Single-Dose Vaccination of a Recombinant Parainfluenza Virus 5 Expressing NP from H5N1 Virus Provides Broad Immunity against Influenza A Viruses

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Influenza viruses often evade host immunity via antigenic drift and shift despite previous influenza virus infection and/or vaccination. Vaccines that match circulating virus strains are needed for optimal protection. Development of a universal influenza virus vaccine providing broadly cross-protective immunity will be of great importance. The nucleoprotein (NP) of influenza A virus is highly conserved among all strains of influenza A viruses and has been explored as an antigen for developing a universal influenza virus vaccine. In this work, we generated a recombinant parainfluenza virus 5 (PIV5) containing NP from H5N1 (A/Vietnam/1203/2004), a highly pathogenic avian influenza (HPAI) virus, between HN and L (PIV5-NP-HN/L) and tested its efficacy. PIV5-NP-HN/L induced humoral and T cell responses in mice. A single inoculation of PIV5-NP-HN/L provided complete protection against lethal heterosubtypic H1N1 challenge and 50% protection against lethal H5N1 HPAI virus challenge. To improve efficacy, NP was inserted into different locations within the PIV5 genome. Recombinant PIV5 containing NP between F and SH (PIV5-NP-F/SH) or between SH and HN (PIV5-NP-SH/HN) provided better protection against H5N1 HPAI virus challenge than did PIV5-NP-HN/L. These results suggest that PIV5 expressing NP from H5N1 has the potential to be utilized as a universal influenza virus vaccine.

Influenza virus is a negative-stranded RNA virus with a segmented genome (1). Influenza A virus is associated with pandemics and is classified by its two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). There are 17 HA and 9 NA subtypes, differing by $\geq 30\%$ in protein homology, which are used to categorize influenza A viruses into subtypes (e.g., H1N1, H3N2, and H5N1, etc.) (2, 3). Point mutations in the antibody-binding sites of surface glycoproteins allow viruses to evade antibody-mediated immunity and reinfect humans and animals (antigenic drift). When different influenza A virus subtypes infect the same host, exchange of gene segments can occur, resulting in a new virus with a unique combination of viral genomes (antigenic shift), which may give rise to pandemics (1). Influenza A virus causes significant morbidity and mortality each year. Strains currently circulating in humans (i.e., H1N1 and H3N2) infect up to 15% of the world's population and cause an average of 36,000 deaths and 226,000 hospitalizations in the United States (4) as well as millions of deaths worldwide (5). Sporadic outbreaks of pandemic influenza have caused significant mortality over the past century, most notably the Spanish flu of 1918, and have caused over 50 million deaths worldwide (reviewed in reference 6). On the horizon is another potentially pandemic strain of influenza virus, H5N1. This avian influenza virus has most notably emerged in Southeast Asia and led to the destruction of millions of birds; resulted in 608 reported human cases, of which 359 were fatal since 2003 (WHO; http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html); and threatens to become the next pandemic.

Inactivated influenza virus vaccines have been available since the 1940s and are 60 to 80% effective against matched influenza virus strains at reducing hospitalizations but are less effective against antigenic drift variants and are ineffective against different subtypes (1). Thus, annual vaccination is needed to prevent infec-

tions by new strains or subtypes. Current seasonal influenza virus vaccines consists of two influenza A viruses (H1N1 and H3N2) and one or two influenza B viruses. Licensed influenza virus vaccines are produced in chicken eggs, requiring the availability of millions of eggs and significant time between identification of vaccine strains and availability of vaccines. Additionally, this vaccination strategy provides no protection against unexpected strains, outbreaks, or pandemics. New vaccination strategies are needed for the prevention and control of influenza virus infection (7).

A vaccine that can provide broad protection against different subtypes of influenza A viruses would be ideal. Vaccine candidates targeting conserved influenza virus proteins have been explored as potential universal influenza virus vaccines. The nucleoprotein (NP) of influenza A virus, which encapsidates the viral genome, is well conserved among all influenza viruses, with over 90% homology of amino acid residues (8), and has been used as a component for developing a universal influenza virus vaccine (9, 10). An adenovirus containing NP was shown previously to provide protection against a homologous as well as a heterosubtypic influenza virus challenge (11). Moreover, a recombinant modified vaccinia Ankara (MVA) virus containing NP and M1 of influenza virus induced CD8⁺ T cell responses and reduced symptom severity and virus shedding in humans in phase 1 and 2a trials (12–14), suggesting that NP can be utilized for the development of a potentially broadly protective influenza virus vaccine. Recombinant DNA vaccines expressing influenza virus NP antigen have been

Received 13 January 2013 Accepted 9 March 2013

Published ahead of print 20 March 2013

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doi:10.1128/JVI.00120-13

tested in animal models and were shown to induce protective antibody and T cell responses (15–18); however, the need for repeated administration of DNA can be a hurdle for the use of a DNA-based vaccine against a rapidly spreading influenza virus pandemic.

Parainfluenza virus 5 (PIV5), a single-stranded, nonsegmented, negative-stranded RNA virus, is a member of the genus *Rubulavirus* of the family *Paramyxoviridae*, which also includes mumps virus (MuV), human parainfluenza virus 2 (HPIV2), and HPIV4 (19). PIV5 exhibits several characteristics that demonstrate it as a good viral vector for vaccine development. First, PIV5 is considered a safe viral vector. It is believed that PIV5 may contribute to kennel cough in dogs (20–24). Even though infection of dogs with PIV5 alone did not lead to kennel cough (25, 26), kennel cough vaccines containing live PIV5 have been used on dogs for over 30 years without any safety concern being raised for dogs or humans. As a result, humans have likely been exposed to PIV5 due to the wide use of kennel cough vaccines containing live PIV5. Dogs often sneeze during intranasal vaccination with kennel cough vaccine and can shed virus after vaccination (25). PIV5 is also considered a safe vector because it does not have a DNA phase in its life cycle, which would prevent the possible unintended consequences of genetic modifications of host cell DNA through recombination or insertion. Second, the genome structure of PIV5 is stable. A recombinant PIV5 expressing green fluorescent protein (GFP) was maintained for more than 10 generations (the duration of the experiment) in tissue-cultured cells without loss of the inserted GFP gene (27). Third, PIV5 can be grown to high titers (over 10^8 PFU/ml) in tissue-cultured cells, indicating its potential as a cost-effective vaccine vector. Currently, kennel cough vaccines are mass produced by major veterinary vaccine companies. Fourth, a PIV5-vectored vaccine has been shown to be efficacious in animals (28–32). In our recent studies, we have found that a single-dose intranasal (i.n.) inoculation of as little as 10^3 PFU of PIV5 expressing HA of influenza A virus H5N1 protected against lethal H5N1 challenge in mice (30). Finally, a PIV5-based vaccine was shown to induce protective immunity in dogs that were exposed to PIV5. Levels of immunity were comparable in dogs with and those without prior PIV5 exposure, indicating that preexisting anti-PIV5 immunity does not negatively affect the immunogenicity of a PIV5-based vaccine (28).

In this work, we generated recombinant PIV5 containing the NP gene from an H5N1 highly pathogenic avian influenza (HPAI) virus (A/Vietnam/1203/2004) and tested their efficacy in protecting against lethal homologous as well as heterosubtypic influenza virus challenges in mice.

MATERIALS AND METHODS

Cells. Monolayer cultures of MDBK, MDCK, and Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin. BHK and BSR-T7 cells were maintained in DMEM containing 10% FBS and 10% tryptose phosphate broth (TPB). G418 was added to BSR-T7 cells. All cells were incubated at 37°C in 5% CO₂. Virus-infected cells were cultured in medium containing reduced FBS (2%). Plaque assays of PIV5 strains were performed by using BHK cells as described previously (27). Fifty-percent tissue culture infectious dose (TCID₅₀) assays of influenza virus were performed by using MDCK cells as described previously (10).

Influenza viruses. A/Puerto Rico/8/34 (H1N1) (PR8); X-31, a reassortant between H3N2 A/Aichi/2/68 and PR8 which contains HA and NA

from H3N2 but all other genes from PR8 (33); and rgA/VN-PR8 (H5N1) (kindly provided by Ruben Donis, CDC, Atlanta, GA), which contains HA and NA from H5N1 but all other genes from PR8, were propagated in the allantoic cavity of embryonated hen eggs at 37°C for 48 to 72 h. Highly pathogenic A/Vietnam/1203/2004 (H5N1) (kindly provided by Richard Webby, St. Jude Children's Research Hospital, Memphis, TN) was propagated in the allantoic cavity of embryonated hen eggs at 37°C for 24 h. All viruses were aliquoted and stored at –80°C. All experiments using live, highly pathogenic A/Vietnam/1203/2004 were reviewed and approved by the institutional biosafety program at the University of Georgia and were conducted in enhanced biosafety level 3+ (BSL3+) containment according to guidelines for the use of select agents approved by the CDC.

Mice. Six- to eight-week-old female BALB/c mice (Charles River Labs, Frederick, MD) were used for all studies. Mice were anesthetized via intraperitoneal administration of 2,2,2-tribromoethanol (Avertin) prior to all intranasal vaccinations and influenza virus challenges. Mouse immunizations and studies with BSL2 viruses were performed in enhanced BSL2 facilities in HEPA-filtered isolators. Mouse HPAI virus infections were performed in enhanced BSL3 facilities in HEPA-filtered isolators according to guidelines approved by the institutional biosafety program at the University of Georgia and according to guidelines for the use of select agents approved by the CDC. All animal studies were conducted under guidelines approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Construction of recombinant plasmid. To generate a plasmid containing an NP insertion between the HN and L genes in the PIV5 genome (PIV5-NP-HN/L), plasmid BH311 containing the full-length genome of PIV5 and an extra GFP gene between the HN and L genes was used (27). The open reading frame (ORF) of NP from the highly pathogenic H5N1 influenza virus (A/Vietnam/1203/2004) was cloned from virions by reverse transcription-PCR (RT-PCR). To generate plasmids containing an NP insertion between M and F (PIV5-NP-M/F), F and SH (PIV5-NP-F/SH), or SH and HN (PIV5-NP-SH/HN), plasmid BH276 containing the full-length genome of PIV5 was used (27). To obtain the NP gene, HPAI H5N1 virus RNA was extracted, and cDNA was generated. The cDNA was amplified to generate the NP gene by using NP-specific primers. The expression plasmid pET-15b-NP, encoding a His-tagged H5N1-NP protein, was constructed by using the pET-15b vector. Sequences of the primers for the PIV5-NP-HN/L and pET-15b-NP plasmids are available upon request.

Virus rescue and sequencing. The PIV5-NP-M/F, PIV5-NP-F/SH, PIV5-NP-SH/HN, or PIV5-NP-HN/L plasmid encoding the full-length genome of PIV5 with the NP gene insertion at the indicated gene junction and three helper plasmids, pPIV5-NP, pPIV5-P, and pPIV5-L, encoding NP, P, and L proteins, respectively, were cotransfected into BSR-T7 cells at 95% confluence in 6-cm plates with Jetprime (Polyplus-transfection, Inc., New York, NY). The amounts of plasmids used were as follows: 5 µg full-length PIV5-NP plasmids, 1 µg pPIV5-NP, 0.3 µg pPIV5-P, and 1.5 µg pPIV5-L. After 72 h of incubation at 37°C, the media were harvested, and cell debris was pelleted by low-speed centrifugation (3,000 rpm for 10 min). Plaque assays were used to obtain single clones of recombinant viruses.

The full-length genome of plaque-purified PIV5-NP strains was sequenced. Total RNA from the media of PIV5-NP-infected Vero cells was purified by using a viral RNA extraction kit (Qiagen, Inc., Valencia, CA). cDNAs were prepared by using random hexamers, and aliquots of the cDNA were then amplified by PCR using appropriate oligonucleotide primer pairs, as described previously (30). PCR products were sequenced.

Detection of viral protein expression. Immunofluorescence (IF) and immunoprecipitation (IP) assays were used to detect expression of viral proteins. For IF assays, MDBK cells in 24-well plates were mock infected or infected with PIV5 or PIV5-NP-HN/L at a multiplicity of infection (MOI) of 0.1. At 2 days postinfection (dpi), the cells were washed with phosphate-buffered saline (PBS) and then fixed in 0.5% formaldehyde. The cells were permeabilized in a 0.1% PBS-Saponin solution and then

incubated for 30 min with monoclonal anti-PIV5-V/P or anti-H5N1-NP antibodies. The cells were washed with PBS–1% bovine serum albumin (BSA) and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody. The cells were incubated for 30 min, washed, and examined and photographed by using a fluorescence microscope (Advanced Microscopy Group).

For IP, MDBK cells in 6-well plates were mock infected or infected with PIV5 or PIV5-NP-HN/L at an MOI of 5. At 22 h postinfection (hpi), the cells were labeled with [³⁵S]Met-Cys Promix (100 µCi/ml) for 2 h. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and aliquots were immunoprecipitated by using monoclonal anti-PIV5-V/P or anti-H5N1-NP antibodies. The precipitated proteins were resolved by 15% SDS-PAGE and examined by autoradiography using a Storm Phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

Expression levels of H5-NP in virus-infected cells were compared by using MDBK cells in 6-well plates that were mock infected or infected with PIV5, PIV5-NP-M/F, PIV5-NP-F/SH, PIV5-NP-SH/HN, or PIV5-NP-HN/L at an MOI of 5. The cells were collected at 2 dpi and fixed with 0.5% formaldehyde for 1 h. The fixed cells were resuspended in FBS-DMEM (50:50) and then permeabilized in 70% ethanol overnight. The cells were washed once with PBS and then incubated with mouse anti-H5N1-NP antibody or anti-PIV5-V/P antibody in PBS–1% BSA (1:200) for 1 h at 4°C. The cells were stained with anti-mouse antibody labeled with phycoerythrin for 1 h at 4°C in the dark and then washed once with PBS–1% BSA. The fluorescence intensity was measured by using a flow cytometer (BD LSR II).

Growth of viruses *in vitro* and *in vivo*. MDBK cells in 6-well plates were infected with PIV5, PIV5-NP-M/F, PIV5-NP-F/SH, PIV5-NP-SH/HN, or PIV5-NP-HN/L at an MOI of 0.1. The cells were then washed with PBS and maintained in DMEM–2% FBS. Medium was collected at 0, 24, 48, 72, 96, and 120 hpi. The titers of viruses were determined by plaque assays on BHK cells.

To compare the growth of viruses in mice, 6-week-old wild-type BALB/c mice were vaccinated with 10⁵ PFU of PIV5, PIV5-NP-M/F, PIV5-NP-F/SH, PIV5-NP-SH/HN, or PIV5-NP-HN/L in a 50-µl volume intranasally. Mice were euthanized, and lungs were harvested to determine virus titers at 3 days postinfection.

ELISA. To generate purified influenza virus NP protein, the pET-15b-NP plasmid was transformed into *Escherichia coli* BL21(DE3)/pLysS competent cells. The recombinant 6×His-NP protein was purified by using Ni-charged resin (Novagen) and examined by SDS-PAGE and Coomassie blue staining. For the generation of immune serum, mice were vaccinated with 10⁶ PFU of PIV5 or PIV5-NP-HN/L or 10⁵ PFU of X31 intranasally, and blood samples were collected on day 21 postvaccination. Ninety-six-well plates were coated purified NP protein at 2 µg/ml at 4°C overnight. An enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions (KPL, Inc.). Serial dilutions of serum samples from PIV5-, PIV5-NP-HN/L-, and X31-inoculated mice were added to coated plates. Goat anti-mouse IgG conjugated to alkaline phosphatase (AP; KPL, Inc.) was added, and plates were developed. The optical density (OD) at 405 nm was measured on a Bio-Tek Powerwave XS plate reader.

IFN-γ ELISpot assay. To detect cytotoxic T lymphocyte (CTL) responses in spleens of vaccinated mice, a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assay was performed. Mice were vaccinated with PBS; 10⁷ PFU of PIV5, PIV5-NP-F/SH, PIV5-NP-SH/HN, or PIV5-NP-HN/L; or 0.1 50% lethal dose (LD₅₀) of PR8 intranasally. At day 21 postvaccination, mice were sacrificed, and spleens were collected. Spleens were homogenized and washed with Hanks balanced salt solution (HBSS). Gey's solution was added to remove the red blood cells. Splenocytes in complete tumor medium (CTM) were added to a 96-well plate (catalog number MAIPSWU10; Millipore) pretreated with 70% ethanol and coated with anti-mouse IFN-γ monoclonal antibody (Mab) AN18 (Mabtech, Inc.). Cells were mock restimulated or restimulated with a peptide (TYQRTRALV) corresponding to amino acid resi-

FIG 1 Schematic of PIV5-NP viruses. The PIV5 genome contains seven genes in the order of 3′-NP-V/P-M-F-SH-HN-L-5′, with leader and trailer regions located at the ends of the genome. The H5N1-NP gene was inserted into the PIV5 genome at the indicated gene junctions.

dues 147 to 155 of the NP protein of influenza virus, the Ebola virus GP peptide P2 (EYLFVDNL) as an irrelevant peptide, or phorbol myristate acetate (PMA)-ionomycin. Cultures were incubated at 37°C in 5% CO₂ for 48 h. Splenocytes were removed, and plates were washed and incubated with biotinylated anti-mouse IFN-γ Mab R4-6A2 (Mabtech, Inc.) at room temperature for 1 h. After washing, plates were incubated with streptavidin-alkaline phosphatase (KPL, Inc.) and were incubated at room temperature for 1 h. Plates were developed by using a BCIP (5-bromo-4-chloro-3-indolylphosphate)–nitroblue tetrazolium (NBT) (KPL, Inc.) solution. Spots were counted by using an AID ViruSpot reader (Cell Technology, Inc.). Results are presented as the mean number of cytokine-secreting cells subtracted by the mean number of mock stimulation per 10⁶ splenocytes.

Infection of mice with influenza A viruses. (i) H1N1. Mice were immunized with a single dose of PBS, 10⁶ PFU of PIV5 or PIV5-NP-HN/L, or 10⁵ PFU of X31 intranasally. At day 21 postvaccination, mice were challenged with 10 LD₅₀ of A/Puerto Rico/8/34 (H1N1). On day 3 postchallenge, groups of mice were euthanized, and the lungs were collected and homogenized. A TCID₅₀ assay was used to determine virus titers in the cleared homogenate.

(ii) H5N1. Mice were immunized with a single intranasal administration of PBS; 10⁷ PFU of PIV5, PIV5-NP-HN/L, PIV5-NP-F/SH, or PIV5-NP-SH/HN; or 2,000 PFU of rgA/VN-PR8. At day 21 postvaccination, mice were challenged with 10 or 20 LD₅₀ of H5N1 HPAI virus, as indicated.

Following challenge, mice were monitored daily for weight loss and survival. Mice were scored based upon clinical signs of infection (ruffled fur, hunched posture, and dyspnea, 1 point each; <25% weight loss, 1 point; 25 to 35% weight loss, 2 points; >35% weight loss, 3 points; neurological symptoms, 3 points). Animals were humanely euthanized upon reaching 3 points. Challenges involving A/Vietnam/1203/2004 were conducted in animal BSL3+ (ABSL3+) containment. All animal studies were conducted under guidelines approved by the Institutional Animal Care and Use Committee of the University of Georgia.

RESULTS

Generation and analysis of PIV5-NP-HN/L. To test whether recombinant PIV5 expressing NP from H5N1 HPAI virus can provide protection against different subtypes of influenza virus challenges in mice, the H5N1 HPAI virus NP gene was inserted into the cDNA of PIV5 between the HN and L genes (Fig. 1). The plasmid containing the PIV5 genome with the NP gene inserted between the HN and L genes was transfected along with three

FIG 2 Expression of H5-NP and PIV5 proteins. (A) IF assay to detect H5N1-NP and PIV5-V/P protein expression in MDBK cells. MDBK cells in 24-well plates were mock infected or infected with PIV5 or PIV5-NP-HN/L at an MOI of 0.1. At 2 dpi, the cells were fixed, permeabilized, and then incubated with monoclonal anti-PIV5-V/P or anti-H5N1-NP antibodies. The cells were photographed by using a fluorescence microscope (Advanced Microscopy Group). (B) An IP assay was used to detect H5N1-NP and PIV5 protein expression in MDBK cells. MDBK cells were mock infected or infected with PIV5 or PIV5-NP-HN/L at an MOI of 5. At 22 hpi, the cells were labeled with [^{35} S]Met-Cys Promix, lysed, and immunoprecipitated with monoclonal anti-PIV5-V/P or anti-H5N1-NP antibodies. The precipitated proteins were resolved by 15% SDS-PAGE and examined by autoradiography using a Storm Phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

helper plasmids encoding PIV5 NP, P, and L genes into BSR-T7 cells to recover infectious virus, as described previously (34). Infectious PIV5-NP-HN/L was plaque purified, and the full-length genome sequence of PIV5-NP-HN/L was determined by RT-PCR sequencing as described previously (30). One plaque-purified clone matching the exact cDNA of the genome sequence was used for all further experiments.

Expression of NP from PIV5-NP-HN/L-infected cells was confirmed by immunofluorescence (Fig. 2A) and immunoprecipitation (Fig. 2B) assays. H5N1-NP protein was detected in PIV5-NP-HN/L-infected cells but not in mock- or PIV5-infected cells. To test whether NP expression affected PIV5 protein expression levels, PIV5 proteins were immunoprecipitated by using a PIV5-V/P antibody. No difference in PIV5 protein expression levels was observed between PIV5 and PIV5-NP-HN/L (Fig. 2B).

To compare growth of PIV5 and PIV5-NP-HN/L in tissue-cultured cells, multiple-step growth curves were performed by using MDBK cells. PIV5-NP-HN/L grew slightly more slowly than PIV5 (Fig. 3A). It is possible that NP of influenza virus, which encapsidates the influenza virus RNA genome, encapsidates the PIV5 RNA genome, resulting in downregulation of PIV5 gene expression.

To examine growth of PIV5 and PIV5-NP-HN/L *in vivo*, BALB/c mice were vaccinated with 10^5 PFU of PIV5 or PIV5-NP-HN/L intranasally. Titers of virus in the lungs of vaccinated mice were determined at 3 days postinfection. The titers in the lungs of PIV5-NP-HN/L-vaccinated mice were lower than those of PIV5-vaccinated mice; however, there was no significant difference between the two groups (Fig. 3B).

Immune responses to PIV5-NP-HN/L inoculation in mice.

To investigate whether PIV5-NP-HN/L could generate NP-specific antibodies *in vivo*, mice were vaccinated intranasally with PIV5, PIV5-NP-HN/L, or X31, which contains HA and NA from H3N2 but all the rest of genes, including NP, from PR8. X31 served as a positive control for the effects of NP. At day 21 post-vaccination, blood samples were collected, and sera were pre-

pared. Purified His-tagged NP protein from bacteria was used to coat 96-well plates. Serially diluted serum samples were added to the plates. PIV5-NP-HN/L vaccination induced robust anti-NP serum IgG titers comparable to those induced by X31, a live influ-

FIG 3 Growth of PIV5 and PIV5-NP-HN/L *in vitro* and *in vivo*. (A). Multiple-step growth curves of PIV5 and PIV5-NP-HN/L in tissue-cultured cells. MDBK cells were infected with PIV5 or PIV5-NP-HN/L at an MOI of 0.1, and the media were collected at 24-h intervals. Virus titers were determined by plaque assays on BHK cells. All *P* values were calculated by using a *t* test. (B). Mice were vaccinated with 10^5 PFU of PIV5 or PIV5-NP-HN/L intranasally. Mice were euthanized on day 3 postvaccination to determine lung virus titers.

FIG 4 Induction of humoral and cellular responses by PIV5-NP-HN/L. (A). NP antibody levels induced by PIV5-NP-HN/L in mice. Mice were vaccinated with 10^6 PFU of PIV5 or PIV5-NP-HN/L or 10^5 PFU of X31 intranasally. At day 21 postvaccination, blood samples were collected, and sera were prepared. An ELISA was performed according to the manufacturer's instructions (KPL, Inc.), using purified H5N1-NP. (B). T cell response induced by PIV5-NP-HN/L in mice. Mice were vaccinated with PBS, 10^7 PFU of PIV5 or PIV5-NP-HN/L, or 0.1 LD₅₀ of PR8 intranasally ($n = 5$ per group). At day 21 postvaccination, mice were sacrificed, and spleens were collected. Splenocytes were restimulated with Flu-NP, Ebola virus GP P2 as a negative control, or PMA-ionomycin as a positive control. Results are presented as the mean number of cytokine-secreting cells per 10^6 splenocytes. The P value is 0.11 between PIV5 and PIV5-NP-F/SH in Flu-NP stimulation.

enza virus (Fig. 4A). However, a caveat for this experiment is that the purified NP was from an H5N1 strain. The NP proteins of H5N1 and X31 (the same as PR8) are highly conserved (93% identical and 97% homologous).

To examine whether PIV5-NP can induce a cellular immune response, mice were vaccinated with PBS, PIV5, PIV5-NP-HN/L, or PR8 intranasally. At day 21 postvaccination, mice were euthanized, and an IFN- γ ELISpot assay was performed. PIV5-NP-HN/L-vaccinated mice induced a similar level of NP-specific CD8⁺ T cell responses compared to PR8-vaccinated mice (Fig. 4B).

FIG 5 PIV5-NP-HN/L protection against H1N1 challenge. Mice were vaccinated with PBS ($n = 10$), 10^6 PFU of PIV5 ($n = 10$) or PIV5-NP-HN/L ($n = 10$), or 10^5 PFU of X31 ($n = 9$) intranasally. At day 21 postvaccination, mice were challenged with 10 LD₅₀ of A/Puerto Rico/8/34 (H1N1). Weight loss (A) and survival (B) were monitored daily for 14 days following challenge. Weight loss is presented as the average percentage of original weight (the day of challenge). (C) Lung titers of mice challenged with H1N1. Mice ($n = 5$) were sacrificed at 3 days postchallenge. The titers were determined by using a TCID₅₀ assay using MDCK cells. The P value was >0.05 for PBS versus PIV5-NP-HN/L and for PIV5-NP-HN/L versus X31; the P value was <0.05 for PBS versus X31.

Determining efficacy of PIV5-NP-HN/L against heterosubtypic H1N1 challenge in mice. To examine if PIV5-NP-HN/L could provide cross-protection against a heterosubtypic H1N1 challenge, mice were immunized with a single dose of PIV5-NP-HN/L intranasally. X31, which contains the same NP as PR8, was used as a positive control. At day 21 postvaccination, mice were challenged with 10 LD₅₀ of A/Puerto Rico/8/34 (H1N1). PBS- and PIV5-immunized mice lost body weight, and all mice died by day 10 after challenge. In contrast, all mice immunized with PIV5-NP-HN/L showed no significant weight loss during the time of the experiment, and all mice survived challenge (Fig. 5A and B). This was comparable to the X31-primed positive-control group. Influenza virus was detected in the lungs of PIV5-NP-HN/L-immunized mice at 3 days postchallenge (Fig. 5C), and although the virus titers in the PIV5-NP-HN/L-immunized group were lower than those in the PBS group, there was no statistically significant difference between the two groups.

Determining efficacy of PIV5-NP-HN/L against H5N1 HPAI virus challenge in mice. To examine if PIV5-NP-HN/L could provide protection against a homologous H5N1 HPAI virus challenge, mice were immunized with a single dose of PIV5-NP-HN/L intranasally. Since HPAI H5N1 virus is the most virulent influenza virus in mice, we increased the vaccination dose. At day 21 postvaccination, mice were challenged with 10 LD₅₀ of H5N1 HPAI virus. All PBS- and PIV5-immunized mice lost body weight and succumbed to the infection. Mice vaccinated with PIV5-NP-HN/L exhibited 50% survival following challenge, with surviving mice losing less than 20% of their original body weight (Fig. 6).

Generation and analysis of PIV5 expressing H5N1-NP at different locations within the PIV5 genome. In our previous work, we demonstrated that an insertion site within the PIV5 genome affected the immunogenicity of inserted antigens (30). To investigate whether insertion of NP at different locations within the PIV5 genome will result in improved efficacy of the vaccine, NP was inserted between different gene junctions within the PIV5 genome. NP insertion between the leader sequence and the NP gene did not result in a viable infectious virus. Because the insertion of a foreign gene upstream of the M gene within the PIV5 genome affected virus growth *in vitro* and *in vivo* (30), we inserted NP at the junction regions downstream of the M gene within the PIV5 genome. PIV5-NP-M/F, PIV5-NP-F/SH, PIV5-NP-SH/HN, and PIV5-NP-HN/L grew similarly to each other and with a reduction in titer compared to PIV5 (Fig. 7A). The ratio of the mean fluorescence intensity (MFI) of NP to that of PIV5-V/P

FIG 6 PIV5-NP-HN/L protection against H5N1 HPAI virus challenge. Mice were vaccinated with PBS ($n = 9$) or 10^7 PFU of PIV5 ($n = 7$), PIV5-NP-HN/L ($n = 6$), or rgA/VN-PR8 ($n = 10$). At day 21 postvaccination, mice were challenged with 10 LD_{50} of H5N1 HPAI virus. Weight loss (A) and survival (B) were monitored for 16 days following influenza virus challenge.

was examined by flow cytometry to determine H5N1-NP protein expression levels. PIV5-NP-F/SH produced the highest ratio, while PIV5-NP-SH/HN and PIV5-NP-HN/L produced similar ratios. PIV5-NP-M/F yielded the lowest ratio. These results suggest that PIV5-NP-F/SH induces the highest expression levels of NP (Fig. 7B).

The ability of these viruses to replicate in mice was also compared. Although lung virus titers in mice vaccinated with PIV5-NP-F/SH, PIV5-NP-SH/HN, and PIV5-NP-HN/L were slightly lower than those in mice vaccinated with PIV5 only, there were no significant differences at day 3 after vaccination (Fig. 7C).

Cellular response to PIV5-NP infection. To determine

FIG 8 PIV5-NP viruses prime T cell responses. Mice were vaccinated with PBS, 10^7 PFU of PIV5 or PIV5-NP viruses, or 0.1 LD_{50} of PR8 intranasally ($n = 5$ per group). At day 21 postvaccination, mice were sacrificed, and spleens were collected. Splenocytes were restimulated with Flu-NP, Ebola virus GP P2, or PMA-ionomycin. Results are presented as the mean number of cytokine-secreting cells per 10^6 splenocytes ($P = 0.08$ for PIV5 versus PIV5-NP-F/SH; $P = 0.29$ for PIV5 versus PIV5-NP-SH/HN; $P = 0.43$ for PIV5 versus PIV5-NP-HN/L after Flu-NP stimulation).

whether expression levels of NP affected the immune responses as expected, mice were vaccinated with PBS, PIV5, PIV5-NP-F/SH, PIV5-NP-SH/HN, PIV5-NP-HN/L, or PR8 intranasally. At day 21 postvaccination, mice were euthanized, and IFN- γ ELISpot assays were performed. PIV5-NP-vaccinated mice induced a higher level of NP-specific CD8 $^{+}$ T cell responses than did PIV5-vaccinated mice. PIV5-NP-F/SH-vaccinated mice produced the highest level of NP-specific CD8 $^{+}$ T cell responses compared to mice vaccinated with other PIV5-NP viruses and PR8 (Fig. 8), although the difference between PIV5 and PIV5-NP viruses or between PIV5-NP viruses and PR8 was not statistically significant.

Determining efficacy of PIV5-NP against H5N1 HPAI virus challenge in mice. To investigate whether PIV5-NP-F/SH and PIV5-NP-SH/HN immunization could provide better protection against H5N1 HPAI virus challenge, mice were immunized with a single dose of PIV5-NP viruses intranasally. At day 21 postvaccination, mice were challenged with 20 LD_{50} of H5N1 HPAI virus. This higher challenge dose was used to maximize any possible differences among the different vaccine candidates. All PBS- and PIV5-immunized mice lost body weight and succumbed to infec-

FIG 7 Analysis of recombinant PIV5 expressing NP. (A) Multiple-step growth curves of PIV5 and PIV5-NP viruses. MDBK cells were infected with PIV5 or PIV5-NP viruses at an MOI of 0.1, and the media were collected at 24-h intervals. Virus titers were determined by plaque assays using BHK cells. (B) H5N1-NP expression levels in PIV5-NP-infected cells. MDBK cells were infected with PIV5 or PIV5-NP viruses at an MOI of 5. The ratios of MFI of H5-NP to that of PIV5-VP were examined by flow cytometry. (C) Growth of PIV5 and PIV5-NP viruses *in vivo*. Mice were vaccinated with 10^5 PFU of PIV5 or PIV5-NP viruses intranasally. Mice were euthanized on day 3 postvaccination to determine lung virus titers.

FIG 9 PIV5-NP protection against H5N1 HPAI virus challenge. Mice were vaccinated with PBS ($n = 10$), 10^7 PFU of PIV5 ($n = 10$) or PIV5-NP viruses ($n = 10$, except $n = 9$ for PIV5-NP-F/SH), or 2,000 PFU of rgA/VN-PR8 intranasally ($n = 7$). At day 21 postvaccination, mice were challenged with 20 LD₅₀ of H5N1 HPAI virus. Weight loss (A) and survival (B) were monitored for 14 days following influenza virus challenge. Weight loss is graphed as an average percentage of the original weight (the day of challenge).

tion by 10 days postchallenge. In contrast, 20% of mice vaccinated with PIV5-NP-HN/L, 30% of mice vaccinated with PIV5-NP-SH/HN, and 67% of mice vaccinated with PIV5-NP-F/SH survived challenge, indicating that insertion of NP between F and SH within PIV5 provided the best protection (Fig. 9).

DISCUSSION

Viral vectors have been widely explored for developing vaccines. Adenovirus (AdV)- and vaccinia virus (VV)-based vectors are among the most extensively studied. While AdV expressing a single NP gene (AdV-NP) of influenza A virus is protective, its protection has been less than ideal: the best result reported to date using a single inoculation of AdV-NP as a dose of 10^{10} virus particles was 80% protection of mice from lethal H1N1 challenge, but the mice lost close to 30% of their body weight (11). There is no report of success of a single inoculation of AdV-NP against H5N1 HPAI virus challenge in mice. The recent failure of an AdV-based HIV vaccine candidate in clinical trials has also dampened enthusiasm for this viral vector (35). MVA expressing a single NP gene did not provide any immunity against lethal influenza virus H1N1 challenge (36). Our work, to the best of our knowledge, is the first report of a live viral vector-based vaccine expressing a single NP gene that was completely protective against lethal H1N1 challenge and provided substantial protection (67%) against a highly lethal H5N1 challenge in a robust challenge model (challenge with 20 LD₅₀) in mice, indicating that PIV5 is a more efficacious viral vector than AdV and VV for an NP-based vaccine.

Protective immunity against influenza A virus generated by NP is generally thought to be cell mediated, with antibodies against

NP being nonessential for NP-mediated protection (9). Recent reports, however, indicated that anti-NP antibody may play a role in protective immunity (37, 38). Consistent with anti-NP antibodies being noncritical, we did not observe a correlation between anti-NP serum titers and weight loss (data not shown). This lack of correlation, however, does not exclude a contribution of antibody to protective immune responses. In our work, PIV5-NP-HN/L generated a robust NP-specific CD8⁺ T cell response, as demonstrated by an IFN- γ ELISpot assay. PIV5-NP-HN/L-immunized mice induced a similar level of NP-specific CD8⁺ T cell responses as sublethal H1N1 infection (Fig. 4B). Consistent with the observation that cell-mediated immunity does not inhibit growth of influenza virus, titers of challenge influenza viruses in the lungs of PBS-treated and immunized mice were similar (Fig. 5C). PIV5-NP-F/SH-immunized mice, which had the most NP-specific CD8⁺ T cells, had the highest survival rate (67%) after H5N1 HPAI virus challenge among the groups of mice immunized with different recombinant PIV5 expressing NP. The mechanism of protection by NP-mediated immunity is not clear. We speculate that robust T cell responses may have limited damage to lungs and cleared infected cells, resulting in survival of the mice.

Transcriptional polarity is found in the order *Mononegavirales*, which contains a single *de facto* promoter in the 3' end of the genome, i.e., the leader sequence. Viral genes closer to the 3' end of the genome are transcribed in greater abundance than those toward the 5' end. In the previous work, insertion of HA from H5N1 HPAI virus between SH and HN in PIV5 (ZL46; PIV5-H5-SH/HN) resulted in better protection against H5N1 HPAI virus challenge than insertions at the junctions of NP and V/P, V/P and M, or HN and L of PIV5 (ZL48; PIV5-H5-HN/L), presumably because PIV5-H5-SH/HN produced the highest levels of HA expression without negatively affecting virus growth (30). To improve the efficacy of our PIV5-based NP vaccine, the NP gene was inserted upstream of the NP gene of PIV5, between the M and F genes, F and SH genes, and SH and HN genes. As reported previously (30), insertion upstream of the NP gene of PIV5 did not lead to a viable recombinant PIV5, suggesting that insertion of a foreign gene upstream of the NP gene of PIV5 is lethal. Interestingly, PIV5-NP-F/SH-infected cells produced the highest levels of NP expression. PIV5-NP-M/F, which contained NP inserted closest to the 3'-end leader sequence and should have had the highest expression levels of NP, had the lowest expression level of NP (Fig. 7B). The junction between M and F is the longest among all the junctions within the PIV5 genome, and the readthrough transcript from M to F is the highest among the junction regions of PIV5 (39). We speculate that disruption of the M-F junction negatively affected the expression of the inserted gene. PIV5-NP-SH/HN- and PIV5-NP-HN/L-vaccinated mice induced similar levels of NP-specific CD8⁺ T cell responses as PR8 (H1N1), and PIV5-NP-F/SH-vaccinated mice induced a slightly higher level of T cell responses, although the difference was not statistically significant (Fig. 8). Intriguingly, the NP expression levels produced in PIV5-NP-infected cells correlated with the levels of NP-specific CD8⁺ T cell responses, suggesting that increasing the expression level of a foreign gene may lead to a higher level of cellular immune response generated by the foreign gene. It is known that PIV5 does not replicate well in mice, likely due to PIV5's inability to block IFN signaling by targeting STAT1 for degradation in mouse cells. It is thus possible and likely that a PIV5-based vaccine will generate more robust cellular immune responses in animals and hu-

mans, in which PIV5 can block IFN signaling by targeting STAT1 for degradation.

We could further improve the efficacy of our vaccine candidates by increasing the inoculation dose and/or by using a prime-boost regimen. In the case of AdV-NP, immunization with AdV-NP along with AdV expressing M2, another highly conserved protein of influenza A virus, protected mice much better than immunization with AdV-NP or AdV-M2 alone. Thus, combining PIV5-NP with other influenza virus antigens such as M2 may further enhance its efficacy, leading to an influenza virus vaccine that is more efficacious than PIV5-NP alone.

ACKNOWLEDGMENTS

We are grateful to Ruben Donis and Richard Webby for providing the VN-H5N1-PR8/CDC-RG (H5N1) (rgA/VN-PR8) and A/Vietnam/1203/2004 viruses, respectively. We appreciate helpful discussion and technical assistance from all the members of Biao He's laboratory.

This work was supported by grants from the National Institute of Allergy and Infectious Diseases (R01AI070847) to B.H.

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