDCK cells, 293 cells COS cells, and CEK cells, including 293T cells, COS7 cells. Commonly, co-cultures including two of the above cell lines, e.g., MDCK cells and either 293T or COS cells are employed at a ratio, e.g., of 1:1, to improve replication efficiency. Typically, cells are cultured in a standard commercial culture medium, such as Dulbecco's modified Eagle's medium supplemented with serum (e.g., 10% fetal bovine serum), or in serum free medium, under controlled humidity and CO_2 concentration suitable for maintaining neutral buffered pH (e.g., at pH between 7.0 and 7.2). Optionally, the medium contains antibiotics to prevent bacterial growth, e.g., penicillin, streptomycin, etc., and/or additional nutrients, such as L-glutamine, sodium pyruvate, non-essential amino acids, additional supplements to promote favorable growth characteristics, e.g., trypsin, β-mercaptoethanol, and the like.

Procedures for maintaining mammalian cells in culture have been extensively reported, and are known to those of skill in the art. General protocols are provided, e.g., in Freshney (1983) Culture of Animal Cells: Manual of Basic Technique, Alan R. Liss, New York; Paul (1975) Cell and Tissue Culture, 5th ed., Livingston, Edinburgh; Adams (1980) Laboratory Techniques in Biochemistry and Molecular Biology-Cell Culture for Biochemists, Work and Burdon (eds.) Elsevier, Amsterdam. Additional details regarding tissue culture procedures of particular interest in the production of influenza virus in vitro include, e.g., Merten et al. (1996) Production of influenza virus in cell cultures for vaccine preparation. In Cohen and Shafferman (eds) Novel Strategies in Design and Production of Vaccines, which is incorporated herein in its entirety. Additionally, variations in such procedures adapted to the present invention are readily determined through routine experimentation.

Cells for production of a virus can be cultured in serum-containing or serum free medium. In some case, e.g., for the preparation of purified viruses, it is desirable to grow the host cells in serum free conditions. Cells can be cultured in small scale, e.g., less than 25 ml medium, culture tubes or flasks or in large flasks with agitation, in rotator bottles, or on microcarrier beads (e.g., DEAE-Dextran microcarrier beads, such as Dormacell, Pfeifer & Langen; Superbead, Flow Laboratories; styrene copolymer-tri-methylamine beads, such as Hillex, SoloHill, Ann Arbor) in flasks, bottles or reactor cultures. Microcarrier beads are small spheres (in the range of 100-200 microns in diameter) that provide a large surface area for adherent cell growth per volume of cell culture. For example a single liter of medium can include more than 20 million microcarrier beads providing greater than 8000 square centimeters of growth surface. For commercial production of viruses, e.g., for vaccine production, it is often desirable to culture the cells in a bioreactor or fermenter. Bioreactors are available in volumes from under 1 liter to in excess of 100 liters, e.g., Cyto3 Bioreactor (Osmonics, Minnetonka, Minn.); NBS bioreactors (New Brunswick Scientific, Edison, N.J.); laboratory and commercial scale bioreactors from B. Braun Biotech International (B. Braun Biotech, Melsungen, Germany).

Virtually any heterologous gene sequence may be constructed into the viruses of the invention for use in vaccines. Preferably, epitopes that induce a protective immune response to any of a variety of pathogens, or antigens that bind neutralizing antibodies may be expressed by or as part of the viruses. For example, heterologous gene sequences that can be constructed into the viruses of the invention for use in vaccines include but are not limited to epitopes of human immunodeficiency virus (HIV) such as gp120; hepatitis B virus surface antigen (HBsAg); the glycoproteins of herpes virus (e.g. gD, gE); VP1 of poliovirus; antigenic determinants of non-viral pathogens such as bacteria and parasites, to name but a few. In another embodiment, all or portions of immunoglobulin genes may be expressed. For example, variable regions of anti-idiotypic immunoglobulins that mimic such epitopes may be constructed into the viruses of the invention. In yet another embodiment, tumor associated antigens may be expressed.

Either a live recombinant viral vaccine or an inactivated recombinant viral vaccine can be formulated. A live vaccine may be preferred because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confers substantial, long-lasting immunity. Production of such live recombinant virus vaccine formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in the allantois of the chick embryo followed by purification.

Many methods may be used to introduce the vaccine formulations described above, these include but are not limited to introduction intranasally, intratracheally, orally, intradermally, intramuscularly, intraperitoneally, intravenously, and subcutaneously. It may be preferable to introduce the virus vaccine formulation via the natural route of infection of the pathogen for which the vaccine is designed, or via the natural route of infection of the parental virus.

A vaccine of the present invention, comprising a mutant CIV could be administered once. Alternatively, a vaccine of the present invention, comprising a mutant CIV, could be administered twice or three or more times with a suitable interval between doses. Alternatively, a vaccine of the present invention, comprising a mutant CIV, could be administered as often as needed to an animal, preferably a mammal.

Methods

The invention provides a method for treating or preventing canine influenza infection or a CIV-related disease or disorder. In one embodiment, the method comprises administering an immunological composition comprising a mutant CIV. In one embodiment, the method comprises administering an immunological composition comprising a mutant CIV comprising one or more mutations in segment 8, to a subject in need thereof.

In certain embodiments, the mutant CIV induces an enhanced immune response as compared to an inactivated CIV. For example, in certain embodiments, the induced immune response of mutant CIV is 2-fold more, 3-fold more, 5-fold more, 10-fold more, 15-fold more, 20-fold more, 50-fold more, 100-fold more, 500-fold more, or 1000-fold more, than inactivated CIV. The immune response induced the mutant CIV can be measured using standard assays. For example, in certain embodiments, the immune response induced by mutant CIV is measured by detecting the amount of CIV-specific antibodies produced in the subject following administration of mutant CIV.

The therapeutic compositions of the invention may be administered prophylactically or therapeutically to subjects suffering from, or at risk of, or susceptible to, developing the disease or condition. Such subjects may be identified using standard clinical methods. In the context of the present invention, prophylactic administration occurs prior to the manifestation of overt clinical symptoms of disease, such that a disease or disorder is prevented or alternatively delayed in its progression. In the context of the field of medicine, the term “prevent” encompasses any activity which reduces the burden of mortality or morbidity from disease. Prevention can occur at primary, secondary and tertiary prevention levels. While primary prevention avoids the development of a disease, secondary and tertiary levels of prevention encompass activities aimed at preventing the progression of a disease and the emergence of symptoms as well as reducing the negative impact of an already established disease by restoring function and reducing disease-related complications.

In certain embodiments, the subject is a mammal. For example, the subject may include, but is not limited to, a human, primate, cow, horse, sheep, pig, dog, cat, or rodent. In one embodiment, the subject is a dog. The method may be used to treat or prevent CIV or CIV-related pathology in any breed or species of dog. In certain embodiments, the relative amount of active ingredient in a single dose, or the frequency of doses, will vary depending on the age, sex, weight, or breed of subject (e.g. dog).

The composition may be combined with an adjuvant. An adjuvant refers to a compound that enhances the immune response when administered together (or successively) with the immunological composition. Examples of suitable adjuvants include cholera toxin, salmonella toxin, alum and such, but are not limited thereto. Furthermore, a vaccine of this invention may be combined appropriately with a pharmaceutically acceptable carrier. Examples of such carriers are sterilized water, physiological saline, phosphate buffer, culture fluid and such. Furthermore, the vaccine may contain as necessary, stabilizers, suspensions, preservatives, surfactants and such. The vaccine is administered systemically or locally. Vaccine administration may be performed by single administration or boosted by multiple administrations.

Administration

In one embodiment, the methods of the present invention comprise administering an immunological composition of the invention directly to a subject in need thereof. Administration of the composition can comprise, for example, intranasal, intramuscular, intravenous, peritoneal, subcutaneous, intradermal, as well as topical administration.

Furthermore, the actual dose and schedule can vary depending on whether the compositions are administered in combination with other pharmaceutical compositions, or depending on inter-individual differences in pharmacokinetics, drug disposition, and metabolism. One skilled in the art can easily make any necessary adjustments in accordance with the exigencies of the particular situation.

Pharmaceutical Compositions

The present invention envisions treating or preventing CIV or CIV-related pathology in a mammal by the administration of a therapeutic composition of the invention to a mammal in need thereof. Administration of the composition in accordance with the present invention may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the compositions of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated. The amount administered will vary depending on various factors including, but not limited to, the composition chosen, the particular disease, the weight, the physical condition, and the age of the mammal, and whether prevention or treatment is to be achieved. Such factors can be readily determined by the clinician employing animal models or other test systems which are well known to the art.

The present invention encompasses pharmaceutical compositions comprising a mutant CIV to be used as anti-viral agents or as agents against CIV-related diseases and disorders. The pharmaceutical compositions have utility as an anti-viral prophylactic and may be administered to a subject at risk of getting infected or is expected to be exposed to a virus. For example, subjects traveling to parts of the world where CIV is prevalent can be administered a pharmaceutical composition of the invention. In certain embodiments, subjects who are expected to be in contact with other subjects at risk, can be administered a pharmaceutical composition of the invention.

The mutant CIV of the invention may be engineered using the methods described herein to express proteins or peptides which would target the viruses to a particular site. In one embodiment, where the site to be targeted expresses a receptor to a growth factor, e.g., VEGF, EGF, or PDGF, the LACIV may be engineered to express the appropriate growth factor or portion(s) thereof. Thus, in accordance with the invention, the mutant CIV may be engineered to express any target gene product, including peptides, proteins, such as enzymes, hormones, growth factors, antigens or antibodies, which will function to target the virus to a site in need of anti-viral, antibacterial, anti-microbial or anti-cancer activity.

Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The pharmaceutical compositions of the present invention may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, in a preferred embodiment it may be desirable to introduce the pharmaceutical compositions of the invention into the lungs by any suitable route. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In certain embodiments, the pharmaceutical composition is a veterinary pharmaceutical composition suitable for administration to a veterinary subject, including but not limited to a canine subject. Exemplary canine subjects include dogs, wolves, foxes, coyotes, and jackals.

In certain embodiments, the veterinary pharmaceutical composition is "palatable," meaning an oral veterinary composition that is readily accepted by canines, including dogs, without any coaxing or with some coaxing. Palatable compositions are compositions that score at least 2 using a palatability assessment method wherein dog owners score the composition from 0 to 3, wherein dogs scoring 0 do not consume the composition; dogs scoring 1 consume the composition after some time; dogs scoring 2 consume the composition with some coaxing and dogs scoring 3 consume the composition readily. A skilled person is well-versed in these palatability standards and scoring regimes. In another embodiment, the daily dose for dogs may be around 100 mg/kg. Veterinary pharmaceutical agents that may be included in the compositions of the invention are well-known in the art (see e.g. Plumb' Veterinary Drug Handbook, 5th Edition, ed. Donald C. Plumb, Blackwell Publishing, (2005) or The Merck Veterinary Manual, 9th Edition, (January 2005)).

In yet another embodiment, the pharmaceutical composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980, Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla. (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger & Peppas, 1983, J. Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351 (1989); Howard et al., 1989, J. Neurosurg. 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the composition's target, i.e., the lung, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, 1984, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138). Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

The pharmaceutical compositions of the present invention comprise a therapeutically effective amount of the attenuated virus, and a pharmaceutically acceptable carrier. In a specific embodiment, the term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeiae for use in animals, and more particularly in humans. The term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water and the like. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. These compositions can be formulated as a suppository. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in “Remington's Pharmaceutical Sciences” by E. W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

The amount of the pharmaceutical composition of the invention which will be effective in the treatment or prevention of a particular disease or disorder will depend on the nature of the disease or disorder, and can be determined by standard clinical techniques. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: Canine influenza viruses with modified NS1 proteins for the development of live-attenuated vaccines

Influenza non-structural (NS) segment 8 encodes the non-structural protein 1 (NS1) from the full-length transcript, as well as the nuclear export protein (NEP) from pre-mRNA splicing (Hale et al., 2008, The Journal of general virology, 89:2359-2376, Lamb et al., 1980, Proceedings of the National Academy of Sciences, 77:1857-1861). NS1 is a multifunctional protein that is mainly involved in counteracting the antiviral type I interferon (IFN) response (Garcia-Sastre et al., 1998, Virology, 252:324-330, Hale et al., 2008, The Journal of general virology, 89:2359-2376, Steidle et al., 2010, J Virol, 84:12761-12770), and also contributes to viral virulence and pathogenesis (Geiss et al., 2002, Proceedings of the National Academy of Sciences, 99:10736-10741, Nogales et al., 2014, J Virol, 88:10525-10540). Because of NS1's ability to hijack the host immune response, a variety of potential vaccine strategies have been developed that are based on the use of modified NS1 proteins as a mean for virus attenuation (Falcon et al., 2005, The Journal of general virology, 86:2817-2821, Ferko et al., 2004, J Virol, 78:13037-13045, Quinlivan et al., 2005, J Virol, 79:8431-8439, Richt et al., 2009, Current topics in microbiology and immunology, 333:177-195, Steel et al., 2009, J Virol 83:1742-1753, Vincent et al., 2007, Vaccine 25:7999-8009). Equine (Quinlivan et al., 2005, J Virol, 79:8431-8439), swine (Richt et al., 2006, J Virol 80:11009-11018, Solorzano et al., 2005, J Virol, 79:7535-7543, Vincent et al., 2007, Vaccine, 25:7999-8009), avian (Choi et al., 2015, Archives of virology, 160:1729-1740, Steel et al., 2009, J Virol, 83:1742-1753, Wang et al., 2008, Vaccine, 26:3580-3586), and human (Baskin et al., 2007, J Virol, 81:11817-11827, Pica et al., 2012, J Virol, 86:10293-10301) IAVs with partial truncations or deletions in the viral NS1 protein are all attenuated in vitro and in vivo (Pica et al., 2012, J Virol, 86:10293-10301, Quinlivan et al., 2005, J Virol, 79:8431-8439, Steel et al., 2009, J Virol, 83:1742-1753, Vincent et al., 2007, Vaccine, 25:7999-8009). These NS1 mutant IAVs are also able to induce protective immune response in mice (Hai et al., 2008, J Virol, 82:10580-10590, Pica et al., 2012, J Virol, 86:10293-10301, Talon et al., 2000, Proceedings of the National Academy of Sciences, 97:4309-4314), horses (Quinlivan et al., 2005, J Virol, 79:8431-8439), pigs (Richt et al., 2006, J Virol, 80:11009-11018, Solorzano et al., 2005, J Virol, 79:7535-7543, Vincent et al., 2007, Vaccine, 25:7999-8009), birds (Choi et al., 2015, Archives of virology, 160:1729-1740, Steel et al., 2009, J Virol, 83:1742-1753, Wang et al., 2008, Vaccine, 26:3580-3586), and macaques (Baskin et al., 2007, J Virol, 81:11817-11827) and therefore, they represent LAIV candidates for prevention of IAV infections.

Canine Influenza Virus (CIV) H3N8 is the causative agent of canine influenza, a common and contagious respiratory disease of the dog. CIV originated from the transfer of H3N8 Equine Influenza Virus (EIV) into canine populations around 1999. Since that time CIV has continued to infect and spread among dog populations. Currently, only inactivated influenza vaccines (IIV) are commercially available for the prevention of CIV H3N8. However, live-attenuated influenza vaccines (LAIV) are known to provide better immunogenicity and protection efficacy than IIVs. The influenza virus non-structural protein 1 (NS1) is a virulence factor that offers an attractive target for the preparation of attenuated viruses for use as LAIVs. Described herein is the development and manufacture of a genetically engineered A/canine/NY/dog23/2009 in order to generate LAIV candidates. To this end, reverse genetics technology was used to generate viruses containing C-terminal truncations of NS1 (NS1-73, NS1-99 and NS1-126) or an entire NS1 deletion (ΔNS1). All recombinant viruses replicated efficiently in MDCK cells in spite of being impaired in their ability to inhibit the type I interferon (IFN) response. Compared with wild-type H3N8 CIV, a single intranasal inoculation of the NS1 mutant viruses resulted in lower levels of replication *in vivo*, while retaining immunogenicity and conferring homologous protection against wild type virus challenge. Immunogenicity and protection efficacy was also better than that observed with a commercially available IIV (Nobivac). This is the first description of a LAIV for the prevention and control of H3N8 CIV in dogs.

The materials and methods employed in these experiments are now described.

Cells and viruses

Human embryonic kidney 293T (293T; ATCC CRL-11268) and Madin-Darby canine kidney (MDCK; ATCC CCL-34) cells were grown at 37°C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc.), 10% fetal bovine serum (FBS), and 1% PSG (penicillin, 100 units/ml; streptomycin 100 μg/ml; L-glutamine, 2 mM) (Nogales et al., 2014, J Virol, 88:10525-10540).

Influenza A/canine/NY/dog23/2009 H3N8 WT (Feng et al., 2015, J Virol, 89:6860-6873) and NS1 truncated (NS1-73, NS1-99 and NS1-126) or deleted (ΔNS1) NS1 mutants were grown in MDCK cells at 33°C. For infections, virus stocks were diluted in phosphate buffered saline (PBS), 0.3% bovine albumin (BA) and 1% PS (PBS/BA/PS). After viral infections, cells were maintained in DMEM with 0.3% BA, 1% PSG, and 1 μg/ml TPCK-treated trypsin (Sigma) (Martinez-Sobrido et al., 2010, Journal of visualized experiments, doi: (42)10.3791/2057). Recombinant Newcastle Disease Virus (rNDV) expressing the green fluorescent protein (GFP), rNDV-GFP, was also used in this experiment (Martinez-Sobrido et al., 2006, J Virol, 80:9192-9199, Park et al., 2003, J Virol, 77:1501-1511).

Construction of plasmids

The ambisense pDZ plasmid (Quinlivan et al., 2005, J Virol, 79:8431-8439) encoding the NS gene (Feng et al., 2015, J Virol, 89:6860-6873) was used to engineer the four NS1 mutants: pDZ-NS1-73 (encoding the first 73 amino acids of NS1), pDZ-NS1-99 (encoding the first 99 amino acids of NS1), pDZ-NS1-126 (encoding the first 126 amino acids of NS1) and pDZ-ΔNS1 (deletion of the entire

NS1). Truncations were generated by inverse PCR using primers designed to introduce deletions together with stop codons into the recombinant NS segments (Figure 1A and Figure 1B) (Quinlivan et al., 2005, J Virol, 79:8431-8439). The presence of introduced mutations was confirmed by sequencing.

The nucleotide sequences encoding the NS1 mutants are provided in SEQ ID NOs: 1-4, while the amino acid sequences of the NS1 truncation mutants are provided in SEQ IDs NOs 5-7 (Figure 9 through Figure 12).

Rescue of recombinant H3N8 CIVs

Viruses were rescued as previously demonstrated (Martinez-Sobrido et al., 2010, Journal of visualized experiments, doi: (42)10.3791/2057). Briefly, co-cultures (1:1) of 293T/MDCK cells (6-well plate format, 10^6 cells/well) were co-transfected in suspension, using Lipofectamine 2000 (Invitrogen), with 1 µg of the seven-ambisense WT plasmids (43) (pDZ-PB2, -PB1, -PA, -HA, -NP, -NA, -M,) plus the ambisense WT NS plasmid (pDZ-NS) or the NS1 mutant constructs (pDZ-ΔNS1, -NS1-73, -NS1-99 and -NS1-126). At 12 h post-transfection, medium was replaced with DMEM supplemented with 0.3% BA, 1% PSG, and 1.0 µg/ml TPCK-treated trypsin (Sigma). Virus-containing tissue culture supernatants (TCS) were collected 2-3 days post-transfection, clarified, and used to infect fresh MDCK cells. At 3 days post-infection (p.i), recombinant H3N8 CIVs were plaque purified and grown in MDCK cells (Nogales et al., 2014, J Virol, 88:10525-10540). Virus stocks were titrated by standard plaque assay (plaque forming units, PFU/ml) in MDCK cells (Nogales et al., 2014, J Virol, 88:10525-10540).

RT-PCR

Total RNA from mock or CIV-infected MDCK cells (multiplicity of infection [MOI] 3) was collected at 20 h p.i and purified using TRIzol reagent (Invitrogen) according to the manufacturer's specifications. cDNA synthesis for NS or NP viral (v)RNAs was performed using SuperScript® II Reverse Transcriptase (Invitrogen) and specific primers. cDNA synthesis of canine GAPDH mRNA was performed using a dT oligonucleotide (Invitrogen). cDNAs were used as templates for semi-quantitative PCR with primers specific for the NS and NP vRNAs and the cellular GAPDH mRNA.

Virus growth kinetics and plaque assays

Confluent monolayers of MDCK cells (12-well plate format, triplicates, 5 x 10^5 cells/well) were infected (MOI 0.001) and placed at 33°C or 37°C. Tissue culture supernatants were collected at various times p.i and viral titers were determined by immunofocus assay (fluorescent forming units, FFU]/ml) in MDCK cells (Nogales et al., 2014, J Virol, 88:10525-10540). The mean value and standard deviation was calculated using Microsoft Excel. For plaque assays, confluent MDCK cell monolayers (6-well plate format, 106 cells/well) were infected with the indicated CIVs. One hour after infection, monolayers were overlaid with agar and incubated for 3 days at 33°C. Cells were then fixed with 4% paraformaldehyde (PFA), and the overlays removed. Fixed cells were permeabilized (0.5% Triton X-100 in PBS for 15 minutes at room temperature) and used for immunostaining (Nogales et al., 2014,Virology, 476C:206-216) using the anti-NP monoclonal antibody (MAb) HB-65 (ATTC) and the Vectastain ABC kit and DAB HRP Substrate Kit (Vector), according to manufacturer's specifications.

Bioassay to assess interferon production

The levels of type I interferon (IFN) produced in CIV-infected cells were determined by using confluent monolayers of MDCK cells (12-well format, triplicates, 5 x 10^5 cells/well) constitutively expressing GFP-CAT and firefly luciferase (FFluc) under the control of the IFNβ promoter (MDCK IFNβ GFP-CAT/FFluc) (Hai et al., J Virol, 82:10580-10590). These cells were mock infected or infected (MOI 3) with either WT or NS1 mutant H3N8 CIVs. Infection levels were evaluated by immunofluorescence using the anti-NP MAb HB-65. At 12 h p.i., activation of the IFNβ promoter was determined by assessing GFP expression under a fluorescence microscope, and also by quantifying FFluc activity from cell lysates using a luciferase reporter assay (Promega) and a Lumicount luminometer. Supernatants of infected MDCK cells were also collected and viruses were inactivated by exposure to shortwave (254 nm) UV radiation for 10 min at a distance of 6 cm (Nogales et al., 2014, J Virol, 88:10525-10540). Fresh MDCK cells seeded in 96-well plates (5 x 10^4 cells/well, triplicates) were treated with UV-inactivated supernatants for 24 h and then infected (MOI 3) with rNDV-GFP (Nogales et al., 2014, J Virol, 88:10525-10540). GFP intensity was measured 14 h p.i. using a microplate reader (DTX880; Beckman Coulter). MDCK cells were used as experimental controls and were mock treated or treated with 100 or 1000 units (U) of universal IFN (PBL Assay Science). GFP expression of mock-treated cells infected with rNDV-GFP were determined to be 100%. Mean values and SDs were calculated using Microsoft Excel.

Mice experiments

Viral replication, immunogenicity and protection efficacy, was evaluated using 5 to 7-week-old female C57BL/6 mice, which were purchased from the National Cancer Institute (NCI) and maintained under specific pathogen-free conditions for one week. Mice were anesthetized intraperitoneally (i.p.) with 2,2,2-tribromoethanol (Avertin; 240 mg/kg of body weight) and inoculated intranasally (i.n.) with the indicated amounts of H3N8 CIVs in a final volume of 30 µl. Alternatively, 100µl of a commercially available, inactivated CIV H3N8 vaccine ("Nobivac", Merck Animal Health) was inoculated intramuscularly (i.m). CIV H3N8 replication was determined by measuring viral titers in the lungs of infected mice at days 2 and 4 p.i. To that end, three mice from each group were euthanized and lungs were collected and homogenized. Mice were euthanized by administration of a lethal dose of avertin and exsanguination. Virus titers were determined by immunofocus assay (FFU/ml). Mouse sera were collected by submandibular bleeding 24 hours prior to viral challenges and evaluated for the presence of influenza virus total antibodies.

ELISA

Enzyme-linked immunosorbent assays (ELISAs) were performed (Nogales et al., 2014, J Virol, 88:10525-10540) by coating 96-well plates for 16 hours at 4°C with lysates from mock-, or CIV WT infected MDCK cells. The coated wells were blocked with PBS containing 1% BSA, and then plates were incubated with 1:2 fold dilutions of serum (starting dilution of 1:50) for 1 hour at 37°C. After incubation, plates were washed with PBS, and incubated with HRP-conjugated goat anti-mouse IgG (1:2,000; Southern Biotech) for 1 hour at 37°C. Reactions were developed with tetramethylbenzidine (TMB) substrate (BioLegend) for 10 minutes at room temperature, quenched with 2N H2SO4, and read at 450 nm (Vmax kinetic microplate reader; Molecular Devices).

HAI assays

Hemagglutination inhibition (HAI) assays were used to assess the presence of neutralizing antibodies (NAbs). Mouse sera was treated with receptor-destroying enzyme (RDE; Denka Seiken) and heat inactivated for 30 minutes at 56°C. Sera was then serially 2-fold diluted in 96-well V-bottom plates and mixed 1:1 with 4 hemagglutinating units (HAU) of H3N8 CIV for 30 min at room temperature. The HAI titers were determined by adding 0.5% turkey red blood cells (RBCs) to the virus-antibody mixtures for 30 min on ice (Nogales et al., 2014, J Virol, 88:10525-10540). The GMT and SD from individual mice (n = 4) was calculated from the last well where hemagglutination was inhibited, using Microsoft Excel.

Canine tracheal explants preparation

Dog tracheas were collected from 3 healthy Beagles (Charles River Laboratories) used as negative controls in other, unrelated studies. Briefly, tracheas were aseptically collected immediately upon euthanasia and transported in pre-warmed medium as previously described (Gonzalez et al., 2014, J Virol, 88(16): 9208-9219). Tracheas were washed 6 times over a period of 4 hours and maintained at 33°C, 5% CO_2, and 95% humidity between washes. The connective tissue was then removed and the trachea was open lengthwise. Each tracheal ring was divided in four 0.5- by 0.5-cm explants and placed onto a sterile section of filter paper on top of an agarose plug, epithelium facing upwards.

Explants infection and virus quantification

Explants were infected with a dose of 200 PFU 24 hours after dissection (designed as day 0). Culture medium was used for mock-infected explants. Inoculated explants were sampled for bead clearance, histology, and viral replication at days (D) 0, 1, 3 and 5 p.i. Viral titers were determined by standard plaque assays on MDCK cells and revealed by immunostaining of plaques.

Estimation of bead clearance time

Ciliary beating of the tracheal explants was checked at indicated times p.i. Five microliters of polystyrene microsphere beads (Polysciences, Northampton, UK) was placed on the apical surface of the explants and bead clearance was evaluated by eye every 5 min. Ciliary beating was considered efficient when the beads were completely cleared to one side of the explants by coordinated cilia movement.

Histological analysis and immunohistochemistry

After collection, the explants were fixed in 10% buffered formalin for a minimum of 48 hours. Subsequently, 4μm sections of paraffin embedded tissue were either stained with Haematoxylin and Eosin or deparaffinized and hydrated for viral nucleoprotein (NP) staining using standard procedures. Briefly, sections were incubated overnight at 4°C with a mouse MAb anti-NP (HB-65) diluted in 10% normal goat serum. Immunohistochemistry was performed using the Dako supervision system according to the manufacturer protocol, and slides were counterstained with Mayer's haematoxylin. Histological images were captured with the cellD software (Olympus).

The results of the experiments are now described.

Generation of recombinant H3N8 CIVs with truncated NS1 proteins

H3N8 CIVs containing a full-length (WT), truncations (NS1-73, NS1-99, and NS1-126) or a deletion (ΔNS1) of the NS1 protein (Feng et al., 2015, J Virol, 89:6860-6873, Martinez-Sobrido et al., 2010, Journal of visualized experiments, doi: (42)10.3791/2057) were generated by reverse genetics (Figure 1A). Both deletions and stop codons in the NS1 open reading frame were included, as well as unique restriction sites into each modified viral NS segment (Figure 1B) (Quinlivan et al., 2005, J Virol, 79:8431-8439). Importantly, the open reading frame of the H3N8 CIV NEP was not altered in any of the recombinant CIVs. The evaluation of NS1 protein expression levels by Western blot was demonstrated using a polyclonal antibody generated against the first 73 amino acids of NS1 A/swine/Texas/4199-2/98 (Solorzano et al., 2005, J Virol, 79:7535-7543). Detection of NS1 expression from WT and NS1-126 CIVs was demonstrated, likely due to low protein stability and/or concentration in the latter clones (Quinlivan et al., 2005, J Virol, 79:8431-8439, Solorzano et al., 2005, J Virol, 79:7535-7543). The identities of the recombinant H3N8 CIVs were confirmed by analyzing the expression of the viral NS vRNA by RT-PCR, revealing products with sizes of 890 (WT), 812 (NS1-126), 731 (NS1-99), 653 (NS1-73) and 424 (ΔNS1) nucleotides (Figure 1C), corresponding to the different recombinant NS segments. The influenza NP vRNA and the canine GAPDH mRNA were also evaluated by RT-PCR from the same samples, which served as controls.

Altogether, the data demonstrated the nature of the generated recombinant NS1 mutant H3N8 CIVs.

Growth properties of recombinant H3N8 CIVs in tissue culture

The replicative properties of the mutant NS1 H3N8 CIVs were examined using multicycle growth curves at 33°C or 37°C in MDCK cells infected at a low MOI (0.001) (Figure 2). Viruses in culture supernatants collected at 24, 48, 72 and 96 h p.i. were titrated using immunofocus assay (fluorescent forming units, FFU/ml). All the viruses containing truncated versions of NS1 (CIV NS1-126, NS1-99 and NS1-73) displayed replication kinetics comparable to that of WT H3N8 CIV at both 37°C and 33°C (Figure 2A and Figure 2B, respectively). In contrast, ΔNS1 H3N8 CIV replication was significantly affected at 37°C (Figure 2A) but not at 33°C (Figure 2B) for NS1 deficient influenza viruses (Falcon et al., 2005, The Journal of general virology, 86:2817-2821). Plaque sizes of WT and NS1-truncated H3N8 CIVs in MDCK cells at 33°C were consistent with the virus growth kinetics at that temperature (Figure 2C). Comparable plaque sizes for the WT and NS1 truncated H3N8 CIVs were observed, while the ΔNS1 CIV showed smaller plaques. The evaluation of the plaque phenotype of the recombinant H3N8 CIVs at 37°C could not be demonstrated, since none of the viruses plaque at this temperature. The data demonstrates that the NS1 mutant H3N8 CIVs can be propagated to levels comparable to those of WT virus in MDCK cells at permissive (33°C) temperatures.

Induction of IFN by recombinant H3N8 CIVs

One of the main functions of influenza NS1 protein is to counteract the type I IFN response during viral infection (Garcia-Sastre et al., 1998, Virology, 252:324-330, Hale et al., 2008, Virology, 89:2359-2376). The ability of NS1 mutant H3N8 CIVs to counteract the IFN response was demonstrated by two complementary bioassays (Figure 3A). The cell-based assay involved MDCK cells constitutively expressing GFP-CAT and FFluc reporter genes under the control of the IFNβ promoter (MDCK IFNβ GFP-CAT/FFluc) (Hai et al., 2008, J Virol, 82:10580-10590). Those cells were mock infected or infected (MOI 3) with either WT or mutant H3N8 CIVs, and IFNβ promoter activation was evaluated. Further, IFN in culture supernatants from the same virus-infected MDCK cells was assessed using a virus-based assay, where inhibition of rNDV-GFP infection was evaluated (Nogales et al., 2014, J Virol, 88:10525-10540). Comparable levels of viral infection were verified by immunofluorescence using an anti-NP HB-65 MAb (Figure 3B). For the cell-based assay, at 12 h p.i, activation of the IFNβ promoter in MDCK IFNβ GFP-CAT/FFluc cells was assessed by evaluating GFP expression (Figure 3C) and FFluc activity (Figure 3D). GFP expression was detected only in cells infected with the NS1 mutant CIVs, but not in mock-infected or WT H3N8 CIV-infected cells (Figure 3C), indicating that CIV WT infection (and NS1 protein) is able to counteract the IFN response during viral infection. This result with GFP was further confirmed and quantified by assessing FFluc activities from infected-cells lysates (Figure 3D). Differences in the activation of the IFNβ promoter and induction of FFluc during infection with the NS1 truncated H3N8 CIVs were observed. A stronger activation of the IFNβ promoter was observed in H3N8 CIV ΔNS1 infected cells, followed by NS1-126, NS1-99 and NS1-73 CIVs. The data demonstrated that CIVs encoding truncated or a deleted NS1 protein have different capabilities to counteract IFN activation. These results were confirmed by evaluating the presence of IFN in supernatants of virus-infected cells using virus-based bioassay (Nogales et al., 2014, J Virol, 88:10525-10540). In this assay, the level of IFN in supernatants from virus-infected cells resulted in inhibition of NDV infection and, therefore, GFP expression (Martinez-Sobrido et al., 2009, J Virol, 83:11330-11340, Martinez-Sobrido et al., 2006, J Virol, 80:9192-9199, Park et al., 2003, J Virol, 77:1501-1511). In cells pre-treated with culture supernatants from mock- and WT H3N8 CIV-infected cells, rNDV-GFP replicated efficiently (Figure 3E). The levels of rNDV-GFP infection decreased in MDCK cells pre-treated with culture supernatants from NS1 mutant H3N8 CIV-infected cells. NDV inhibition was stronger in cells pre-treated with supernatants from H3N8 CIV ΔNS1 infected MDCK cells, followed by NS1-126, NS1-99 and NS1-73, (Figure 3E). The data demonstrated that infection with the H3N8 CIV NS1 mutants induced different levels of IFN, where ΔNS1 induced higher levels of IFN than the NS1 truncated CIVs.

CIV H3N8 NS1 mutants are attenuated in vivo

As the different NS1 truncated H3N8 CIVs presented defects in counteracting the innate immune IFN response (Figure 3) it was next demonstrated whether the NS1 mutant H3N8 CIVs was attenuated in mice. No signs or symptoms of CIV infection were detected after intranasal (i.n) administration of 10^5 PFU WT

H3N8 CIV. Viral attenuation was determined by the viral titers of H3N8 CIV (and the NS mutants) at days 2 (N=3) and 4 (N=3) p.i in the lungs of infected mice. All H3N8 CIV NS1 mutants replicated efficiently in the lungs of infected mice, although ~ 1 log lower than WT H3N8 CIV (Figure 4). ΔNS1 H3N8 CIV was highly defective and was only detected at day 2 p.i. (~ 2.5 log lower titers than H3N8 CIV WT). These results demonstrate that NS1 mutant H3N8 CIVs are attenuated in mice, as compared to WT CIV, with ΔNS1 H3N8 CIV showing the highest level of attenuation.

Vaccination with H3N8 NS1 mutant CIVs elicits protective immunity against WT H3H8 CIV in mice

Inoculated mice (N=6) i.n. with 10^3 PFU of WT or NS1 mutant (NS1-73, NS1-99, NS1-126 and ΔNS1) H3N8 CIVs or mock vaccinated with PBS was used to demonstrate the immunity generated in mice. A group of mice (N=6) were vaccinated i.m. with 100 µl of Nobivac as a control (Deshpande et al., 2009, Veterinary therapeutics: research in applied veterinary medicine, 10:103-112). The humoral immune responses induced upon vaccination with the NS1 truncated H3N8 CIVs (Figure 5A) was determined. Serum samples taken two weeks post-vaccination were evaluated by ELISA using cell lysates from mock- or CIV H3N8-infected MDCK cells (Figure 5A). All mice vaccinated with the NS1-truncated H3N8 CIVs induced significant antibody titers against WT H3N8 CIV, similar to those induced by WT H3N8 CIV infection (Figure 5A). It was also demonstrated that total antibody titers from mice vaccinated with the ΔNS1 H3N8 CIV were lower than those observed with the NS1 truncated CIVs. Titers induced by vaccination with the truncated or deleted NS1H3N8 CIVs were higher than those obtained with the commercial vaccine (Figure 5A). Similar results were demonstrated when the HA inhibiting titers were evaluated against CIV H3N8 using a conventional HAI assay (Table 3). The HAI titers in mice infected with the NS1-truncated H3N8 CIV were slightly lower than those observed in WT CIV H3N8 infected mice, but higher than those observed with the inactivated vaccine (Table 3).

It was then demonstrated that a single immunization with the mutant H3N8 CIVs induced protection against challenge with WT H3N8 CIV. Mice vaccinated with the NS1 mutant H3N8 CIVs were challenged two weeks after vaccination, and then evaluated for the challenge virus at days 2 (N=3) and 4 (N=3) p.i (Figure 5B). In PBS inoculated mice it was detected high titers of CIV WT H3N8 at days 2 (~ 106) and 4 (~ 105) p.i. In contrast, virus in mice vaccinated with the NS1-truncated viruses at either time point (Figure 5B) were undetected. Mice vaccinated with the H3N8 ΔNS1 CIV or the inactivated vaccine showed similar viral titers at day 2 p.i, and those were slightly lower than the titers in the PBS vaccinated group. At day 4 p.i, the presence of WT H3N8 CIV in the lungs of IIV-vaccinated mice was not detected, but the presence of WT H3N8 CIV in the lungs of the ΔNS1 H3N8 CIV vaccinated group (Figure 5B) was. The protection results correlate with the ability of these NS1 truncated H3N8 CIV to induce total (Figure 5A) or neutralizing (Table 3) immune responses.

<u>CIV H3N8 NS1 mutants are attenuated *ex vivo*</u>

To determine the effect of NS1 truncations in CIV H3N8 pathogenesis at the site of infection within the natural host, dog tracheal explants were infected with CIV WT and mutants, as described above. The viruses were titrated at different times p.i., histological lesions changes in ciliary function were assessed and the presence of virus in the tissues was determined using immunohistochemistry. While mock-infected explants kept their normal morphology throughout the study period, infected explants showed major histopathological changes, including loss of cilia and destruction of the epithelium followed by desquamation of cells and subsequent decrease in epithelium thickness (Figure 6A). Changes were evident by day 1 p.i. for CIV H3N8 WT. H3N8 CIV NS1-126, and to less extent the NS1-99, resembled the WT virus in terms of histological damage (Figure 6A) and viral antigen expression (Figure 6B). In contrast, ΔNS1 and the NS1-73 mutant H3N8 CIVs showed an attenuated phenotype as the epithelium exhibited less destruction of cilia and cells (Figure 6A). Moreover, immunostaining for the viral nucleoprotein (NP) showed that fewer cells were infected with NS1-73 and ΔNS1 H3N8 CIVs (Figure 6B). Consistent with this, ciliary function was significantly reduced for WT, NS1-126 and 1-99 H3N8 CIVs (Figure 6C), but only slightly affected compared to mock-infected tracheal explants for NS1-73 and ΔNS1 H3N8 CIVs (Figure 6C). In terms of virus replication, all H3N8 CIV NS1 mutants were able to replicate in the dog trachea, although viral kinetics between them and WT H3N8 CIV differed (Figure 6D). H3N8 CIV WT virus was detected from day 1 and peaked at day 3 p.i., whereas infectious virus was only detectable from day 3 p.i. for all H3N8 CIV NS1 mutants. In addition, the peak of virus growth of NS1-73, NS1-99 and ΔNS1 H3N8 CIVs was significantly lower than for WT H3N8 CIV. These results indicate that NS1 truncations attenuate H3N8 CIV *ex vivo* and that the level of attenuation is influenced by the magnitude of the NS1 truncation.

H3N8 NS1 mutant CIV can provide protection against H3N2 CIV

Experiments were conducted to examine if the H3N8 NS1 mutant CIV can provide protection against H3N2 CIV. First, experiments were conducted to evaluate the induction of humoral responses by NS1-truncated CIV vaccination. Female 6- to-8-week-old C57BL/6 mice were immunized intranasally with the H3N8 CIV inactivated vaccine (Nobivac; 100ul intramuscular), or with 1x10^3 PFU of H3N8 CIV wild-type (WT) or NS1-truncated viruses (ΔNS1, NS1-73, NS1-99 and NS1-126); or mock vaccinated with PBS. At 14 days post-infection, mice were bled and the sera were collected and evaluated by ELISA for IgG antibodies against total influenza virus protein using cell extracts of MDCK cells infected with CIV H3N2 wild-type (A/Ca/IL/41915/2015). Mock-infected cell extracts were used to evaluate the specificity of the antibody response. It was observed that, replicating-competent, NS1 deficient or truncated CIV induce better immune responses that the inactivated H3N8 CIV inactivated vaccine (Figure 7). Next, experiments were conducted to evaluate the protection efficacy of H3N8 NS1 mutant CIV against H3N2 CIV. Female 6- to-8-week-old C57BL/6 mice (n=6) were immunized intranasally with the H3N8 CIV inactivated vaccine (Nobivac; 100ul intramuscular), or with 1x10^3 PFU of CIV wild-type (WT) or ΔNS1 or mock vaccinated with PBS. Two weeks post-vaccination, mice were challenged with 1x10^5 PFU of CIV H3N2 wild-type (A/Ca/IL/41915/2015). To evaluate viral lung replication, mice were sacrificed at days 3 (n=3) post-challenge and lungs were harvested, homogenized, and used to quantify viral titers by immunofocus assay (FFU/ml) using an anti-NP MAb (HB-65). Dotted black lines indicate limit of detection (200 FFU/ml). Even ΔNS1, inducing a weaker immune response as compared to mice intranasally infected with the NS1-truncated viruses NS1-73, NS1-99 and NS1-126; is able to confer better protection against the new H3N2 CIV than the inactivated influenza vaccine (Figure 8).

Vaccination with H3N8 NS1 mutant CIVs elicits protective immunity against WT H3H8 CIV in mice

Currently, there are two subtypes of CIV co-circulating in the US, an equine-origin CIV H3N8, and an avian-origin CIV H3N2. H3N8 CIV has been circulating widely in the dog population for at least 16 years (Crawford et al., 2005, Science, 310:482-485, Yoon et al., 2005, Emerging infectious diseases, 11:1974-1976), particularly in animal shelters (Crawford et al., 2005, Science, 310:482-485, Holt et al., 2010, Journal of the American Veterinary Medical Association, 237:71-73, Pecoraro et al., 2013, Journal of veterinary diagnostic investigation, 25:402-406). Although CIV H3N2 appeared to be limited to Asia, in 2015 there was an outbreak of CIV H3N2 in the Chicago area that has been reported later on in more than 25 states in the US (Newbury et al., 2016, Journal of the American Veterinary Medical Association, 248:1022-1026). The emergence and establishment of these two viral strains create many opportunities for CIV exposure to humans and other species. Moreover, IAVs that belong to the H3 subtype are the most ubiquitous as they have been found in various different hosts including humans, pigs, horses, dogs, cats, seals, poultry and wild aquatic birds (Bean et al., 1992, J Virol, 66:1129-1138, Bush et al., 1999, Molecular biology and evolution, 16:1457-1465, Parrish et al., 2015, J Virol, 89:2990-2994, Song et al., 2008, Emerging infectious diseases, 14:741-746, Song et al., 2015, The Journal of general virology, 96:254-258). Human IAVs have not become established in dogs despite serological evidence of exposure and infection (Dundon et al., 2010, Emerging infectious diseases, 16:2019-2021, Ramirez-Martinez et al., 2013, Influenza and other respiratory viruses, 7:1292-1296). It has been demonstrated that various human influenza viruses such as PR8 (H1N1), Udorn/72 (H3N2) and A/California/04/09 (pdm09, H1N1) replicate in dog tracheas at levels similar to those observed for CIV H3N8 (Gonzalez et al., 2014, J Virol, 88:9208-9219), and viable reassortments generated by reverse genetics between CIV H3N8 and pdm09 viruses have been reported (Gonzalez et al., 2014, J Virol, 88:9208-9219). Therefore, dogs act as an intermediate host and “mixing vessels” for genetic reassortment between human and avian viruses, facilitating the generation of novel human influenza virus strains and the initiation of influenza pandemics.

Currently, there are only inactivated vaccines to control H3N8 CIV in dogs. The vaccine is intended as an aid in the control of the disease associated with viral infection. Although the vaccine may not completely prevent H3N8 CIV infection, efficacy trials have shown that it may significantly decrease the signs, severity and spread of viral infection, including the level of damage to the lungs or the duration and degree of viral shedding (Deshpande et al., 2009, Veterinary therapeutics: research in applied veterinary medicine, 10:103-112). These benefits are similar to those provided by IIV used in other species, including humans (De Villiers et al., 2009, Vaccine, 28:228-234). CIV LAIV may afford better and faster protection, as it has been shown with other influenza vaccines (Belshe et al., 2007, The New England journal of medicine, 356:685-696, Gorse et al., 1991, Scandinavian journal of infectious diseases, 23:7-17, Pica et al., 2013, Annual review of medicine, 64:189-202.).

Influenza NS1 protein is a multifunctional viral factor which displays several regulatory functions during virus infection and can antagonize the IFN response (Randall et al., 2008, The Journal of general virology, 89:1-47). In these experiments, for the first time, it has been demonstrated that the generation of CIVs with truncations or a deletion of the NS1 protein constitute LAIV candidates.

It was demonstrated that H3N8 CIV WT, NS1 mutant viruses all displayed similar growth kinetics at 33°C, while the CIV ΔNS1 demonstrated a slightly reduced growth. These results correlated with the virus plaque size phenotypes. At 37°C, the NS1-truncated viruses demonstrated comparable replication kinetics to that of H3N8 CIV WT, but the ΔNS1 replication was highly impaired. Cells infected with ΔNS1 CIV induced IFN production at higher levels, followed by the NS1-126, NS1-99 and NS1-73 viruses, and the WT H3N8 CIV. Similar truncations of the NS1 protein in swine or equine influenza viruses generated comparable results (Quinlivan et al., 2005, J Virol, 79:8431-8439, Solorzano et al., 2005, J Virol, 79:7535-7543). It has been demonstrated that the different levels of attenuation observed between the NS1 mutants is that the truncated constructs display different RNA and/or protein stability, affecting the levels of NS1 and NEP expression (Quinlivan et al., 2005, J Virol, 79:8431-8439, Solorzano et al., 2005, J Virol, 79:7535-7543). In vivo the ΔNS1 H3N8 CIV demonstrated the most attenuation in viral growth in mice, followed by the NS1 truncated viruses. These results illustrate the differences observed in pigs with swine influenza viruses encoding the same truncated NS1 proteins (NS1-73, NS1-99 and NS1-126) (Solorzano et al., 2005, J Virol, 79:7535-7543).

The NS1 mutant viruses and the inactivated commercial vaccine conferred protection against challenge with WT CIV H3N8, as demonstrated by the induction of antibodies specific for the virus and reduced virus titers in the vaccinated mice. Importantly, virus replication was not detected in the lungs of mice immunized with the NS1 truncated H3N8 CIV. Furthermore, the lungs from animals vaccinated with ΔNS1 virus or the inactivated vaccine demonstrated high viral titers at day 2 post-challenge (similar to those of mock-vaccinated mice), demonstrating that vaccinated animals shed and transmit virus at early times after natural infection. It should be noted that H3N8 CIV was cleared by day 4 post-challenge in animals vaccinated with Nobivac. Vaccination also induced high and comparable levels of IgG antibodies in sera against the parental CIV WT and the NS1-truncated viruses. The ΔNS1 did not elicit high levels of antibodies and the sera from mice immunized with Nobivac elicited intermediate values, demonstrating that the mutants lacking regions of NS1 protein induce better immune B cell responses in mice than those obtained with current commercial inactivated vaccines.

This report is the first description of a CIV H3N8 LAIV generated by reverse genetics whose attenuation mechanism is based on truncations or total deletion of the NS1 protein. The viruses demonstrated attenuation, while retaining immunogenicity (Hussain et al., 2010, Vaccine, 28:3848-3855, 15).

TABLE 3: Immunogenicity of NS1-truncated viruses

Immunization and dose[a]		Mean (SD) serum HAI titer[b]
PBS	-	≤ 8 (ND)
WT	10^3 PFU	215.3 (64)
NS1-73	10^3 PFU	128 (0)
NS1-99	10^3 PFU	107.6 (32)
NS1-126	10^3 PFU	90.5 (36.9)
ΔNS1	10^3 PFU	22.6 (9.23)
Nobivac	100 µl	26.9 (8)

[a] Virus was administered intranasally to anesthetized mice (*n* = 4), Nobivac was administered intramuscularly, and sera were collected at 14 days postinfection.

[b] Four HAU of the WT virus was incubated with 2-fold serial dilutions of the indicated sera.
ND, not determined.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. An immunological composition comprising a canine influenza virus (CIV), wherein the CIV comprises one or more mutations in segment 8 of the viral genome.

2. The composition of claim 1, wherein the segment 8 comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4

3. The composition of any of claims 1-2, wherein the CIV comprises one or more mutations in segment 8, which encodes a truncation mutant of NS1.

4. The composition of claim 3, wherein the truncation mutant of NS1 is selected from the group consisting of NS1-126, NS1-99, and NS1-73.

5. The composition of any of claims 3-4, wherein the truncation mutant of NS1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7.

6. The composition of any of claims 1-2, wherein the CIV comprises one or more mutations in segment 8 such that NS1 is not expressed.

7. The composition of any of claims 1-6, wherein the CIV is derived from H3N8 subtype of influenza A virus.

8. The composition of any of claims 1-7, wherein the composition is used for the treatment or prevention of canine influenza in a subject.

9. A method for treating or preventing canine influenza in a subject, the method comprising administering to the subject an immunological composition

comprising a canine influenza virus (CIV), wherein the CIV comprises one or more mutations in segment 8 of the viral genome.

10. The method of claim 9, wherein the segment 8 comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4

11. The method of any of claims 9-10, wherein the CIV comprises one or more mutations in segment 8, which encodes a truncation mutant of NS1.

12. The method of claim 11, wherein the truncation mutant of NS1 is selected from the group consisting of NS1-126, NS1-99, and NS1-73.

13. The method of any of claims 11-12, wherein the truncation mutant of NS1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7.

14. The method of any of claims 9-10, wherein the CIV comprises one or more mutations in segment 8 such that NS1 is not expressed.

15. The method of any of claims 9-16 wherein the CIV is derived from H3N8 subtype of influenza A virus.

16. The method of any of claims 9-15, wherein the subject does not have canine influenza, and wherein the method induces immunity against one or more of: influenza A virus subtype H3N8 and influenza A virus subtype H3N2.

17. The method of any of claim 9-15, wherein the subject is infected with at least one or more of: influenza A virus subtype H3N8 and influenza A virus subtype H3N2; and wherein the method induces a therapeutic immune response.

18. The method of any one of claims 9-17, wherein the immunological composition is administered intranasally, intratracheally, orally, intradermally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

19. The method of any one of claims 9-18, wherein the subject is a dog.

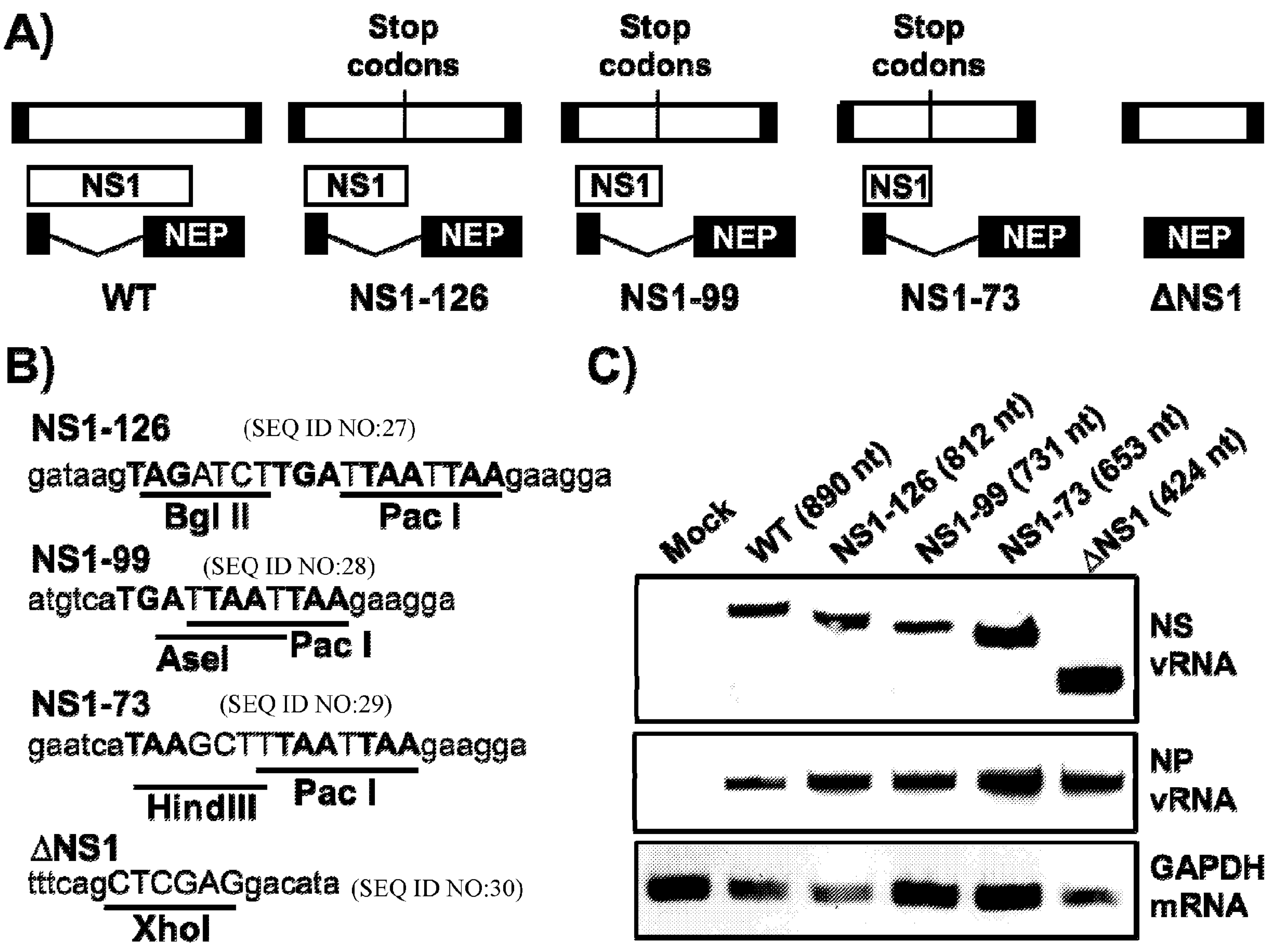

Figure 1A – Figure 1C

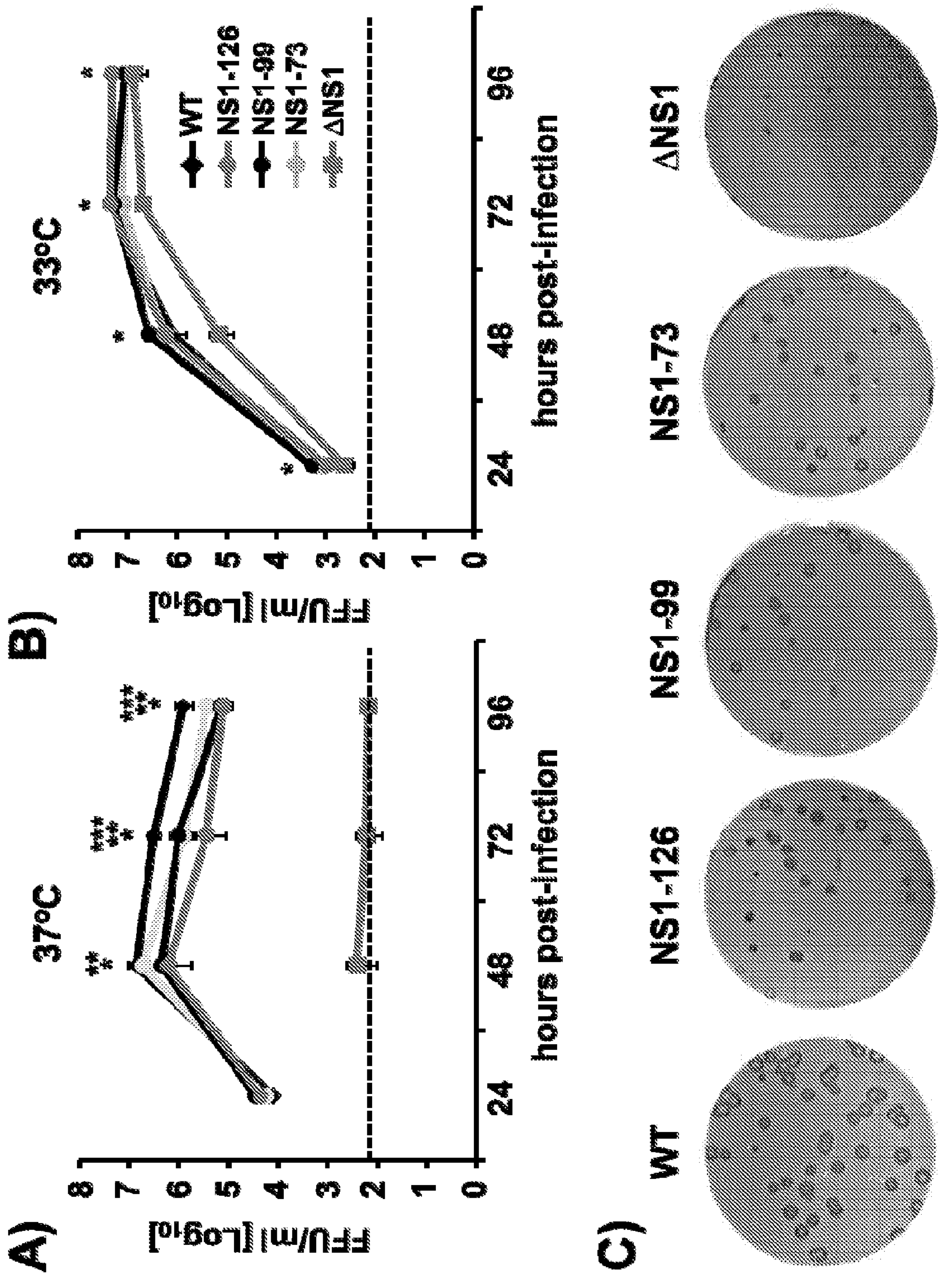

Figure 2A – Figure 2C

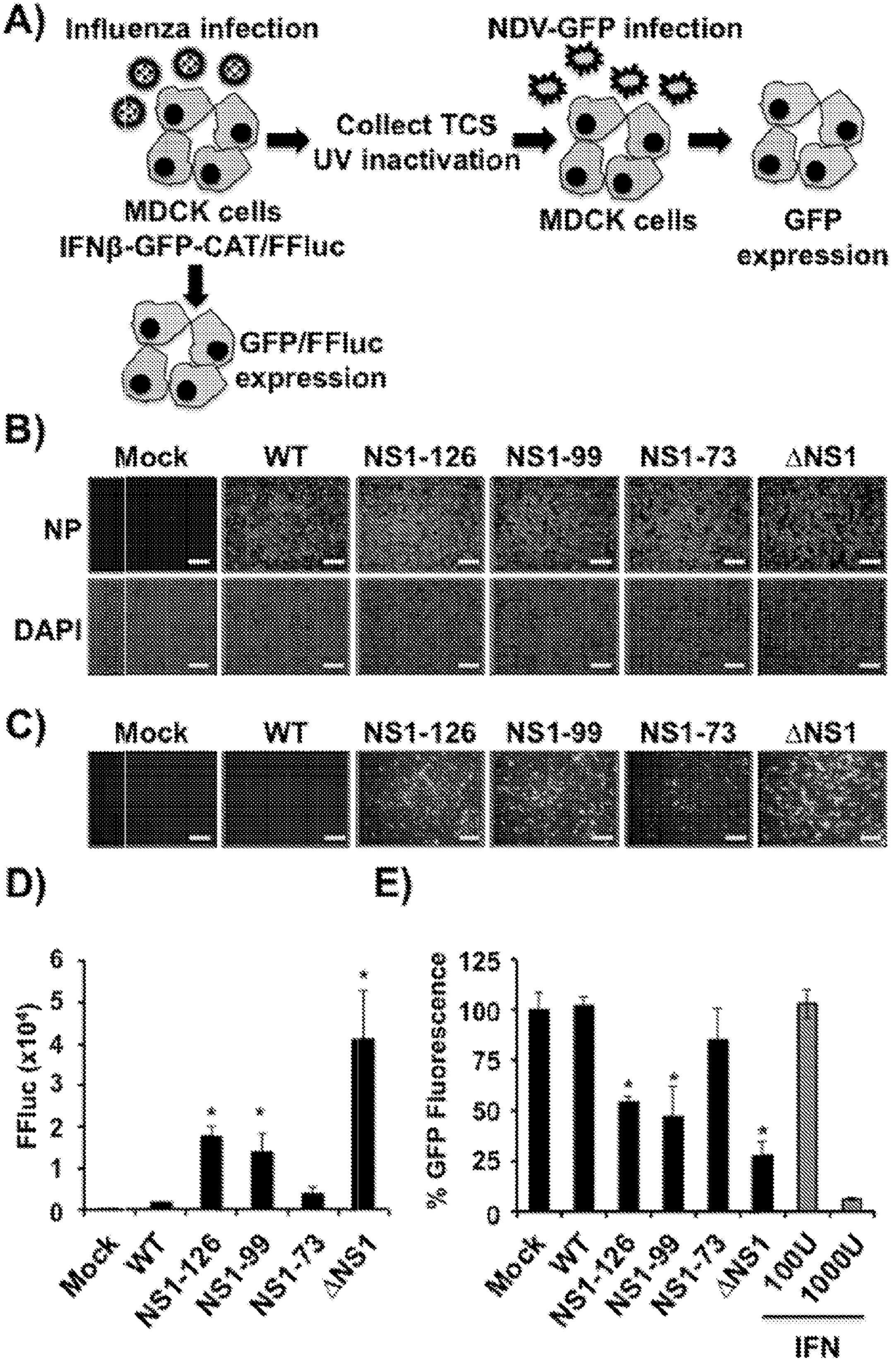

Figure 3A - Figure 3E

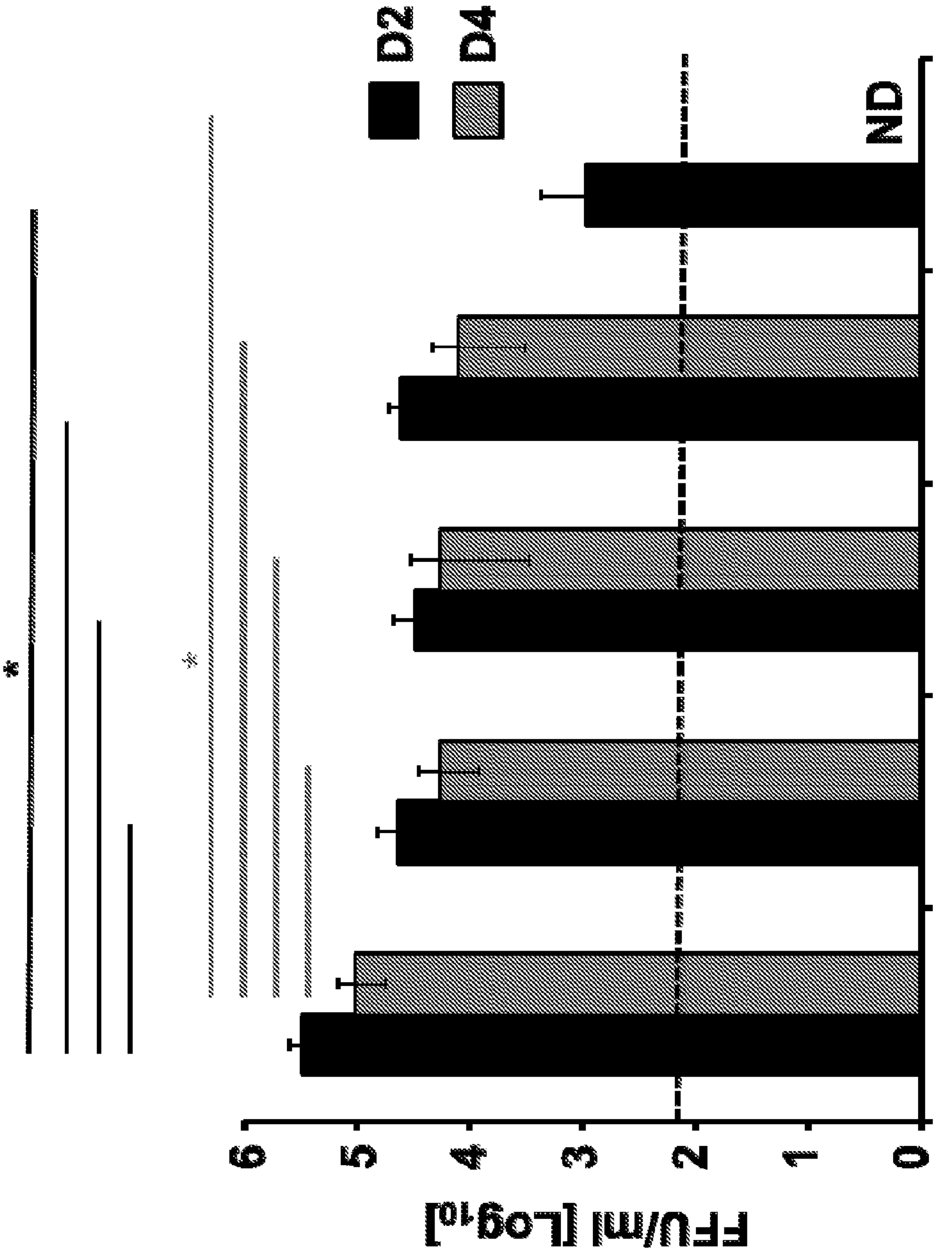

Figure 4

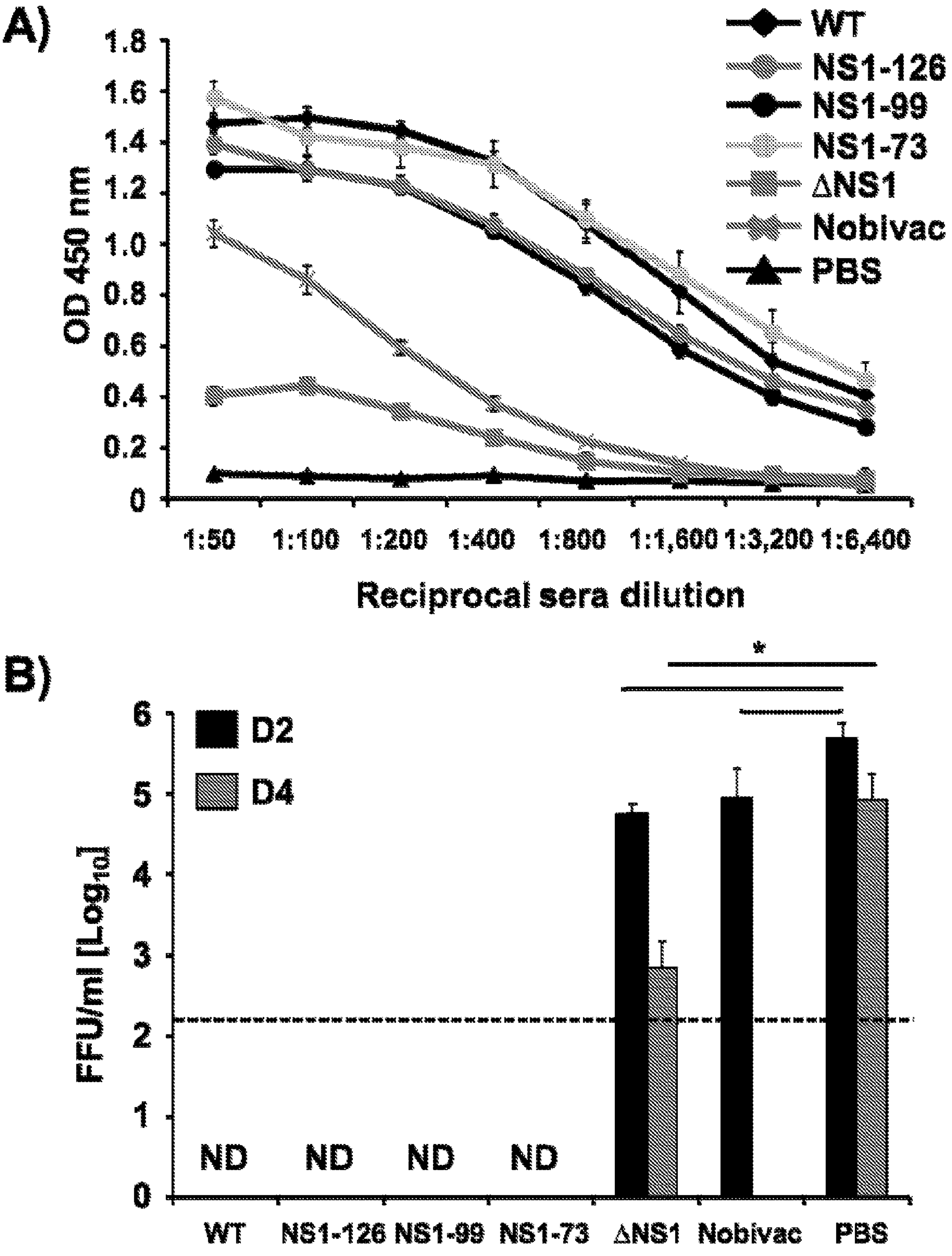

Figure 5A - Figure 5B

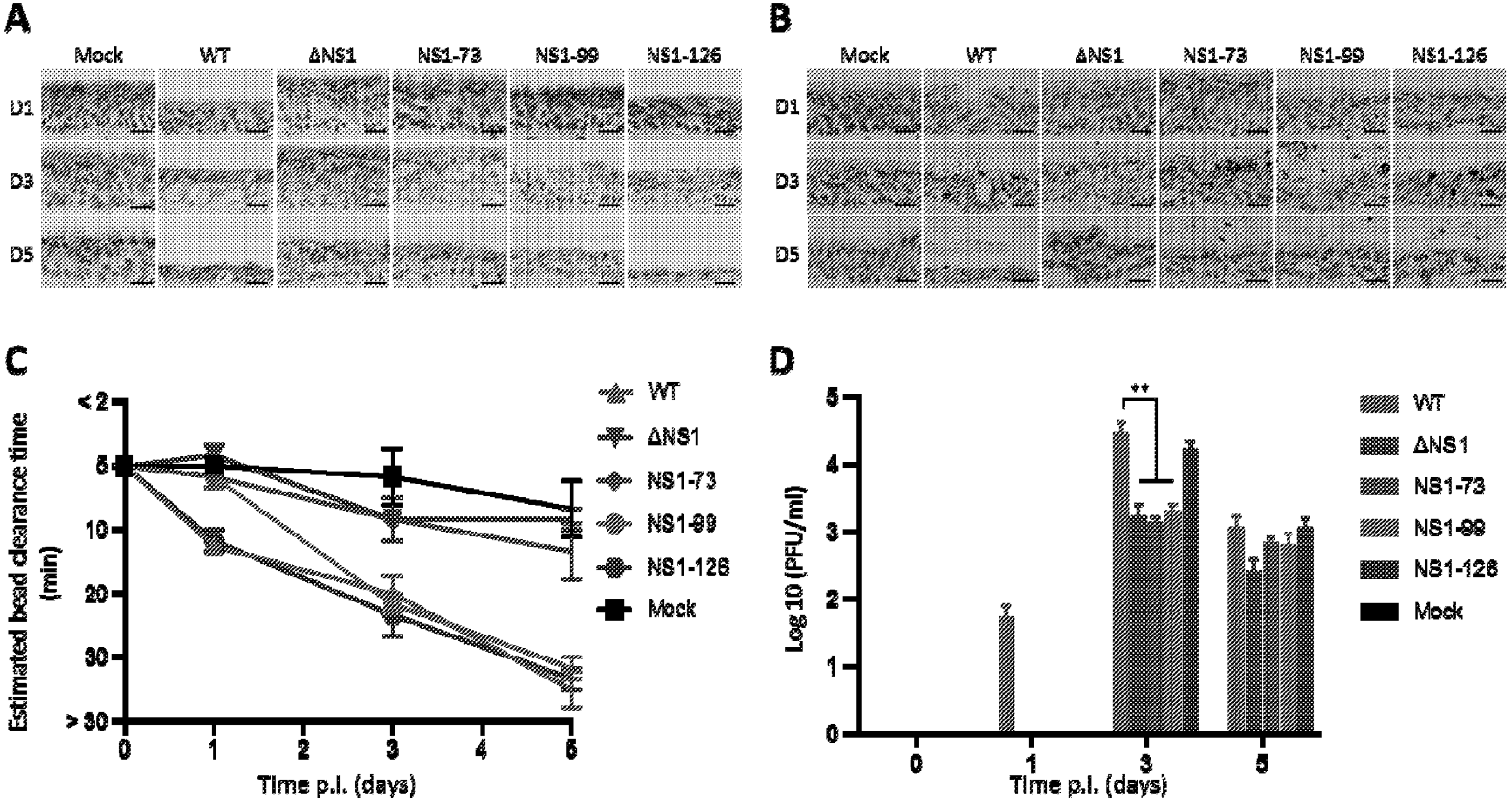

Figure 6A – Figure 6D

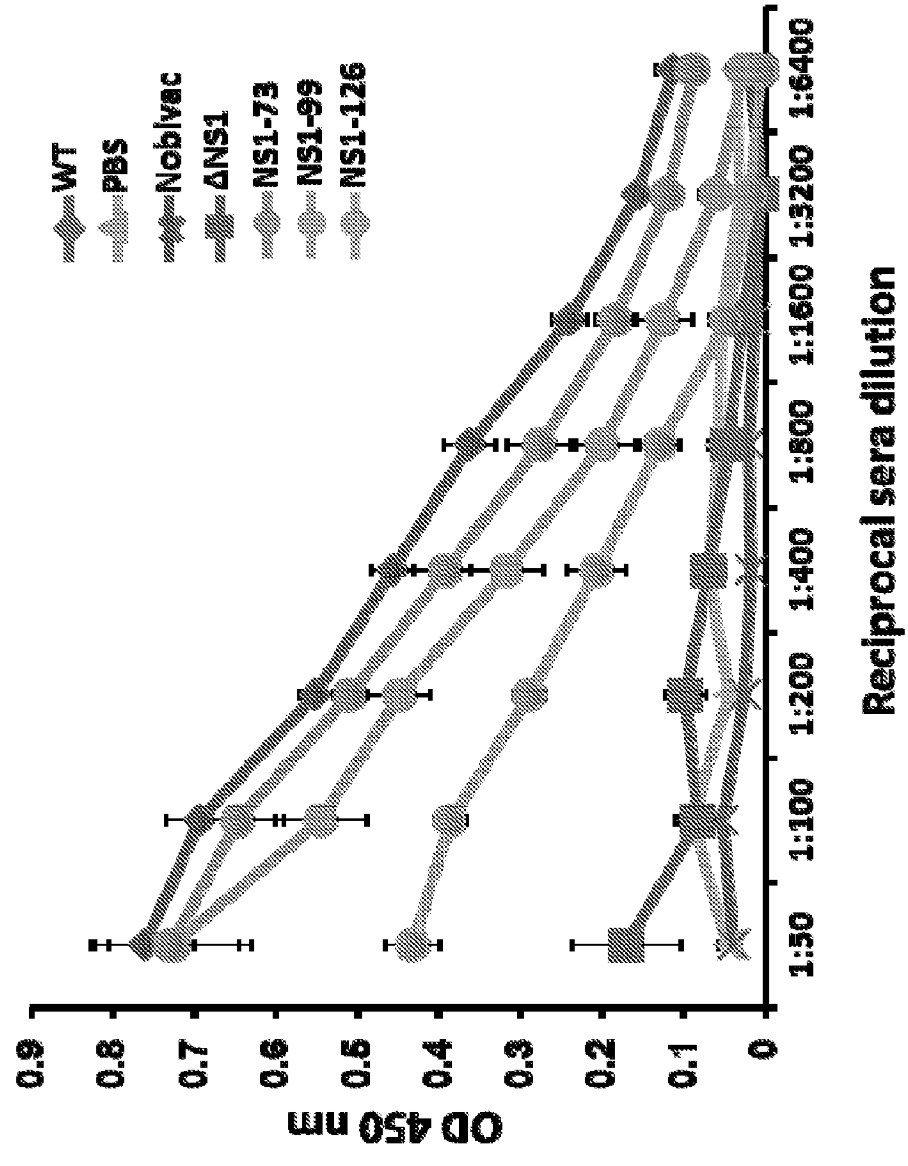
OD 450 nm
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1:50
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400
Reciprocal sera dilution
WT
PBS
Nobivac
ΔNS1
NS1-73
NS1-99
NS1-126

Figure 7

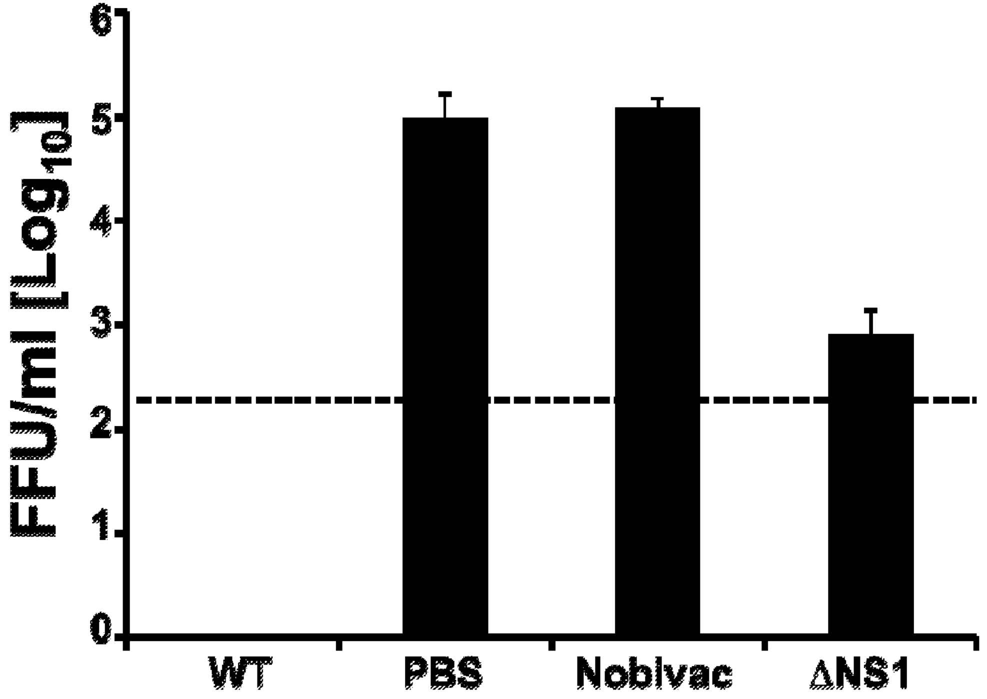

Figure 8

ΔNS1 - Nucleotide sequence of modified segment 8 - (SEQ ID NO: 1)

agcaaaagcagggtgacaaaaacataatggattccaacactgtgtcaagct
ttcaggacatactaatgaggatgtcaaaaatgcaattggggtcctcatcgg
aggatttaaatggaatgataatacggttaaaatctctgaaactctacagag
attcgcttggagaagcagtcatgagaatgggagaccttcactcccttcaaa
gcagaaacgaaaaatggagagaacaattaagccagaaatttgaagaaataa
gatggttgattgaagaagtgcgacatagactgaaaaatacagaaaatagtt
ttgaacaaataacatttatgcaagccttacaactattgcttgaagtagaac
aagagataagaactttctcgtttcagcttatttaatgataaaaaacaccct
tgtttctact

Figure 9

NS1-126 - Nucleotide sequence of modified segment 8 (SEQ ID NO: 2)

```
agcaaaagcagggtgacaaaaacataatggattccaacactgtgtcaagct
ttcaggtagactgttttctttggcatgtccgcaaacaattcgcagaccaag
aactgggtgatgccccattccttgaccggcttcgccgagaccagaagtccc
taaggggaagaggtagcactcttggtctggacatcgaaacagccactcatg
caggaaagcagatagtggagcagattctggaaaaggaatcagatgaggcac
ctaaaatgaccattgcctctgttcctgcttcacgctacttaactgacatga
ctcttgatgagatgtcaagagactggttcatgctcatgcccaagcaaaaag
taacaggctccctatgtataagaatggaccaggcaatcatggataagtaga
tcttgattaattaagaaggagcaatcgttggcgaaatttcaccattacctt
ctcttccaggacatactaatgaggatgtcaaaaatgcaattggggtcctca
tcggaggatttaaatggaatgataatacggttaaaatctctgaaactctac
agagattcgcttggagaagcagtcatgagaatgggagaccttcactccctt
caaagcagaaacgaaaaatggagagaacaattaagccagaaatttgaagaa
ataagatggttgattgaagaagtgcgacatagactgaaaaatacagaaaat
agttttgaacaaataacatttatgcaagccttacaactattgcttgaagta
gaacaagagataagaactttctcgtttcagcttatttaatgataaaaaaca
cccttgtttctact
```

Figure 10A

NS1-126 - Amino Acid Sequence (SEQ ID NO: 5)

```
MDSNTVSSFQVDCFLWHVRKQFADQELGDAPFLDRLRRDQKSLRGRGSTLG
LDIETATHAGKQIVEQILEKESDEAPKMTIASVPASRYLTDMTLDEMSRDW
FMLMPKQKVTGSLCIRMDQAIMDK
```

Figure 10B

NS1-99 - Nucleotide sequence of modified segment 8 (SEQ ID NO: 3)

agcaaaagcagggtgacaaaaacataatggattccaacactgtgtcaagct
ttcaggtagactgtttctttggcatgtccgcaaacaattcgcagaccaag
aactgggtgatgccccattccttgaccggcttcgccgagaccagaagtccc
taaggggaagaggtagcactcttggtctggacatcgaaacagccactcatg
caggaaagcagatagtggagcagattctggaaaaggaatcagatgaggcac
ctaaaatgaccattgcctctgttcctgcttcacgctacttaactgacatga
ctcttgatgagatgtcatgattaattaagaaggagcaatcgttggcgaaat
ttcaccattaccttctcttccaggacatactaatgaggatgtcaaaaatgc
aattggggtcctcatcggaggatttaaatggaatgataatacggttaaaat
ctctgaaactctacagagattcgcttggagaagcagtcatgagaatgggag
accttcactcccttcaaagcagaaacgaaaaatggagagaacaattaagcc
agaaatttgaagaaataagatggttgattgaagaagtgcgacatagactga
aaaatacagaaaatagttttgaacaaataacatttatgcaagccttacaac
tattgcttgaagtagaacaagagataagaactttctcgtttcagcttattt
aatgataaaaaacacccttgtttctact

Figure 11A

NS1-99- Amino Acid Sequence (SEQ ID NO: 6)

MDSNTVSSFQVDCFLWHVRKQFADQELGDAPFLDRLRRDQKSLRGRGSTLG

LDIETATHAGKQIVEQILEKESDEAPKMTIASVPASRYLTDMTLDEMS

Figure 11B

NS1-73 - Nucleotide sequence of modified segment 8 (SEQ ID NO: 4)

agcaaaagcagggtgacaaaaacataatggattccaacactgtgtcaagct
ttcaggtagactgtttctttggcatgtccgcaaacaattcgcagaccaag
aactgggtgatgccccattccttgaccggcttcgccgagaccagaagtccc
taaggggaagaggtagcactcttggtctggacatcgaaacagccactcatg
caggaaagcagatagtggagcagattctggaaaaggaatcataagctttaa
ttaagaaggagcaatcgttggcgaaatttcaccattaccttctcttccagg
acatactaatgaggatgtcaaaaatgcaattggggtcctcatcggaggatt
taaatggaatgataatacggttaaaatctctgaaactctacagagattcgc
ttggagaagcagtcatgagaatgggagaccttcactcccttcaaagcagaa
acgaaaaatggagagaacaattaagccagaaatttgaagaaataagatggt
tgattgaagaagtgcgacatagactgaaaaatacagaaaatagttttgaac
aaataacatttatgcaagccttacaactattgcttgaagtagaacaagaga
taagaactttctcgtttcagcttatttaatgataaaaaacacccttgtttc
tact

Figure 12A

NS1-73 - Amino Acid Sequence (SEQ ID NO: 7)

MDSNTVSSFQVDCFLWHVRKQFADQELGDAPFLDRLRRDQKSLRGRGSTLG
LDIETATHAGKQIVEQILEKES

Figure 12B