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Influenza virus vaccine expressing fusion and attachment protein epitopes of respiratory syncytial virus induces protective antibodies in BALB/c mice

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ABSTRACT

Respiratory syncytial virus (RSV) is an important viral pathogen that causes life-threatening respiratory infections in both infants and the elderly; no vaccines are at present available. In this report, we examined the use of influenza virus as a vehicle for production of an experimental RSV vaccine. We used reverse genetics to generate a recombinant influenza A virus with epitopes from the RSV fusion (F) and attachment (G) proteins (rFlu/RSV/F+G) in the influenza virus nonstructural (NS1) protein gene. Expression of RSV F+G epitope proteins was confirmed by Western blotting, and no changes in viral morphology were evident following examination by electron microscopy. BALB/c mice immunized intranasally with rFlu/RSV/F+G showed viral-specific antibody responses against both influenza and RSV. Total IgG, IgG1, IgG2a and IgA were measured in mice immunized with rFlu/RSV/F+G, revealing robust cellular and mucosal immune responses. Furthermore, we found that rFlu/RSV/F+G conferred protection against subsequent influenza and RSV challenges, showing significant decreases in viral replication and obvious attenuation of histopathological changes associated with viral infections. These findings suggest that rFlu/RSV/F+G is a promising vaccine candidate, which should be further assessed using cotton rat and primate models.

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1. Introduction

RSV is the leading cause of lower respiratory tract infections in infants, the elderly, and susceptible adults worldwide (Robinson et al., 2012). Therapeutic options are currently limited to a single FDA-approved medication, Synagis (palivizumab) (MedImmune, USA), was has been approved for prophylactic use in high-risk infants.

While prophylactic therapies have proven useful in high-risk populations, vaccination remains the most effective method available to control disease. Despite decades of efforts, there are currently no licensed vaccines available for prevention of RSV. An alum-precipitated formalin-inactivated RSV (FI-RSV) vaccine was developed in the 1960s (Sawada et al., 2011), but was abandoned following observations of enhanced illness characterized by exacerbated T_H2-like immune responses, along with insufficient neutralizing antibodies, and a poor mucosal immunity.

Since these initial studies, a number of alternative strategies have been employed, including live attenuated vaccines, live viral vector vaccines, subunit vaccines and DNA vaccines. Despite preliminary evidence demonstrating the potential efficacy of an RSV live attenuated vaccine candidate (rA2cp248/404/1030ΔSH)

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(Schickli et al., 2012), significant obstacles remain, with approval in the near future unlikely. Therefore, alternative strategies for the development of an effective RSV vaccine remain urgently needed.

Influenza viruses are capable of eliciting strong humoral, cellular, and mucosal immune responses, making them attractive candidates for use as viral vaccine vectors. These viruses are easily manipulated using reverse-genetics techniques (Liniger et al., 2007), and can be administered needle-free at mucosal sites. Furthermore, influenza virus is safer than other viral vectors, such as adenoviruses and retroviruses, as it is unable to form DNA intermediate products, which can be integrated into the host DNA during replication. This improved safety profile has been demonstrated in vaccine studies covering a variety of exogenous genes from a wide range of diseases, including chlamydia, tuberculosis, malaria, and cancer (He et al., 2007; Miyahira et al., 1998; Sereinig et al., 2006; Strobel et al., 2000).

The RSV F and G proteins are the major antigens expressed on the virion surface, and contain multiple epitopes recognized by T cells and neutralizing antibodies. $CD8^+$ cytotoxic T lymphocytes (CTLs) are known to play a role in both lung pathology and viral clearance (Singh et al., 2007); however, mice primed with adjuvant-combined whole F or G protein were instead found to exhibit imbalanced T_H1/T_H2 responses, as well as pulmonary eosinophilia following exposure to RSV, similar to that seen in the failed RSV vaccine trial. We therefore hypothesized that recombinant influenza virus containing F and G epitopes would induce strong RSV-specific immune responses, leading to protective immunity. In this research, we describe the construction and characterization of a recombinant influenza virus vector expressing the F and G protein epitopes of RSV, termed as rFlu/RSV/F+G. Using reverse genetics, a recombinant influenza virus was generated using influenza virus PR8 as the core of the virus, with the RSV F and G protein epitopes inserted into the location of nonstructural protein NS1. The immune response and protective immunity against influenza and RSV were determined in BALB/c mice immunized with rFlu/RSV/F+G.

2. Materials and methods

2.1. Viruses and cells

Influenza virus wild-type (WT) strain A/PR/8/34 (PR8) was injected into the allantoic cavity of 9-day-old SPF chicken embryos (Laboratory Animal Center, Beijing, China). Allantoic fluids containing virus were harvested 3 days after inoculation and stored at −80 °C until use. An RSV subgroup A WT strain was obtained from the American Type Culture Collection (ATCC). Virus was grown in human laryngeal epithelial (HEp-2) cells (ATCC, Manassas, Virginia, USA) cultured in media containing 40% Dulbecco's modified Eagle's medium, 40% F-12 medium (GIBCO, USA), and 10% fetal bovine serum (FBS). African green monkey kidney cells transformed by SV40 (COS-1) and Madin–Darby canine kidney (MDCK) cells (ATCC, Virginia, USA) were maintained in DMEM supplemented with 10% FBS. Media were supplemented with 10,000 IU/mL penicillin and 10,000 μg/mL streptomycin.

2.2. Construction of recombinant plasmid NS1-F+G

RSV protective antigen epitopes of genes F (F205-223 : PIVNKQSCRI SNIETVIEF; F255-278 : SELLSLIN DMPITNDQKK LMSNNV) and G (G142-204 : QPSKPTTKQR QNKPPNKPNN DFHFEVFNFV PCSICSNNPT CWAICKRIPN KKPGKKTTTK PTK) were linked via GPG (rigid linker) and inserted into the NS1 gene of PR8 influenza virus. F and G epitopes were inserted in-frame with the NS1 gene, and used as a fusion protein. Of note, an overlapping TAATG stop-start code cassette was introduced into the NS gene as previously described (Beier et al., 2001; Christian et al., 2005). The coding sequences of either RSV F+G epitopes or green fluorescent protein (GFP) was blunt end cloned downstream of the stop-start cassette, and the plasmids designated NS1-F+G and NS1-GFP, respectively. The NS1-F+G or NS1-GFP sequence was synthesized by Shanghai Sangon Company. The cDNA of recombinant plasmid NS1-F+G expressed in vector pHW2000 was constructed by *Bsm*BI enzyme digestion, ligation, transformation and PCR; the sequence of the resulting clone was confirmed by DNA sequencing (Chen et al., 2010). Positive control plasmids carrying the eight individual gene segments of the laboratory strain PR8 virus (pHW191 to pHW198) were identical to those used by Hoffmann et al. (2000).

2.3. Rescue of the infectious recombinant rFlu/RSV/F+G from cDNA

The recombinant influenza virus rFLU/RSV/F+G were rescued expressing the NS1-F+G recombinant gene along with the remaining gene segments derived from the PR8 strain (pHW191, pHW192, pHW193, pHW194, pHW195, pHW196 and pHW197). Briefly, COS-1 cells and MDCK cells were cultured together in six-well plates at a ratio of 2:1 and adsorbed into the cell monolayer. Transfection of plasmid cDNA was performed by mixing 0.2 μg of each plasmid with 8 μL of Effectene transfection reagent (QIAGEN China, Shanghai) diluted in 100-μL DMEM. Recombinant influenza virus rFlu/RSV/F+G was generated by reverse genetics, based on the eight influenza gene segments of strain A/PR/8/34, as described by Yang et al. (2011). When a hemagglutination-positive result was observed, recombinant rFlu/RSV/F+G virus supernatant and cell lysates were collected and stocked. Viruses were propagated in SPF chicken embryos, concentrated by ultrafiltration (PALL, USA), purified on a 20–30–60% discontinuous sucrose gradient, and diluted with phosphate-buffered saline (PBS). Infective titers were determined and expressed as hemagglutination titers. The 50% tissue culture infective dose in MDCK cells ($TCID_{50}$/mL) was calculated using the method of Reed and Muench (Neumann and Kawaoka, 2001).

2.4. Western blotting and electron microscopy

Recombinant rFlu/RSV/F+G viruses were characterized by Western blotting and electron microscopy. Concentrated, purified rFlu/RSV/F+G virus was resolved by SDS–PAGE, and NS1-F+G protein detected using the method of Sawada et al. (2011). Polyclonal mouse anti-respiratory syncytial virus antibody (Millipore, USA) was used to probe RSV protein epitopes of NS1-F+G. For morphology and size determinations, negative staining of rFlu/RSV/F+G was performed, followed by visualization by transmission electron microscopy.

2.5. Immunofluorescent microscopy

MDCK cells were infected with rFLU/RSV/F+G virus at an MOI of 0.01 and harvested at 24 h.p.i. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 1% BSA in PBS for 1.5 h and treated with NS1-23-1 monoclonal antibody (diluted 1: 2000; SANTA CRUZ) or nuclear export protein (NEP) polyclonal antibody (diluted 1: 1000; GeneTex; Catalog Number: GTX125953) in 1% BSA/PBS overnight at 4 °C. Cells were washed four times with PBS and then treated with Alexa Fluor 546 (diluted 1:250; Invitrogen)-conjugated goat anti-mouse secondary antibodies in 1% BSA/PBS for 1 h at room temperature. Images were obtained with a Zeiss LSM710 laser-scanning microscope (Carl Zeiss MicroImaging).

2.6. Virus growth

To assess viral growth kinetics, MDCK and A549 cells were infected with rFlu/RSV/F+G or wild-type PR8 (multiplicity of

infection (MOI) = 0.002) viruses, and the plates were incubated at 35 °C in 5% CO_2. Cell culture supernatants were harvested every 12 h, and virus titers were assayed and expressed as PFU/mL.

2.7. Quantitative real-time PCR

RNA was extracted from MDCK cells infected with rFlu/RSV/F+G using the RNeasy Mini Kit (Qiagen). We conducted real-time RT-PCR on an ABI 7500 Real-Time PCR system (Applied Biosystems) with primers and TaqMan one-step RT-PCR master mix (Applied Biosystems). Herein, the forward primer prior to the splice site and the reverse primer after the splice site were used. The specific primers that could detect the NEP gene used were as follows: forward, 5′-GTTGGCGAAA TTTCACCATT GCCTTCTCT-3′, reverse, 5′-TTAAATAAGC TGAAATGAGA AAGTTCT-3′. Samples were normalized to the control of GAPDH. Relative amounts of mRNA were calculated by using the comparative C_T method.

2.8. Immunization and challenge in BALB/c mice

Six-to-eight-week-old female specific-pathogen-free BALB/c mice (Animal Experimental Center, AMMS) were used in this study. Groups of mice were immunized intranasally twice with either 10^4 or 10^5 $TCID_{50}$ of rFlu/RSV/F+G at 4-week intervals. Blood samples were collected before immunization and on days 28 and 42 after priming. Five mice were sacrificed 2 weeks after the second immunization. Meanwhile, nasal and lung lavages were collected for measurement of mucosal sIgA titer. Tissue samples were stored at −20 °C until use. For viral challenge, naïve or vaccinated mice were isoflurane-anesthetized and infected intranasally with live RSV A2 (10^6 PFU) in 50 μL of PBS 2 weeks after boost. Mice were observed daily and changes in body weight were recorded. All animal experiments were conducted under the guidelines of the Academy of Military Medical Sciences Institutional Animal Care and Use Committee.

2.9. Neutralization assays

To detect recombination influenza virus rFlu/RSV/F+G-specific neutralizing antibodies, a hemagglutination inhibition (HI) assay was performed using standard methods. Briefly, four hemagglutination units (HAU) of influenza virus PR8 were added to a v-bottom 96-well microtiter plate containing 0.5% turkey erythrocytes (Webster et al., 1991). HI titers were expressed as the reciprocal of the highest serum dilution that completely inhibited the agglutination of the chicken erythrocytes by influenza virus PR8.

Neutralization tests (NT) against RSV strain A2 were performed using a 50% plaque reduction assay, as described previously (Jones et al., 2012). Mice sera were serially diluted at a ratio of 1:2, mixed with an equal volume of RSV (100 PFU), and inoculated on monolayer of HEp-2 cells in 24-well plates. Plaques were counted, and NT antibody titers were calculated as the reciprocal of the serum dilution that showed a 50% reduction in plaque number.

2.10. RSV protein-specific ELISA

The solid-phase enzyme-linked immunosorbent assay (ELISA) was performed in a microtiter plate as described previously (Farnos et al., 2006). Plates for the detection of RSV specific IgG, IgG1 and IgG2a in serum and secretory IgA (sIgA) in nasal and lung lavage fluids were coated with 5 μg/mL inactivated RSV virions. Samples were serially diluted 1:2 in PBS containing 1% bovine serum albumin (BSA) (Serva, BRD), and added to the coated plates. Bound antibodies were detected using goat anti-mouse IgG, IgG1, IgG2a and IgA (Sigma, USA) conjugated to horseradish peroxidase (HRP) and diluted 1:20,000. Plates were stained with TMB (Sigma, USA) as a substrate and read at 450 nm. Each sample was assayed in duplicate.

2.11. Weights and viral titers of mice following RSV challenge

Following rFlu/RSV/F+G immunization, BALB/c mice were intranasally challenged with RSV strain A2 for evaluation of protective immunity. Mice were monitored for weight change throughout the 14-day observation period. At day 6 post-challenge, individual lungs were collected for pathological examination and recovery of RSV infectious particles. Viral titers of RSV isolated from tissue homogenates of nasal turbinate and lung were determined by 50% plaque reduction assay using HEp-2 cells (Krause et al., 2011).

2.12. Bronchoalveolar lavage and histopathological analysis

The right lobes of collected lungs were isolated, fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin using standard histological techniques (Jones et al., 2012). Total inflammatory cells and cells specific for RSV were counted in bronchoalveolar lavage fluid (BALF) using a hemocytometer and Diff-Quik cell stain (Baso, China), as described previously (An et al., 2009).

2.13. Statistics

The GraphPad Prism 5 software was used for all data analysis (GraphPad Software Inc., San Diego, CA). Groups were compared using analysis of variance (ANOVA); a value of $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Generation of constructs

The F and G antigen epitopes from RSV were inserted into the coding region of the influenza NS1 gene, producing recombinant plasmid NS1-F+G (Fig. 1A). To verify NS1 and NEP were expressed properly in cells, we constructed NS1-GFP plasmid in which the stop codon of the stop-start cassette terminates the translation of NS1 after 417nt and the start codon reinitiates the translation of GFP (Fig. 1B). As demonstrated by fluorescence microscope analysis, the NS1-GFP was able to express GFP in infected MDCK cells at 24 h post-transfection, whereas pHW198 plasmid was not (Fig. 1C and D). The recombinant influenza virus rFLU/RSV/F+G was generated by reverse genetics, expressing the NS1-F+G recombinant gene, along with the remaining gene segments derived from the PR8 laboratory virus strain (pHW191–pHW198). Rescued virus was fully sequenced and was identical to the plasmids used in its creation.

3.2. Characteristics of recombinant influenza virus

Recombinant rFLU/RSV/F+G containing RSV F and G glycoprotein antigen epitopes was produced as described in the Section 2. Expression of protein NS1-F+G was confirmed by Western blotting using an antibody against RSV (Fig. 1E). Recombinant rFLU/RSV/F+G viral particles were examined by electron microscopy, revealing spherical structures ~80–120 nm in size, containing a characteristic lipid membrane bilayer on their outer surface (Fig. 1F and G). rFlu/RSV/F+G viruses are therefore similar to influenza virus in both morphology and size, suggesting that no major differences were conferred by expression of the fusion construct. Moreover, the antigenic properties of rFLU/RSV/F+G were stable for at least 1 year at −80 °C (data not shown).

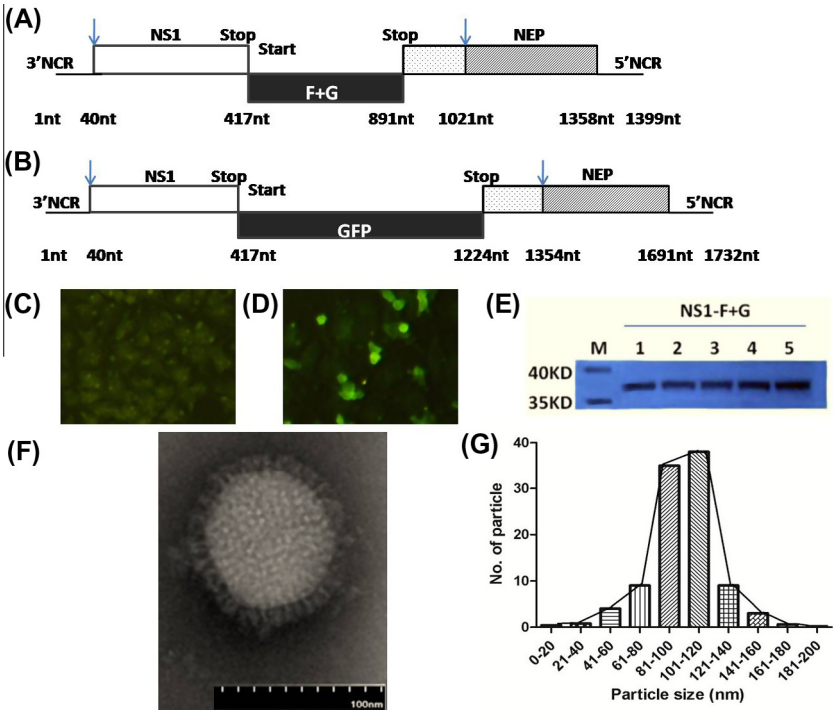

Fig. 1. Construction of influenza vaccine vectors encoding RSV F and G protein epitopes. The characteristics of rFLU/RSV/F+G were confirmed by expression of specific proteins and visualization of virus particles shapes and their size distribution. (A–B) Schematic representation of the NS1 gene segments of recombinant influenza viruses. RSV F and G epitopes or GFP were inserted into the coding region of the influenza virus NS1 gene. An overlapping stop-start pentanucleotide cassette (TAATG) was introduced. The coding sequence of RSV F and G epitopes or GFP was cloned downstream of the stop–start cassette. Hatched boxes represented non-translated regions. Arrows indicated NEP splicing sites. (C–D) GFP expression levels. MDCK cells were transfected with pHW198 (C) and NS1-GFP (D) plasmid alone, respectively. At 24 h post-transfection, fluorescence was analyzed using microscopy (Magnification ×200). (E) Expression of NS1-F+G protein was examined by Western blotting. Concentrated and purified rFLU/RSV/F+G protein was resolved by SDS–PAGE, and probed using a polyclonal anti-RSV antibody. (F) Recombinant rFLU/RSV/F+G viral particles containing RSV/F+G were observed using electron microscopy. (G) Viral particle size distribution; 73% of rFLU/RSV/F+G viral particles were 80–120 nm in diameter.

We then performed immunofluorescent assay to test the expression of NS1, NEP protein of rFLU/RSV/F+G virus. As shown in Fig. 2A and B, the rFLU/RSV/F+G virus was able to express NS1 and NEP in infected MDCK cells at 24 h post-infection, whereas mock group was not (Fig. 2C). Notably, the staining could be detected in the cytoplasm only. To further assess the relationship between cytopathicity and virus replication in the tested cell lines, we compared the growth kinetics of the rFlu/RSV/F and PR8 viruses. Recombinant rFlu/RSV/F+G virus reached a peak titer of $10^{7.7}$ PFU/mL in 48 h after infection of MDCK cells (Fig. 2D). Similarly with the wild-type PR8 virus, the rescued viruses could also effectively replicate in A549 cells (Fig. 2E). Additionally, in MDCK cells, rFlu/RSV/F+G produced high copies of viral mRNA at 12 h post-infection as PR8 virus did (Fig. 2F).

3.3. Immunogenicity of rFlu/RSV/F+G

Female BALB/c mice were inoculated intranasally with recombinant rFlu/RSV/F+G viruses to confirm the immunogenicity of the inserted RSV F and G protein epitopes. Influenza virus and RSV-specific antibody responses in all animals were measured using HI and neutralization assay (Fig. 3A and B, respectively). nAb titers against RSV increased in a dose-dependent manner after each immunization (prime and boost). Similar responses were detected against influenza virus, with small increases in HI titers seen in the higher inoculum group, and after the second immunization. Following two immunizations with rFlu/RSV/F+G, BALB/c mice demonstrated RSV specific antibody responses, whereas immunization with PR8 control viruses did not (data not shown). From these results we concluded that rFlu/RSV/F+G is immunogenic *in vivo*.

3.4. Enhanced T_H1-type anti-RSV immunity following immunization with rFlu/RSV/F+G

RSV-specific total serum IgG, IgG1, and IgG2a antibody levels (Fig. 4A–C) were determined following both prime and boost. Total IgG and IgG2a titers of mice immunized with rFlu/RSV/F+G were significantly higher after boost compared with those after prime; IgG1 titers were significantly lower than total IgG or IgG2a, and showed only modest increases following boost. As shown in Fig. 4D, IgG1 to IgG2a ratios were significantly lower than after restimulation by RSV strain A2. These results demonstrate that rFlu/RSV/F+G are highly immunogenic and preferentially induce a T_H1-type cellular immune response in BALB/c mice.

3.5. Improved mucosal anti-RSV immune response following immunization with rFlu/RSV/F+G

Mice immunized intranasally with recombinant rFlu/RSV/F+G induced potent sIgA antibody responses in the mucosa. sIgA antibodies were detected by ELISA in lung and nasal lavage fluids (Fig. 5A and B) restimulated by RSV A2, whereas only trace antibody levels were detected in control groups. These results suggest that intranasal administration enhances mucosal antibody production in response to rFlu/RSV/F+G vaccination.

3.6. rFlu/RSV/F+G confers protection against live RSV A2 virus challenge

To evaluate the level of protective immunity conferred by rFlu/RSV/F+G immunization, mice were challenged intranasally with live RSV A2 virus. Changes in body weight were measured

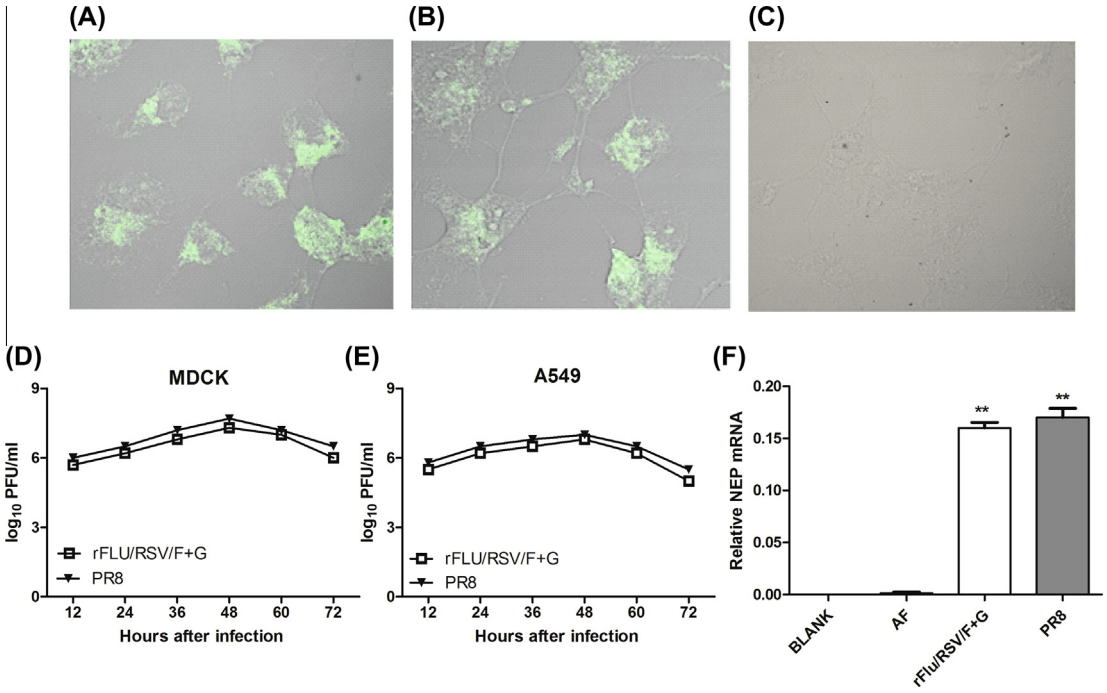

Fig. 2. The expression of NS1, NEP protein and growth kinetics of rFLU/RSV/F+G. MDCK cells were infected with rFLU/RSV/F+G virus at an MOI of 0.01 and fixed at 24 h.p.i. Fixed cells were treated with NS1-23-1 monoclonal antibody (A) or NEP polyclonal antibody (B), secondary Alexa Fluor 546-conjugated goat anti-mouse antibody. Mock-infected cells (C) were also prepared (Magnification ×1000). In another experiments, MDCK (D) and A549 (E) cells were infected with rFLU/RSV/F+G or PR8 virus at a MOI of 0.002. Culture supernatants were obtained every 12 h post-infection. Infectivity values are shown as mean PFU/mL in MDCK and A549 cells. (F) MDCK cells were infected with rFLU/RSV/F+G, PR8 virus, the mock-infected control AF or blank at 0.01 MOI. Virus mRNA at 12 h was quantified by qRT-PCR. The mRNA levels were normalized to GAPDH. $^{**}p < 0.001$.

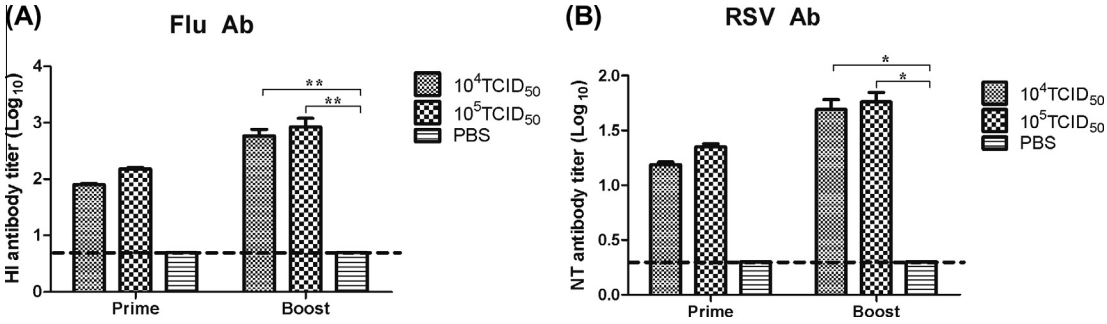

Fig. 3. Development of HI antibodies against influenza virus PR8 and NT antibodies against RSV strain A2 following intranasal vaccination in BALB/c mice with 10^4 or 10^5 $TCID_{50}$ rFLU/RSV/F+G, or PBS. Sera were collected 4 weeks after prime and 2 weeks after boost. (A) HI antibody titers were detected using 0.5% turkey erythrocytes. (B) NT antibodies were assayed using RSV strain A2 (Subgroup A). A 50% plaque reduction assay of NT titers using HEp-2 cells; results are expressed as 10^n. $^{*}p < 0.01$ and $^{**}p < 0.001$. The dashed horizontal line indicates the lower limit of detection.

throughout the 14-day observation period; the greatest percentage change in body weight was observed at 8 days post-infection in control mice, whereas body weight was unchanged in mice immunized with rFlu/RSV/F+G (Fig. 6A). Further studies were conducted to determine whether vaccination with rFlu/RSV/F+G enhanced viral clearance after lower respiratory tract (LRT) challenge with RSV. Viral loads in lung and nasal tissues harvested 6 days post-challenge were determined by plaque assay. A significant decrease in viral load was detected in mice immunized with rFlu/RSV/F+G compared with unvaccinated controls (Fig. 6B), consistent with enhanced viral clearance.

3.7. Absence of RSV-induced inflammatory responses in mouse lungs

For histopathological examination, lung tissues were obtained 6 days after challenge with RSV strain A2; the results of H&E staining are shown in Fig. 7. Compared with normal mice (Fig. 7A), the PBS-immunized mice challenged with RSV strain A2 showed prominent interstitial pneumonia, thickening of the alveolar wall, and infiltration of inflammatory mononuclear cells (Fig. 7D). In contrast, only mild inflammatory changes were observed in BALB/c mice immunized with 10^4 and 10^5 $TCID_{50}$ of rFlu/RSV/F+G (Fig. 7B and C). Furthermore, the number of immune cells in BALF collected from control mice was significantly higher after RSV challenge compared to those vaccinated with rFlu/RSV/F+G (Fig. 7E). The increase in total cell number was associated with influx of eosinophils (EOS), neutrophils (NEUT), small mononuclear cells (MONO), macrophages (MAC) and lymphocytes (LYMP).

4. Discussion

RSV is a clinically important pathogen responsible for significant mortality and morbidity in infants, the elderly, and other immunocompromised populations. Worldwide, ~64 million cases of RSV

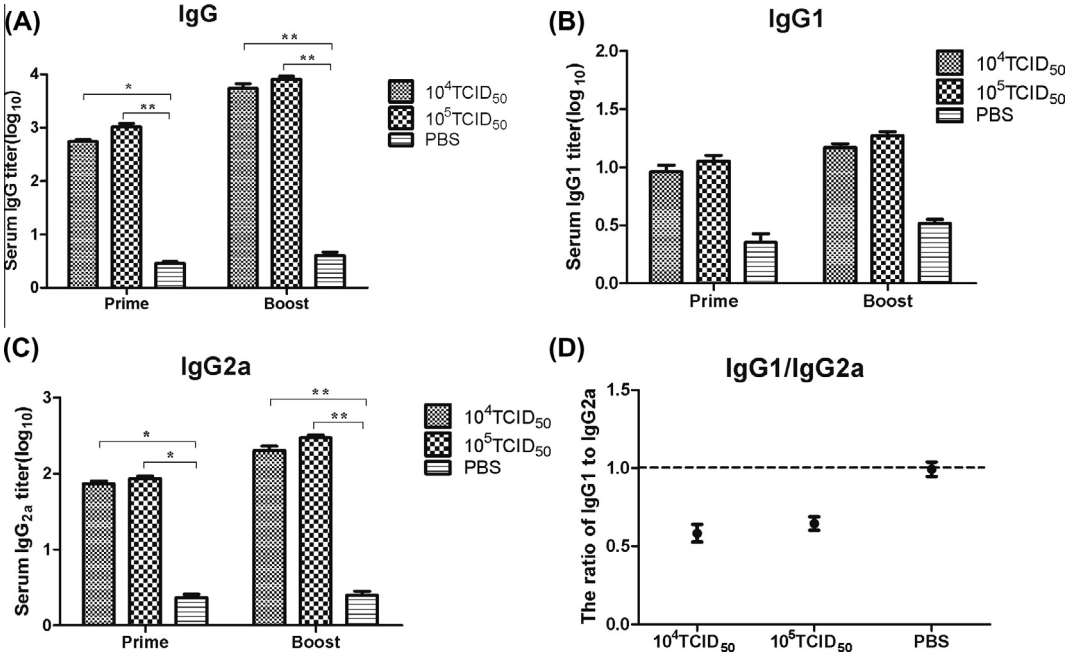

Fig. 4. Female BALB/c mice vaccinated intranasally with rFLU/RSV/F+G induced RSV-specific antibody responses. Mice were immunized with rFLU/RSV/F+G containing 10^4 or 10^5 $TCID_{50}$ viral particles at days 0 and 28. Serum RSV-specific IgG (A), IgG1 (B) and IgG2a (C) levels were assessed by ELISA using purified RSV protein. (D) RSV-specific IgG1/IgG2a ratios in mice immunized with rFLU/RSV/F+G and PBS. Each time point represents the mean of at least five samples ± SEM, and the experiment was repeated with similar results. $^{*}p < 0.01$ and $^{**}p < 0.001$.

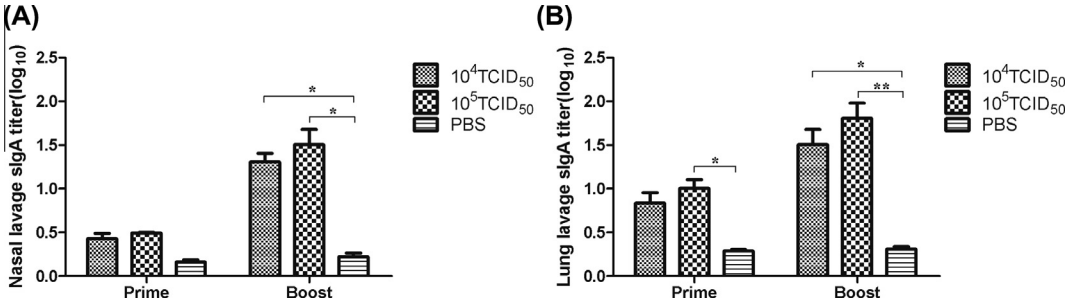

Fig. 5. Mucosal antibody response in BALB/c mice. Secretory IgA against RSV inactivated antigen were assessed by ELISA in nasal (A) and lung (B) lavage fluid from mice immunized intranasally with 10^4 or 10^5 $TCID_{50}$ rFLU/RSV/F+G. Nasal and lung lavages were collected 4 weeks after prime and 2 weeks after boost. The values are means ± SEM from five mice. $^{*}p < 0.01$ and $^{**}p < 0.001$.

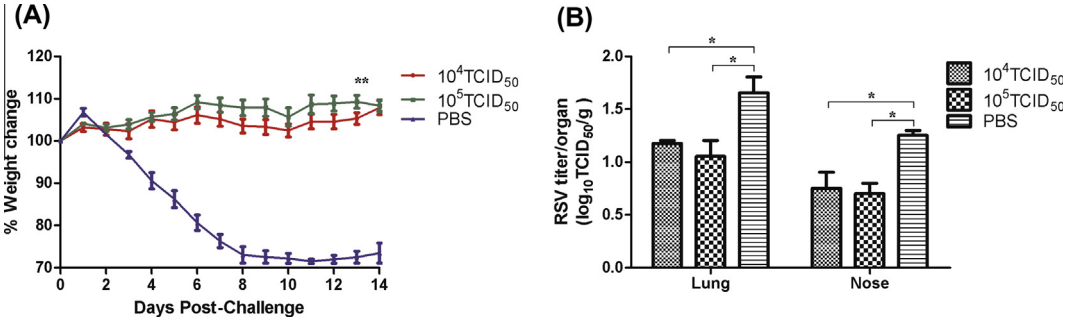

Fig. 6. Protective effect of rFLU/RSV/F+G in BALB/c mice challenged with RSV strain A2. Groups of mice (n = 15/group) were immunized intranasally with 10^4 or 10^5 $TCID_{50}$ rFLU/RSV/F+G, and then challenged with RSV (10^6 PFU) by intranasal inoculation 2 weeks after boost. (A) Mice were monitored for body weight change throughout the 14-day observation period. (B) The nasal turbinates and lungs of five mice in the rFLU/RSV/F+G and PBS groups were collected on day 6 post-challenge. Viral titers in tissue homogenates were determined by 50% plaque reduction assay using HEp-2 cells. $^{*}p < 0.01$ and $^{**}p < 0.001$.

occur every year, resulting in 160,000 deaths (Sawada et al., 2011). A wide range of approaches has been considered for the development of an RSV vaccine. In light of the many obstacles associated with RSV vaccine development, the F and glycoprotein have emerged as the best target for generation of neutralizing and protective antibodies. These epitopes of the F and glycoprotein also have been used in the development of multiple RSV vaccines, including a live vector vaccine, subunit vaccines, and DNA vaccines (de Waal et al., 2004). A re-

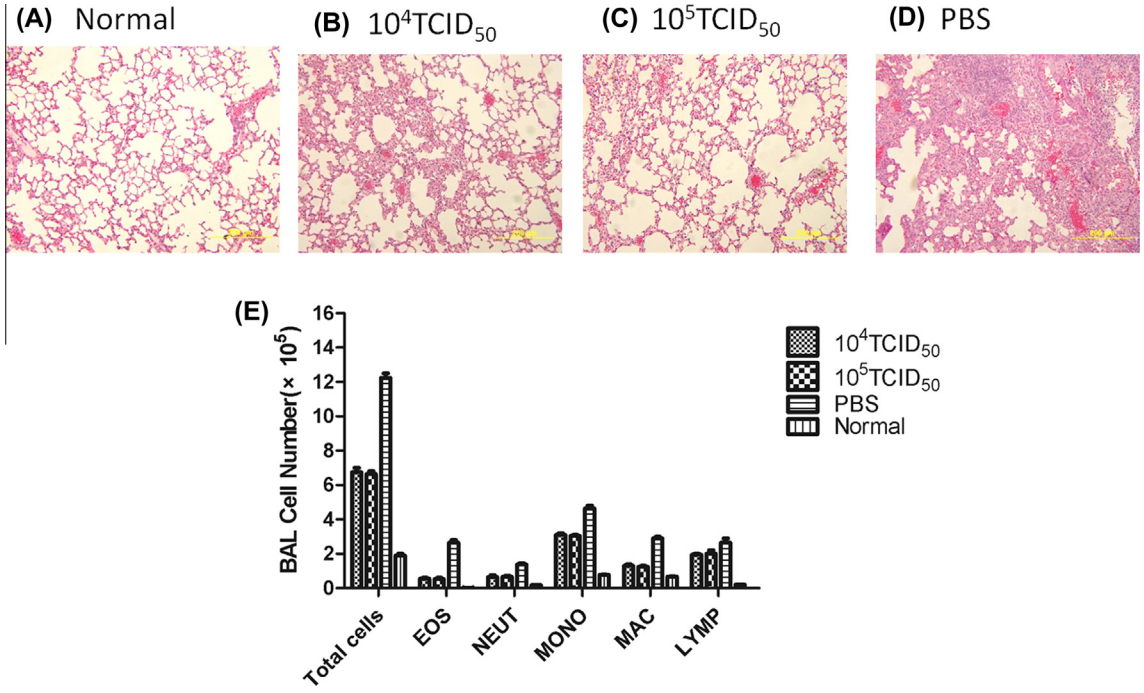

Fig. 7. Absence of RSV-induced inflammatory responses in pathological sections of mouse lung tissue and BALF following immunization with rFLU/RSV/F+G. After two immunizations with rFLU/RSV/F+G, BALB/c mice were challenged intranasally with 10^6 PFU of RSV strain A2. Five mice were sacrificed on day 6 after virus challenge, followed by collection of lung sections and BALF. Histological examination was performed by H&E staining of lung tissues; (A) normal, (B) 10^4 $TCID_{50}$, (C) 10^5 $TCID_{50}$ and (D) PBS. Original magnification ×200. (E) The absolute numbers of eosinophils (EOS), neutrophils (NEUT), monocytes (MONO), macrophages (MAC) and lymphatic cells (LYMP) were determined using a hemocytometer. Differential staining was observed in the BALF of unvaccinated, rFLU/RSV/F+G-vaccinated, and PBS-vaccinated mice at day 6 post-infection. $^*p < 0.05$ and $^{**}p < 0.001$.

combinant parainfluenza virus chimeric expressing RSV F protein (Tang et al., 2005) did not lead to enhanced disease in hamsters, and has been proven to be safe in phase I clinical trial in seronegative children (Bernstein et al., 2012).

In recent years, influenza virus vaccines have been proposed as potential viral vectors for mucosal immunization against a variety of infectious diseases, and even as a treatment for certain cancers (Bergmann et al., 2001; Ferko et al., 2001). Regarding the benefits of a dual-purpose vaccine, in particular for RSV and influenza, the vectored influenza reassortant virus induces robust antibody and T cell responses against both influenza and RSV infection. One of the most important determinants for inducing a robust immune response is the amount of antigen presented to the immune system. We have directly assessed the effect of our gene insertion location on the level of antigen expression. In this study, a reassortant influenza virus as a vector carrying RSV F and G protein epitopes (rFlu/RSV/F+G) was rescued using reverse genetics. We also constructed two reassortant influenza viruses expressing a single F (rFlu/RSV/F) or G epitope (rFlu/RSV/G). Compared with rFlu/RSV/F+G group, lower antibody responses and induction of protective immunity in mice was observed for both the rFlu/RSV/F and rFlu/RSV/G constructs. While immune responses were overall lower in these groups, all mice were able to produce protective immune response against RSV, while no immunity was seen in any of the PR8-immunized control groups. In addition, the genetic stability of rFlu/RSV/F, rFlu/RSV/ G, and rFlu/RSV/F+G was examined, and all constructs were shown to be stable over multiple passages (data not shown).

Recombinant rFlu/RSV/F+G viruses were administered intranasally and triggered an immune response in BALB/c mice after vaccination. Upon vaccination of mice with rFlu/RSV/F+G virus, specific antibody responses were induced. HI antibody titers against influenza were investigated using WT influenza virus PR8, and NT antibody titers against RSV were investigated using RSV strain A2. After two intranasal immunizations with rFlu/RSV/F+G, BALB/c mice developed RSV specific antibody responses, whereas the PR8 immunized group did not (data not shown). Meanwhile, influenza virus specific HI antibodies were also induced by vaccination with rFlu/RSV/F+G. Thus, immunization with rFlu/RSV/F+G resulted in influenza- and RSV-specific antibody responses, confirming that the recombinant influenza virus was immunogenic in mice, further supporting its use an effective vector for vaccine development.

In view of the importance of neutralizing antibodies and a balanced T_H1/T_H2 response to both safety and efficacy, an RSV vaccine capable of inducing such a response may be necessary to provide protective immunity. To determine whether rFlu/RSV/F+G virus was able to induce relevant immune responses in a mouse model, the levels of IgG, IgG1, IgG2a and sIgA antibodies specific for RSV were measured in mice immunized intranasally with rFlu/RSV/F+G. Detection of IgG1 and IgG2a antibodies is important, both in the context of the T_H1/T_H2 paradigm, and in the known contributions of these antibody isotypes towards clearance of viral infections. Based on the serum IgG1/IgG2a ratio, we hypothesized that rFlu/RSV/F+G would induce a T_H1-biased cellular immune response in BALB/c mice. Of particular importance was the observation that mice immunized intranasally with rFlu/RSV/F+G developed antibodies detectable in mucosal secretions from both the nose and lungs. Virus-specific IgA, restimulated by RSV strain A2, was the predominant isotype detected in mucosal secretions. These results suggest that recombinant rFlu/RSV/F+G induced a T_H1-like immune response characterized by significant induction of virus-specific serum IgG and IgG2a, with the ratio of IgG1 to IgG2a <1. Furthermore, the intranasal administration route used in this study enhanced the efficiency of sIgA antibody production in mucosal immune responses to rFlu/RSV/F+G vaccination.

After challenge with RSV strain A2, the control mice displayed prominent interstitial pneumonia characterized by substantial inflammatory cell infiltration, particularly inflammatory mononu-

clear cells and airway eosinophilic granulocytes, which are a hallmark of vaccine-enhanced disease (Castilow et al., 2007). In comparison, rFlu/RSV/F+G-vaccinated mice exhibited only mild inflammatory changes in BALF, with no evidence of enhanced airway eosinophilia.

A recombinant influenza virus PR8/NA–F (85–93) containing the RSV F_{85-93} epitope in its neuraminidase stalk has recently been shown to yield a significant reduction in the lung viral load upon subsequent challenge with RSV (De Baets et al., 2013). Here, we described the rFlu/RSV/F+G virus, which expresses RSV epitopes $F_{205-223;\ 255-278}$ and $G_{142-204}$ in the NS1 fragment. Of note, the TAATG start-stop codon is introduced for the NS1-F+G construct. To make sure the splice site for NEP would be function efficiently as expected and avoid a frame-shift mutation, we confirmed the proper expression of NS1 and NEP protein by IFA. What's more, as shown in Fig2F, a qPCR assay was made with the forward primer prior to the splice site and the reverse primer after the splice site.

PBS-immunized mice challenges with RSV A2 exhibited significantly increased viral loads in both the lung and nasal mucosa, along with a significant decrease in body weight over the course of the study. In stark contrast to these findings, BALB/c mice immunized with two doses of rFlu/RSV/F+G had substantially lower viral loads in both the lung and nasal mucosa compared to control groups, with minimal changes in body weight. Thus, RSV strain A2 was readily cleared from BALB/c mice immunized with rFlu/RSV/F+G following challenge, with minimal pathological findings associated with an active infection.

Data presented here also suggest that recombinant rFlu/RSV/F+G would provide protective immunity against WT influenza virus. Only weak inflammation was seen in pathological sections of lung tissue from rFlu/RSV/F+G vaccinated mice, along with minimal weight loss, compared to death of all control mice (data not shown). These results demonstrate that recombinant influenza virus rFlu/RSV/F+G expressing RSV F and G epitopes is capable of inducing protective immunity against both RSV and influenza virus in BALB/c mice.

In conclusion, recombinant influenza virus rFLU/RSV/F+G effectively induced of humoral and cell-mediated immune responses in BALB/c mice, along with a potent mucosal immune response against both influenza and RSV. These findings not only demonstrate the efficacy of recombinant influenza vaccines expressing RSV proteins, but also suggest new approaches to development of dual or multi-purpose respiratory vaccines. We therefore conclude that rFlu/RSV/F+G is a promising vaccine candidate, which warrants further examination in cotton rat and primate models.

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