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Newcastle disease virus-vectored Nipah encephalitis vaccines induce B and T cell responses in mice and long-lasting neutralizing antibodies in pigs

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ABSTRACT

Nipah virus (NiV), a member of the Paramyxoviridae family, causes deadly encephalitis in humans and huge economic losses to the pig industry. Here, we generated recombinant avirulent Newcastle disease virus (NDV) LaSota strains expressing the NiV G and F proteins respectively (designated as rLa-NiVG and rLa-NiVF), and evaluated their immunogenicity in mice and pigs. Both rLa-NiVG and rLa-NiVF displayed growth properties similar to those of LaSota virus in chicken eggs. Co-infection of rLa-NiVG and rLa-NiVF caused marked syncytia formation, while intracerebral co-inoculation of these viruses in mice showed they were safe in at least one mammalian species. Animal immunization studies showed rLa-NiVG and rLa-NiVF induced NiV neutralizing antibody responses in mice and pigs, and F protein-specific CD8+ T cell responses in mice. Most importantly, rLa-NiVG and rLa-NiVF administered alone or together, induced a long-lasting neutralizing antibody response in pigs. Recombinant rLa-NiVG/F thus appear to be promising NiV vaccine candidates for pigs and potentially humans.

Introduction

NiV, a member of the genus *Henipavirus* genus in the family *Paramyxoviridae*, causes Nipah encephalitis, a deadly disease in humans. The first outbreak of NiV was reported in Malaysia and Singapore in 1998. NiV then emerged in Bangladesh and India between 2001 and 2012 (Anonymous, 2010, 2011, 2012; Hsu et al., 2004; Luby et al., 2006). The clinical case mortality rate ranges from 9% to 100% (Anonymous, 2012; Lo and Rota, 2008; Wacharapluesadee et al., 2010).

NiV has the potential to be used as a bio-terrorism agent and is listed as a BSL-4 pathogen due to its virulence in humans. Its wide host spectrum includes pigs, dogs, cats, ferrets, African green monkeys and horses; the fruit bat (flying fox) is its natural reservoir (Halpin et al., 2011). During the initial NiV outbreaks in Malaysia and Singapore, most human NiV cases were caused by close contact with pigs. In subsequent outbreaks during 2001 to 2012 in Bangladesh and India, drinking fresh date palm sap which is contaminated by fruit bat's droppings, urine and saliva and close contact with infected humans were found to be the major source of NiV infection. However, pigs are highly susceptible to NiV and may serve as an amplifying host of the virus. Further, the findings that most infected pigs do not show serious clinical signs but nonetheless could shed large amount of virus make them a dangerous infection source for humans. Of note, about 90% of total human infections to date could be attributed to close contact with infected pigs in the 1998 outbreak, suggesting that pig-to-human transmission may be more efficient for NiV infection. Several Nipah vaccine candidates have been reported to induce immune responses in immunized animals or to provide protection against NiV infection, such as recombinant canarypox-vectored vaccines (Weingartl et al., 2006), recombinant vaccinia-vectored vaccines (Guillaume et al., 2004), and soluble G protein as a subunit vaccine (McEachern et al., 2008; Mungall et al., 2006; Pallister et al., 2011).

Newcastle disease virus (NDV) is a member of the genus *Avulavirus* in the family *Paramyxoviridae*. NDV strains are classified as nonvirulent (lentogenic), moderately virulent (mesogenic) or highly virulent (velogenic) for poultry (Alexander, 1989). This virulence is determined mainly by the amino acid sequence of the protease cleavage site of the fusion (F) protein precursor (Peeters et al., 1999). Lentogenic strains contain fewer basic amino acids at this site and can only be cleaved by trypsin-like extracellular proteases that are largely confined to the respiratory tract, whereas highly virulent strains are cleaved by ubiquitous

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intracellular proteases, potentially resulting in systemic infection (Panda et al., 2004). Currently, lentogenic strains, such as the LaSota strain, are used as live vaccines against NDV in poultry (Alexander, 1997).

NDV has been actively developed and evaluated as a vaccine vector for the control of human and animal diseases (Bukreyev and Collins, 2008; Bukreyev et al., 2006; DiNapoli et al., 2010a, 2010b; Ge et al., 2007; Ge et al., 2010; Khattar et al., 2010; Kortekaas et al., 2010). It has potential as a vaccine vector for many reasons, including that it does not usually result in a productive infection in mammals and the pre-existing immunity and maternal antibody to mammalian paramyxoviruses does not interfere with the replication of NDV as NDV is antigenically distinct from the mammalian paramyxoviruses. Its safety in many animal models, including African green and rhesus monkeys, as well as humans, has been demonstrated (Bukreyev and Collins, 2008; Bukreyev et al., 2006; DiNapoli et al., 2010a, 2010b; Ge et al., 2007; Ge et al., 2010; Khattar et al., 2010; Kortekaas et al., 2010).

The glycoprotein (G) and fusion protein (F) are two major NiV surface glycoproteins that stimulate protective immune responses (Guillaume et al., 2004; Weingartl et al., 2006). Here, we have used an established reverse genetics system to generate two recombinant NDV LaSota viruses that express either NiV G or F protein and we have evaluated the immunogenicity of these two recombinant viruses in mice and pigs.

Materials and methods

Viruses, cells and purified G and F proteins

BHK-21 and 293 T cells used for virus rescue and titration were maintained in complete Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Recombinant NDV strains were grown and titrated in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs by inoculation of the allantoic cavity. The Vesicular Stomatitis Virus (VSV) pseudotyped assay system (Takada et al., 1997) was kindly provided by Dr. Michael Whitt (University of Tennessee Health Science Center). The pseudotyped VSVΔG*GFP virus particle, which packaged the Nipah virus G and F proteins on the viral envelope (designated as VSVΔG*GFP-NiVG/F), were prepared and used as a substitute for live NiV in the serum neutralization test. The titer of VSVΔG*GFP-NiVG/F pseudotyped virus was determined by infection of BHK21 cells and counting the number of GFP expressing cells under a fluorescence microscope as previously described (Wang et al., 2006a), and calculated by using the method of Reed and Muench (Reed and Muench, 1938). Stocks of prepared pseudotyped virus were stored at −70 °C. Recombinant baculovirus expressing G-his or F-his fusion protein (G or F was fused with a histag segment HHHHHH at its C-terminus) was generated and used to infect Sf9 insect cells. At 96 h post-infection, cells were harvested and lysed, and the G-his or F-his protein released into the lysing buffer was purified using Ni-NTA agarose beads (QIAGEN, Valencia, CA). Purified proteins were characterized by Coomassie blue staining and Western blot.

Plasmid construction and virus rescue

To construct the full-length recombinant genomic cDNA, the cDNA of the NiV G and F genes were amplified from synthesized G and F cDNA (Invitrogen, Shanghai, China) by using the following primers: to amplify the G gene, 5'-GACT**GTTTAAAC**ttagaaaaaa-TacgggtagaaC*gccacc*ATGCCGGCAGAAAACAAGAAAGTTAG-3' and 5'–CTGA**GTTTAAAC**GTTATGTACATTGCTCTGGTATC-3'; and to amplify the F gene, 5'-GACT**GTTTAAAC**ttagaaaaaaTacgggtagaaC*gccacc*ATGGTAGTTATACTTGACAAGAGATG-3' and 5'- CTGA**GTTTAAAC**GCTATGTCCCAATGTAGTAGAG-3'. Both the G and F genes were flanked by the restriction endonuclease *Pme* I (boldface letters); In order to form a NDV L protein recognizable transcription unit, the NDV gene start sequence (GS, 5'-acgggtagaa-3') and gene end sequence (GE, 5'-ttagaaaaaa-3') were presented before the optimal Kozak sequence (italic lowercase letters) and the NiV G or F gene sequences (underlined uppercase letters). The amplified G and F genes were digested by *Pme* I and then inserted into the P-M intergenic region at nucleotide position 3165 of the NDV genome, as described previously (Ge et al., 2007). The resultant plasmids were designated as prLa-NiVG and prLa-NiVF and were used for virus rescue by means of reverse genetics as described previously (Ge et al., 2007). The presence of recombinant NDV expressing NiV G and NiV F was confirmed using IFA and RT-PCR. The resultant recombinant viruses were designated as rLa-NiVG and rLa-NiVF.

Immunofluorescence

NDV infection of cultured cells was detected by means of an IFA with chicken serum against NDV as previously described (Ge et al., 2007). For confocal assays, BHK-21 cells were grown in 24-well plates or plated on cover slips in 35-mm diameter dishes and infected with NDV rLa, rLa-NiVG, or rLa-NiVF. At 24 h post-infection, cells were fixed in pre-chilled 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, washed with PBS three times then blocked with PBS containing 1% (wt/vol) bovine serum albumin (BSA) at room temperature for 1 h. Cells were then incubated with mouse monoclonal antibodies (G3E9 for G protein, F2G1 for F protein, prepared in our lab) or chicken serum against NDV for 1 h at room temperature. Cells were then washed three times with PBS containing 0.05% Tween 20 and stained with an FITC-conjugated goat anti-mouse antibody (Sigma) or a TRITC-conjugated rabbit anti-chicken antibody (Sigma) for 30 min. Cells were washed three times with PBS then their nuclei were stained with DAPI. Cells were analyzed with a fluorescence microscope or confocal laser microscope. Images were acquired with a Zeiss (Thornwood, N.Y.) Axioscop microscope equipped for epifluorescence with a Sensys charge-coupled device camera (Photometrics, Tucson, Ariz.) by using IPLab software (Scanalytics, Vienna, Va.).

Assessment of virus pathogenicity

To determine the pathogenicity of rLa-NiVG and rLa-NiVF in poultry, the mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) were determined in embryonated SPF chicken eggs or in SPF chickens according to the OIE Manual(OIE, 2011). To assess the pathogenicity of the recombinant viruses in mice, two groups of 10 six-week-old female Balb/c mice (Vital River, Beijing, China) were intramuscularly (*i.m.*) injected with 0.1 ml of diluted allantoic fluid containing 10^8 EID_{50} (50 percent Embryo Infectious Dose) rLa-NiVG/F (a mix of rLa-NiVG and rLa-NiVF) or rLa and intranasally (*i.n.*) inoculated with 0.03 ml of diluted allantoic fluid containing 3×10^7 EID_{50} rLa-NiVG/F or rLa. The third group of 10 mice were *i.m.* injected with 0.1 ml and *i.n.* inoculated with 0.03 ml PBS as mock infection control. Mice were monitored daily for weight changes. To test whether co-infection of rLa-NiVG and rLa-NiVF was safe, 4 groups of 10 three-week-old female Balb/c mice were intracerebrally (*i.c.*) inoculated with 0.03 ml of diluted allantoic fluid containing 3×10^7 EID_{50} rLa, rLa-NiVG, rLa-NiVF or rLa-NiVG/F. Another group of 10 mice were *i.c.* inoculated with 0.03 ml of PBS as mock infection control. Mice

were again observed daily for 3 weeks for signs of illness, weight loss, or death.

Immunization of mice and pigs

For mouse immunization, 6-week-old female Balb/c mice (Vital River, Beijing, China) were *i.m.* vaccinated with each recombinant virus at a dose of 10^8 EID_{50} in 100 μl of diluted allantoic fluid. Four weeks after the first dose, the mice were boosted with the same vaccine at the same dose and via the same route. To assess F protein-specific CD8+ T cell responses, we prepared splenocytes from rLa-NiVF-immunized mice 10 day after the first or second vaccine dose. For the serological assay, two weeks after each dose, mouse blood samples were collected from the retro-orbital sinuses under isoflurane inhalation anesthesia.

For pig immunization, five groups (five each) of four-week-old crossbred Landrace pigs were obtained and housed at the Experimental Animal Center of Harbin Veterinary Research Institute. Three groups were *i.m.* inoculated with rLa-NiVG, rLa-NiVF, or a mix of rLa-NiVG and rLa-NiVF (rLa-NiVG/F) at a dose of 2×10^9 EID_{50} in 2 ml of diluted allantoic fluid. The fourth group was inoculated *i.m.* with rLa at a dose of 2×10^9 EID_{50} in 2 ml of diluted allantoic fluid, and the fifth group was *i.m.* immunized with 2 ml of PBS. Four weeks after the first dose, the pigs were boosted with the same vaccine at the same dose and via the same route. Serum was collected before vaccination and at week 2, 3, 4, 5, 7, 9, 13, 21, and 29 after prime.

ELISA

NiV G and F protein-specific antibodies were measured in individual mouse or pig sera by means of an enzyme-linked immunoabsorbent assay (ELISA). For mouse serum, 96-well ELISA plates were coated overnight at 4 °C with purified His-tagged G or F protein at a concentration of 4 μg/ml. The plates were then washed and blocked with 2% BSA-PBST (PBS containing 0.05% Tween-20 (v/v) and 2% bovine serum albumin (BSA, wt/v)) at room temperature for 1 h. Serially diluted serum was added to the ELISA plate, and incubated at room temperature for 1 h. Plates were washed three times with PBST, then a 1:4000 dilution of HRP-labeled goat anti mouse IgG (Southern Biotech, Birmingham, AL) was added and incubated for another 1 h at room temperature. The plates were washed thoroughly five times with PBST, and any remaining fluid was decanted completely from each plate. For visualization, 50 μl of 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate (Sigma) was added to each well for 5 min at room temperature; 50 μl of 0.2M hydrochloric acid was added to stop the reaction. O.D values were determined with a Model 680 microplate reader (Biorad) at 450 nm. A standard curve was generated by coating each ELISA plate with serial 2-fold dilutions of mouse IgG (Southern Biotech, Birmingham, AL) at known concentrations. A linear equation was obtained based on the 2-fold decreased standard IgG concentration and their O.D values, thus the concentration of NiV G and F protein-specific antibodies in each sample could be calculated according to the linear equation by their O.D values and expressed as the amount of antigen-specific IgG per ml of serum (ng/ml). The protocol of ELISA for pig serum is basically the same as the ELISA for mouse serum. The differences were all pig sera were used at 1:1000 fixed dilution, and due to the lack of purified pig IgG to build standard curves, the pig sera ELISA results were expressed as the relative O.D values to the negative controls.

Neutralization assay

NiV specific neutralizing antibody levels were determined using pseudotyped VSV in place of live NiV as described by Wang et al. (Wang et al., 2006a). Pseudotyped VSV (VSVΔG*GFP) has the VSV envelope protein G gene replaced by the green fluorescent protein (GFP) gene and complemented with the VSV G protein expressed *in trans*. VSVΔG*GFP-NiVG/F pseudotyped virus was prepared by co-transfecting 293T cells with pCAGG-G and pCAGG-F and then infecting those cells with VSVΔG*GFP. The recombinant pseudotyped virus VSVΔG*GFP-NiVG/F has GFP and either the NiV G or F protein incorporated into its VSV envelope. VSVΔG*GFP-NiVG/F can mimic the infection of real NiV, entering NiV permissive cells by F- and G-mediated fusion. The titer of VSVΔG*GFP-NiVG/F was determined in BHK-21 cells by counting the fluorescent cells in an infectivity assay, and calculated by using the method of Reed and Muench. The titer is typically around 3×10^6 $TCID_{50}$/ml. VSVΔG*GFP-NiVG/F was aliquoted and stored at −80 °C. To perform the neutralization assay, 25 μl of 2-fold serially diluted serum (heat inactivated at 56 °C for 30 min before use, initial dilution was 1:4) was mixed with 25 μl of DMEM containing 5×10^2 $TCID_{50}$ VSVΔG*GFP-NiVG/F and incubated at 37 °C for 1 h. After the incubation, 50 μl of the mixture was added to the BHK-21 cells in triplicate wells of a 96-well plate. The GFP-expressing cells were counted at 16 h post-infection under a fluorescence microscope. Neutralization titers were expressed as the reciprocal of the highest dilution of serum that

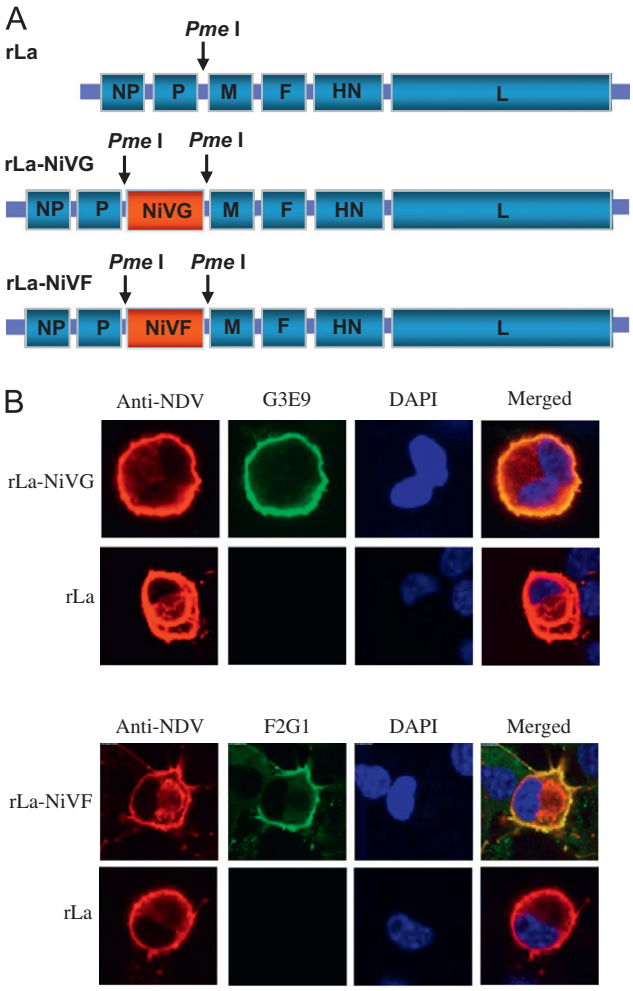

Fig. 1. Generation of recombinant NDV expressing the NiV G and F genes. (A) Schematic representation showing the rLa genome with the restriction endonuclease *Pme I* site introduced between the P and M genes and the NiV G and F genes inserted into the *Pme* I site. (B) Indirect immunofluorescence staining of rLa-NiVG- and rLa-NiVF-infected BHK-21 cells with chicken anti-NDV serum and mouse monoclonal antibodies to the NiV G (G3E9) or NiV F (F2G1) protein and observed with a confocal laser microscope.

showed at least a 50% reduction in the number of fluorescent cells as compared with the negative control.

To assess the level of the neutralizing antibodies against the NDV vector virus, a recombinant NDV LaSota virus expressing the enhanced green fluorescent protein (designated as rLa-EGFP) was constructed and titrated before use. Briefly, 25 μl of 2-fold serially diluted serum (initial dilution=1:50) was mixed with 25 μl of medium containing 2×10^3 EID_{50} of rLa-EGFP at 37 °C for 1 h. After the incubation, 50 μl of the mixture was added to the BHK-21 cell monolayer in triplicate wells of a 96-well plate. The GFP-expressing cells were counted at 16 h post-infection under a fluorescence microscope. Neutralization titers were expressed as the reciprocal of the highest dilution of serum that showed at least a 50% reduction in the number of fluorescent cells as compared with the negative control.

Flow cytometric analysis of the mouse CD8+ T cell response

The NiV F protein-specific CD8+ T cell response in Balb/c mice was determined by using flow cytometry with established protocols as previously described (Wen et al., 2009). rLa-NiVF-immunized mice were sacrificed on day 10 after the first and second immunizations. Mouse splenocytes were prepared as reported by Ye et al. (Ye et al., 2004). Briefly, spleens were removed from the euthanized mice, cut into small pieces, and then homogenized by gently rubbing. After low-speed centrifugation, the supernatant was removed and the cells were gently resuspended in red blood cell lysing buffer (Sigma) and incubated on ice for one min. Splenocytes were stimulated with 20 μg/ml of NiV F-specific CD8 peptide (VYFPILTEI, $H2K^d$, unpublished) or an unrelated peptide (AMQMLKETI, which corresponds to a epitope in the HIV Gag protein) for 6 h in presence of 10 ng/ml of Brefeldin A (eBioscience, San Diego, CA) to assess the CD8+ T cell response. A positive control was prepared by stimulating the splenocytes with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma) and 500 ng/ml of ionomycin (Sigma). After stimulation, the cells were washed twice with PBS containing 3% fetal calf serum and then stained with Peridinin-Chlorophyll-Protein-Complex (PerCP)-conjugated rat anti-mouse CD8 and phycoerythrin (PE)-conjugated rat anti-mouse CD3 antibodies (BD Pharmingen, San Diego, CA). Cells were then fixed and permeabilized with Fix&Perm buffers (eBioscience, San Diego, CA) and stained for intracellular interferon-gamma (IFN-γ) with an allophycocyanin (APC)-conjugated rat anti-mouse IFN-γ antibody (BD Pharmingen, San Diego, CA). The levels of CD8+ T cell responses were determined by using flow cytometry on a BD FACSAria Station (BD Immunocytometry Systems, San Jose, CA). Data were analyzed with FlowJo software (Treestar Inc, Ashland, OR).

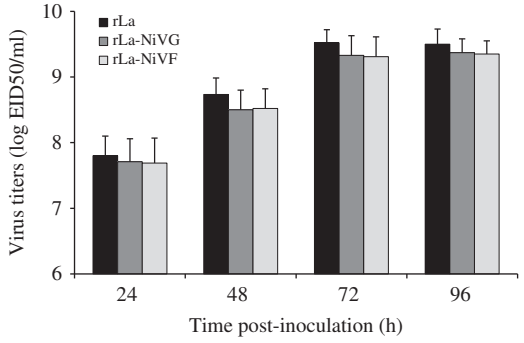

Fig. 2. Growth properties of recombinant viruses in embryonated eggs. rLa, rLa-NiVG, and rLa-NiVF were inoculated into the allantoic cavities of 10-day-old embryonated eggs at a dose of 100 EID_{50} in 0.1 ml, and the allantoic fluid of six eggs from each group was harvested at the time points indicated and pooled for EID_{50} determination in eggs. The data shown were acquired from three independent experiments and presented as the mean ± SD of 10 eggs for each group.

Statistical analysis

Data on virus titers, antibody titers and mouse T cell responses were analyzed by using the Student's t test with the Excel program (Microsoft, Redmond WA).

Results

Generation of recombinant NDV expressing the NiV G and F genes

Recombinant NDV expressing the NiV G and F genes were generated by inserting the NiVG or NiVF gene between the P and M genes in the genome cDNA of NDV (Fig. 1A). The presence of the inserted gene was confirmed by RT-PCR and the expression of

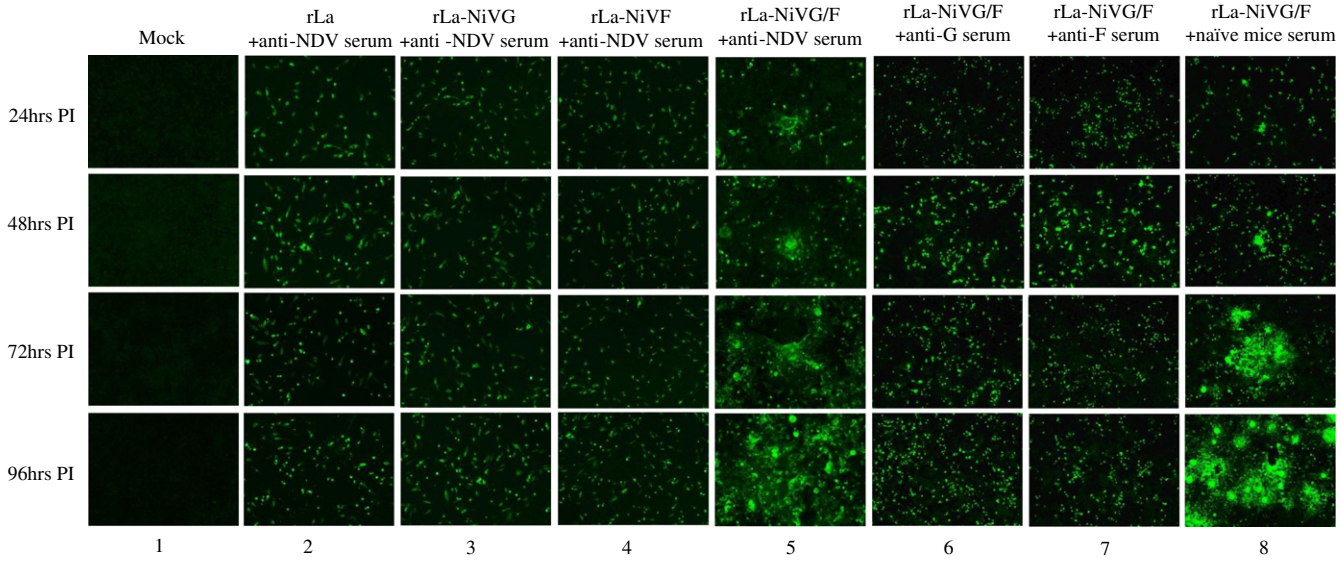

Fig. 3. Syncytia formation and inhibition assay. Confluent monolayers of BHK-21 cells were infected with rLa, rLa-NiVG, rLa-NiVF, or rLa-NiVG/F at a MOI of 0.05. Chicken anti-NDV serum, mouse anti-NiVG or mouse anti-NiVF serum was added to the supernatant 1 h post-infection at a dilution of 1:50. Cells were fixed at different time points and detected with chicken anti-NDV serum, then incubated with an FITC-conjugated rabbit anti-chicken antibody.

the G and F genes confirmed by indirect confocal immunofluorescence staining of rLa-NiVG- and rLa-NiVF-infected BHK-21 cells. rLa-NiVG- and rLa-NiVF-infected BHK-21 cells stained with the NiVG protein-specific monoclonal antibody G3E9 and the NiVF protein-specific monoclonal antibody F2G1, respectively, whereas rLa-infected BHK-21cells were not stained by either G3E9 or F2G1 (Fig. 1B). NDV antigens and NiV G or F protein also co-localized on the surface of the BHK-21 cells, thus confirmed the surface expression of NiV G and F protein (Fig. 1B).

The growth properties of rLa, rLa-NiVG, and rLa-NiVF were examined in embryonated chicken eggs. All three viruses grew to similar levels; rLa-NiVG and rLa-NiVF reached peak titers of 9.7 $\log EID_{50}$/ml and 9.65 $\log EID_{50}$/ml respectively at 72 h post-inoculation (Fig. 2). The genetic stability of the NiV G or F gene within rLa-NiVG or rLa-NiVF was assessed by serially passaging the virus in SPF chicken eggs 10 times. After 10 passages, both the presence and expression of the NiV G or F gene was confirmed by RT-PCR and immunofluorescence (data not shown).

As a low pathogenicity NDV strain, rLa infected individual cells but did not spread to adjacent BHK-21 cells in the absence of exogenous trypsin. Therefore, as shown in Fig. 3, the percentage of infected cells at 96 h post-infection was almost identical to that at 24 h post-infection. This property was not altered by the insertion and expression of the NiV G or F gene. Co-infection of rLa-NiVG and rLa-NiVF resulted in syncytia formation on BHK-21

A

Viruses	Pathogenicity in eggs and chickens		
	MDT	ICPI	IVPI
rLa	>120	0.4	0
rLa-NiVG	>120	0	0
rLa-NiVF	>120	0	0

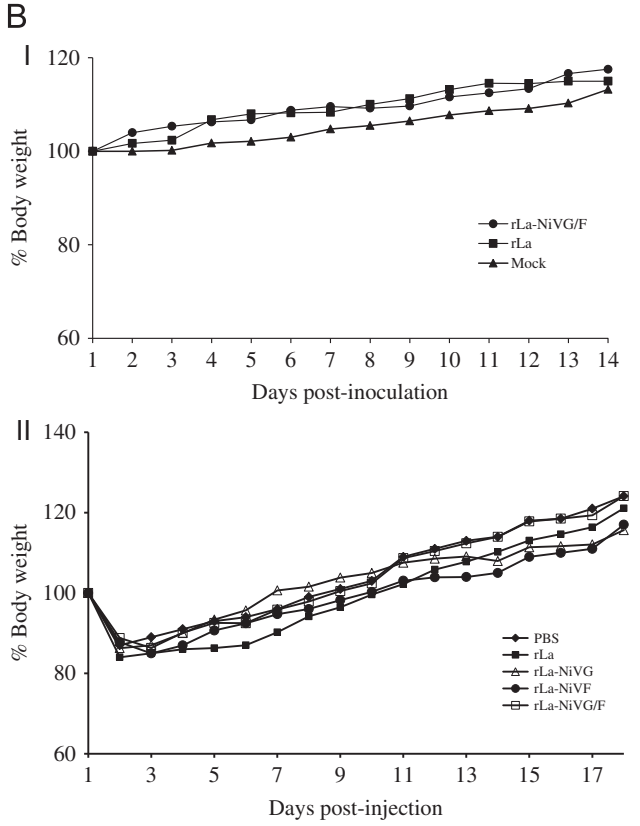

Fig. 4. Pathogenicity of the recombinant viruses in chickens and mice. (A) Pathogenicity assay in SPF eggs and chickens. The mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) were determined as described in the Materials and methods. (B) Weight change in mice inoculated with recombinant and vector viruses. Groups of 10 mice were inoculated (I) *i.m.* with 10^8 EID_{50} (in 100 μl) of rLa-NiVG/F, rLa and PBS and (II) *i.c.* with 3×10^7 EID_{50} (in 30 μl) of rLa, rLa-NiVG, rLa-NiVF or rLa-NiVG/F. Mice were observed and weighed daily for 14 (I) and 18 (II) days, respectively. All mice survived the duration of the experiment. Body weight changes for each group are shown as ratios of the body weight at day 0, which was set as 100.

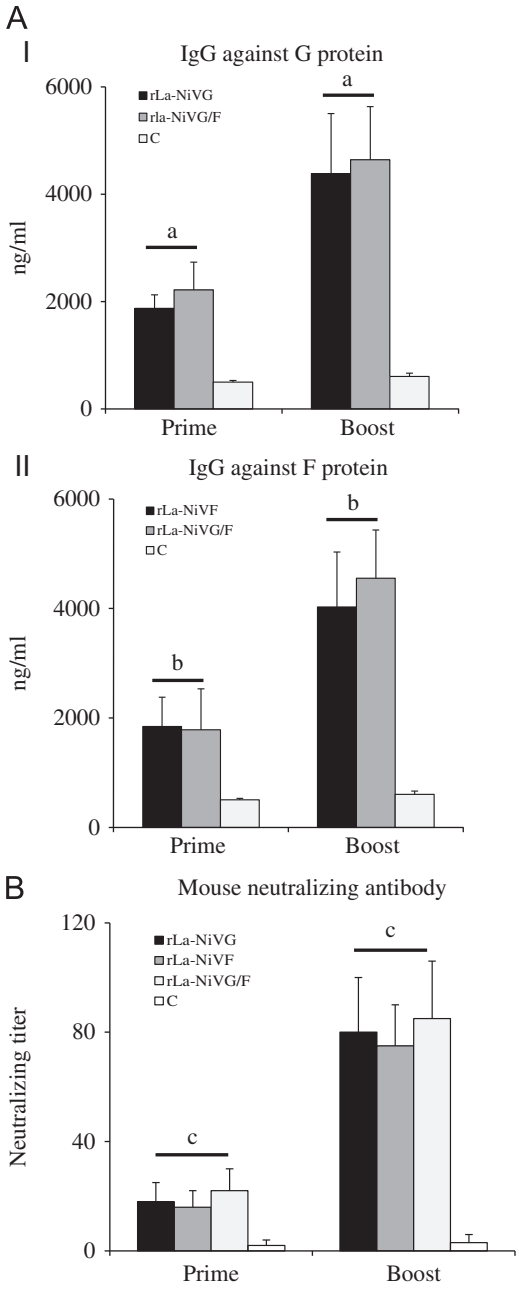

Fig. 5. Immunization of mice. Groups of ten mice each received two doses of 10^8 EID_{50} of rLa-NiVG, rLa-NiVF, or rLa-NiVG/F *i.m.* with a 4-week interval. Blood samples were collected two weeks after the first dose (prime) and the second dose (boost) for antibody assays. The ELISA antibody (A) against the NiV G protein (I) and the NiV F protein (II) and the neutralization antibody against NiV (B) for each group were determined as described in the Materials and methods. Data are presented as the mean $\pm$SD of 10 mice for each group. (a), (b), and (c): $P<0.05$, significance of the difference in antibody amount after the second dose compared with the corresponding value after the first dose of virus.

cells that increased in size with time (Fig. 3). The addition of 1:50 diluted chicken anti-NDV serum to the cell culture supernatant had no effect on syncytia formation or growth (Fig. 3). In contrast, anti-NiVG or -NiVF serum (1:50 dilution) from pCAGG-NiVG- or pCAGG-NiVF-immunized mice (Wang et al., 2006a) inhibited syncytia formation when added to the supernatant of rLa-NiVG/F pre-infected BHK-21 cells.

Expression of the NiV G and F proteins does not increase the virulence of the NDV vector in chickens and mice.

To investigate whether the expression of the NiV G or F genes alters the pathogenicity of the NDV vector, we compared the biological character and pathogenicity of rLa-NiVG and rLa-NiVF with their vector, rLa, in chickens and mice. The MDT, ICPI, and IVPI tests are internationally accepted methods for assessing the pathogenicity of NDV strains in poultry. NDV strains are categorized into three groups on the basis of their MDT (mean death time): velogenic (<60 h), mesogenic (60–90 h), and lentogenic (>90 h). The MDT values for rLa, rLa-NiVG, and rLa-NiVF were all greater than 120 h, indicating that these three viruses belong to the lentogenic NDV (Fig. 4A). The ICPI (intracerebral pathogenicity index) values for rLa, rLa-NiVG, and rLa-NiVF were 0.4, 0, and 0, respectively; and the IVPI (intravenous pathogenicity index) values for rLa, rLa-NiVG, and rLa-NiVF were 0, 0, and 0 respectively. After being inoculated with rLa, rLa-NiVG, or rLa-NiVF, all of the chickens remained healthy during the observation period. These results indicate that rLa-NiVF and rLa-NiVG are of low pathogenicity to poultry, suggesting that the insertion of the NiV G and F genes did not increase the virulence of the NDV vector.

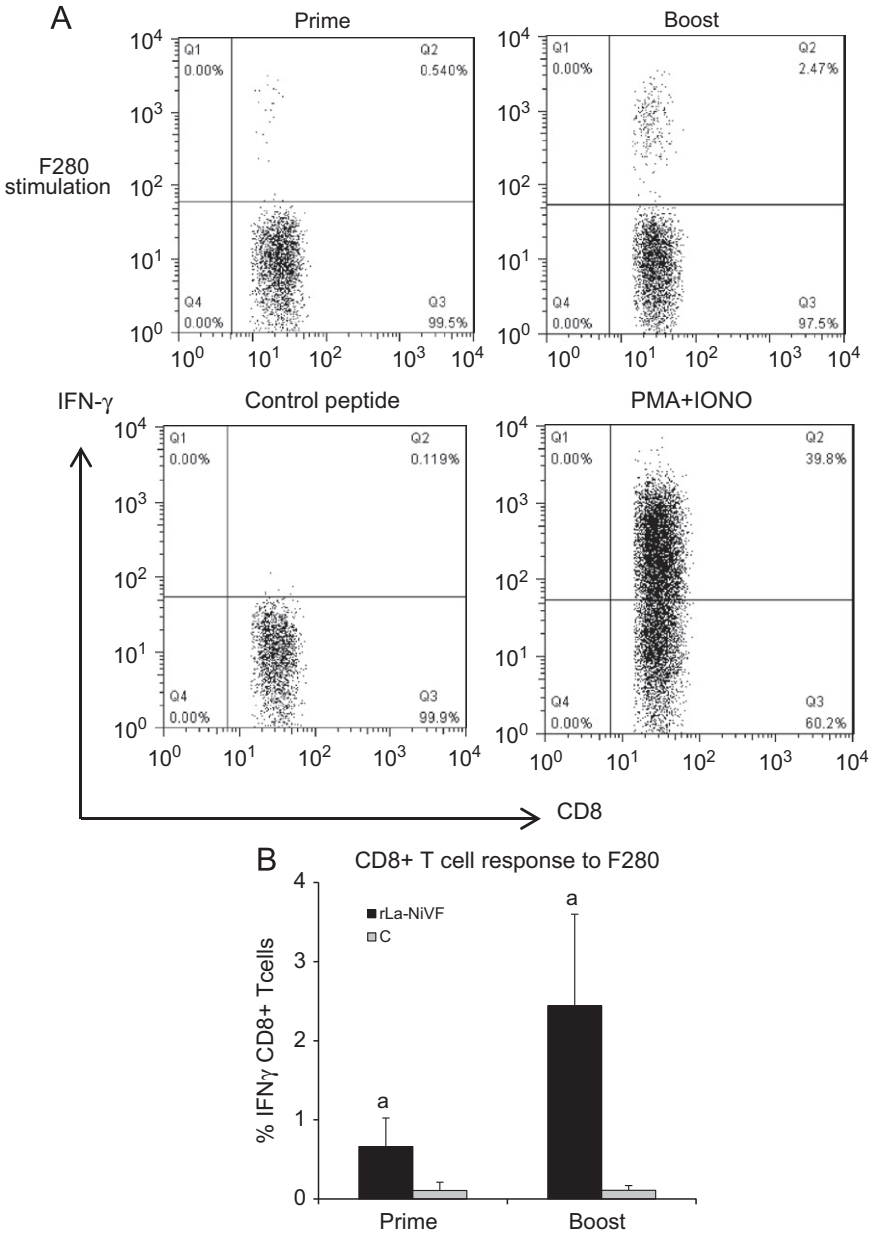

Fig. 6. CD8+ T cell responses in mice. Ten days after the first dose (prime) and the second dose (boost), five rLa-NiVF-immunized mice were sacrificed and their splenocytes prepared and stimulated with F280, control peptide, or PMA/ionomycin. The cells were then stained for cell surface CD8 as well as intracellular IFN-γ protein before undergoing flow cytometry analysis. F280, a peptide corresponding to a CD8+ T cell epitope in the NiV F protein; control peptide, a peptide corresponding to a CD8+ T cell epitope in the HIV Gag protein; PMA/ionomycin, T cell activation stimulators (positive control). The representative (A) and statistical (B) results of percentages of IFN-γ-producing CD8+ T cells stimulated by the different peptides are shown. Data are presented as the mean ± SD of five mice for each group. (a): $P<0.05$, the value after the second dose compared with the corresponding value after the first dose.

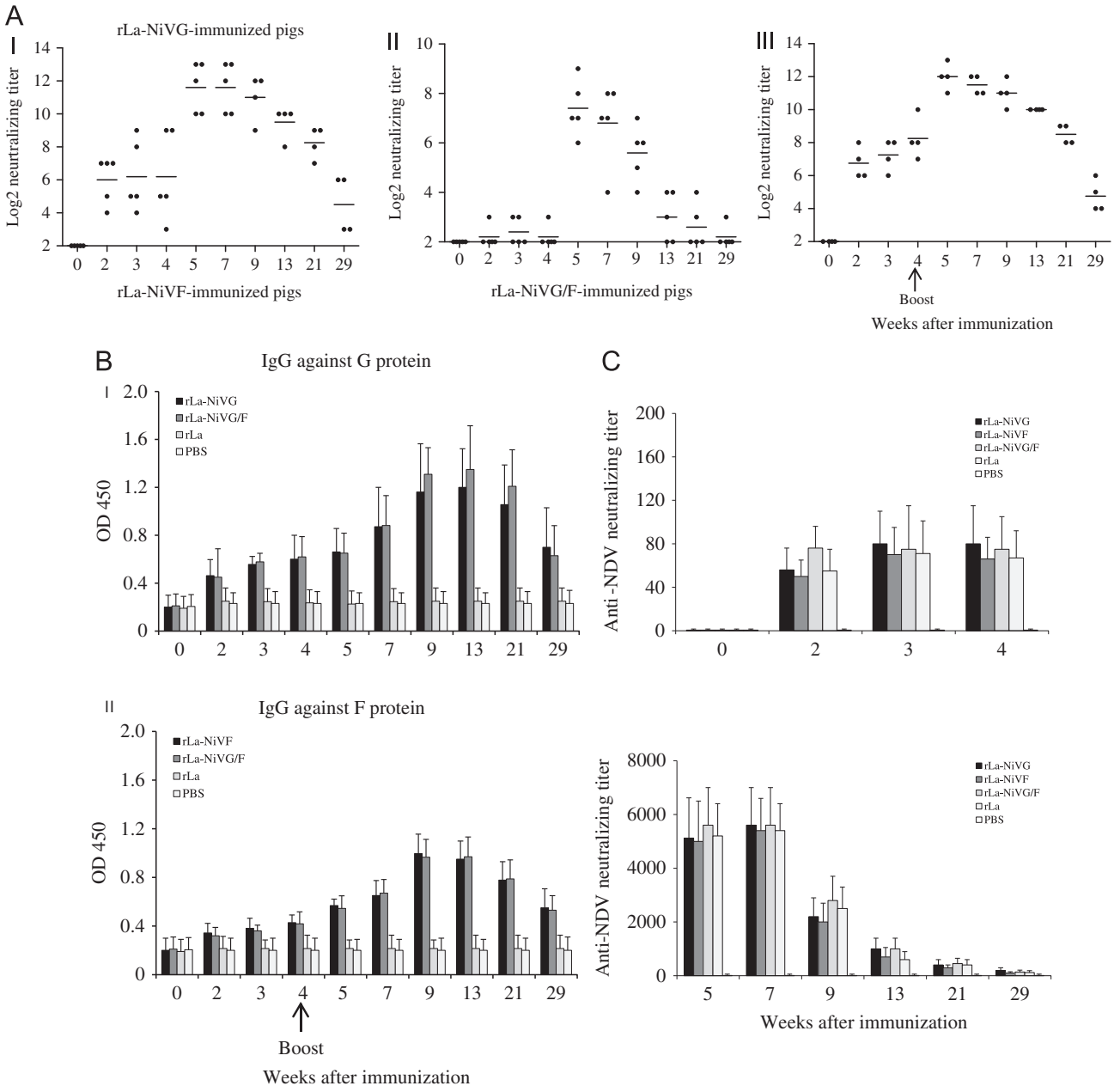

Fig. 7. Neutralizing antibodies and ELISA antibodies in pigs. Pigs were immunized with two doses of 2×10^9 EID_{50} of recombinant viruses with a 4-week interval. Blood samples were collected at the time points indicated for the neutralizing assay. The sera neutralization titers to NiV (A) and to NDV (C) in the different groups were analyzed as described in the Materials and Methods. Data for the neutralization titers to NiV were presented individually, and the data for the neutralization titers to NDV were presented as the mean $\pm$ SD of five pigs for each group. The ELISA antibodies (B) against the NiV G protein (I) and the NiV F (II) protein for each group were determined as described in the Materials and methods. Data are presented as the mean $\pm$ SD of five pigs for each group.

To investigate the safety of the recombinant viruses in mammals, we inoculated mice *i.m.* with 10^8 EID_{50} of rLa or a mix of rLa-NiVG and rLa-NiVF (rLa-NiVG/F) in 100 μl of diluted allantoic fluid, and at the same time inoculated the mice *i.n.* with 3×10^7 EID_{50} of rLa or rLa-NiVG/F in 30 μl of diluted allantoic fluid. The mice were observed daily for two weeks for signs of weight change or sickness. rLa- and rLa-NiVG/F-infected mice showed similar body weight changes to those of mock-infected mice and no signs of disease were observed (Fig. 4B I). Because NiV infections in animals can cause severe encephalitis, largely due to syncytia formation induced by the NiV G and F proteins, we further tested whether *i.c* inoculation of rLa-NiVG/F was safe for 3-week-old mice. After *i.c* inoculation with 3×10^7 EID_{50} of rLa, rLa-NiVG, rLa-NiVF, or rLa-NiVG/F, mice from each group endured a period of weight loss from day 2 to day 7, with maximum weight loss (less than 15%) observed at days 2 and 3 (Fig. 4B II). Mice started gaining weight from day 7 and almost recovered fully by day 8. Thereafter, all of the mice continued to gain weight normally. The weight change patterns of the mice inoculated with rLa-NiVG, rLa-NiVF, and rLa-NiVG/F were almost identical to those of rLa- and PBS-inoculated mice. This result demonstrates the safety of the recombinant viruses in at least one mammalian species.

The recombinant viruses rLa-NiVG and rLa-NiVF induces Nipah-specific humoral and CD8+ T cell responses in mice

To evaluate the potential of rLa-NiVG and rLa-NiVF as NiV vaccines, four groups of mice (10 mice each) were immunized *i.m.* with 10^8 EID_{50} of rLa-NiVG, rLa-NiVF, a mix of rLa-NiVG and rLa-

NiVF (rLa-NiVG/F), or with rLa in 100 μl of diluted allantoic fluid. Four weeks after the first dose, the mice were boosted with the same dose via the same route. Serum samples were collected 2 weeks after the first dose and 2 weeks after the second dose for ELISA and neutralizing assays. NiV G- (Fig. 5A I) and F- (Fig. 5A II) specific IgG from each group was detected by ELISA. The IgG antibody levels were significantly boosted after the second dose ($P<0.05$). The serum neutralizing antibodies were then analyzed using a NiV G/F pseudovirus neutralization assay. As shown in Fig. 5B, rLa-NiVG, rLa-NiVF, and rLa-NiVG/F induced similar levels of NiV neutralizing antibodies after the first dose, although the levels were not high; whereas after the second dose the neutralizing antibodies were boosted significantly ($P<0.05$).

To investigate whether the recombinant viruses could elicit T cell responses (especially CD8+ T cell responses), a NiV F protein mouse CD8 epitope-F280 (VYFPILTEI, $H2K^d$, unpublished) was identified by using bioinformatics prediction and flow cytometry verification. Using the same approach, however, we were unable to find any CD8 epitopes in the G protein from fourteen synthesized peptides. Therefore we could only evaluate the rLa-NiVF-induced CD8+ T cell responses. As shown in Figs. 6A and B, rLa-NiVF immunization induced significant F280-specific IFN-γ-producing CD8+ T cell responses after the first dose and these responses were significantly boosted after the second dose ($p<0.05$).

rLa-NiVG and rLa-NiVF elicit high-level, long-lasting NiV virus-specific neutralizing antibodies in pigs

The levels and duration of the recombinant virus-induced neutralizing antibody response are shown in Fig. 7. For rLa-NiVG- (Fig. 7A I) and rLa-NiVG/F- (Fig. 7A III) immunized pigs, NiV-neutralizing antibodies were detected shortly after priming and were boosted after the second dose. The neutralizing antibodies lasted for at least 21 weeks (Fig. 7A I and III) and had reduced to a very low level by 29 weeks. No significant difference in neutralizing antibody titers was found between the rLa-NiVG and rLa-NiVG/F groups at any time point. The level of neutralizing antibody to NiV in the rLa-NiVF group (Fig. 7A II) was lower than that in the rLa-NiVG and rLa-NiVG/F groups for the overall 29-week observation period. Serum antibodies against purified His-tagged NiV G or F proteins were measured by ELISA after the primary and secondary immunization. Compared with the neutralizing antibodies, the ELISA antibodies were also boosted, and gradually increased after the second dose (Fig. 7B). NDV neutralizing antibodies were detected after the primary immunization and were boosted after the second dose (Fig. 7C) but no statistical difference in NDV neutralization antibody titers was seen between the rLa-NiVG, rLa-NiVF, and rLa-NiVG/F groups during the 29-week observation period.

Discussion

Nipah virus is a deadly emerging infectious pathogen that poses a huge public health threat. Development of NiV vaccines is important both to protect susceptible animals and to reduce transmission from animals to humans (Guillaume et al., 2004; McEachern et al., 2008; Mungall et al., 2006; OIE, 2011; Weingartl et al., 2006). In this study, we used reverse genetics to construct recombinant NDV, rLa-NiVG and rLa-NiVF, which express the NiV glycoprotein (G) and fusion (F) proteins respectively. rLa-NiVG and rLa-NiVF maintained high growth titers in embryonated eggs and low pathogenicity in poultry and mice. The recombinant viruses induced significant NiV-specific neutralizing antibodies in mice and long-lasting NiV-specific neutralizing antibodies in pigs. Our findings thus show that rLa-NiVG and rLa-NiVF are safe and promising candidate vaccines against NiV infection.

Interestingly, rLa-NiVG was better able than rLa-NiVF to induce neutralizing antibodies, which is similar to observations from previous studies on other paramyxovirus, such as NDV, SeV, NiV and PPRV(Chen et al., 2010; Orvell and Norrby, 1977; Seto et al., 1974; Umino et al., 1984; Wang et al., 2006a; Weingartl et al., 2006) Biosafety level-4 containment is required to work with Nipah virus due to its pathogenicity. This made it impossible for us to do challenge studies or the neutralization assay using live NiV. The VSV pseudotyped virus system has been successfully used in neutralization assays for enveloped viruses such as Ebola, Nipah, and Hantavirus (Ito et al., 2001; Ogino et al., 2003; Takada et al., 1997; Tamin et al., 2009). In this study, we used VSVΔG*GFP-NiVG/F pseudotyped virus to mimic authentic NiV in a serum neutralization test. The pseudotyped particles are easy to prepare and render high level biosecurity containment unnecessary. In addition the assays are sensitive and rapid.

The role of CD8+ CTLs in controlling viral infections has been well documented. One advantage of using live vectored vaccines is that they elicit good cellular responses (Mathew et al., 2005; Song et al., 2011; Wang et al., 2006b). Splenocytes from rLa-NiVF immunized mice elicited high level CD8+ T cell responses when stimulated with an F protein-derived Balb/c mouse CD8+ T cell epitope. To our knowledge, this is the first identification of a CD8 epitope of F protein in Balb/c mice. Unfortunately, due to the unclear MHC background of the crossbred pigs, we were unable to identify pig CD8+ T cell epitopes in the NiV G or F protein, and, therefore, we could not measure epitope-specific CD8+ T cell responses in immunized pigs.

The co-expression of the NiV G and F proteins in permissive cells causes syncytia formation. It is essential to evaluate whether co-infection of two viruses is safe in mammalian systems. Our results showed that even when a high dose of rLa-NiVG/F was injected into a 3-week-old mouse brain, the weight change pattern and post-injection behavior were almost exactly the same as those of groups that were injected with PBS, rLa, rLa-NiVG alone or rLa-NiVF alone. In addition, there was no significant difference in serum NiV-specific IgG or neutralizing antibodies between rLa-NiVG- and rLa-NiVG/F-vaccinated mice and pigs, and the levels of NDV-neutralizing antibody induced by co-infection of rLa-NiVG/F in pigs were similar to those induced by rLa-NiVG or rLa-NiVF alone. These results indicate that the co-infection with rLa-NiVG/F is safe and did not result in enhanced virus replication in vaccinated animals.

Weingartl *et al.* showed that canarypox vectored NiV vaccines (ALVAC-G and ALVAC-F) were effective in eliciting antibodies against NiV and could provide complete protection for pigs against lethal NiV challenge (Weingartl et al., 2006). The success of canarypox-based NiV vaccines demonstrated protection against NiV infection in pigs can be achieved by live-attenuated vectored-NiV vaccines. Compared with canarypox virus, NDV-vectored vaccines have several advantages: they are easy to culture and grow to high titers in chicken eggs; they do not require complicated cell culture equipment and are thermostable when lyophilized. These features make NDV-based Nipah vaccines feasible and accessible especially to rural areas. In areas frequented by fruit bats, rLa-NiVG and rLa-NiVF could be used to decrease the susceptibility of pigs and other animals to NiV infection. During outbreaks of NiV, infection of pigs or other animals that are in close contact with humans may facilitate human infection by NiV and lead to sustainment of the outbreak. Therefore, application of these recombinant vaccines in these susceptible animals will be important for protecting them from NiV infection and thus preventing sustained NiV transmission to humans from these animal hosts. In addition, given their safety

and versatile applications, rLa-NiVG and rLa-NiVF maybe have the potential to serve as human NiV vaccines, such as health care people and researchers who face the threat of NiV infection in the future.

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