Recombinant Parainfluenza Virus 5 Expressing Hemagglutinin of Influenza A Virus H5N1 Protected Mice against Lethal Highly Pathogenic Avian Influenza Virus H5N1 Challenge

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A safe and effective vaccine is the best way to prevent large-scale highly pathogenic avian influenza virus (HPAI) H5N1 outbreaks in the human population. The current FDA-approved H5N1 vaccine has serious limitations. A more efficacious H5N1 vaccine is urgently needed. Parainfluenza virus 5 (PIV5), a paramyxovirus, is not known to cause any illness in humans. PIV5 is an attractive vaccine vector. In our studies, a single dose of a live recombinant PIV5 expressing a hemagglutinin (HA) gene of H5N1 (rPIV5-H5) from the H5N1 subtype provided sterilizing immunity against lethal doses of HPAI H5N1 infection in mice. Furthermore, we have examined the effect of insertion of H5N1 HA at different locations within the PIV5 genome on the efficacy of a PIV5-based vaccine. Interestingly, insertion of H5N1 HA between the leader sequence, the *de facto* promoter of PIV5, and the first viral gene, nucleoprotein (NP), did not lead to a viable virus. Insertion of H5N1 HA between NP and the next gene, V/phosphoprotein (V/P), led to a virus that was defective in growth. We have found that insertion of H5N1 HA at the junction between the small hydrophobic (SH) gene and the hemagglutinin-neuraminidase (HN) gene gave the best immunity against HPAI H5N1 challenge: a dose as low as 1,000 PFU was sufficient to protect against lethal HPAI H5N1 challenge in mice. The work suggests that recombinant PIV5 expressing H5N1 HA has great potential as an HPAI H5N1 vaccine.

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 population and cause an average of 36,000 deaths and 226,000 hospitalizations in the United States (1), as well as millions of deaths worldwide (2). H5N1, an avian influenza virus, has emerged in southeast Asia and resulted in the destruction of millions of birds and 608 reported human cases, causing 359 fatalities since 2003 (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html). Recent works show that as few as five mutations enable transmission of H5N1 among ferrets (3, 4), a well-accepted human influenza virus infection model, and that two of these mutations are commonly found in H5N1 viruses in nature (4), highlighting the potential of H5N1 as a pandemic threat.

Currently, the only FDA-approved vaccine against H5N1 has serious limitations, particularly as it has to be given twice and requires substantially higher concentrations of the vaccine to achieve a moderate level of efficacy compared to conventional influenza vaccines. Conventional vaccines utilizing the hemagglutinin (HA) and neuraminidase (NA) of H5N1 viruses have been poorly immunogenic and have safety and production issues (reviewed in reference 5). A live-attenuated H5N1 vaccine has been generated by reverse genetics (6), but the risk of generating a reassortant prohibits use of this vaccine in most instances. Inactivated virus vaccines have also been derived by reverse genetics (7, 8) and produced in large quantities, but preliminary results from NIAID clinical trials suggest that efficacy will require both multiple immunizations and 6 times the standard influenza virus antigen dose, i.e., 90 instead of 15 µg of antigen, while only providing protection in a subset (~50%) of vaccinated individuals (9, 10). Nonetheless, the FDA has approved the inactivated H5N1 vaccine for use in people between 18 and 64 years, an age group that is not

the most vulnerable to influenza virus infection. Thus, there is a clear need for new vaccine strategies that provide increased immunogenicity and safety.

Parainfluenza virus 5 (PIV5), formerly known as simian virus 5 (SV5), is a member of the *Rubulavirus* genus of the family *Paramyxoviridae*, which includes mumps virus and human parainfluenza virus types 2 (HPIV2) and 4 (HPIV4) (11). The origin and natural host of PIV5 is not clear. PIV5 was first isolated from monkey cells as a contaminant in 1956, hence the original name SV5 (12). However, subsequent serological testing of wild monkeys indicated no exposure to this virus. In contrast, monkeys in captivity at an animal facility rapidly seroconverted, suggesting they were exposed to the virus in captivity (13, 14). Thus, all evidence to date indicates that PIV5 is not a simian virus. The virus was renamed parainfluenza virus 5 (PIV5) by the ITCV in 2009. It is believed that PIV5 contributes to kennel cough in dogs (15–19). Even though infection of dogs with PIV5 did not lead to kennel cough (20, 21), kennel cough vaccines containing live-attenuated PIV5 have been used on dogs for more than 30 years without raising any safety concerns for dogs or humans.

PIV5, a negative nonsegmented single-stranded RNA virus (NNSV), is a good viral vector candidate for vaccine development because it does not have a DNA phase in its life cycle, thus the possible unintended consequences of genetic modifications of

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host cell DNA through recombination or insertion are avoided. Compared to positive-strand RNA viruses, the genome structure of PIV5 is stable. A recombinant PIV5 expressing green fluorescent protein (GFP) has been generated, and the GFP gene was maintained for more than 10 generations (the duration of the experiment) (22). Thus, PIV5 is better suited as a vaccine vector than positive-strand RNA viruses, since the genomes of positive-strand RNA viruses recombine and often delete the inserted foreign genes quickly. PIV5 infects a large range of cell types, including primary human cells as well as established human cell lines (11, 23), and in spite of extensive testing, we have not found a cell line that is resistant to PIV5 infection. However, PIV5 has very little cytopathic effect (CPE) on most infected cells (24, 25). PIV5 also infects a large number of mammals without being associated with any diseases except kennel cough in dogs (15–19). PIV5 can be grown in MDBK cells for more than 40 days as well as in Vero cells, a WHO-approved cell line for vaccine production, to high titers and is released in the media at a titer of up to 8×10^8 PFU/ml, indicating its potential as a cost-effective and safe vaccine vector that can be used in mass production. At present, major veterinary vaccine makers market kennel cough vaccines containing live PIV5.

We have previously shown that vaccination with recombinant PIV5, containing the HA gene of an H3N2 virus inserted between HN and the large (L) gene, induces influenza virus neutralizing antibodies and prevents disease associated with nonlethal influenza virus infection (26). Here, we extend that work, testing the effectiveness of recombinant PIV5 viruses with HA transgenes inserted sequentially at sites proximal to the leader sequence. Moreover, we use PIV5 to express the HA of H5N1 highly pathogenic avian influenza virus (HPAI) and tested its effectiveness as a vaccine candidate in an established mouse model of lethal HPAI challenge.

MATERIALS AND METHODS

Cells. Monolayer cultures of BSR T7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB), and 400 μ g/ml G418. Monolayer cultures of Vero cells, MDBK cells, MDCK cells, and BHK cells were maintained in DMEM containing 10% FBS, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. All cells were incubated at 37°C in 5% CO₂. Virus-infected cells were grown in DMEM containing 2% FBS. Plaque assays of PIV5 were performed using BHK cells, and plaque assays of influenza virus were performed using MDCK cells.

Influenza viruses. Influenza A viruses used include reverse-genetic VN/H5N1-PR8/CDC-RG (H5N1; rgVN-PR8; kindly provided by Ruben Donis, CDC, Atlanta, GA) and A/Vietnam/1203/04 (H5N1; kindly provided by Richard Webby, St. Jude Children's Research Hospital, Memphis, TN). A/VN-PR8 was propagated in the allantoic cavity of embryonated hen eggs at 37°C for 48 to 72 h. β -Propiolactone (BPL)-inactivated A/Vietnam/1203/04 was provided by Richard Webby from St. Jude Children's Research Hospital (Memphis, TN). A/Vietnam/1203/04 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 avian influenza viruses were reviewed and approved by the institutional biosafety program at the University of Georgia and were conducted in biosafety level 3 (BSL3) enhanced containment by following the guidelines for use of select agents approved by the CDC.

Mice. Female 6- to 8-week-old BALB/c mice (Charles River Laboratories, Frederick, MD) were used for all studies. Mouse immunizations and studies with BSL2 viruses were performed in enhanced BSL2 facilities in HEPA-filtered isolators. Mouse HPAI infections were performed in en-

hanced BSL3 facilities in HEPA-filtered isolators by following guidelines approved by the institutional biosafety program at the University of Georgia and for the use of select agents approved by the CDC. All animal studies were conducted under protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Construction of recombinant plasmids. To generate ZL48 (rPIV5-H5-HN/L) plasmid, the coding sequence of GFP in the plasmid BH311 containing the full-length PIV5 genome and an extra enhanced GFP gene insertion between the HN and L genes was replaced with an HA gene of H5N1. To generate ZL46 (rPIV5-H5-SH/HN), ZL209 (rPIV5-H5-NP/VP), and ZL215 (rPIV5-H5-Le/NP) plasmids, the plasmid BH276, containing the full-length genome of PIV5, was used as the vector. To generate ZL47 (rPIV5-H5-VP/M) plasmid, the plasmid pSV5-M-NS (from Anthony Schmitt at Penn State University) containing the full-length genome of PIV5 was used as the vector. The plasmid containing the H5N1 HA gene without the cleavage site was used as the DNA template for PCR amplification using appropriate oligonucleotide primer pairs. Sequences of all primers for plasmid construction and complete genomes of ZL48, ZL46, ZL47, ZL209, and ZL215 are available on request.

Rescue of recombinant PIV5. The rescue of infectious recombinant PIV5 was performed as described before (22). Briefly, the plasmids pZL48, encoding the full-length genome of PIV5 with an HA gene insertion between the HN and L genes, pZL46, encoding the full-length genome of PIV5 with an HA gene insertion between the SH and HN genes, pZL47, encoding the full-length genome of PIV5 with an HA gene insertion between the V/P and matrix (M) genes, pZL209, encoding the full-length genome of PIV5 with an HA gene insertion between nucleoprotein (NP) and V/P genes, and pZL215, encoding the full-length genome of PIV5 with an HA gene insertion between leader and NP genes, as well as 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 Plus and Lipofectamine (Invitrogen). The amounts of plasmids used were 5 μ g pZL48/ZL46/ZL47/ZL209/ZL215, 1 μ g pPIV5-N, 0.3 μ g pPIV5-P, and 1.5 μ g pPIV5-L. After 3 h of incubation, the transfection media were replaced with DMEM containing 10% FBS and 10% TPB. After 72 h of incubation at 37°C, the media were harvested and cell debris was pelleted by low-speed centrifugation (3,000 rpm, 10 min). Plaque assays were used to obtain a single clone of recombinant viruses. The full-length genomes of the plaque-purified single clone of ZL48, ZL46, ZL47, and ZL209 viruses were sequenced as described before (27, 28). Total RNAs from the media of ZL48, ZL46, ZL47, and ZL209 virus-infected Vero cells were purified using the viral RNA extraction kit (Qiagen Inc., Valencia, CA). cDNAs were prepared using random hexamers, and aliquots of the cDNA were then amplified in PCRs using appropriate oligonucleotide primer pairs.

Growth of recombinant PIV5 *in vitro* and *in vivo*. MDBK cells in 6-well plates were infected with PIV5, ZL48, ZL46, or ZL47 at a multiplicity of infection (MOI) of 0.1. The cells were then washed with phosphate-buffered saline (PBS) and maintained in DMEM–2% FBS. Media were collected at 0, 24, 48, 72, 96, and 120 h postinfection (hpi). The titers of viruses were determined by plaque assay on BHK cells.

To analyze the growth of viruses in mice, 6-week-old wild-type BALB/c mice were infected with 10^6 PFU of PIV5, ZL48, ZL46, or ZL47 in a 100- μ l volume intranasally (i.n.). Mice were euthanized 4 days postinfection, and the lungs were collected to determine virus titers.

Detection of protein expression. Immunoblotting was performed on MDBK cells in 6-well plates that were infected with PIV5 and ZL48 at an MOI of 5. At 24 hpi, the cells were lysed with whole-cell extract buffer (WCEB) (50 mM Tris-HCl [pH 8], 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, and 10% glycerol). The lysates were run on an SDS-PAGE gel and immunoblotted with anti-H5N1 HA and anti-PIV5 antibody.

Immunofluorescence of H5N1 HA expression was carried out in MDBK cells in 24-well plates that were infected with PIV5 and ZL48 at an

MOI of 0.1. At 2 days postinfection (dpi), the cells were washed with PBS and then were fixed in 0.5% formaldehyde. The cells were permeabilized in 0.1% PBS-Saponin solution and incubated for 30 min with polyclonal anti-PIV5-VP or anti-H5N1 HA at 1:200 dilution, and then fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody was added to the cells. The cells were incubated for 30 min and were examined and photographed using a Nikon FXA fluorescence microscope.

Expression levels of H5N1 HA in virus-infected cells was analyzed using MDBK cells in 6-well plates that were mock infected or infected with PIV5, ZL48, ZL46 or ZL47 at an MOI of 1. The cells were collected at 24 hpi and fixed with 0.5% formaldehyde for 1 h. The fixed cells were pelleted by centrifugation and then resuspended in 500 µl of solution containing fetal bovine serum (FBS)-DMEM (50:50). The cells were permeabilized in 70% ethanol overnight. The cells were washed once with PBS and then incubated with mouse anti-H5N1 HA antibody in PBS-1% bovine serum albumin (BSA) (1:200) for 1 h at 4°C. The cells were stained with anti-mouse antibody labeled with phycoerythrin (1:200) for 1 h at 4°C in the dark and then washed once with PBS-1% BSA. The fluorescence intensity was measured using a flow cytometer.

ELISA. HA (H5N1 HA)-specific serum antibody titers were measured using an IgG enzyme-linked immunosorbent assay (ELISA). Immulon 2 HB 96-well microtiter plates (ThermoLabSystems) were coated with 2 µg/ml recombinant H5N1 HA protein and incubated at 4°C overnight. Plates were then washed with KPL wash solution (KPL, Inc.), and the wells were blocked with 200 µl KPL wash solution with 5% nonfat dry milk and 0.5% BSA (blocking buffer) for 1 h at room temperature. Serial dilutions of serum samples were made (in blocking buffer), transferred to the coated plate, and incubated for 1 h. To detect bound serum antibodies, 100 µl of a 1:1,000 dilution of alkaline phosphatase-labeled goat anti-mouse IgG (KPL, Inc.) in blocking buffer was added per well and incubated for 1 h at room temperature. Plates were developed by adding 100 µl pNPP phosphatase substrate (KPL, Inc.), and the reaction was allowed to develop at room temperature. Optical density (OD) was measured at 405 nm on a Bio-Tek Powerwave XS plate reader. The IgG titer was determined to be the lowest serum dilution with an OD greater than that of the mean of naïve serum plus 2 standard deviations above the mean OD.

Infection of mice with PIV5. For vaccination with PIV5 and rPIV5-H5, 10⁶ PFU PIV5, rPIV5-ZL46, or rPIV5-ZL48 in 50 µl PBS was administered intranasally to mice anesthetized with 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Aldrich Chemical Co). For sublethal rgVN-PR8 infection, 2,000 PFU of virus in 50 µl PBS was administered as described for PIV5 vaccination. For BPL-inactivated A/VN/1203/04 vaccination, 256 hemagglutination units (HAU)/ml in 50 µl PBS was injected into each of the caudal thigh muscles. Blood was collected on day 21 postimmunization. If boosted, this process was repeated on day 28 after the prime. Mice were monitored daily, and for some experiments body weights were recorded every other day.

Measurement of neutralizing antibody titer. Influenza virus neutralizing antibody titers were measured in serum by a microneutralization assay with an ELISA endpoint. Heat-inactivated serum was serially diluted in DMEM with 1% BSA, antibiotic/antimycotic, and 1 µg/ml tosylsulfonyl phenylalanyl chloromethyl ketone trypsin. Diluted serum was then incubated with 1,000 50% tissue culture infectious doses (TCID₅₀) of rgA/VN-PR8 or rgA/Anhui-PR8 for 2 h at 37°C. MDCK cells were then added and incubated at 37°C for 18 to 24 h. At the end of the incubation, wells were fixed with ice-cold methanol and acetone (80:20, respectively), and an ELISA was performed as described above. The neutralization titer was determined to be the lowest serum dilution capable of neutralizing 1,000 TCID₅₀ rgA/VN-PR8 or rgA/Anhui-PR8, as determined by an OD readout two times above the background OD.

Cellular responses. Enzyme-linked immunosorbent spot (ELISpot) assays to detect T-cell responses in lymphocytes to inactivated A/VN/1203/04 were performed as described previously (29). Cells were restimulated with inactivated A/VN/1203/04 (the equivalent of 10 HAU per well), with Ebola GP P2 EYLFEVDNL as an irrelevant peptide (1 µg/ml),

FIG 1 Schematics of recombinant PIV5 expressing H5. (A) Schematics of recombinant PIV5 expressing H5N1 HA. The cleavage site that contains polybasic amino acid residues is deleted and the sequences are shown. (B) Titers of recombinant PIV5 expressing H5N1 HA stocks. The plaque-purified viruses were grown in MDBK cells and titrated in BHK cells.

and with phorbol myristate acetate-ionomycin (25 and 1.25 ng/ml, respectively) in 50 µl complete tumor medium (CTM). Spots were counted using an AID ViruSpot Reader (Cell Technology, Inc.).

Infection of mice with influenza A virus. BALB/c mice were first vaccinated with wild-type PIV5, rPIV5-ZL46, rPIV5-ZL48 intranasally (i.n.), or inactivated A/VN/1203/04 intramuscularly (i.m.) as described above. Twenty-one days postvaccination, the mice were bled for serum analysis via the tail vein. On day 24 postvaccination, mice were anesthetized and inoculated intranasally with 10 50% lethal infectious doses (LD₅₀) A/Vietnam/1203/04 diluted in 50 µl PBS. Mice were then monitored daily for morbidity and mortality with body weights measured every other day. On day 3 postchallenge, groups of mice were euthanized and their lungs collected into 1.0 ml PBS and homogenized. Homogenate was then cleared by centrifugation. A TCID₅₀ assay was then used to determine virus titers in cleared homogenate as described previously (30).

RESULTS

Generating and analyzing recombinant PIV5 expressing HA of H5N1 (rPIV5-H5) *in vitro*. To test whether recombinant PIV5 expressing HA of H5N1 can protect mice against lethal challenge with HPAI H5N1, we have inserted the HA gene of HPAI H5N1 without the polybasic cleavage site (31, 32) into a plasmid containing the full-length cDNA genome of PIV5 at the junction between the HN and L genes of PIV5 (ZL48) (Fig. 1A). Infectious virus ZL48 (rPIV5-H5) was recovered by transfecting the plasmid into BSR T7 cells along with plasmids carrying NP, P, and L as described before (28). Recovered virus was plaque purified and grown in Vero cells. The full-length genome of plaque-purified virus was sequenced using direct reverse transcription-PCR (RT-PCR) sequencing. A large stock of the virus was grown in MDBK cells. The titer of the virus was 10⁸ PFU/ml (Fig. 1B). Expression of

FIG 2 Generation and analysis of recombinant PIV5 expressing H5N1 HA between HN and L of the PIV5 genome. (A) Confirmation of H5N1 HA expression using immunoblotting. MDBK cells were infected with ZL48 and lysed at 24 hpi. The lysates were run on an SDS-PAGE gel and immunoblotted with anti-H5N1 HA. (B) Confirmation of H5N1 HA expression using immunofluorescence (IF). MDBK cells were infected with ZL48 and stained with anti-H5N1 HA. Antibodies used for IF are shown on the left side of the panel. (C) Growth rate of rPIV5-H5. MDBK cells were infected with PIV5 or ZL48 at an MOI of 0.1. Media were collected at 24-h intervals. The titers of viruses in the media were determined using plaque assay.

H5N1 HA from ZL48-infected cells was confirmed using immunoblotting (Fig. 2A) and immunofluorescence (Fig. 2B). ZL48 grew similarly to wild-type PIV5, as shown in a low-MOI growth curve (Fig. 2C).

Immunogenicity of rPIV5-H5 in mice. To determine whether ZL48 could generate HA-specific immunity *in vivo*, mice were infected intranasally with a single dose of 10^6 PFU of ZL48 or wild-type PIV5, and immune responses were compared to those of mice infected with an H5N1 reverse-genetic vaccine construct (rgVN-PR8) or immunized with inactivated H5N1 virus (iA VN). Mice were bled at 21 days postinoculation/immunization. Serum levels of anti-HA IgG were determined using ELISA (Fig. 3A and

B). The infection of ZL48 generated balanced levels of IgG1 and IgG2a that were comparable to those of inactivated H5N1 virion and recombinant H5N1 that contain internal genes from PR8 and HA and NA from H5N1. The neutralizing antibody (NAb) titers of the sera from ZL48-infected mice were low (Fig. 3C). However, a boost at 21 dpi enhanced the levels of serum NAb to a level that is considered protective. Cellular responses were examined using an ELISpot assay. ZL48-infected mice generated cellular responses (Fig. 3D). While the Th1 (IFN- γ -producing T cell) response was limited compared to that of mice infected with the reverse-genetic H5N1 virus, these mice had responses to the entire influenza virus, including immunodominant antigens contained in internal and

FIG 3 Immune responses in mice inoculated with rPIV5-H5. Mice were infected with PIV5 or ZL48 at a dose of 10^6 PFU via the intranasal route. (A) ELISA titers of anti-HA. Mice were infected with 10^6 PFU of ZL48 or PIV5. At 21 dpi, mice were bled. Titers of anti-HA were determined using ELISA. (B) Boost of anti-HA titers. The mice depicted in panel A were boosted at 28 dpi and bled at 35 dpi. Anti-HA titers were measured using ELISA. (C) Neutralization titers. Titers of NABs in sera of mice vaccinated with PIV5 or ZL48 against H5N1 were determined as described in Materials and Methods. (D) Cell-mediated responses. IFN- γ -producing lymphocytes (pools of $n = 3$ mice per group) in the mediastinal lymph nodes on day 12 postvaccination as determined by ELISpot analysis. Data are presented as means $\pm$ standard errors of the means.

FIG 4 Efficacy of rPIV5-H5 against rgVN-PR8 (H5N1) challenge in mice. The mice were inoculated with PBS, PIV5, or ZL48 ($n = 10$ per group) at a dose of 10^6 PFU per mouse. At 21 dpi, the mice were challenged with rgVN-PR8 (H5N1) at a dose of 1,000 TCID₅₀. The lungs were collected at 4 days postchallenge. Titers of rgPR8H5N1 in the lungs of mice were determined using a plaque assay.

nonstructural proteins (33), similar to those of ZL48, which had only the influenza virus HA, and the lymphocytes were restimulated with whole virus.

Efficacy of rPIV5-H5 against recombinant H5N1 influenza virus challenge in mice. Because of the cost and the relatively low NAb titer, the efficacy of ZL48 against the homotypical virus challenge was first examined in mice using a recombinant influenza virus, rgVN-PR8 (H5N1), that contains all internal genes from PR8 and HA and NA from HPAI H5N1 (with the polybasic cleavage site within HA removed). This virus is less virulent in mice than wild-type HPAI H5N1 and can be used in BSL2 biocontainment. Mice were immunized with a single dose of 10^6 PFU of ZL48 or wild-type PIV5 via the intranasal route. A separate group of mice received the inactivated H5N1 virus (iA VN) as a positive control. The mice were challenged with 1,000 TCID₅₀ rgA-PR8 (H5N1) at 21 days postimmunization. Because rgVN-PR8 (H5N1) does not cause mortality in mice, the efficacy of ZL48 immunization was examined using titers of the challenge virus in the lungs of the mice. No rgVN-PR8 (H5N1) virus was detected in the lungs of ZL48-immunized mice at 4 days postchallenge (Fig. 4), suggesting that ZL48 was effective in preventing H5N1 infection.

Efficacy of rPIV5-H5 against HPAI H5N1 challenge in mice. The efficacy of ZL48 against HPAI H5N1 was examined in mice with the A/Vietnam/1203/2004 strain (34). Mice were immunized with a single dose of 10^6 PFU of ZL48 via the intranasal route. The

mice were challenged with H5N1 at 21 days postimmunization. PIV5-immunized mice lost substantial amounts of weight, 90% of them were dead by day 10 after challenge, and all had died at day 14 after challenge (Fig. 5B). In contrast, all mice immunized with ZL48 survived challenge, and no weight loss (Fig. 5A) was observed during the time of the experiment. Furthermore, no challenge virus was detected in the lungs of ZL48-immunized mice (Fig. 5C), indicating that ZL48 is effective in preventing H5N1 infection in mice.

Generating recombinant PIV5 expressing HA of H5N1 (rPIV5-H5) at different locations within PIV5 genome and analyzing them *in vitro* and *in vivo*. The distance to the leader sequence, the only *de facto* promoter for PIV5, inversely affects gene expression levels. The gene junction between the HN and L genes where we inserted H5N1 HA in ZL48 is the most distant from the leader sequence in PIV5 (Fig. 1). Moving H5N1 HA from the HN-L gene junction closer to the leader sequence will increase the level of expression of H5N1 HA protein. We reasoned that increasing the expression level of H5N1 HA would increase the efficacy of the vaccine. We first tried to insert the H5N1 HA gene between the leader sequence and the NP gene. Unfortunately, while we were able to generate a plasmid with an insertion between the leader sequence and the NP gene, the plasmid was not able to generate a viable infectious virus (Fig. 1), suggesting that the insertion is detrimental to the virus life cycle. The H5N1 HA gene was then inserted at the next gene junction, the NP and V/P

FIG 5 Efficacy of rPIV5-H5 against HPAI H5N1 challenge in mice. The mice were inoculated with PBS, PIV5, or ZL48 ($n = 15$ per group) at a dose of 10^6 PFU per mouse. At 21 dpi, the mice were challenged with HPAI H5N1 at a dose of 10 LD₅₀. (A) Weights of mice challenged with H5N1. Weights were monitored daily after challenge for 15 days. Weight is graphed as the average percentages of original weight (the day of challenge). (B) Survival rate. (C) Lung titers of mice challenged with H5N1. Mice ($n = 5$) were sacrificed at 4 days after H5N1 challenge. The titers were determined using plaque assays in MDCK cells.

FIG 6 Analysis of recombinant PIV5 expressing H5N1 HA. (A) Analysis of H5N1 HA expression in cells infected with recombinant PIV5 expressing H5N1 HA. Cells were infected with ZL46, ZL47, and ZL48 as well as PIV5 at an MOI of 1. The expression levels of H5N1 HA in infected cells were determined using flow cytometry as described in Materials and Methods. (B) Growth rate of the recombinant viruses in tissue culture cells. Cells were infected with viruses at an MOI of 0.1. The media from infected cells were collected at 24-h intervals and used for plaque assays to determine titers of viruses.

gene junction (ZL209). While recombinant viruses were recovered from plasmid ZL209, the viruses did not grow well in tissue culture cells. In addition, the viruses contained mutations at the other sites (Fig. 1B and data not shown). The H5N1 HA gene was inserted at the next gene junction, the V/P and the M gene junction (ZL47) (Fig. 1), as well as at the junction of SH and HN (ZL46) (Fig. 1). ZL46 and ZL48 grew similarly to wild-type PIV5, while ZL47 had a slight reduction in titer (Fig. 6A). The expression level of H5N1 HA was highest in ZL46-infected cells, while in ZL47-infected cells the level of H5N1 HA was similar to that in ZL48-infected cells (Fig. 6B). The abilities of these viruses to replicate in mice were compared by determining titers of virus in the lungs of infected mice. The titers of PIV5 and ZL46 were similar at 4 days postinfection (Fig. 7). The titers in the lungs of ZL48- and ZL47-infected mice were lower than those from PIV5- or ZL46-infected mice. On average, ZL47 titers were the lowest (Fig. 7) (however, the difference between ZL47 and ZL48 is not statistically significant).

Determining efficacy of recombinant PIV5H5 expressing H5N1 HA against HPAI H5N1 challenge in mice. As all recombinant PIV5 vaccines expressing H5N1 HA provided complete protection against H5N1 challenge in mice after a single high inoculation dose (10^6 PFU; data not shown), a dose-response study was performed to determine if the location of the H5N1 HA gene within the PIV5 genome modified the efficacy of the vaccine. Mice were infected with ZL46, ZL47, and ZL48 at a dose of 10^3 , 10^4 , or 10^5 PFU via the intranasal route. Mice were bled at 21 dpi and sera were analyzed (data not shown).

All mice inoculated with $\geq 10^4$ PFU of ZL46, ZL47, or ZL48

FIG 7 Growth of the recombinant viruses *in vivo*. Mice ($n = 5$) were infected with viruses at a dose of 10^6 PFU via the intranasal route. At 4 dpi, the lungs were collected and used for plaque assays to determine titers of viruses.

survived lethal H5N1 challenge (data not shown). However, low-dose immunization revealed distinct outcomes. One thousand (10^3) PFU of ZL46 protected 100% of mice against a lethal challenge with HPAI H5N1, while ZL48 protected 70% of immunized animals and ZL47 only protected 30% of mice (Fig. 8A), suggesting that the insertion of the H5N1 HA gene between SH and HN enabled the most potent priming of protective immune responses. We observed a similar trend for weight loss. At an inoculum of 10^3 PFU, mice immunized with ZL47 had the greatest weight loss, while those immunized with ZL46 had the least weight loss (Fig. 8B). At an inoculum of 10^4 PFU, ZL48 and ZL46 similarly protected mice from weight loss, while ZL47-immunized mice still lost 10 to 15% of their starting weight (Fig. 8C). At immunization doses of 10^5 PFU and higher, all of the mice were similarly protected from weight loss due to HPAI infection (Fig. 8D and data not shown).

DISCUSSION

Inactivated influenza virus vaccines have been available since the 1940s and are 60 to 80% effective against matched influenza virus strains, but they are less effective against antigenic drift variants and are ineffective against different subtypes (35). Moreover, vaccination coverage and production continue to be problems worldwide. Current 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 (36). Viral vectors such as adenovirus (AdV) are being tested in clinical trials for HIV. Similarly, viral vector-based H5N1 vaccines have been widely explored. AdV has been modified to express influenza A virus antigens; however, the recent failure of an AdV-based HIV vaccine candidate in a clinical trial has dampened enthusiasm for this viral vector (37). Recombinant DNA vaccines expressing influenza antigens have been tested in animal models and shown to induce protective antibody and T cell responses (38–41). However, the need for repeated administration of DNA can be a hurdle for using the DNA-based vaccine for rapidly spreading influenza virus infection or general immunization compliance (i.e., ensuring individuals receive the full vaccination schedule). Previously, a recombinant PIV5 expressing HA of H3 subtype of influenza A virus protected against a homotypical influenza virus H3N2 chal-

FIG 8 Efficacy of recombinant PIV5 expressing H5N1 HA against HPAI H5N1 challenge. Mice were infected with PIV5 or ZL48 at a dose of 10^3 , 10^4 , or 10^5 PFU via the intranasal route. At 24 dpi, the mice were challenged with 10 LD₅₀ of H5N1. The weights of the mice were monitored daily. (A) Log-rank survival analysis of mice challenged with 10 LD₅₀ of A/Vietnam/1203/04. Relative weights of mice vaccinated with 10^3 PFU (B), 10^4 PFU (C), or 10^5 PFU (D) and challenged with 10 LD₅₀ of HPAI H5N1. Mice vaccinated with rgVN-PR8 received 2,000 PFU of virus.

challenge. However, the challenge model was not robust: H3N2 does not cause death in infected mice (26). In this work, recombinant PIV5 expressing HA of H5N1 was tested against challenge with the most virulent influenza virus in mice, HPAI H5N1. This recombinant vaccine was efficacious in protecting mice against HPAI H5N1 challenge even at very low doses, suggesting that PIV5 is a viable vector for development of H5N1 vaccine. Currently, the only FDA-approved vaccine against H5N1 has serious limitations, particularly as it has to be given twice and requires substantially higher concentrations of the vaccine to achieve a moderate level of efficacy compared to conventional influenza virus vaccines. Conventional vaccines utilizing the HA and NA of H5N1 viruses have been poorly immunogenic and have safety and production issues (reviewed in reference 5). A PIV5-based H5N1 vaccine has advantages over the current FDA-approved H5N1 vaccine, because a PIV5-based H5N1 vaccine, such as ZL46, can generate protective immunity with a single, low dose (10^3 PFU per mouse), while the vaccine can be grown to 10^8 PFU/ml in tissue culture cells without using specific pathogen-free eggs, and the production of the vaccine does not pose a health risk to workers. Furthermore, because of the cost-effective nature of PIV5-based H5N1 vaccines, it is possible to use it on animal carriers of H5N1 such as chickens.

Paramyxoviruses such as PIV5 initiate transcription only at the leader sequence. Thus, the distance to the leader sequence is in an inverse proportion to levels of the viral gene. The gene junction between the HN and L genes is the most distant from the leader sequence in PIV5. It has been used often to insert foreign genes to avoid any potential adverse effects of inserting foreign genes (22, 26) (Fig. 1). Moving H5N1 HA from the HN-L gene junction closer to the leader sequence should increase the level of gene

expression. It is interesting that insertion of a foreign gene between the leader sequence and the NP gene did not lead to a viable virus, suggesting that the insertion is detrimental to the virus life cycle and that the region between the leader and NP genes is critical for PIV5 replication. While insertion of H5N1 HA between NP and V/P generated a viable virus, the virus was defective in its growth and mutations rose in the other regions of the recovered viruses, suggesting that the region is important for virus replication as well. It has been reported before that the ratio of NP expression to V/P gene expression is critical for optimal virus replication in a minigenome system (42). It is likely that insertion of H5N1 HA between NP and V/P disrupted the ratio of NP to V/P, resulting in the defects in virus replication. This result is similar to the insertion of GFP between N and P in vesicular stomatitis virus (42). While ZL46, ZL47, and ZL48 all provided complete protection against H5N1 challenge in mice after a single inoculation dose as low as 10^4 PFU per mouse, it is surprising that insertion of H5N1 HA between V/P and M did not provide better protection than ZL48 (insertion between HN and L) or ZL46 (insertion between SH and HN). It is possible that the insertion between V/P and M negatively affected replication of recombinant virus *in vitro* (Fig. 6) and *in vivo* (Fig. 7), resulting in a less efficacious vaccine candidate. In our accompanying article, we have found that live recombinant PIV5 is needed for efficacious vaccination (43). These results indicate that fitness of virus *in vivo* and the insertion site within the PIV5 genome has a major impact on the efficacy of the vaccine candidate.

Neutralizing antibody against influenza virus is the hallmark of protective immunity. However, at lower doses of inoculation with recombinant PIV5 expressing H5N1 HA of H5N1, low levels of

NAB were barely detected, yet the mice were completely protected. The results suggest that cell-mediated immune responses from a live vaccine can contribute to the protection against highly pathogenic influenza virus challenge. This is supported by studies utilizing the NP protein of influenza virus as a vaccine antigen (44). It is interesting that the boost of ZL48 enhanced immunity in mice, suggesting that prime inoculation did not prevent reimmunization or infection of PIV5-based vaccine. This is consistent with the report that in mice neutralizing antibodies against PIV5 do not prevent PIV5 infection (45). While people have been exposed to PIV5 (46), PIV5 is not associated with human diseases, suggesting that PIV5 is safe to use in humans. However, it raises the question of whether a PIV5-based vaccine can be effective in humans. Further analysis of the impact of immunity against the vector is under way in dogs, which are routinely vaccinated with kennel cough vaccines that contain live PIV5.

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