

12-2014

Recombinant Influenza Virus Carrying the Conserved Domain of Respiratory Syncytial Virus (RSV) G Protein Confers Protection Against RSV Without Inflammatory Disease

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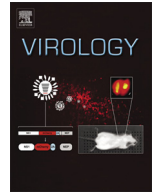
Recommended Citation

Recombinant influenza virus carrying the conserved domain of respiratory syncytial virus (RSV) G protein confers protection against RSV without inflammatory disease. Y. N. Lee, H. S. Hwang, M. C. Kim, Y. T. Lee, M. K. Cho, Y. M. Kwon, J. S. Lee, R. K. Plempner and S. M. Kang. *Virology*, 476 217-25. doi: <http://dx.doi.org/10.1016/j.virol.2014.12.004>

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ARTICLE INFO

Article history:

Received 24 September 2014

Returned to author for revisions

24 November 2014

Accepted 2 December 2014

Available online 30 December 2014

Keywords:

Influenza virus

Respiratory syncytial virus

Recombinant

Viral vector

G protein

ABSTRACT

Respiratory syncytial virus (RSV) is one of the most important causes for viral lower respiratory tract disease in humans. There is no licensed RSV vaccine. Here, we generated recombinant influenza viruses (PR8/RSV.HA-G) carrying the chimeric constructs of hemagglutinin (HA) and central conserved-domains of the RSV G protein. PR8/RSV.HA-G virus showed lower pathogenicity without compromising immunogenicity in mice. Single intranasal inoculation of mice with PR8/RSV.HA-G induced IgG2a isotype dominant antibodies and RSV neutralizing activity. Mice with single intranasal inoculation of PR8/RSV.HA-G were protected against RSV infection as evidenced by significant reduction of lung viral loads to a detection limit upon RSV challenge. PR8/RSV.HA-G inoculation of mice did not induce pulmonary eosinophilia and inflammation upon RSV infection. These findings support a concept that recombinant influenza viruses carrying the RSV G conserved-domain can be developed as a promising RSV vaccine candidate without pulmonary disease.

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Introduction

Respiratory syncytial virus (RSV) is the most important cause of viral respiratory tract infections in children, and the second leading cause of death from respiratory viral infections in the elderly (Falsey et al., 2005; Nair et al., 2010). The first RSV vaccine, formalin-inactivated RSV (FI-RSV) formulated with alum, was not protective, associated with enhanced respiratory disease, and raised concerns that non-live vaccines including FI-RSV and subunit protein vaccines might also predispose to vaccine enhanced respiratory disease (ERD) (Kapikian et al., 1969). Replicating vaccinia virus-vectored vaccines expressing full-length RSV G or F proteins, and purified subunit vaccines with adjuvants have been tested with little success due to potential safety concerns (Castilow et al., 2008a, 2008b; Chang, 2011; Hancock et al., 2001; Murphy et al., 1990). Previous studies suggest that purified RSV F or G protein vaccination also may prime for ERD upon subsequent RSV challenge, albeit less severe than that observed with FI-RSV (Hancock et al., 1996; Johnson et al., 1998; Murphy et al., 1990). Live attenuated RSV vaccine candidates also suffer from genetic instability, protective immunogenicity, residual virulence, safety concerns in young infants, and lack of long-term

immunity (Graham, 2011). Despite extensive efforts to develop RSV vaccines, there is no licensed RSV vaccine to date (Graham, 2011).

RSV F and G glycoproteins are the protective antigens inducing neutralizing antibodies against RSV infection. The central region of RSV G (aa130–230) is relatively well conserved and contains 5 human B cell epitopes called protectopes (Plotnicky-Gilquin et al., 1999), including a highly immunogenic peptide (aa174–187) (Trudel et al., 1991), and a T helper epitope (aa184–198) (Tebbey et al., 1998). Antibodies to the central RSV G epitopes were shown to be protective without ERD (Haynes et al., 2009; Plotnicky-Gilquin et al., 1999; Radu et al., 2010). Importantly, monoclonal antibody 131-2G that recognizes a G peptide with 13 amino acids (aa164–176) was shown to neutralize RSV *in vivo*, to block RSV G binding to CX3CR1, and to prevent ERD (Boyoglu-Barnum et al., 2013; Haynes et al., 2009; Radu et al., 2010). Mice that were immunized with G-deleted FI-RSV showed similar levels of ERD but higher RSV lung viral titers (Johnson et al., 2004). These results suggest that G-specific immune responses are important for protection and that other RSV components have similar potential to induce ERD.

Influenza vaccines either in the form of inactivated viral split or as a live attenuated viral platform, have been safely used in humans for many years. Beyond protection against influenza, influenza virus emerges as a promising vaccine vector candidate due to its protective immune responses (Krejtz et al., 2011) and the availability of a reverse genetics system that allows the expression of foreign genes (Hoffmann

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et al., 2000). Li et al. (1992) generated recombinant influenza A virus containing chimeric hemagglutinins (HAs) in which the six-amino-acid loop of antigenic site B was replaced by the corresponding structures of the HA of H2 or H3 subtypes. Moreover, it was also reported that the insertion of foreign epitopes from human immunodeficiency virus-1 (Li et al., 1993) into an antigenic site B of the HA. Furthermore, there is no risk that the viral genome would integrate into the host genome when influenza virus is used as a vaccine vector because there is no DNA phase during the life-cycle of negative-sense RNA viruses (Kittel et al., 2005). Compared to other DNA-based viral vectors with the potential to integrate their viral DNA into the host genome, influenza virus-based vaccine vectors therefore promise high safety for human use. Here, as a proof-of-concept, we explored a recombinant influenza virus as a live viral vector for mucosal delivery of the RSV G protein conserved domain (aa131–230). We produced recombinant influenza viruses carrying a conserved-domain of the RSV G protein in the HA, and tested their protective efficacy against RSV and safety in comparison with FI-RSV and live RSV.

Materials and methods

Cells and viruses

293T cells (DuBridgde et al., 1987) and HEp2 cells (Quan et al., 2011) were obtained from ATCC and maintained in DMEM media. The RSV strain A2 was originally gifted from Dr. Barney Graham. Influenza virus A/PR/8/1934 (H1N1, abbreviated as PR8) was grown in 10-day-old embryonated hen's eggs at 37 °C for 2 days. The allantoic fluid was harvested and stored at –70 °C until used. The viruses were inactivated by mixing the virus with formalin at a final concentration of 1:4000 (v/v) as described previously (Quan et al., 2008). The viruses were purified by using discontinuous sucrose gradient ultracentrifugation with layers of 20 and 60% (wt/vol) as previously described (Song et al., 2011).

Construction of PR8/RSV.HA-G1 and PR8/RSV.HA-G2

Recombinant viruses were rescued using the pHW2000-based eight-plasmid system that was kindly provided by Dr. Webster as described by Hoffmann et al. (2000). The sequence encoding RSV G protein aa131–230 was inserted between the 3' end of the HA signal peptide sequence and the nucleotide sequences encoding the N-terminal domain of the HA1 ectodomain of pHW2000-HA plasmid as described (Li et al., 2005). The inserted sequence was followed by a GGGGS or AAAPGAA peptide linker to facilitate the proper folding of the inserted polypeptides as independent domains, respectively (Fig. 1A).

To generate recombinant viruses PR8/RSV.HA-G1 and PR8/RSV.HA-G2, 293T cells were cotransfected with eight pHW2000 plasmids of influenza virus gene segments including the chimeric HA-G constructs (Fig. 1A). After 48 h, the medium was collected and inoculated to embryonated chicken eggs. After 72 h, the presence of the rescued recombinant viruses in the allantoic fluids was confirmed by hemagglutination of chicken red blood cells. Purified viruses (2×10^8 PFU/ml) were prepared by discontinuous sucrose gradient ultracentrifugation of each RSV stock (2×10^6 PFU/ml) and then by resuspending in PBS to have the same PFU amount of virus. To determine the incorporation of recombinant HA-G chimeric proteins, the reactivity to RSV G protein monoclonal antibody (Clone 131-2G, Millipore) was analyzed using equal amounts of virus.

Immunizations and RSV challenge of mice

For animal experiments, 6- to 8-week-old female BALB/c mice ($n=5$; Harlan Laboratories) were intranasally inoculated with phosphate-buffered saline (PBS) or 500 EID₅₀ dose (50% egg infective dose,

EID₅₀) of PR8/RSV.HA-G1, PR8/RSV.HA-G2, or PR8 wild-type (PR8 WT) or 2×10^5 PFU of RSV A2 strain under isoflurane anesthesia. The FI-RSV control group ($n=5$) was intramuscularly immunized with 50 μ l of FI-RSV (2 μ g) adsorbed to aluminium hydroxide adjuvant (2 mg/ml) (Prince et al., 2001). Blood samples were collected at 7 weeks after immunization. All immunized mice were challenged with RSV A2 strain (2×10^5 PFU) at 8 weeks after immunization. The individual lungs, spleens, and bronchoalveolar lavage fluid (BALF) samples were removed aseptically at day 5 post-challenge (p.c.), and lung homogenates were prepared as described (Kwon et al., 2014). All animal experiments presented in this study were approved by the Georgia State University IACUC review boards (IACUC A11026).

Assays for antibody responses and virus titration

RSV G protein-specific antibodies (IgG, IgG1, and IgG2a) were determined in samples by enzyme-linked immunosorbent assay (ELISA) as previously described (Kim et al., 2012). Briefly, the extracellular domain of RSV G protein with over 95% purity (200 ng/ml, Sino biological, Beijing, China) or inactivated influenza virus (4 μ g/ml) was used as a coating antigen. The wells were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with PBST containing 3% BSA for 2 h at 37 °C. Serially diluted serum samples were added and incubated for 1.5 h at 37 °C then horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, and IgG2a (Southern Biotechnology) were used as secondary antibodies. The tetramethylbenzidine (TMB) peroxidase substrate (Sigma-Aldrich, St. Louis, MO) was used to develop color and optical density was read at 450 nm. RSV-specific neutralizing antibody titers in mouse sera were measured by a slightly modified version of a standard method as described previously (Anderson et al., 1988). Briefly, mouse sera were heat-inactivated at 56 °C for 45 min and serially diluted two-fold in growth medium. Equal volumes of diluted sera were mixed with RSV A2 to yield 300 PFU/well. RSV with or without immune serum mixture was incubated at 33 °C, 5% CO₂ for 1 h before incubation in the HEp2 monolayers. The next steps were followed by an immunoplaque assay procedure as described (Quan et al., 2011). After fixing with ice-cold acetone-methanol and air drying, individual plaques were visualized using anti-RSV F monoclonal antibody (131-2A, Millipore), HRP conjugated anti-mouse IgG antibody, and 3,3'-diaminobenzidine tetrahydrochloride substrate (Invitrogen).

Analysis of cytokines

Cytokine levels in BALF were determined using ELISA kits for IL-5 (eBioscience) and eotaxin (R&D Systems, Minneapolis, MN) according to the manufacturers' instructions in duplicate against a standard curve.

Flow cytometric analysis

For analyzing phenotypes of cell population, BAL cells were collected and then stained with fluorochrome-conjugated antibodies (anti-CD3, CD45, CD11b, CD11c, and SiglecF antibodies) as described in previous studies (Lee et al., 2014). The lung tissues were homogenized and cells were then passed through strainer and spun on 44 and 67% Percoll gradients at 2800 rpm for 20 min. A band of cells was harvested and washed with PBS. To determine intracellular cytokine production, lung cells were stimulated with 5 μ g/ml of peptides corresponding to the CD4 T cell epitope G_{183–195} peptide (WAICKRIPNKKPG) in the presence of Brefeldin A (BFA) (20 μ g/ml) at 37 °C for 5 h. Then stimulated lung cells were surface stained using anti-CD45-peridinin chlorophyll protein complex, anti-CD4-allophycocyanin (APC) and anti-CD8 α -r-phycoerythrin (PE) antibodies and then were permeable by using the Cytofix/Cytoperm kit (BD Biosciences). Intracellular cytokines were revealed by staining the cells

with or anti-IL-4-fluorescein isothiocyanate or anti-IFN- γ -APC-Cy7 antibodies. All antibodies were purchased from eBiosciences or BD Bioscience. Stained BAL and lung cells were analyzed using LSRFortessa (BD Biosciences) and FlowJo software (Tree Star Inc.).

Lung histology of RSV-infected mice

For histological analysis, lung samples were fixed in 10% neutral buffered formalin for 48 h, transferred to 70% ethanol, embedded paraffin blocks, sectioned into a thickness of 5 μ m and stained with hematoxylin and eosin (H&E), periodic acid-Schiff stain (PAS) or hematoxylin and congo red (H&CR) as described (Meyerholz et al., 2009). At least eight sections per mouse were obtained for histopathologic analysis.

Statistical analysis

All results are expressed as the mean \pm standard error of the mean (SEM). Significant differences among treatments were evaluated by 1-way or 2-way ANOVA where appropriate. *p*-Values of less than or equal to 0.05 were considered statistically significant.

Results

Generation of recombinant influenza virus containing an RSV G protein conserved-domain

As a proof-of-concept study to explore whether a recombinant influenza virus carrying an RSV G conserved-central domain could provide protection against RSV, we used the PR8 influenza virus reverse genetics system. Two chimeric recombinant PR8/RSV viruses were generated, each containing the RSV G conserved-domain (aa131–230) but with different linkers (PR8/RSV.HA-G1 with a GGGGS linker, PR8/RSV.HA-G2 with AAAPGAA linkers at both conjugate sites, Fig. 1). For quantitative determination of G domain expression on recombinant influenza/RSV viruses, we compared PR8/RSV.HA-G1, -G2, parental PR8 WT, and RSV for its reactivity to G protein-specific monoclonal antibody 131-2G by ELISA (Fig. 1B and C). PR8/RSV.HA-G1 with a shorter linker was found to have 4-fold higher reactivity to 131-2G antibody than RSV. The reactivity of 131-2G antibody to PR8/RSV.HA-G2 was similar to that of RSV. These results suggest that recombinant influenza/RSV viruses contain RSV G domains at

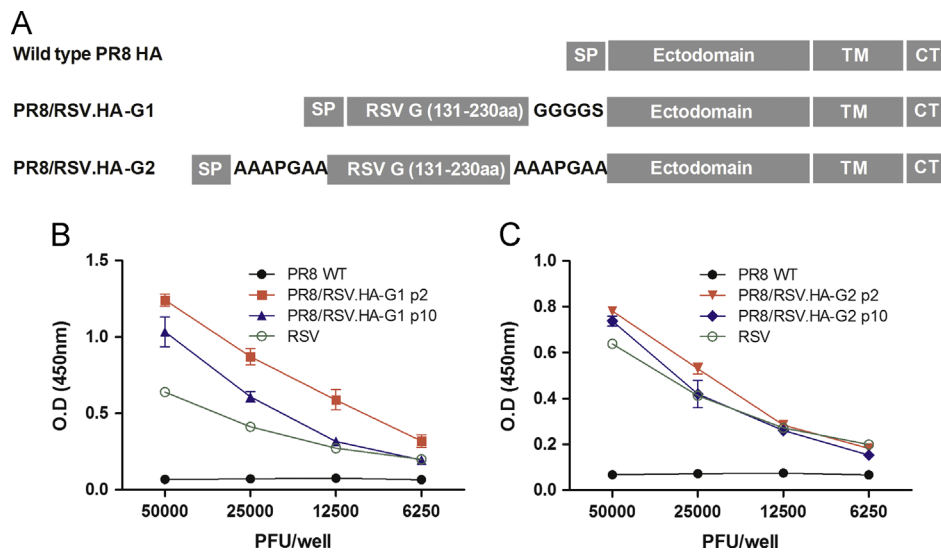


Fig. 1. Characterization of recombinant PR8/RSV.HA-G1 and PR8/RSV.HA-G2 viruses. (A) Schematic representation of WT and mutant HA-G constructs. (B) G protein reactivity to PR8 WT, PR8/RSV.HA-G1, and RSV by ELISA using G protein monoclonal antibody (131-2G) after serial passages. (C) G protein reactivity to PR8 WT, PR8/RSV.HA-G2, and RSV by ELISA using G protein monoclonal antibody (131-2G) after serial passages. The viruses were inactivated and then purified by using discontinuous sucrose gradient ultracentrifugation. CT, cytoplasmic tail; TM, transmembrane domain; WT; wild-type; p2, passage 2; p10, passage 10.

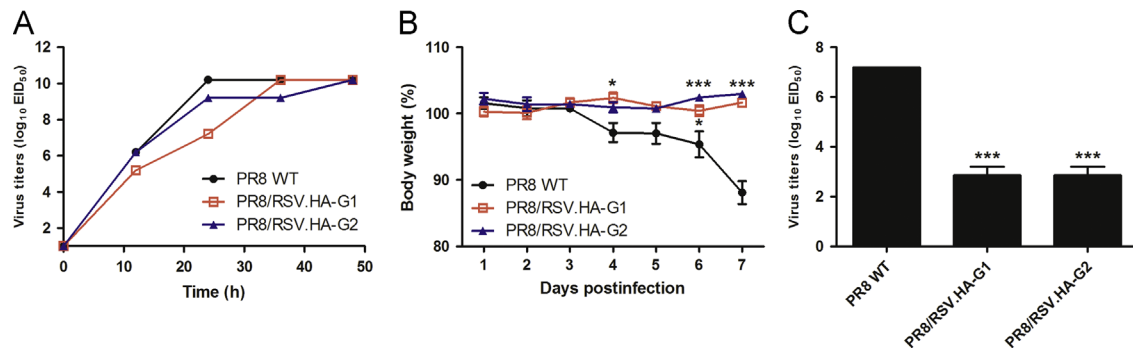


Fig. 2. Growth kinetics and pathogenicity of recombinant PR8/RSV.HA-G viruses. (A) *In vitro* growth kinetics. Eggs were infected at 15 EID₅₀ (50% egg infective dose) of PR8 WT, PR8/RSV.HA-G1 and PR8/RSV.HA-G2 virus. Samples were taken at 0, 12, 24, 36, and 48 h postinfection. The viral titer in the samples was determined by EID₅₀ assay. (B and C) Mice were inoculated intranasally with 1000 EID₅₀ of the PR8 WT and PR8/RSV.HA-G viruses. (B) Body weight changes were monitored daily for 7 days after inoculation. (C) Lung viral titers were determined by EID₅₀ assay at 7 days after inoculation. Statistically significance was determined by 1-way or 2-way ANOVA where appropriate. Asterisks indicate significant differences (**p* < 0.05 and ****p* < 0.001) compared with the results in the PR8 WT group.

higher or similar levels compared to those in WT RSV, which is important. In addition, the stability of the G domain expression in the PR8/RSV.HA-G viruses was ascertained by serially passing the virus in eggs (Fig. 1B and C). The reactivity of 131-2G antibody to PR8/RSV.HA-G viruses slightly decreased after 10 serial passages probably due to a fraction of virus that might not express a conjugate HA-G but the reactivity for RSV G domain contents was still higher than that of RSV.

Recombinant PR8/RSV.HA-G viruses show attenuated phenotypes

To determine *in vitro* viral growth kinetics, eggs were infected at a 15 EID₅₀ of PR8 WT, PR8/RSV.HA-G1 or PR8/RSV.HA-G2. At various times after infection, viral titers in allantoic fluids were quantified by EID₅₀ assay (Fig. 2A). The growth kinetics of PR8/RSV.HA-G1 or PR8/RSV.HA-G2 in eggs was found to be comparable to that of PR8 WT.

To compare replication and pathogenicity of PR8/RSV.HA-G and PR8 WT viruses, we infected BALB/c mice with 1000 EID₅₀ of each virus. Mice infected with PR8 WT virus showed a progressive weight loss over 12% from day 4 to day 7 post-infection. In contrast, mice inoculated with PR8/RSV.HA-G viruses did not display weight losses (Fig. 2B). The recombinant viral vaccine-inoculated mice showed significantly lower lung viral titers compared with those in PR8 WT-inoculated mice ($p < 0.001$, Fig. 2C). Despite similar growth kinetics in eggs, the recombinant PR8/RSV.HA-G viruses were attenuated in terms of viral replication and did not cause weight losses in mice compared to PR8 WT.

Inoculation with PR8/RSV.HA-G viruses induces RSV G specific antibody

BALB/c mice received a single intranasal administration with PR8/RSV.HA-G1, PR8/RSV.HA-G2, or PR8 WT at 500 EID₅₀ dose. Seven weeks after immunization, we measured serum antibody titers (Fig. 3). RSV G-specific IgG antibodies were detected at high levels in the live RSV group (331.3 ± 158.5 ng/ml). RSV G-specific IgG antibody concentrations in the PR8/RSV.HA-G1 (155.6 ± 95.4 ng/ml) and the PR8/RSV.HA-G2 group (90.4 ± 48.9 ng/ml) were approximately 4.6- and 2.5-fold higher than those in the FI-RSV group (33.8 ± 11.6 ng/ml) (Fig. 3A). Moreover, the PR8/RSV.HA-G1 and PR8/RSV.HA-G2 groups showed higher ratios of IgG2a/IgG1 isotype antibodies than the FI-RSV and live RSV group (Fig. 3B). The PR8/RSV.HA-G1 and PR8/RSV.HA-G2 groups showed RSV neutralizing antibody titers of 5.2 ± 0.73 log₂ and 5.8 ± 1.11 log₂, respectively. The FI-RSV and live RSV group showed lower RSV neutralizing titers of 4.3 ± 0.3 and 4.4 ± 0.2 log₂, respectively (Fig. 3C), but there were no significant differences among the groups. A partial domain (aa131–230) of RSV G protein in recombinant viruses might limit the levels of RSV G-specific IgG antibody responses in the PR8/RSV.HA-G groups compared to those in the live RSV group despite comparable RSV neutralizing activity. All mice immunized with recombinant or PR8 WT virus showed similar levels of PR8 virus specific-IgG antibodies (Fig. 3D). These results provide evidence that recombinant influenza/RSV viruses are able to raise RSV neutralizing antibody responses.

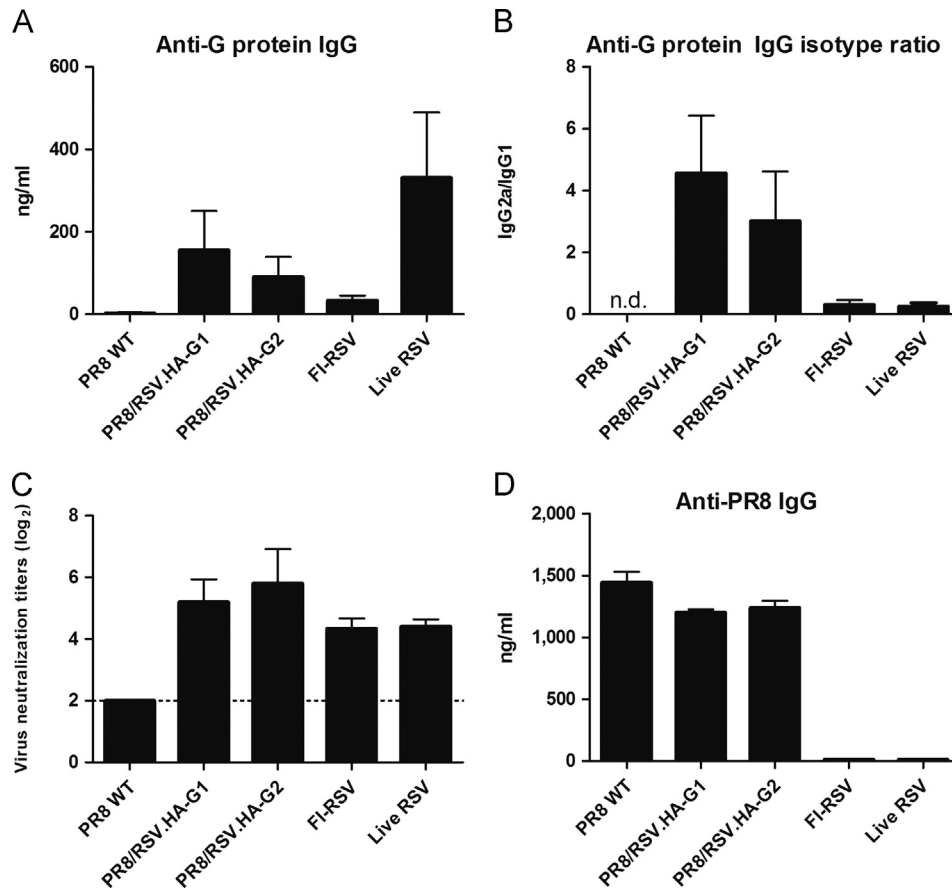


Fig. 3. Immunogenicity of recombinant PR8/RSV.HA-G1 and PR8/RSV.HA-G2 viruses. Mice were inoculated intranasally with PR8 WT, PR8/RSV.HA-G1 and PR8/RSV.HA-G2 virus (500 EID₅₀, $n = 5$). Serum samples were collected at 7 weeks after immunization. (A) IgG antibodies specific to G protein in serum. ELISA was performed for serum antibodies specific for G protein using serially diluted samples. (B) Ratios of IgG2a/IgG1 isotype antibodies specific for the G protein. (C) RSV neutralization titers. (D) IgG antibodies specific for inactivate PR8 virus. ELISA was performed for serum antibodies specific for PR8 virus. Error bars indicates mean \pm SEM. N.d., not determined.

Recombinant PR8/RSV.HA-G virus confers protection against RSV infection

To assess the protective efficacy of recombinant influenza/RSV vaccines, groups of mice were challenged with RSV A2 (2×10^5 PFU/mouse) at 8 weeks after immunization. PBS-immunized mice showed moderate weight loss ($\sim 4\%$) after RSV challenge. By contrast, weight loss was not observed with PR8/RSV.HA-G1, PR8/RSV.HA-G2, FI-RSV, or live RSV-immunized mice, improving clinical outcomes after RSV challenge (Data not shown). Clearance of lung viral loads is an important parameter in assessing the efficacy of protection against RSV infection. Lung tissues were collected from individual mice at day 5 p.c. and viral titers in lung samples were determined using an immunoplaque assay. The highest RSV titer was detected in PBS-immunized mice. FI-RSV, live RSV and recombinant PR8/RSV.HA-G-inoculated mice showed significantly lower lung RSV titers compared with those in PBS-immunized mice ($p < 0.001$, Fig. 4A).

To better understand mechanism of protection by recombinant viral RSV vaccines, RSV G-specific antibody and immune responses in BALF were determined at an early time post challenge. BALF samples from the groups of mice that were immunized with PR8/RSV.HA-G1 and PR8/RSV.HA-G2 showed higher levels of RSV G-specific IgG concentrations than those from PBS, PR8 WT, FI-RSV, and live RSV-immunized mice at day 5 p.c. (Fig. 4B). The levels of IL-5 (Fig. 4C) and eotaxin (Fig. 4D) in mice immunized with FI-RSV were significantly higher than those in the other groups.

To determine T cell responses, we measured IFN- γ or IL-4 cytokine-producing lung cells after *in vitro* stimulation with

G_{183–195} peptide (Fig. 5). FI-RSV immune mice showed the highest levels of IL-4-producing G_{183–195}-specific CD4⁺ T cells whereas the PR8/RSV.HA-G1 and PR8/RSV.HA-G2 groups did not induce RSV specific CD4⁺ T cell responses (Fig. 5A). IFN- γ -producing G_{183–195}-specific CD4⁺ T cells were not significantly induced in the recombinant PR8/RSV vaccine groups compared with the FI-RSV or PR8 WT group ($p < 0.001$, Fig. 5B). It is speculated that no induction of RSV-specific T cells might be due to the complete control of lung viral loads by RSV specific antibodies in this live RSV group.

PR8/RSV.HA-G virus does not cause pulmonary inflammation upon RSV infection

Eosinophils with the phenotypes of CD45⁺CD11c⁻CD11b⁺SiglecF⁺ are known to be enriched in inflamed lung tissues (Stevens et al., 2007). At day 5 p.c., the FI-RSV group prominently induced a population with CD45⁺CD11c⁻CD11b⁺SiglecF⁺ cells (Fig. 6A), which was approximately 73% out of the CD45⁺CD11c⁻granulocyte populations (Fig. 6B). Importantly, the group of mice immunized with PR8/RSV.HA-G1, PR8/RSV.HA-G2, PR8 WT, and live RSV did not show such a distinct population of CD11b⁺SiglecF⁺ cells (Fig. 6A and B). Moreover, there was significantly higher cellularity of infiltrating cells in BAL fluids from mice in the FI-RSV group compared to those from other groups (Fig. 6C).

Examination of pulmonary histopathology is important in assessing the safety of RSV vaccine candidates. Lung tissue sections in each group of mice were examined for inflammation at

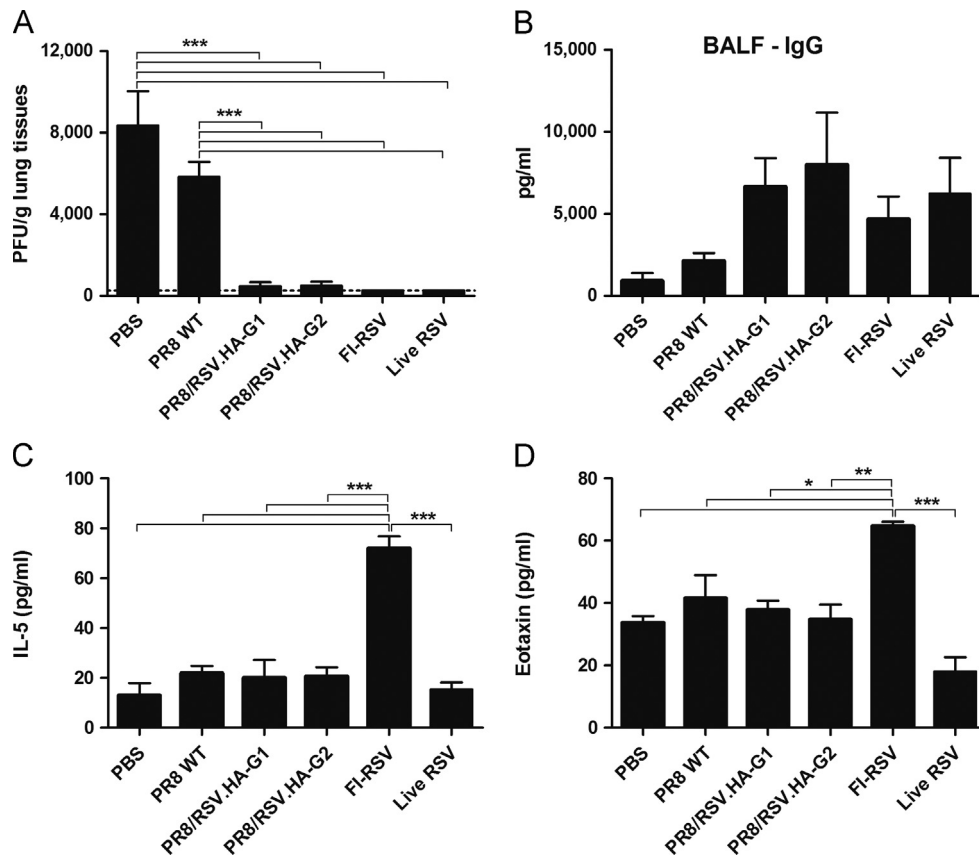


Fig. 4. Recombinant PR8/RSV.HA-G1 and PR8/RSV.HA-G2 confer protection against RSV. Mice that were inoculated intranasally with PR8 WT, PR8/RSV.HA-G1, or PR8/RSV.HA-G2 were challenged with RSV A2 ($n=5$). (A) Lung RSV titers were determined using an immunoplaque assay at day 5 p.c. (B) Levels of G-specific IgG antibodies were determined in BALF collected from immunized mice at day 5 p.c. with RSV. Antibody levels were determined by ELISA using the G protein as a coating antigen. IL-5 (C) and eotaxin (D) in BALF were determined from immunized mice at day 5 p.c. IL-5 and eotaxin were determined by cytokine ELISA kits. Data represent mean \pm SEM. Statistically significance was determined by 1-way ANOVA. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) compared with the results in the PBS and PR8 WT groups (A) or FI-RSV group (C, D).

day 5 p.c. (Fig. 7). FI-RSV-immunized mice displayed a massive influx of inflammatory cells around pulmonary airways and thickened alveolar layers, presenting heavily inflamed lung tissues. In contrast, lung tissues from mice immunized with PR8/RSV.HA-G1, PR8/RSV.HA-G2, or live RSV did not show an obvious sign of inflammation (Fig. 7A). Alveolar epithelium appeared to be normal in lung tissue histology from recombinant influenza/RSV or live

RSV-immunized mice. PBS or PR8 WT control mice infected with RSV showed a slight level of interstitial pneumonia as indicated by infiltrates of inflammatory cells and thickened alveolar layers in the H&E staining of lung tissue sections.

Lung sections were stained with PAS to visualize mucus production. Representative PAS-stained sections of lungs from the mice are shown in the middle row of Fig. 6A. These sections were scored for

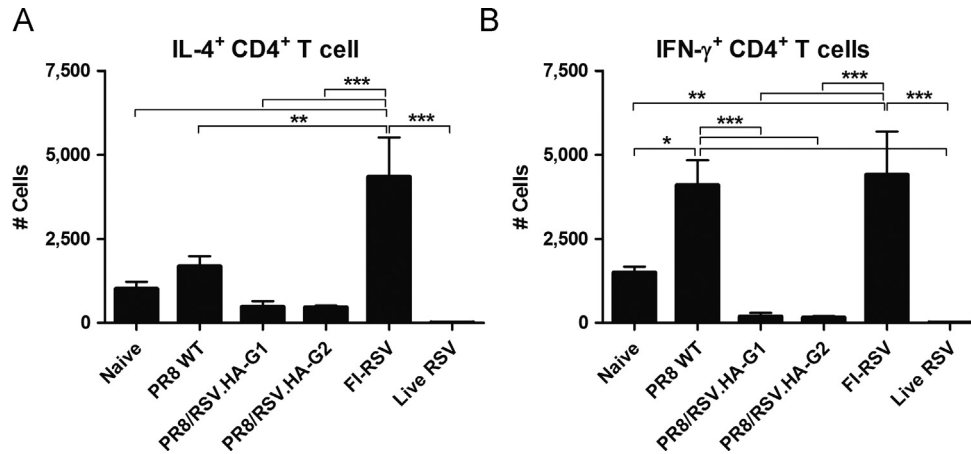


Fig. 5. Recombinant PR8/RSV.HA-G viruses do not induce RSV-specific T cell responses. Lung cells were harvested, stimulated with G_{183–195}, and stained with CD45, CD4, and CD8 α surface marker antibodies and intracellularly stained with IL-4 (A) or IFN- γ (B) antibodies, and then analyzed by flow cytometry. Data represent mean number plus SEM of IL-4- or IFN- γ -secreting CD4⁺ T cells per lung from five individual mice. Statistically significance was determined by 1-way ANOVA. Asterisks indicate significant differences ($p < 0.05$, $**p < 0.01$ and $***p < 0.001$) compared with the results in the FI-RSV.

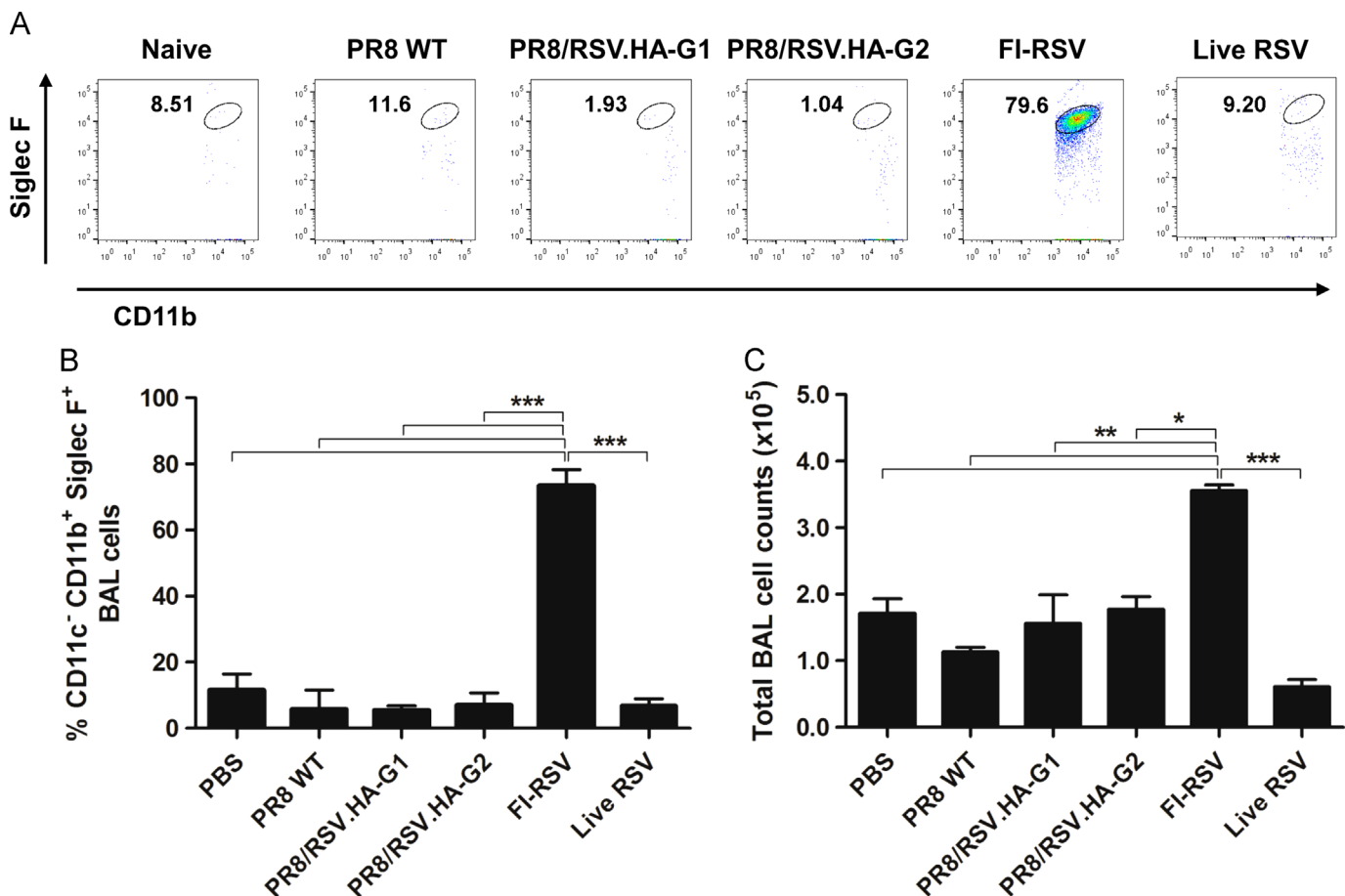


Fig. 6. PR8/RSV.HA-G1 and PR8/RSV.HA-G2 viruses do not induce eosinophil infiltration upon RSV challenge. Cells in BALF samples collected at 5 day p.c. were stained with anti-CD45, CD11b, CD11c, and Siglec-F antibodies. (A) Representative dot plots of CD11b⁺ Siglec F⁺ cells (eosinophils). Number in the dot plots indicates percentages among CD45⁺ CD11c⁻ granulocytes. (B) The mean percentage data are presented as mean \pm SEM. (C) Total BAL cell counts were determined. Data represent mean \pm SEM. Statistically significance was determined by 1-way ANOVA. Asterisks indicate significant differences ($p < 0.05$, $**p < 0.01$, and $***p < 0.001$) compared with the results in the FI-RSV group.

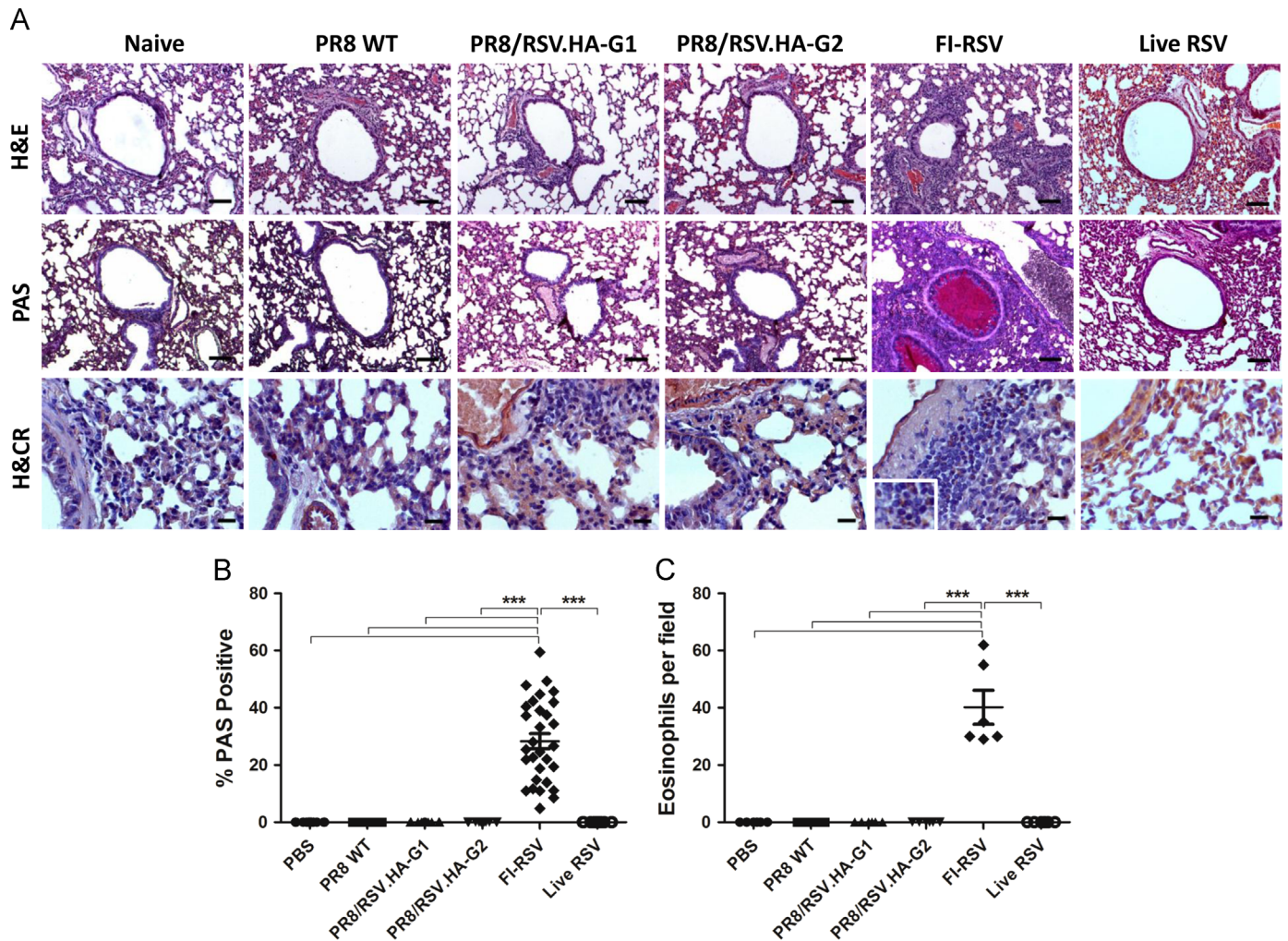


Fig. 7. Histopathological changes in lungs from mice after RSV challenge. (A) Photomicrographs of H&E, PAS, and H&CR stained lung tissue sections from mice at day 5 p.c. Scale bars for H&E and PAS indicate 100 μ m and for H&CR indicate 20 μ m. The insets in H&CR images are details of eosinophil infiltration in lungs. (B) Percentages of PAS positive staining. Tissue sections stained with PAS were scored as percentages of 10 individual airways in each mouse. Each symbol represents one airway. (C) Inflammation scores of H&CR staining. Pulmonary eosinophils per 40 \times field counts in two different regions of each mouse. Data represent mean \pm SEM. Statistically significance was determined by 1-way ANOVA. Asterisks indicate significant differences (***) ($p < 0.001$) compared with the results in the FI-RSV group.

percentages of airway linings showing PAS staining (Fig. 7B). The recombinant influenza/RSV immunized-mice showed significantly less PAS staining than the FI-RSV-immunized mice ($p < 0.001$). To estimate the degree of lung eosinophilia, we examined H&CR stained lung sections (bottom row, Fig. 7C). The accumulation of H&CR positive eosinophils was significantly greater in lungs of FI-RSV immunized-mice than in those of animals that were vaccinated with recombinant influenza/RSV.HA-G, PR8 WT or live RSV ($p < 0.001$).

Discussion

The majority of protective neutralizing antibodies generated by influenza vaccines recognizes the HA glycoprotein on the viral surface (Mozdzanowska et al., 2003). This study presents results supporting influenza virus HA as a possible carrier molecule for the delivery of protective antigens in a replicating viral vector. HA has also been reported to have adjuvant-like effects when co-administered with virus-like particles (Cox et al., 2004; Kang et al., 2004). Since the N-terminal regions of HA1 can differ in length, there may be sufficient structural flexibility in this HA location to accommodate extra polypeptide segments without interfering with HA receptor binding and fusion functions (Nobusawa et al., 1991). To

test this hypothesis, we molecularly designed and constructed new recombinant influenza viruses containing the conserved-domain of the RSV G protein at the N-terminus of influenza virus HA. We demonstrated that the conserved-domain of the RSV G protein can be incorporated as an insert into the HA protein without significant effects on HA immunogenicity (Fig. 3D) or replication (Fig. 2A). Despite the fact that recombinant viruses of influenza/RSV were moderately attenuated based on their low lung viral titers and no body weight changes in mice after inoculation, these recombinant vaccines were found to confer protection against influenza virus (data not shown). Our data provide proof-of-concept that these recombinant virus vaccines can be safe, immunogenic, and confer protection against influenza and RSV after a single mucosal delivery.

Purified full-length ectodomain G protein vaccines were demonstrated to induce Th2 type immune responses (Hancock et al., 1996; Johnson et al., 1998). Conjugate vaccines consisting of the G protein central domain (aa130–230) and the albumin-binding domain of streptococcal protein G (BBG2Na) were shown to be protective without obvious lung disease, but to induce undesirable IL-13-producing T cells and pulmonary eosinophilia (De Waal et al., 2004; Power et al., 2003; Siegrist et al., 1999). In contrast, our results demonstrated that recombinant influenza/RSV viral vaccines induced favorable Th1 type antibody responses. Therefore, a restricted domain of the RSV G

protein in a platform that presents this G fragment in a safe live vector may influence the pattern of host immune responses, which may confer protection against RSV without pulmonary RSV disease. In support of this concept, protection against RSV was provided by two immunizations with recombinant influenza virus harboring multiple RSV F and G-derived epitopes that were introduced into the non-structural (NS1) protein (Bian et al., 2014). Probably due to the nature of intracellular expression of RSV G and F epitopes together with NS1 genes, protective efficacy of single G or F epitope-containing recombinant influenza virus was reported to be low (Bian et al., 2014). Results in our study showed that G domains in recombinant PR8/RSV.HA-G viruses were incorporated into virions at a comparable or higher level as in RSV and a single dose may be sufficient to confer protection. Therefore, the insertion of the foreign proteins into the HA proteins is a promising vector platform for presenting viral vaccine antigens. It would be expected that recombinant influenza virus vaccines with RSV fusion (F) or G plus F epitopes in a form of HA conjugates might be even more effective in conferring protection against RSV.

Considering a previous report that immunization with recombinant vaccinia viruses expressing the full-length wild type or secreted RSV G protein caused severe RSV lung disease (Johnson et al., 1998), it is noticeable to underscore that PR8/RSV.HA-G conferred protection against RSV without undesirable lung disease. It was reported that ERD was associated with eosinophils' infiltration (Kim et al., 1969), elicitation of aberrant T cell responses (Castilow and Varga, 2008), and immune complex deposition in small airways (Polack et al., 2002). It is possible that induction of neutralizing antibodies without significant RSV-specific T cell responses by recombinant PR8/RSV.HA-G might have contributed to preventing RSV lung disease. In contrast, the FI-RSV group showed high levels of IL-5, eotaxin, and eosinophils as well as IL-4-producing T cells. Therefore, our strategy of developing recombinant influenza virus containing the conserved RSV G protein domain may be a promising approach to induce neutralizing antibodies against RSV, preventing the induction of undesirable cellular responses to RSV. Both recombinant PR8/RSV.HA-G1 and -G2 viruses were attenuated in mice as evidenced by low lung viral replication and no body weight loss. Nonetheless, we would not consider using the PR8 wild-type genetic backbone tested in this study for potential translation into humans. Live attenuated influenza vaccine (LAIV) has been approved in the United States, Canada, and Europe for vaccination against influenza (Moore, 2013; Scott et al., 2012). Therefore, testing chimeric HA-G and HA-F neutralizing epitope constructs in the backbone of LAIV should be an important objective for future studies.

In conclusion, recombinant influenza viruses containing the conserved domain of the RSV G protein were found to provide protection without the risk of potentially priming for enhanced disease in mice after RSV challenge. Recombinant PR8/RSV.HA-G vaccines elicited Th1-biased G-specific IgG2a antibodies and significantly reduced lung viral RSV replication. These findings in this study not only demonstrate the efficacy of recombinant influenza vaccines expressing RSV proteins, but may also become instrumental for improving the molecular approaches of developing bivalent or multivalent respiratory viral vaccines. Our results provide evidence for compelling proof-of-concept that recombinant influenza/RSV.HA-G viruses can be developed into a promising RSV vaccine candidate, which may combine an appropriate balance of inducing protective neutralizing antibodies and avoiding undesirable RSV cellular immune responses responsible for lung immunopathology.

Acknowledgments

This work was supported by NIH/NIAID grants AI105170 (S.M.K.) and AI093772 (S.M.K.).

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