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Influenza B Virus NS1-Truncated Mutants: Live-Attenuated Vaccine Approach▽

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Type B influenza viruses can cause substantial morbidity and mortality in the population, and vaccination remains by far the best means of protection against infections with these viruses. Here, we report the construction of mutant influenza B viruses for potential use as improved live-virus vaccine candidates. Employing reverse genetics, we altered the NS1 gene, which encodes a type I interferon (IFN) antagonist. The resulting NS1 mutant viruses induced IFN and, as a consequence, were found to be attenuated in vitro and in vivo. The absence of pathogenicity of the NS1 mutants in both BALB/c and C57BL/6 PKR$^{-/-}$ mice was confirmed. We also provide evidence that influenza B virus NS1 mutants induce a self-adjuvanted immune response and confer effective protection against challenge with both homologous and heterologous B virus strains in mice.

Influenza B viruses belong to the family *Orthomyxiviridae*, the members of which contain eight negative-sense, single-stranded segmented RNAs as genomes (27). Their epidemiology and evolutionary characteristics are distinct from those of the other influenza viruses (19, 37). Influenza B viruses are known to infect mostly humans and occasionally seals (25). It is still a mystery as to why influenza B viruses do not have as many natural hosts as the influenza A viruses. Influenza B viruses also continuously undergo antigenic drift (1, 12, 22), yet with a lower evolutionary rate than that of influenza A viruses (24), and reassortment among different influenza B virus strains occurs regularly (18, 36). Although influenza B viruses have only one recognized subtype, they have evolved into three major lineages, the earliest B/Lee/40 virus-like variants, B/Victoria/2/87-like variants, and B/Yamagata/16/88-like strains (22, 28). The disease caused by influenza B viruses is generally milder than that caused by influenza A viruses; however, in some cases B virus infections can lead to severe disease which requires hospitalization (23). The morbidity caused by infections with influenza B viruses may also have substantial social and economic impacts.

Vaccination is by far the best means to protect people against disease from influenza virus infections (26). Live-attenuated cold-adapted virus and inactivated virus vaccines have been licensed by the FDA. Live-attenuated vaccines have some advantages over inactivated influenza virus vaccines; i.e., (i) they are easy to administer through intranasal delivery, and they mimic natural infection; (ii) they can provide a broader immunological response, including mucosal immunity and cellular immunity, than inactivated virus vaccines; and (iii) they can possibly provide longer-lasting immunity than inactivated virus vaccines. Although the current cold-adapted live-attenuated influenza A and B virus vaccine is effective (26), there is room for improvement (20). One strategy is to make a new live-attenuated influenza virus vaccine through modifying the influenza virus nonstructural protein 1 (NS1), which is an interferon (IFN) antagonist (34). This approach has proved to be successful in different animal models for influenza A virus infection (8, 30, 33). The NS1 of influenza B viruses has also been shown to be an IFN antagonist (4, 5, 7), and IFN enhances the production of immunoglobulins and activates dendritic cells required for antigen presentation (13–15). The modification of the NS1 gene of influenza B viruses by truncation and deletion would ensure that the mutant viruses are attenuated in their replication and enhanced in their induction of IFN compared to the wild-type (WT) virus (8, 30, 33). Hence, we hypothesize that immunization with NS1 mutant viruses would deliver an enhanced immune response compared with the responses induced by the current conventional live-attenuated or inactivated virus vaccines (34).

Here, we report the novel construction of NS1 mutant viruses in an influenza B/Yamagata/16/88 virus background through reverse genetics. We also introduce a transgenic mouse model (C57BL/6 protein kinase R-deficient [PKR$^{-/-}$] mice) in which non-mouse-adapted WT influenza B virus strains are able to cause morbidity and mortality. Using this mouse model, we demonstrated that the NS1 mutant viruses are attenuated and that they are safe in vivo. Finally, we demonstrated their efficacy against challenges with homologous and heterologous viruses in the mouse model.

MATERIALS AND METHODS

Cells and viruses. 293T and MDCK cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) and minimal essential medium (both from GIBCO, Carlsbad, CA), respectively, each supplemented with 10% fetal calf serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (GIBCO).

Influenza B/Yamagata/16/88 virus and rescued recombinant WT (rWT) vi-

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ruses were propagated in 8-day-old embryonated chicken eggs for 3 days at 33°C. Recombinant NS1 mutant viruses deltaNS1 (expressing amino acids 1 to 16 of NS1), NS1-80 (expressing NS1 amino acids 1 to 80), and NS1-110 (expressing NS1 amino acids 1 to 110) were propagated in IFN-deficient 7-day-old embryonated chicken eggs for 3 days at 33°C (10).

Construction of plasmids. The eight reverse genetics plasmids used for rescue of recombinant influenza B/Yamagata/88 virus, pDZ-Ya88-PB2, pDZ-Ya88-PB1, pDZ-Ya88-PA, pDZ-Ya88-NP, pDZ-Ya88-HA, pDZ-Ya88-NA, pDZ-Ya88-M, and pDZ-Ya88-NS, were constructed by the strategy described previously (11, 30). In the system, influenza virus cDNA was inserted between the RNA polymerase I (Pol I) promoter and terminator sequences. This entire RNA Pol I transcription unit was flanked by an RNA Pol II promoter and a polyadenylation site. The orientation of the two promoters allowed the synthesis of negative-sense viral RNA and positive-sense mRNA from one viral cDNA template. A genetic tag was inserted into the NS-encoding plasmid by site-specific mutagenesis. Nucleotides 580 to 585 (5′-GGAACA-3′) were replaced with 5′-GGTACC-3′, resulting in two silent mutations and the creation of a novel KpnI restriction enzyme site. The sequence of all PCR inserts was confirmed, and nucleotide changes that had been introduced by PCR were corrected by using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA).

The plasmids of different NS1 mutants, pDZ-Ya88-delta NS1, pDZ-Ya88-NS1-80, and pDZ-Ya88-NS1-110, were derivatives of the WT NS segment. Briefly, the pDZ-Ya88-delta NS1 plasmid was constructed by inserting two sequential tandem stop codons right after the codon corresponding to amino acid 16 of the WT NS1 protein through QuikChange XL site-directed mutagenesis. For pDZ-Ya88-NS1-80 and pDZ-Ya88-NS1-110, two sequential tandem stop codons were inserted after the codons corresponding to amino acids 80 and 110 of the WT NS1 protein, respectively. In addition, a deletion of 100 nucleotides after each insertion was engineered (see Fig. 1A). The open reading frame of the nuclear export protein (NEP) was not altered in these constructs. All primer sequences are available upon request.

Rescue of recombinant influenza B viruses. The rescue of influenza B viruses from plasmid DNA was performed as described previously (9, 30, 33). Briefly, for the generation of the rWT influenza B/Yamagata/88 virus, 293T-MDCK cell cocultures were cotransfected with 1 μg of each of the eight plasmids by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 12 h posttransfection, the medium was replaced by DMEM containing 0.3% bovine serum albumin, 10 mM HEPES, and 1.5 μg of TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)-treated trypsin/ml. At 3 days posttransfection, virus-containing supernatant was inoculated into 8-day-old embryonated chicken eggs. Allantoic fluid was harvested after 3 days of incubation at 33°C and assayed for the presence of virus by the hemagglutination of chicken red blood cells and by plaque formation in MDCK cells. The different NS1-truncated mutant viruses were generated as described above, but the pDZ-Ya88-NS plasmid was replaced by the corresponding mutant plasmids in order to recover the deltaNS1, NS1-80, and NS1-110 mutant viruses. The virus-containing supernatant was inoculated into 7-day-old embryonated chicken eggs. Allantoic fluid was harvested after 3 days of incubation at 33°C and assayed for the presence of virus by the hemagglutination of chicken red blood cells. Because the mutant viruses do not readily form visible cytolytic plaques, we used an alternative plaque assay which involved indirect immunofluorescence staining of infected MDCK cells with the nucleoprotein (NP)-specific monoclonal antibody B017 (Abcam, Cambridge, MA) as described previously (21).

Western blot analysis. One well of a six-well dish of 90% confluent MDCK cells was infected (multiplicity of infection [MOI] of 1) with WT B/Yamagata/88 virus, rescued WT B/Yamagata/88 virus, or the different recombinant NS1 mutant viruses or mock infected with phosphate-buffered saline (PBS) for 1 h at 33°C. At 12 h postinfection (hpi), cells were lysed as described previously (30). Cell lysates were subjected to Western blot analysis by using monoclonal antibody against NP (B017), polyclonal antibody against NS1 (kindly provided by T. Wolff), or monoclonal antiactin (Sigma, St. Louis, MO). The final Western blot bands were visualized using an enhanced chemiluminescence protein detection system (PerkinElmer Life Sciences, Boston, MA).

Indirect immunofluorescence analysis. Confluent monolayers of MDCK cells on 15-mm coverslips were infected with recombinant viruses at an MOI of 2. At 12 hpi, cells were fixed and permeabilized by treatment with methanol-acetone (ratio, 1:1) at −20°C for 20 min. After being blocked with 1% bovine serum albumin in PBS containing 0.1% Tween 20, cells were incubated for 1 h with a monoclonal antibody directed against NP (B017) or a polyclonal NS1 antibody as mentioned above. After three washes with PBS containing 0.1% Tween 20, cells were incubated for 1 h with Alexa Fluor 594-conjugated anti-mouse immunoglobulin G (IgG; Invitrogen, Carlsbad, CA) and with Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA). After one additional wash, cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen, Carlsbad, CA). Following the final two washes, infected cells were analyzed by fluorescence microscopy with an Olympus IX70 microscope.

Generation of a stable MDCK cell line expressing GFP-CAT and firefly luciferase under the control of the IFN-β promoter. An MDCK cell line expressing the green fluorescence protein (GFP) fused to the chloramphenicol acetyltransferase (CAT) protein under the control of the IFN-β promoter was generated by cotransfection with the pIFNβ GFP-CAT reporter plasmid (16) and the pHCB7 hygromycin resistance vector. Twenty-four hours posttransfection, cells were diluted and incubated in the presence of hygromycin B (Invitrogen) until individual clones were selected. The presence of the IFN-β reporter plasmid was detected by infection with the well-described IFN inducer Sendai virus, strain Cantell. Cells were maintained in DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin in the presence of 1 mg of hygromycin B/ml. The stable MDCK cell line expressing, in addition to GFP-CAT, the firefly luciferase gene under the control of the IFN-β promoter was generated by the transfection of the above-described cell line with the plasmid pGL4.17 IFNβ firefly-luciferase. Then the cells were selected in the presence of Geneticin (Invitrogen). Hygromycin B and Geneticin resistance clones were tested for reporter gene expression by infection with Sendai virus. Clone 41L/23, positive for GFP and firefly luciferase expression upon Sendai virus infection, was selected for these studies and maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1 mg of hygromycin B/ml, and 2 mg of Geneticin/ml.

Bioassay to measure IFN production. To evaluate the levels of IFN produced in cells infected with influenza B viruses, a modified bioassay was applied (30). Briefly, six-well dishes of confluent MDCK pIFNβ cells were infected at an MOI of 2 with different recombinant influenza B/Yamagata/88 viruses for 12 h, with PBS as the mock infection control. The expression of GFP was visualized by fluorescence microscopy with an Olympus IX70 microscope. Cell lysates were prepared for the luciferase assay and Western blot analysis. The supernatants were also collected, and any live virus present was UV inactivated as described previously (6). Flat-bottomed 12-well plates of confluent MDCK cells were treated with the inactivated supernatants for 24 h and infected with vesicular stomatitis virus (VSV) expressing GFP (VSV-GFP; kindly provided by J. Hiscott) at an MOI of 2. At 8 hpi, the cells were examined for GFP.

Growth curves of recombinant viruses. To analyze viral replication, confluent IFN-competent MDCK and IFN-deficient Vero cells were infected at an MOI of 0.5 and incubated at 33°C in minimal essential medium containing 0.3% bovine albumin and 1.5 μg of TPCK-treated trypsin/ml. Viral titers in supernatants were determined by indirect immunofluorescence staining of infected MDCK cells with the influenza B virus NP-specific monoclonal antibody B017 (Abcam, Cambridge, MA) as described previously (21).

Mouse immunization and challenge. Eight-week-old female C57BL/6 $PKR^{-/-}$ (2, 38), C57BL/6 $PKR^{+/+}$, and BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with a mixture of ketamine and xylazine, administered intraperitoneally, and infected intranasally with the amounts of different influenza B viruses (in 50-μl volumes) indicated below. For the determination of lung virus titers, mice were euthanized at either day 3 or day 6 postinfection. Lungs were homogenized and resuspended in 1 ml of sterile PBS, and the titers were evaluated on MDCK cells in the presence of 1.5 μg of TPCK-treated trypsin/ml.

Three weeks after immunization, mice were challenged by intranasal infection with either rWT B/Yamagata/88 virus at 1×10^6 PFU or B/Lee/40 virus at 5×10^5 PFU. All procedures were in accordance with National Institutes of Health guidelines for the care and use of laboratory animals.

ELISA. To assess the amounts of virus-specific antibodies present in immunized mice, enzyme-linked immunosorbent assay (ELISA) analyses of the reactivities of diluted serum samples and nasal and lung wash samples against purified viral antigen were performed. Blood was drawn from mice 3 weeks after immunization and before challenge with WT virus. Blood was spun at 13,000 rpm for 15 min, and the supernatant (serum) was removed and stored at −80°C. Nasal and lung wash samples were taken after the sacrificing of the animals and stored at −80°C. Fifty microliters of sucrose gradient-purified influenza/B/Yamagata/88 or B/Lee/40 virus (10 μg/ml) was used to coat 96-well ELISA plates (Immulon4; Dynex, Chantilly, VA). After being washed with PBS, coated wells were blocked with PBS containing 1% bovine serum albumin and then incubated with diluted serum samples or nasal or lung wash samples. After 3 h of incubation at room temperature, wells were rinsed with PBS and incubated with a secondary anti-mouse IgG or IgA peroxidase (Invitrogen, Carlsbad, CA). Rinsed wells were incubated with colorimetric substrate (4-nitrophenyl phosphate; Invitrogen, Carlsbad, CA) for 30 min and read with an ELISA reader (measuring the optical density at 405 nm; Bio-Tek Instruments, Burlington, VT).

RESULTS

Generation of influenza B/Yamagata/88 virus and different NS1 mutant viruses. In order to generate recombinant influenza B/Yamagata/88 viruses containing different truncations of the NS1 protein, we first set up a reverse genetics system for influenza B/Yamagata/88 virus. A total of eight different segments were cloned into the pDZ rescue plasmid by reverse transcriptase PCR using supernatant from B/Yamagata/88 virus-infected cells. By following the rescue procedure as described before (9, 30, 33), we successfully restored full-length NS1 in rWT B/Yamagata/88 virus. The presence of the KpnI restriction site, which was the genetic marker designed in our rescue system, was identified in the NS1 gene of the rescued samples (data not shown).

For the generation of the different NS1 truncation mutants, a total of three influenza B/Yamagata/88 virus NS1 mutants, deltaNS1, NS1-80, and NS1-110, were constructed by using the pDZ plasmid (30). The coding region of the influenza B/Yamagata/88 virus NS1 gene is 846 nucleotides long (corresponding to 282 amino acids). Deletions and sequential truncations corresponding to the NS1 C terminus were made in this segment, resulting in three constructs encoding amino acids 1 to 16 (deltaNS1), 1 to 80 (NS1-80), or 1 to 110 (NS1-110) of the NS1 protein. The open reading frame of the NEP was not altered in these constructs (Fig. 1A). The three NS1 mutant viruses were rescued in the same manner as the rWT B/Yamagata/88 virus. Seven-day-old eggs were utilized to amplify NS1 mutant viruses. Reverse transcriptase PCR was carried out with RNA extracted from allantoic fluid of hemagglutinin-positive rescued-virus preparations. The sequences of the generated cDNAs and the presence of the specific genetic marker inside the NS segment confirmed the rescue of the specific viruses (data not shown).

Biological characterization of recombinant viruses. To evaluate whether the different NS1 mutant viruses could express the predicted portions of the NS1 protein, Western blot and indirect immunofluorescence analyses were applied.

For Western blot analysis, confluent MDCK cells were infected at an MOI of 2 with the rWT B/Yamagata/88, NS1-110, NS1-80, and deltaNS1 viruses and the parental WT B/Yamagata/88 virus for 15 h. A polyclonal antibody against the amino terminus of the NS1 protein, specific for the parental WT virus and the two derived NS1 mutants, was used to probe for the protein. The expression of the viral NP and that of β-actin were also examined as controls (Fig. 1B). Bands corresponding to the WT NS1 proteins were observed at the expected molecular sizes and similar intensities for WT B/Yamagata/88 and rWT B/Yamagata/88 viruses but not for the other NS1 mutant viruses and the PBS control. We also detected the bands for the NS1 110- and 80-amino-acid mutant forms, even though the expression of the 80-amino-acid NS1 was at a very low level. This result demonstrated that the different mutant viruses were successfully rescued and that they did express the predicted portions of the NS1 protein. The faint bands in the WT B/Yamagata/88 and rWT B/Yamagata/88 virus lanes migrating more slowly than the NS1-110 band are most likely degradation products of the WT NS1 protein.

To further study whether the NS1 truncation would affect the cellular localization of NS1, indirect immunofluorescence analysis was carried out. MDCK cells were infected at an MOI of 1, and at 13 hpi, cells were fixed with methanol-acetone (ratio, 1:1) and stained for the expression of influenza B virus NP and NS1 protein. DAPI was used as the marker for nuclear staining. We detected the viral NP signal in all infected cells (Fig. 1C). The NS1 protein was detected only in rWT virus- and NS1-80 and NS1-110 virus-infected cells (Fig. 1C). The NS1 proteins were located largely in the nucleus. However, the immunofluorescence appearance of the mutant NS1 proteins differed slightly from that of the WT NS1 protein.

Induction of IFN by recombinant influenza B viruses. One of the main functions of the influenza B virus NS1 protein is to counteract the type I IFN response in the infected cells (4, 10). We sought to compare the abilities of the mutated influenza B viruses to counteract the antiviral cytokine response (IFN) by using two different approaches.

First, we took advantage of an MDCK cell line which had a reporter cassette to express GFP-CAT or firefly luciferase under the control of the IFN-β promoter. This approach enabled us to characterize the IFN responses induced by the different viruses by observing the GFP signals following infection and measuring the levels of induction by the luciferase assay (Fig. 2A). We infected this cell line with different recombinant influenza B viruses at an MOI of 1 or with PBS as a mock infection control. At 15 hpi, we observed the GFP signals in infected cells by fluorescence microscopy. The levels of the GFP signals in these cells were a reflection of the amounts of IFN produced in cells infected with the influenza B viruses. The GFP signal was detected in all NS1 mutant virus samples but not in the rWT virus sample (Fig. 2B). This qualitative observation was further evaluated through a firefly luciferase assay. Infection with deltaNS1, NS1-80, and NS1-110 viruses induced various amounts of firefly luciferase versus trace amounts of firefly luciferase in rWT virus-infected cells (Fig. 2C). For the NS1 mutant virus samples, the firefly luciferase levels correlated with the GFP levels observed earlier in the GFP assay (Fig. 2B). Although the differences were not statistically significant, deltaNS1 virus-infected cells induced the largest amount, followed by the cells infected with the mutant viruses. Finally, a Western blot assay was conducted to confirm viral infection and the expression of firefly luciferase (Fig. 2D).

As a second approach to characterize the type I IFN response induced by the different NS1 mutant viruses, we measured the replication of VSV-GFP in the cells treated with supernatants in an IFN bioassay (Fig. 2E) (30). The levels of inhibition of the replication of VSV-GFP in these cells were a reflection of the amounts of IFN present in the supernatants of the cells infected with the different influenza B virus mutants. The VSV-GFP replication pattern confirmed what was observed earlier (Fig. 2B). In cells pretreated with supernatants from mock- and rWT virus-infected cells, VSV-GFP replicated efficiently, as evidenced by high-level GFP expression (Fig. 2F). In contrast, VSV-GFP did not replicate in the NS1 mutant-infected cells, as little or no GFP expression was observed (Fig. 2F). In summary, all the data obtained from both the reporter MDCK cell line and the IFN bioassay demonstrated that infection with the different NS1 mutant viruses induced a type I IFN response.

Growth characteristics of recombinant viruses in vitro. Safety is one of the main considerations in determining

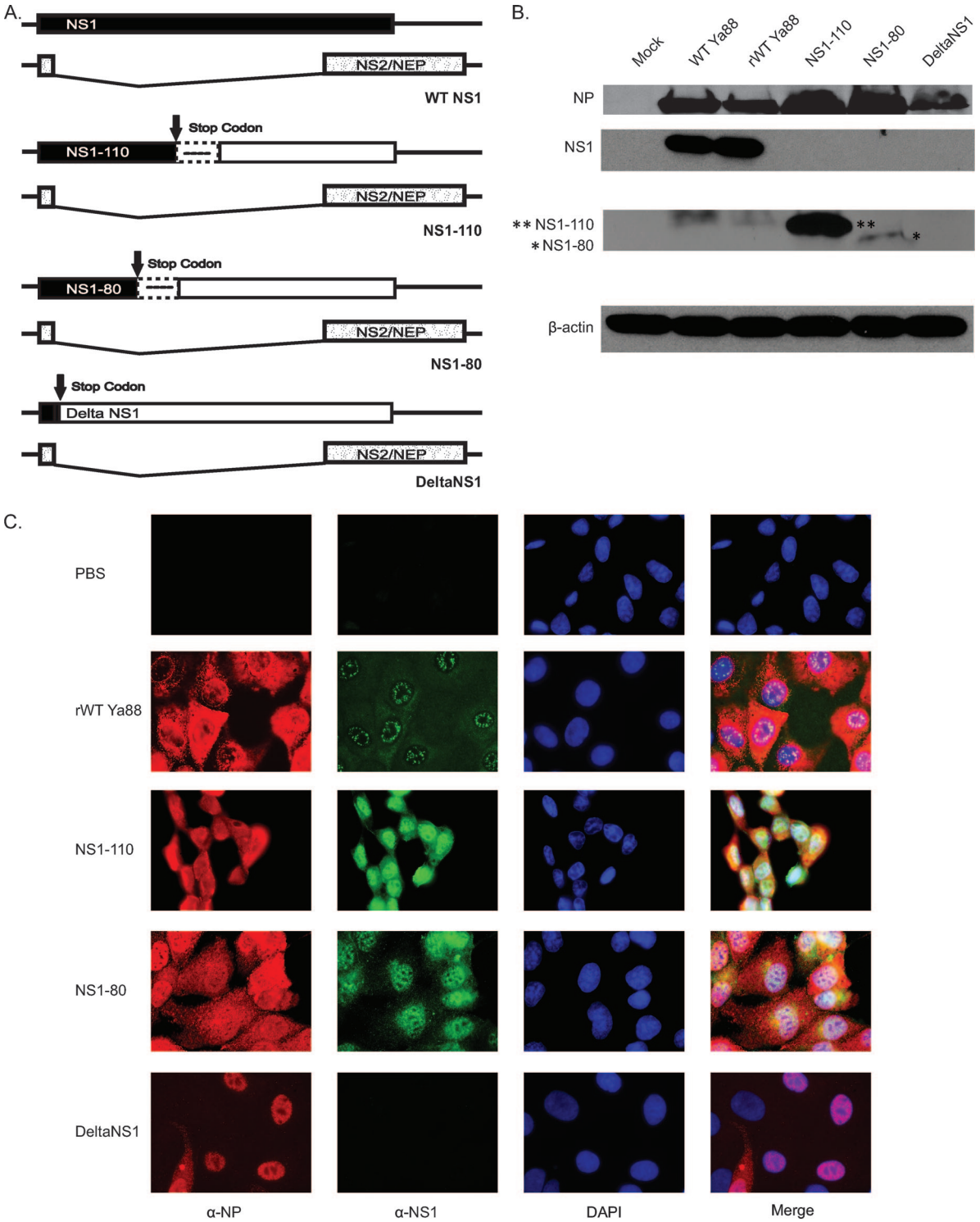

FIG. 1. Generation and characterization of recombinant influenza B/Yamagata/88 viruses with truncated NS1 proteins. (A) Schematic diagram of the influenza B/Yamagata/88 virus WT and NS mutants. Black boxes indicate WT and NS1 deletion constructs. Stippled white boxes indicate the NS2/NEP open reading frame, unaltered in the generation of the recombinant viruses. Mutant viruses NS1-110 and NS1-80 were generated by the introduction of two stop codons (arrows) after the codon corresponding to amino acid 80 or 110 and a deletion of 100 nucleotides (indicated as a box bordered by broken lines). The deltaNS1 virus was constructed by the insertion of two sequential stop codons (arrow) right after the codon corresponding to amino acid 16. (B) Western blot analysis of the rWT and NS1 mutant viruses. Extracts from MDCK cells mock infected or infected with the indicated viruses (16 hpi) were probed with specific antibodies against the viral NP to monitor viral infection, NS1, and β-actin as an internal loading control. Asterisks indicate the NS1-80 and NS1-110 bands, respectively. (C) Immunofluorescence analysis of the rWT and NS1 mutant viruses. Confluent monolayers of MDCK cells were infected with the indicated viruses and, 16 hpi, fixed and stained with a monoclonal antibody against the viral NP (red), a polyclonal antibody against the viral NS1 (green), and DAPI (blue) to detect cellular chromatin. The right panels show merges of the images.

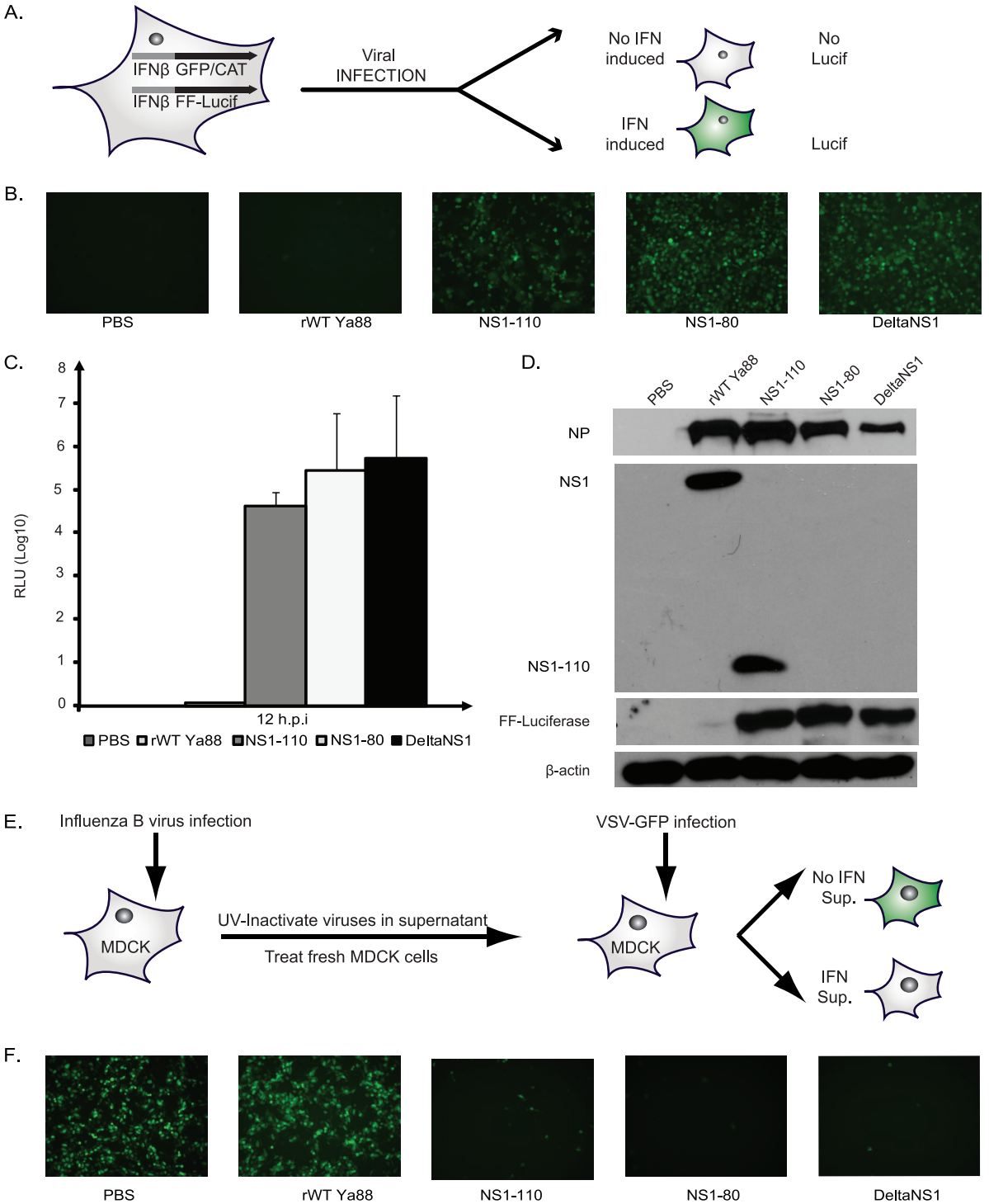

FIG. 2. IFN-α/β induction by the recombinant influenza B viruses. (A) Schematic representation of the IFN induction assay. MDCK cells constitutively expressing the GFP-CAT and firefly luciferase (FF-Lucif) genes under the control of the IFN-β promoter were infected with the recombinant influenza B viruses. (B and C) Fifteen hours postinfection, the activation of the IFN-β promoter was determined by assessing GFP expression under a fluorescence microscope (B) and firefly luciferase activity (C). The saturation plateau of the luciferase assay is 10^8 relative light units (RLU). (D) Cell extracts from the previously described experiment were analyzed for the presence of the viral NP as an infection control, NS1, firefly luciferase (as a measurement of IFN-β promoter activation), and β-actin as a loading control. (E) Schematic diagram of the bioassay for IFN production. Tissue culture supernatants (sup.) of previously infected MDCK cells were collected and, after UV inactivation, were used to treat fresh MDCK cells. (F) After overnight incubation, cells were infected with the recombinant VSV-GFP, and 8 hpi, the VSV-GFP-infected cells were examined by fluorescence microscopy and pictures of representative fields were taken.

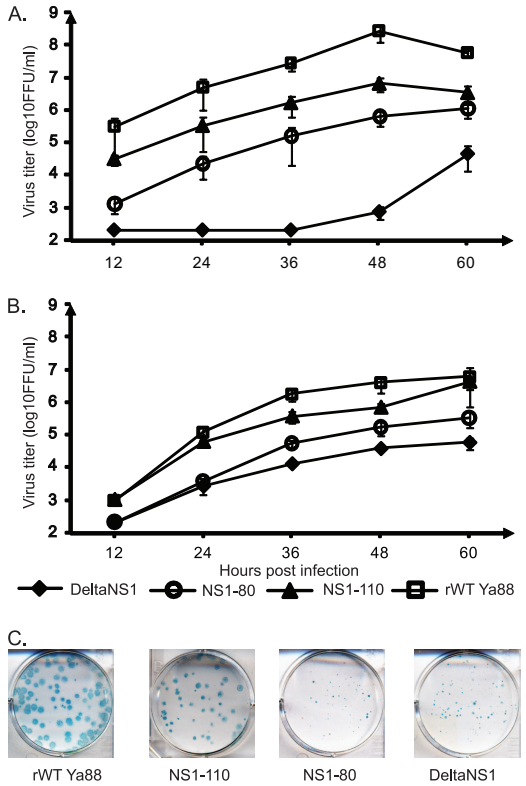

FIG. 3. Characterization of the recombinant B/Yamagata/88 viruses in vitro. (A and B) Multicycle growth curves of the recombinant viruses. IFN-competent MDCK cells (A) and IFN-deficient Vero cells (B) were infected with the rWT and NS1 mutant viruses (MOI of 0.5), and at the indicated time points after infection, virus titers in the supernatant were determined. FFU, fluorescence-forming units. (C) Plaque size phenotypes of the recombinant B/Yamagata/88 viruses in MDCK cells.

whether NS1 mutant viruses can be used as future live-attenuated vaccines. One way to characterize in vitro attenuation is by studying the viral growth kinetics. We infected confluent MDCK cells with different influenza B virus mutants at an MOI of 0.5. Supernatants of infected cells were titrated at different time points postinfection by indirect immunofluorescence staining (21). The growth characteristics of the NS1 mutants in MDCK cells were different from that of rWT virus (Fig. 3A). The results were in agreement with the findings of previous studies using influenza A virus NS1 mutants (30, 32, 33). We hypothesized that in an IFN-deficient system there would be fewer differences between the growth of rWT and NS1 mutant viruses than in an IFN-competent system like MDCK cells. Hence, we used Vero cells as our IFN-deficient model system and evaluated the growth characteristics of the different recombinant influenza B viruses in this system. Surprisingly, the mutant viruses exhibited lower peak titers than the rWT virus in IFN-deficient Vero cells as well as in MDCK cells, although the deltaNS1 virus did grow more efficiently in Vero cells than in MDCK cells (Fig. 3B). In addition to inhibiting type I IFN production, the influenza B virus NS1 protein has been shown previously to perform other functions critical for viral replication (35, 39). The lower peak titers of the NS1 mutant viruses in Vero cells are due possibly to those other functions of the NS1 protein. The plaque sizes of the different viruses in MDCK cells correlated well with their attenuated growth (Fig. 3C). rWT B/Yamagata/88 virus produced plaques that were larger than those formed by all the NS1 mutants. deltaNS1 and NS1-80 viruses showed the smallest plaques.

Pathogenicities of recombinant viruses in vivo. We next investigated the virulence of the NS1 mutant viruses in vivo in a mouse model by measuring viral replication in the lungs and weight loss. One of the challenges in the field of influenza B viruses is the lack of a small-animal model. Without mouse adaptation through tedious passage in mouse lungs, infection with influenza B viruses does not result in severe disease in these animals. Since the PKR gene has been shown previously to play an important role in influenza B virus replication (5), we studied whether infection with non-mouse-adapted B/Yamagata/88 virus could induce a phenotype in C57BL/6 $PKR^{-/-}$ mice.

To evaluate viral replication in the lungs, both C57BL/6 $PKR^{+/+}$ and C57BL/6 $PKR^{-/-}$ mice were infected intranasally with 5×10^5 PFU of the B/Yamagata/88 recombinant viruses indicated in Fig. 4. Viral titers in the lungs of the mice on days 3 and 6 postinfection were measured by immunofluorescence (Fig. 4A and B). On day 3, we detected all of the NS1 mutant viruses in the lungs of the C57BL/6 $PKR^{+/+}$ and C57BL/6 $PKR^{-/-}$ mice. We also noted that lung virus titers in C57BL/6 $PKR^{-/-}$ mice were generally higher than those in C57BL/6 $PKR^{+/+}$ mice, indicating better viral replication in the absence of PKR. On day 6, only rWT virus was detected in C57BL/6 $PKR^{+/+}$ mice while the NS1 mutant viruses were still detected in C57BL/6 $PKR^{-/-}$ mice. For C57BL/6 $PKR^{-/-}$ mice, titers of all the viruses on day 6 postinfection were 1 log lower than those on day 3. The titers of different viruses in the lungs correlated with in vitro growth data (Fig. 3A and B).

To further evaluate the virulence of the NS1 mutant viruses in vivo, C57BL/6 $PKR^{+/+}$ and C57BL/6 $PKR^{-/-}$ mice were infected intranasally with 5×10^5 PFU of each of the viruses and monitored for 14 days (Fig. 4C and D). In C57BL/6 $PKR^{+/+}$ mice, infection with recombinant B/Yamagata/88 viruses resulted in little or no weight loss. This outcome confirmed that infection with non-mouse-adapted influenza B viruses would not result in disease in the WT mouse. In C57BL/6 $PKR^{-/-}$ mice, the rWT virus infection led to weight loss and death for all animals; in contrast, infection with the NS1 mutants did not result in any weight loss.

Vaccination with NS1 mutant viruses elicits antibody responses in C57BL/6 $PKR^{-/-}$ mice. To study the efficacy of NS1 mutant viruses as vaccines to provide protection against homologous challenge, we evaluated antibody responses following vaccination. The C57BL/6 $PKR^{-/-}$ mice were vaccinated with deltaNS1, NS1-80, and NS1-110 viruses at 5×10^5 PFU through nasal infection. Three weeks postinfection, serum samples and nasal and lung wash samples were taken from deltaNS1, NS1-80, and NS1-110 virus-immunized C57BL/6 $PKR^{-/-}$ mice. The IgG and IgA in these samples were analyzed for reactivity against purified influenza B/Yamagata/88 virus by ELISA (Fig. 5). In both serum and lung wash samples, all of the NS1 mutant vaccinations resulted in significant titers of IgG antibody against the parental WT B/Yamagata/88 virus versus the titers in the PBS control group (Fig. 5A and B). IgA

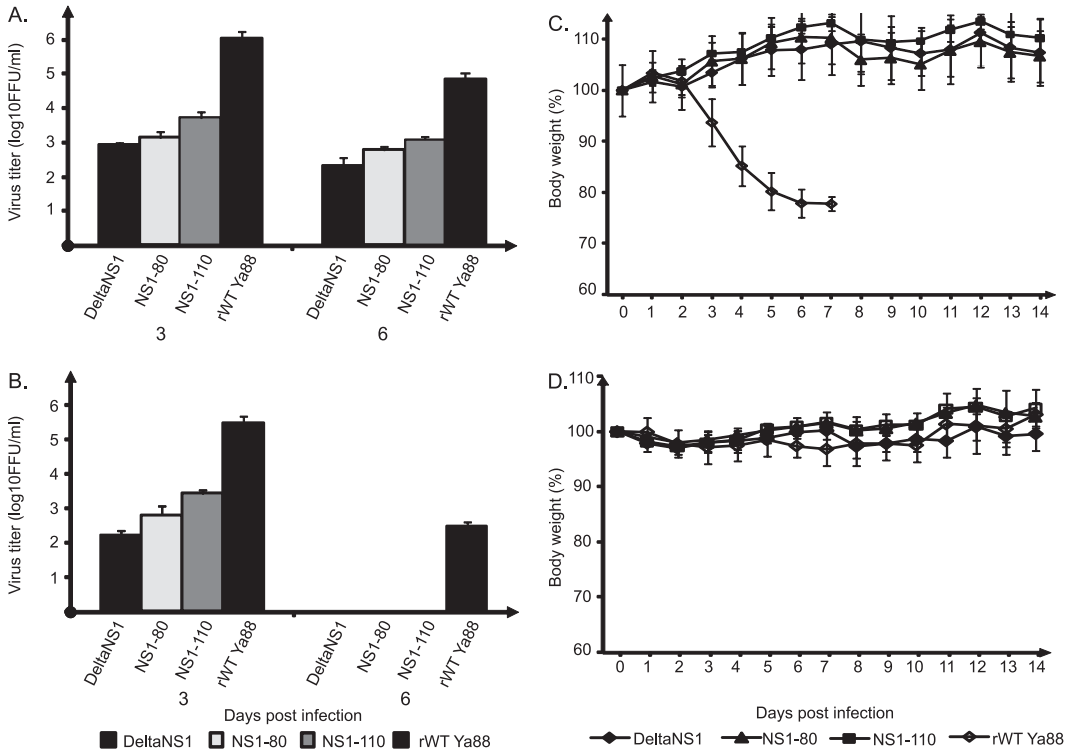

FIG. 4. Pathogenicity of rWT B/Yamagata/88 and NS1 mutant viruses in vivo. Eight-week-old female C57BL/6 PKR$^{-/-}$ (A and C) and WT (B and D) mice, 10 animals per group, were infected intranasally with 5×10^5 PFU of the indicated rescued B/Yamagata/88 WT and NS1 mutant viruses. On days 3 and 6 postinfection, three mice were sacrificed and virus titers in the lungs were determined. Following viral infection, mice were weighed daily, and the average body weights of surviving animals in each group up to day 14 postinfection are indicated as percentages of the original body weights. FFU, fluorescence-forming units.

responses in these samples were negligible, regardless of the NS1 mutant viruses used to vaccinate. In nasal wash samples, we detected a significant increase in IgA antibody titers following vaccination with NS1-80 or NS1-110 virus but not with deltaNS1 virus (Fig. 5C). Only low levels of IgG antibodies were detected in nasal wash samples.

Efficacy of protection of C57BL/6 PKR$^{-/-}$ mice by influenza B virus NS1 mutants against homologous virus challenge. C57BL/6 PKR$^{-/-}$ mice, which were immunized with different NS1 mutant viruses at 5×10^5 PFU, were challenged with parental rWT B/Yamagata/88 virus at 5×10^5 PFU. As shown in Fig. 6, all NS1 mutant virus-vaccinated mice survived the lethal challenge with rWT B/Yamagata/88 virus without showing any symptoms over the course of 14 days postinfection. In contrast, the PBS-immunized mice showed strong reductions in weight and all animals eventually succumbed to infection by day 11, indicating that vaccination with NS1 mutants did protect against a lethal homologous virus challenge.

Vaccination with NS1 mutant viruses elicits antibody responses in BALB/c mice. We next asked the question of whether protection was a mouse strain-specific phenomenon and whether vaccination with the NS1 mutants would give cross protection against heterologous virus challenge. BALB/c mice were immunized with different NS1 mutant viruses and rWT B/Yamagata/88 virus at 10^5 PFU. Three weeks postvaccination, mice were challenged with the parental rWT B/Yamagata/88 virus strain to test the homologous protection efficacy.

Similar to those in the former vaccination study using C57BL/6 PKR$^{-/-}$ mice, the serum, lung wash, and nasal wash samples from the BALB/c mice were collected 21 days postimmunization. They were analyzed for reactivity against purified influenza B/Yamagata/88 or B/Lee/40 virus by ELISA. For the antibodies against the parental influenza B/Yamagata/88 virus in serum samples, the results correlated with those observed before for C57BL/6 PKR$^{-/-}$ mice (Fig. 7A). Vaccination elicited significant IgG responses in sera but no IgA responses (Fig. 7A). In the lung wash samples, all the virus vaccinations elicited both IgG and IgA responses against B/Yamagata/88 virus (Fig. 7B). We also noted that the antibody titers induced by different virus vaccinations correlated with the abilities for viral replication. rWT B/Yamagata/88 virus elicited the highest antibody titer, followed by NS1-110 virus. NS1-80 and delta NS1 viruses induced the lowest-level responses. In the nasal wash samples from all vaccination groups, significant IgA antibody titers were detected (Fig. 7C). IgG antibody titers were detected only in rWT B/Yamagata/88 virus-vaccinated animals. Comparing these data with those obtained for C57BL/6 PKR$^{-/-}$ mice suggested that the antibody responses are not mouse strain specific.

As shown in Fig. 7, we detected very low levels of IgG antibodies against heterologous B/Lee/40 virus in serum samples. In lung wash samples, there were no detectable IgG and IgA responses against B/Lee/40 virus. But in nasal wash samples from B/Yamagata/88, NS1-110, and NS1-80 virus-infected animals, we detected IgA antibody titers which were signifi-

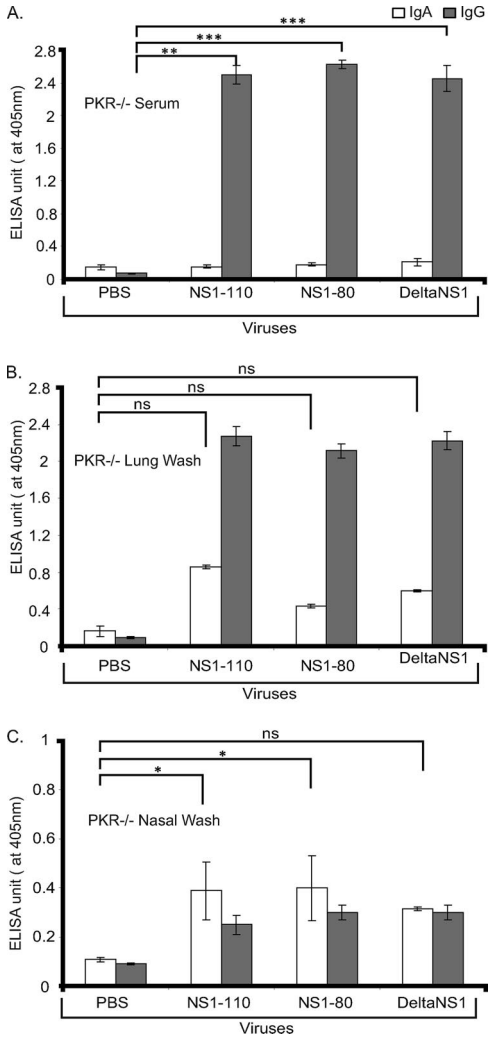

FIG. 5. Virus-specific IgG and IgA antibody responses. Eight-week-old female C57BL/6 PKR$^{-/-}$ mice, three animals per group, were immunized with the indicated viruses or PBS as a negative control, and serum samples (A) and lung (B) and nasal (C) wash samples were collected 21 days postinfection. B/Yamagata/88 virus-specific antibodies were detected by ELISA as described in Material and Methods. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ns, not significant versus PBS control.)

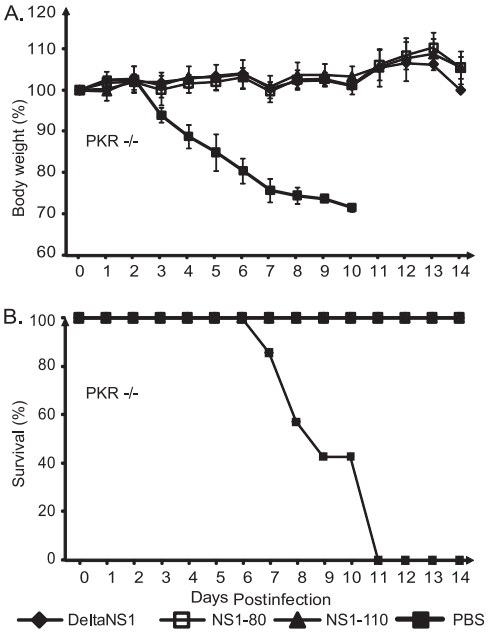

FIG. 6. Vaccination with B/Yamagata/88 virus NS1 mutants protects C57BL/6 PKR$^{-/-}$ mice against lethal challenge with rWT B/Yamagata/88 virus. Eight-week-old female C57BL/6 PKR$^{-/-}$ mice, four animals per group, were vaccinated with 5×10^5 PFU of the indicated B/Yamagata/88 virus NS1 mutants or PBS as a negative control. Three weeks postvaccination, animals were challenged with 5×10^5 PFU of the rWT B/Yamagata/88 virus. Animals were monitored for body weight changes and survival for a total of 14 days postchallenge.

cantly higher than those in samples from deltaNS1 virus-vaccinated animals.

Protective efficacy of the NS1 mutant viruses in BALB/c mice. We next checked virus replication in the lungs of BALB/c mice since there were no severe symptoms in WT mice following infection with rWT B/Yamagata/88 virus. Viral titers in the lungs were used as the index of efficacy against homologous challenge in BALB/c mice. The first set of BALB/c mice was challenged with 10^6 PFU of rWT B/Yamagata/88 viruses 3 weeks postvaccination. The lungs were collected on days 2 and 4 postinfection. The virus titers in lungs were measured by plaque assay. Figure 8 shows that on day 2, the group vaccinated with deltaNS1 demonstrated a detectable virus titer. This titer was around 1,000 times lower than that in the PBS mock vaccination group. On day 4, none of the samples from vaccinated mice contained detectable virus. As expected, virus was detected in lung samples from the PBS control group on both day 2 and day 4 postinfection. This finding indicated that NS1 mutant virus vaccination did confer homologous protection in BALB/c mice. NS1-110 and NS1-80 viruses gave better protection than the deltaNS1 virus since no challenge virus was detected in the lungs of NS1-110 or NS1-80 virus-vaccinated mice at either time point.

For the cross protection study of the NS1 mutant virus-vaccinated animals, we also checked the lung virus titers. Since the B/Lee/40 virus is a mouse-adapted strain, it allowed an evaluation of the efficacy of the vaccine strains. A second set of vaccinated BALB/c mice was challenged with 5×10^5 PFU of B/Lee/40 virus. Viral titers in the lungs of the mice on days 2 and 4 postinfection were measured by plaque assay. As shown in Fig. 8B, on day 2 virus was detected in all NS1 mutant virus-vaccinated groups. The deltaNS1 group showed the highest virus titer, followed by the animals vaccinated with NS1-80 and NS1-110 viruses. It should be noted that even in the delta NS1-vaccinated group, the virus titer was 10 times lower than that in the PBS mock vaccination group. On day 4, no virus was detected in any of the vaccination groups. We also vaccinated one group of mice with 10^4 PFU of B/Lee/40 virus as a positive control. As expected, at both time points virus was not detected in this immunization group. We also examined whether vaccination would protect the animals from severe symptoms following the heterologous challenge with B/Lee/40 virus. Unlike the PBS control animals, all deltaNS1, NS1-80, and NS1-110 virus-vaccinated mice survived the challenge with B/Lee/40

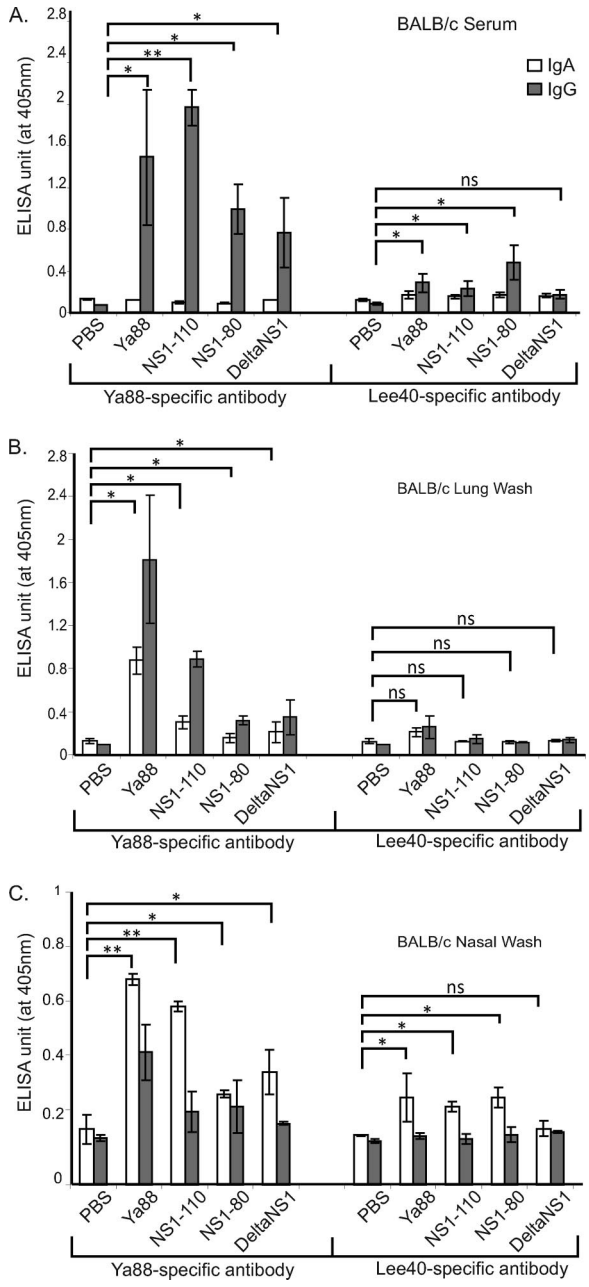

FIG. 7. Virus-specific serum and mucosal antibody responses after intranasal infection of BALB/c mice. Eight-week-old BALB/c mice, three animals per group, were immunized with influenza B/Yamagata/88 WT and mutant viruses, and sera (A) and lung (B) and nasal (C) wash samples were collected 21 days postinoculation. B/Yamagata/88 or B/Lee/40 virus-specific IgA and IgG antibodies were detected by ELISA. (*, $P < 0.05$; **, $P < 0.01$; and ns, not significant versus PBS control.)

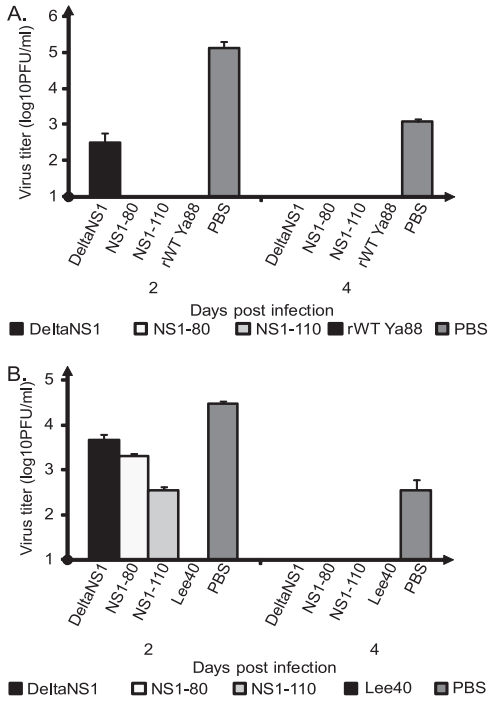

FIG. 8. Replication of WT B/Yamagata/88 and B/Lee/40 viruses in vaccinated BALB/c mice. Eight-week-old female BALB/c mice, six animals per group, were immunized with 10^5 PFU of the indicated rWT and NS1 mutant viruses or 10^4 PFU of influenza B/Lee/40 virus. Three weeks postvaccination, animals were challenged with 10^6 PFU of rWT B/Yamagata/88 virus (A) or 10^5 PFU of influenza B/Lee/40 virus (B). On days 2 and 4 postchallenge, three mice were euthanized and virus titers in the lungs were determined by plaque assay with MDCK cells.

virus (Fig. 9B). The NS1-80 and NS1-110 groups did not show any weight loss over the 14 days postchallenge (Fig. 9A). For the deltaNS1 vaccination group, there was up to 10% weight loss until day 6 postinfection, followed by a gradual recovery beginning on day 7 (Fig. 9A). In contrast, for the PBS control animals, there was a strong reduction in weight until day 8 postinfection, with half of the animals succumbing to infection by day 8 (Fig. 9).

DISCUSSION

The influenza B virus NS1 protein plays an important role as a virulence factor (7, 27). Previous studies have shown that it blocks the activation of the IFN-inducible PKR in vitro (35), the conjugation of ISG15 to its target proteins both in vitro and in virus-infected cells (39), and the induction of type I IFN (4). By the same token, modifications of and mutations in the NS1 protein are associated with the attenuation of viral growth both in vitro and in vivo (4, 34). In the present study, we rescued deltaNS1, NS1-80, and NS1-110 recombinant influenza B/Yamagata/88 virus NS1 mutants by reverse genetics and investigated their potentials as live-attenuated influenza B virus vaccines. All of the mutant viruses replicated in 7-day-old embryonated chicken eggs. The mutants were highly attenuated in IFN-competent MDCK cells compared to rWT virus, and the attenuation correlated with the length of the truncation of the NS1 protein. The deltaNS1 virus displayed the most attenuation in growth, followed by the NS1-80 and NS1-110 viruses. This correlation between NS1 length and attenuation was also observed in vivo.

In the mouse model, we evaluated the homologous and heterologous protection efficacies of different NS1 mutant vi-

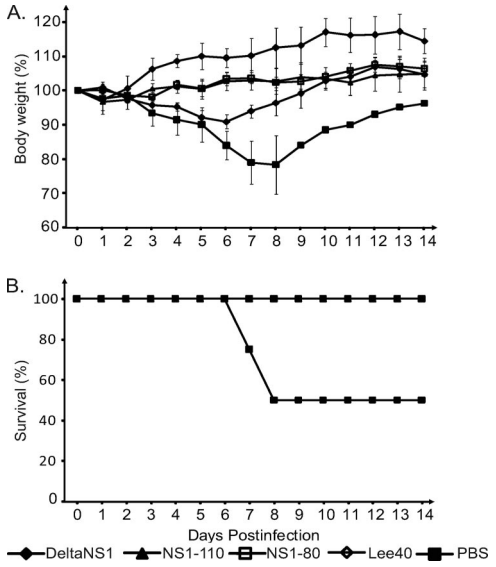

FIG. 9. Vaccination with B/Yamagata/88 virus NS1 mutants protects against lethal viral challenge with WT B/Lee/40 virus. Eight-week-old female BALB/c mice, four animals per group, were vaccinated with 10^5 PFU of the different B/Yamagata/88 virus NS1 mutants, B/Lee/40 virus, and PBS as the negative control. Three weeks postvaccination, mice were challenged with 5×10^5 PFU of influenza B/Lee/40 virus. Mice were monitored for body weight changes and survival for a total of 2 weeks after challenge.

ruses following immunizations. The data indicate that vaccination with NS1 mutant viruses confers protection against challenge with both the parental strain rWT B/Yamagata/88 virus and the heterologous strain B/Lee/40 virus.

The lack of a good small-animal model is one of the obstacles limiting influenza B virus vaccine studies. Only mouse-adapted influenza B viruses result in severe symptoms, such as weight loss and death, in infected mice. Most of the current influenza B viruses are not mouse adapted. To make a strain mouse adapted, the strain has to be either passaged in mice (3) or directly altered in its M1 gene (17). The process is not only cumbersome but also changes the viral genetic background so that the virus is no longer authentic and has become instead a laboratory strain. Here, we evaluated the potential of using C57BL/6 $PKR^{-/-}$ mice as an animal model for studying non-mouse-adapted strains of influenza B viruses, such as B/Yamagata/88. We found that infection with the non-mouse-adapted rWT B/Yamagata/88 virus strain induced severe symptoms in C57BL/6 $PKR^{-/-}$ mice but not in WT mice. The C57BL/6 $PKR^{-/-}$ mice lost weight and succumbed to infection. In contrast, infection with any of the NS1 mutant viruses did not result in severe symptoms and was associated with protection against subsequent viral challenge. The present data indicate that NS1 mutant viruses are much less virulent than WT virus and that they possess characteristics desirable for effective vaccine candidates. Our results also suggest that C57BL/6 $PKR^{-/-}$ mice can be utilized as a suitable animal model for the study of influenza B virus vaccines.

One of the features of live-attenuated influenza virus vaccines is better cross protection against nonhomologous viruses than that provided by inactivated influenza virus vaccines. Live-attenuated virus vaccination enables the host to be more readily protected against heterologous challenge with an antigenic drift strain. Here, we report that NS1 mutant virus vaccinations of mice resulted in both homologous protection against challenge with the parental rWT B/Yamagata/88 virus and heterologous protection against challenge with the B/Lee/40 virus variant. As shown in Fig. 5 and 7, vaccination induced high levels of IgG antibodies against the parental B/Yamagata/88 virus in sera, and these homotypic antibodies were most likely associated with protection. However, the NS1 mutant viruses did not elicit high levels of B/Lee/40 virus-specific IgG and IgA antibodies in serum and lung wash samples (Fig. 7). Nasal wash samples from the NS1-110- or NS1-80-immunized group, but not those from the deltaNS1 vaccination group, had higher levels of IgA antibody against B/Lee/40 virus than the serum and lung wash samples.

Although only low levels of cross-reactive antibodies were detected, the results showed measurable protection against challenge with B/Lee/40 virus. Data from an earlier heterologous protection study with influenza A viruses suggest that the basis of protective immunity may include mucosal polymeric IgA induction and cytotoxic T-lymphocyte responses specific to conserved viral internal proteins, such as NP (29, 31). We speculate that such mucosal IgA and cytotoxic T-lymphocyte immune responses may have been responsible for the protection against heterologous challenge in our influenza B virus system.

Although all our NS1 mutant viruses provided both homologous and heterologous protection (Fig. 8), there were differences among the mutants (i.e., we detected virus at day 2 postinfection with B/Yamagata/88 virus in the deltaNS1-vaccinated animals, as well as a slightly lower antibody response after deltaNS1 vaccination than after vaccination with the other mutants and slight weight loss after heterologous challenge in deltaNS1-immunized BALB/c mice). The protection and immunogenicity data along with the better growth characteristics make either the NS1-110 or the NS1-80 virus a better vaccine candidate than the deltaNS1 virus. Future work will address the question of whether the NS1-based vaccine approach is superior to the use of conventional killed influenza virus vaccines.

In summary, the present study extends our former report on live-attenuated influenza B virus vaccine candidates (34): (i) we have introduced reverse genetics to construct influenza B virus vaccine candidates with different attenuation characteristics; (ii) we have introduced the C57BL/6 $PKR^{-/-}$ mouse for the study of influenza B virus vaccines, allowing the study of current non-mouse-adapted influenza B virus strains; and (iii) we have investigated the cross protection potential of vaccination using NS1 mutant viruses. The data indicate that vaccination gave heterologous protection against challenge with the B/Lee/40 virus which was isolated more than 40 years earlier.

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