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Recombinant Influenza Virus Expressing a Fusion Protein Neutralizing Epitope of Respiratory Syncytial Virus (RSV) Confers Protection without Vaccine-Enhanced RSV Disease

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Abstract

Respiratory syncytial virus (RSV) is the leading cause of viral bronchiolitis in both children and the elderly. There is no vaccine available for the prevention of RSV infection. Here, we generated recombinant influenza virus (PR8/RSV.HA-F) expressing an RSV $F_{243–294}$ neutralizing epitope in the hemagglutinin (HA) as a chimeric protein. Neutralizing antibodies specific for both RSV and influenza virus were induced by a single intranasal immunization of mice with PR8/RSV.HA-F. Mice that were immunized with PR8/RSV.HA-F were protected against RSV infection comparable with live RSV as evidenced by significant reduction of RSV lung viral loads, as well as the absence of lung eosinophilia and RSV-specific cellular immune responses. In contrast, formalin-inactivated RSV-immunized mice showed severe disease and high cellular immune responses in lungs after RSV infection. These findings support a concept that recombinant influenza virus carrying the RSV $F_{243–294}$ neutralizing epitope can be developed as a promising RSV vaccine candidate which induces protective neutralizing antibodies but avoids lung immunopathology.

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Keywords

Influenza virus; respiratory syncytial virus; recombinant; viral vector; F protein; neutralizing epitope vaccine

1. Introduction

Respiratory syncytial virus (RSV) is the leading cause of viral bronchiolitis in infants and young children but also significant health problem in the elderly and immunocompromised individuals (Falsey et al., 2005; Nair et al., 2010). In early clinical trials, vaccination of infants with formalin-inactivated RSV (FI-RSV) formulated with alum resulted in enhanced susceptibility to develop severe pulmonary disease upon subsequent RSV infection (Kim et al., 1969). Replicating vaccinia virus-vectored vaccines expressing the full-length RSV attachment (G) or fusion (F) proteins have been tested but may have safety concerns (Castilow et al., 2008).

The F protein of RSV is highly conserved among RSV strains. Since RSV F protein contains neutralizing epitopes such as antigenic site II (aa 255–275) and IV (aa 422–438) (Arbiza et al., 1992), it is an attractive target as potential RSV vaccines. However, subsequent studies have shown that purified F protein vaccination with alum adjuvant also leads to vaccine-enhanced respiratory disease in cotton rats and mice (Murphy et al., 1990; Vaux-Peretz et al., 1992). In contrast, passive transfer of monoclonal antibodies (palivizumab, motavizumab) recognizing epitopes in the antigenic site II of F suppresses RSV replication *in vivo* and protects against RSV in cotton rats (Wu et al., 2007). Palivizumab, a humanized monoclonal antibody specific for RSV F, has been shown to provide significant prophylactic protection in high-risk infants (Carbonell-Estrany et al., 2010; IMpact-RSV Study Group, 1998). Due to the high cost of antibody prophylaxis, guidelines restrict recommendations for its use to the highest risk subgroups of infants.

Influenza vaccines in a live attenuated viral platform have been safely used in humans for many years. Influenza virus can be an interesting vaccine vector due to its protective immune responses (Kreijtz et al., 2011) and the availability of a reverse genetics system that allows the expression of foreign genes (Hoffmann et al., 2000). Here, as a proof-of-concept, we examined a recombinant influenza virus as a live viral vector for mucosal delivery of the antigenic site II of the RSV F protein. We produced recombinant influenza virus carrying the RSV $F_{243–294}$ neutralizing epitope in the hemagglutinin (HA) and tested its protective efficacy against RSV and safety in comparison with FI-RSV and live RSV.

2. Materials and methods

2.1. Construction of PR8/RSV.HA-F

Cells and viruses including influenza virus A/PR/8/1934 (H1N1, abbreviated PR8) virus and FI-RSV are described in detail in the supplementary information. Recombinant viruses were rescued using the pHW2000-based eight-plasmid system (kindly provided by R.G. Webster, St. Jude Children's Research Hospital, Memphis, TN) as described by Hoffmann et al. (Hoffmann et al., 2000). The RSV $F_{727–882}$ nucleotide fragment (Genbank accession number

FJ614814) was ligated between the 3′ end of the HA signal peptide and the nucleotide encoding the N-terminal domain of the HA1 ectodomain of pHW2000-HA plasmid using a strategy similar to that described by Li et al. (Li et al., 2005). The inserted sequence was followed by an AAAPGAA peptide linker helping to facilitate the proper folding of the inserted fragment as an independent domain (HA-F, Fig. 1A).

To generate recombinant virus PR8/RSV.HA-F, 293T cells were cotransfected with the chimeric HA-F (Fig. 1A) gene along with the remaining gene segments derived from the PR8 strain. After 48 h post-transfection, the supernatant was harvested and then inoculated into embryonated chicken eggs. After 72 h post-inoculation, the presence of the rescued recombinant virus was confirmed by hemagglutination of chicken red blood cells. Characterization of the PR8/RSV.HA-F virus was performed by western blot using mouse anti-HA monoclonal antibody IC5-4F8 (BEI resources, Manassas, VA) and palivizumab (MedImmune, Gaithersburg, MD).

2.2. Immunizations and RSV challenge of mice

For animal experiments, 6- to 8-week-old female BALB/c mice (n = 5; Harlan Laboratories) were intranasally immunized with 500 EID_{50} dose (50% egg infective dose, EID_{50}) of PR8/RSV.HA-F and PR8 wild-type (PR8 WT) or 2×10^5 PFU of RSV A2 strain or phosphate-buffered saline (PBS) under isoflurane anesthesia. The FI-RSV control group was intramuscularly immunized with 50μl of FI-RSV (2μg) precipitated with aluminium hydroxide adjuvant (2 mg/ml) (Prince et al., 2001). Blood samples were collected at 7 weeks after immunization. Immunized mice were challenged with RSV A2 strain (2×10^5 PFU) or a lethal dose ($2xLD_{50}$) of PR8 influenza virus at 8 weeks after immunization. The individual lungs and bronchoalveolar lavage fluid (BALF) samples were collected 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).

2.3. Pulmonary histology of RSV-infected mice

Detailed assays including virus titration, assays for antibody responses, cytokine ELISA, flow cytometry, and statistical analysis are provided in the supplementary materials. For histological analysis of lung tissues, the lungs were fixed in 10% neutral buffered formalin for 24 hrs, transferred to 70% ethanol, embedded in paraffin, sectioned into a thickness of 5 μm and stained with hematoxylin and eosin (H&E), periodic acid-Schiff stain (PAS) or hematoxylin and congo red (H&CR) (Meyerholz et al., 2009). At least ten sections per mouse were obtained for histopathologic analysis. For numerical assessment of histopathology and pneumonia in lung tissues, the bronchioles, vessels and interstitial space were initially scored on a scale of 0 to 3 by blinded observers using a previously described severity scoring system (Meyerholz et al., 2009).

3. Results

3.1. Generation of recombinant influenza virus containing an RSV F neutralizing epitope

As a proof-of-concept, we used the PR8 influenza virus reverse genetics system to explore whether a recombinant influenza virus carrying an RSV F neutralizing epitope could provide protection against RSV. The N-terminus of HA was reported to be a site where relatively long foreign gene segments could be inserted without interfering with the biological function of HA (Hatziioannou et al., 1999). The RSV F domain of amino acids 243–294 ($F_{243–294}$) selected in this study contains the well-characterized RSV F neutralizing epitope amino acids 255 to 275, the antigenic site II of F, which is recognized by palivizumab (Synagis) (McLellan et al., 2011). Since the $F_{225–275}$ epitope has a linear conformation, we propose that a longer length of F fragment might be effective in forming a native-like structure of F epitopes present in RSV. A chimeric recombinant influenza virus containing the RSV $F_{243–294}$ domain in the N-terminal HA after the signal peptide was generated (PR8/RSV.HA-F, Fig. 1). The expression of the chimeric HA-F protein in recombinant PR8/RSV was observed in a slightly lifted position by western blot when probed either by HA specific monoclonal antibody or RSV F epitope specific monoclonal antibody palivizumab (Fig. 1B).

To determine *in vitro* viral growth kinetics, eggs were infected at a 15 EID_{50} of PR8 WT or PR8/RSV.HA-F. The viral titers in allantoic fluids were quantified by an egg infectious dose (EID_{50}) assay at various times after infection (Fig. 1C). The growth kinetics of PR8/RSV.HA-F resembled that of PR8 WT virus. Moreover, PR8/RSV.HA-F maintained the chimeric HA-F expression over multiple passages, indicating its genetic stability (data not shown).

To compare the pathogenicity of PR8/RSV.HA-F virus and PR8 WT, we infected mice with 1,000 EID_{50} of each virus. PR8/RSV.HA-F virus caused slightly less morbidity than the PR8 WT virus (Fig. 1D). The lung viral titers of the PR8/RSV.HA-F group were 21.5-fold lower than those in the PR8 WT group, but there was no significant difference between groups (Fig. 1E). In contrast to similar growth kinetics in eggs, a recombinant PR8/RSV.HA-F virus was slightly less pathogenic than PR8 WT virus in mice.

3.2. PR8/RSV.HA-F virus induces RSV neutralizing antibodies

For immunization studies, we chose a dose of 500 EID_{50} that was found to induce immune responses but did not cause weight loss or disease symptoms (data not shown). Immunogenicity of recombinant PR8/RSV.HA-F virus was determined in mice that received a single intranasal immunization with PR8/RSV.HA-F or PR8 WT. Influenza virus and RSV-specific antibody responses were measured using HI and neutralization assays at 7 weeks after immunization (Fig. 2). PR8 WT and PR8/RSV.HA-F groups showed high titers of HI activity up to 7.6 ± 0.2 log2 and 6.8 ± 0.4, respectively (Fig. 2A). Influenza virus neutralizing titers in sera from PR8 WT and PR8/RSV.HA-F groups were significantly higher than those from the FI-RSV and live RSV groups. Also, mice that were inoculated with PR8 WT or PR8/RSV.HA-F virus were well protected against influenza virus lethal infection (Supplementary Fig. S1).

Moreover, PR8/RSV.HA-F showed high neutralizing antibody titers of 5.0 ± 0.7 $\log_2$ against RSV similar to that of live RSV infection or FI-RSV immunization sera (Fig. 2B). There were no significant differences in RSV neutralizing titers between the recombinant PR8/RSV.HA-F and FI-RSV-immunized groups.

3.3. Recombinant PR8/RSV.HA-F virus confers protection against RSV

To assess the protective efficacy of recombinant PR8/RSV.HA-F vaccine, groups of mice were challenged with RSV A2 ($2x10^5$ PFU/mouse) at 8 weeks after immunization. We chose a dose of $2x10^5$ PFU that was recently reported to be sufficient to assess the efficacy of RSV vaccines (Garg et al., 2014; Johnson et al., 2014; Kim et al., 2014; Murata and Catherman, 2012; Nguyen et al., 2012; Schmidt et al., 2012). We also found that the PBS or PR8 WT group of mice that were infected with $2x10^5$ PFU RSV showed a high titer of approximately up to 10^4 PFU from lungs at day 5 post infection (Fig. 3A). Groups of mice that were intranasally inoculated with recombinant PR8/RSV.HA-F or live RSV or intramuscularly immunized with FI-RSV displayed significantly lower lung RSV titers compared with those in PBS-immunized mice ($p < 0.001$, Fig. 3A). Cytokine and chemokine levels in BALF were determined at day 5 p.c.. The levels of IL-5 (Fig. 3B) and eotaxin (Fig. 3C) in mice immunized with FI-RSV were significantly higher than those in the PR8/RSV.HA-F or other groups.

To determine T cell responses, we measured IFN-γ or IL-4 cytokine-producing lung cells after *in vitro* stimulation with $G_{183–195}$ and $F_{85–93}$ peptide by intracellular cytokine flow cytometry analysis (Fig. 4). Immunization with PR8/RSV.HA-F did not induce $G_{183–195}$-specific IL-4-producing $CD4^+$ T cells at a substantial level in contrast to the FI-RSV group that showed high levels of IL-4-producing $CD4^+$ T cells ($p < 0.001$, Fig. 4A). Furthermore, IFN-γ-producing $F_{85–93}$-specific $CD8^+$ T cells were also low in the PR8/RSV.HA-F group (Fig. 4B). It was reported that IFN-γ-producing F-specific $CD8^+$ T cells were induced in the lungs from PBS-immunized (placebo) mice after RSV infection (De Baets et al., 2013; Garg et al., 2014; Johnson et al., 2013). Concordant with these findings, PBS, PR8 WT, and FI-RSV groups showed small numbers of IFN-γ-producing $F_{85–93}$-specific $CD8^+$ T cells compared to IL-4-producing $CD4^+$ T cells, and there was no significant difference among the groups. Also, the live RSV group did not show significant levels of IL-4- or IFN-γ-producing T cell responses (data not shown).

3.4. PR8/RSV.HA-F immunization does not induce eosinophil infiltration upon RSV infection

Eosinophils are known to have the phenotypes of $CD45^+CD11c^-CD11b^+$Siglec F^+ in inflammatory tissues (Stevens et al., 2007). At day 5 p.c., eosinophils were markedly enhanced in brochoalveolar airway fluids from the FI-RSV group (Fig. 5A and 5B). However, the group of PR8/RSV.HA-F, PR8 WT, or live RSV mice did not show such a distinct population of $CD11b^+SiglecF^+$ cells in BAL fluids (Fig. 5A and 5B). Moreover, cellularity of infiltrating cells in BAL fluids was significantly higher from mice in the FI-RSV group compared to those from recombinant PR8/RSV.HA-F or other groups (Fig. 5C).

3.5. Recombinant PR8/RSV.HA-F virus does not cause pulmonary RSV disease

Pulmonary histopathology is an important parameter for assessing the safety of RSV vaccine candidates in preclinical studies. Lung samples from FI-RSV immunized mice showed a massive influx of inflammatory cells around pulmonary airways (pathology score 1.5, Fig. 6A, 6B), blood vessels (pathology score 2.3, Fig. 6A, 6C), and in the peribronchial and perivasicular spaces (pathology score 0.8, Fig. 6A, 6D) as well as epithelial cell thickening of airway linings. Thus, despite lung viral control, FI-RSV immunization of mice induced severe inflammatory histopathology of lungs upon RSV infection. In contrast, lung tissues from the mice immunized with PR8/RSV.HA-F and live RSV did not show overt inflammation. Alveolar epithelium appeared to be normal in lung tissue histology from mice that were immunized with PR8/RSV.HA-F. PBS-immunized RSV-challenged mice showed a low level of cellular infiltration in the interstitial area (Fig. 6D), as previously reported (Blanco et al., 2014; Castilow and Varga, 2008; Cherukuri et al., 2012; Smith et al., 2012).

Mucus production is another characteristic observed in severe inflammatory RSV lung disease, which can be detected by PAS staining (Jafri et al., 2004). To visualize mucus-associated carbohydrate materials, lung sections from mice were stained with PAS (Fig. 7A). Airway linings showing PAS staining were presented by quantitative scores (Fig. 7B). PR8/RSV.HA-F-immunized mice showed no PAS stained-linings along the airways (Fig. 7B).

To estimate the degree of lung eosinophilia, H&CR-stained eosinophils were examined in the lung sections (lower row, Fig. 7A). The accumulation of H&CR positive eosinophils was significantly high in lungs of FI-RSV immunized-mice but was not observed in the lung tissue sections from mice that were vaccinated with the recombinant PR8/RSV.HA-F or control PR8 WT or live RSV ($p < 0.001$, Fig. 7C).

4. Discussion

Over the past decade, a live-attenuated virus platform is considered one of the most promising RSV vaccine candidates. Live-attenuated vaccines can be administered intranasally, which offers several benefits such as induction of local mucosal immunity and needle-free administration. However, studies with live cold-passaged, temperature-sensitive RSV vaccine candidates that were primarily targeted for use in young infants were shown to induce inadequate protection, being under- or over-attenuated in a variety of animal models (Crowe et al., 1994; Crowe et al., 1993; Piedra et al., 1996) or infants or sero-negative children (Karron et al., 2005; Wright et al., 2006). Other RSV vaccine approaches include recombinant viral vectors, including vaccinia virus (Olmsted et al., 1986; Wyatt et al., 1999), bovine/human parainfluenza virus type 3 (Haller et al., 2003; Liang et al., 2014), and adenovirus (Hsu et al., 1992). Although some of these vaccines showed promising preclinical data, no vaccine has been licensed for human use due to safety concerns and lack of efficacy data. Since influenza vaccines have an extensive safety record in the human population, influenza viruses are now considered potential viral vectors. RSV F protein epitopes as neutralizing antibody targets are supported by studies examining the efficacy of palivizumab, which is a humanized monoclonal antibody specific for the antigenic site II of the F protein (Huang et al., 2010). In this study, we designed and constructed a new

chimeric recombinant influenza virus with the RSV $F_{243–294}$ domain containing the palivizumab-recognized neutralizing epitope. We demonstrated that the RSV $F_{243–294}$ neutralizing epitope can be incorporated as an insert into the N-terminus of influenza virus HA without detrimental effects on HA function and immunogenicity (Figs. 1–2). Results in this study provide a proof-of-concept of developing a safe RSV vaccine based on utilizing recombinant influenza virus as a vaccine vector with an evidence to avoid pulmonary RSV disease after a single mucosal delivery. Therefore, testing chimeric HA-F construct in the backbone of attenuated influenza vaccines should be an important objective of future studies.

Purified full-length RSV F protein vaccines in alum adjuvant were demonstrated to augment the induction of RSV lung disease and Th2 type immune responses similar to FI-RSV upon RSV challenge despite their effective control of lung viral replication (Murphy et al., 1990). In contrast, our results demonstrated that recombinant influenza/RSV.HA-F did not cause inflammatory cytokine IL-5 and chemokine eotaxin secretion in the airways (BALF). It is speculated that a restricted domain containing RSV neutralizing epitopes in a safe live vector may confer protective immune responses without inflammatory viral disease. In support of this concept, protection against RSV was reported by two immunizations with recombinant influenza virus harboring multiple RSV F and G-derived epitopes that were introduced into the nonstructural (NS1) protein (Bian et al., 2014). Probably due to the nature of intracellular expression of RSV G and F epitopes under the NS1 genes, protection by single G or F epitope-containing recombinant influenza virus was low or not effective even after prime-boost immunizations (Bian et al., 2014). In our study, a single immunization of chimeric PR8/RSV.HA-F virus may be sufficient to induce protective immunity to RSV, probably because of expression in a chimeric HA-F protein. Thus, expression of foreign protective epitopes on the surfaces of recombinant influenza viruses in an HA chimeric form would be superior to intracellular expression in conferring protection. In addition, 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). Therefore, recombinant influenza virus with RSV protective epitopes may be an approach to develop a safe RSV vaccine.

It is important to note that PR8/RSV.HA-F conferred protection against RSV without a sign of lung disease. Vaccine-enhanced respiratory disease was suggested to be associated with elicitation of aberrant T cell responses (Castilow and Varga, 2008) and eosinophilic infiltration (Kim et al., 1969). Therefore, induction of a balanced and controlled immune response will be critical for a safe vaccine that would not cause disease upon RSV infection. In this study, the FI-RSV group induced high levels of IL-5, eotaxin, and eosinophils as well as both IFN-γ- and IL-4-producing T cells. It would be desirable to induce neutralizing antibodies without substantial T cell responses that might be responsible for Th2 cytokines and infiltrating granulocytes such as eosinophils contributing to RSV lung disease. A strategy of developing recombinant influenza virus containing the RSV $F_{243–294}$ neutralizing epitope may be an alternative approach to induce neutralizing antibodies against RSV, without inducing cellular responses responsible for RSV disease.

In conclusion, a recombinant influenza virus containing an RSV F neutralizing epitope was found to confer protection against RSV infection without the risk of potentially priming for enhanced disease in mice. These findings in this study not only demonstrate potential promising efficacy of recombinant influenza vaccines expressing an RSV F epitope, but may also become instrumental for improving the design of bivalent or multivalent respiratory vaccines. This study provides evidence for a safe RSV vaccine which would induce protective neutralizing antibodies but not prime lung immunopathology of RSV disease. Overall, our results confer a compelling proof-of-concept that recombinant influenza/RSV may be developed into a promising RSV vaccine candidate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research highlights

- Recombinant PR8/RSV.HA-F virus induces RSV neutralizing antibodies.
- PR8/RSV.HA-F virus was effective in conferring protection against RSV.
- PR8/RSV.HA-F virus did not cause vaccine-enhanced RSV histopathology.

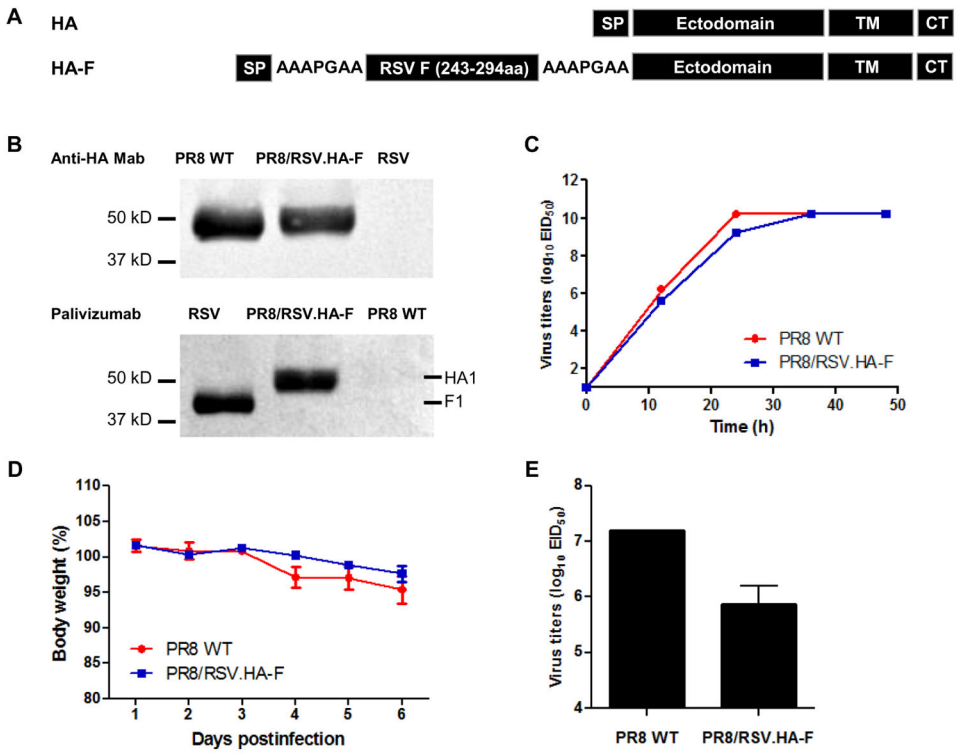

Fig. 1. Characterization of recombinant PR8/RSV.HA-F virus *in vitro* and *in vivo*

(A) Schematic representation of WT HA and mutant HA-F constructs. (B) Western blot of PR8 WT virus, recombinant PR8/RSV.HA-F, and RSV using mouse anti-HA monoclonal antibody (IC5-4F8) or palivizumab under reducing conditions. (C) *In vitro* growth kinetics. Eggs were infected with a 15 EID_{50} (50% egg infectious dose) of PR8 WT and PR8/RSV.HA-F virus. Samples were taken at 0, 12, 24, 36, and 48 h post-infection. The viral titer in the samples was determined by EID_{50} assay. (D–E) Mice were inoculated intranasally with 1,000 EID_{50} of the PR8 WT and PR8/RSV.HA-F virus. (D) Body weight changes were monitored daily for 6 days after inoculation. (E) Lung viral titers were determined by EID_{50} assay at 6 days after inoculation. CT, cytoplasmic tail; TM, transmembrane domain. WT; wild-type.

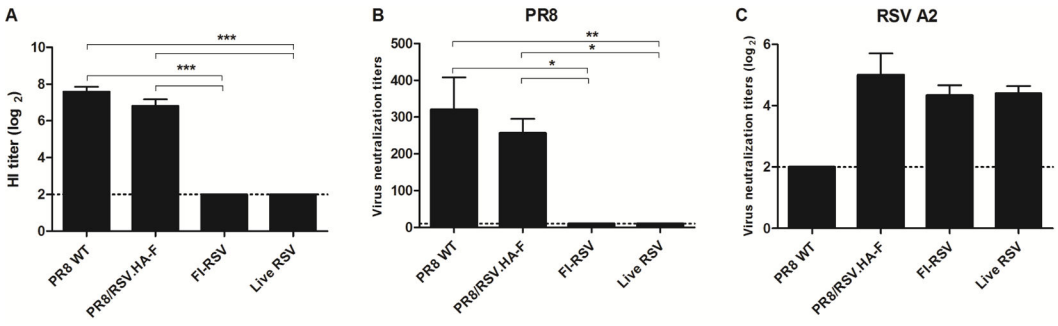

Fig. 2. Immunogenicity of recombinant PR8/RSV.HA-F virus in mice

Mice were inoculated intranasally with 500 EID_{50} of the PR8 WT and PR8/RSV.HA-F virus or 2×10^5 PFU of RSV A2 strain. Serum samples were collected at 7 weeks after immunization. (A) Hemagglutination inhibition (HI) titers. HI titers were determined by standard methods using 4 HA units of inactivated A/PR8 virus and 0.5% chicken erythrocyte suspension. (B) Serum neutralizing titers against A/PR8 virus. (C) RSV neutralization titers. Error bars indicate mean ± SEM.

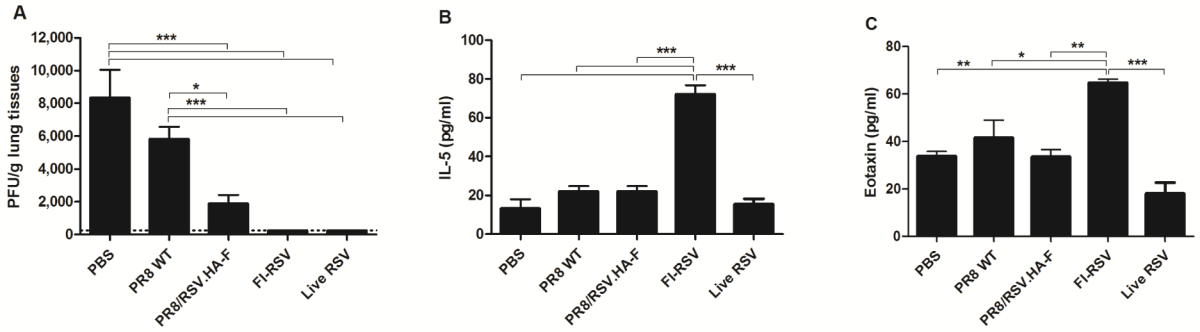

Fig. 3. Recombinant PR8/RSV.HA-F confers protection against RSV

Mice that were inoculated intranasally with PR8 WT or PR8/RSV.HA-F or 2×10^5 PFU of RSV A2 strain or PBS were challenged with RSV A2. (A) Lung RSV titers were determined by an immune-plaque assay at 5 days after RSV challenge. IL-5 (B) and eotaxin (C) were determined in BALF from immunized mice at day 5 p.c.. IL-5 and eotaxin was determined by a cytokine ELISA. Data represent mean ± SEM.

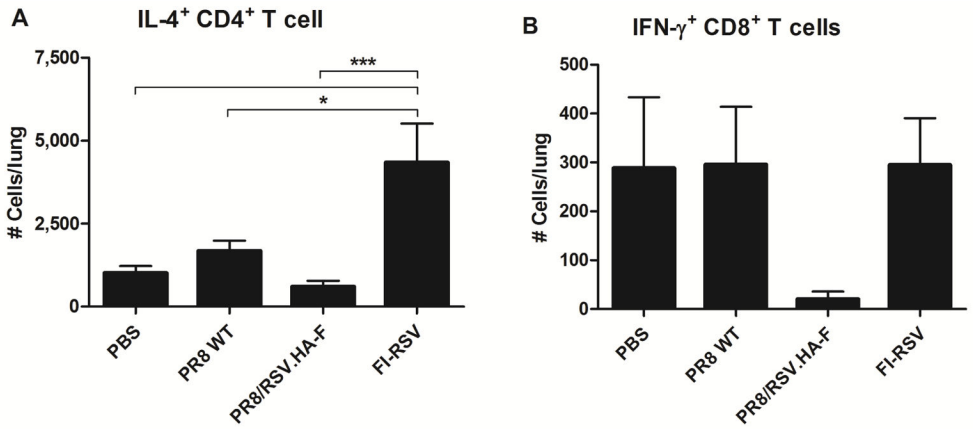

Fig. 4. Recombinant PR8/RSV.HA-F virus does not induce RSV-specific T cell responses

IL-4- (A) secreting $CD4^+$ or IFN-γ– (B) secreting $CD8^+$ T cells in lungs. Mice that were inoculated intranasally with PR8 WT or PR8/RSV.HA-F or 2×10^5 PFU of RSV A2 strain or PBS were challenged with RSV A2. At 5 day p.c., lung cells were harvested, stimulated with $G_{183–195}$ and $F_{85–93}$ peptides, and stained with CD45, CD4, and CD8α surface marker antibodies and intracellularly stained with cytokine IFN-γ and IL-4 antibodies, and then analyzed by flow cytometry. Data represent mean number plus SEM of IL-4-secreting $CD4^+$ or IFN-γ–secreting $CD8^+$ T cells per lung of a mouse (n = 5).

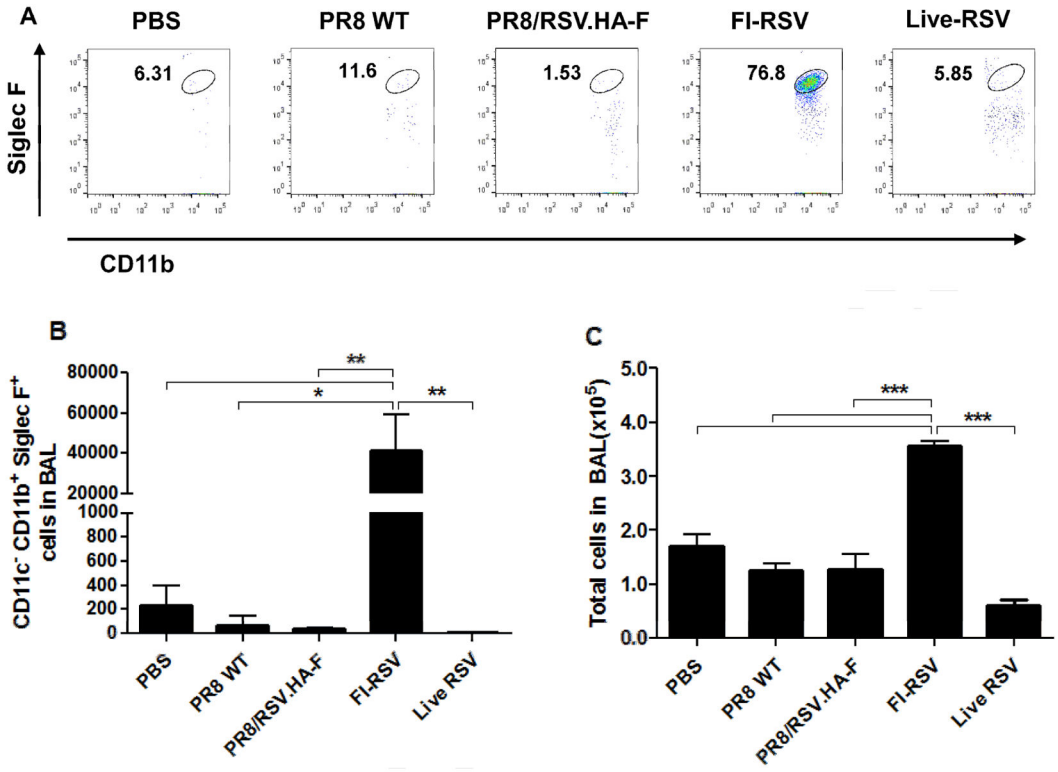

Fig. 5. PR8/RSV.HA-F virus does not cause eosinophil infiltration upon RSV challenge

Cells in BALF samples collected at 5 day p.c. were stained with anti-CD45, CD11b, CD11c, and Siglec-F. (A) Representative dot plots of $CD11b^{+}SiglecF^{+}$ cells (eosinophils). Number in the dot plots indicates percentages among $CD45^{+}$ $CD11c^{-}$ granulocytes. (B) $CD11c^{-}CD11b^{+}SiglecF^{+}$ cell counts were determined. (C) Total BAL cell counts were determined. Data represent mean ± SEM.

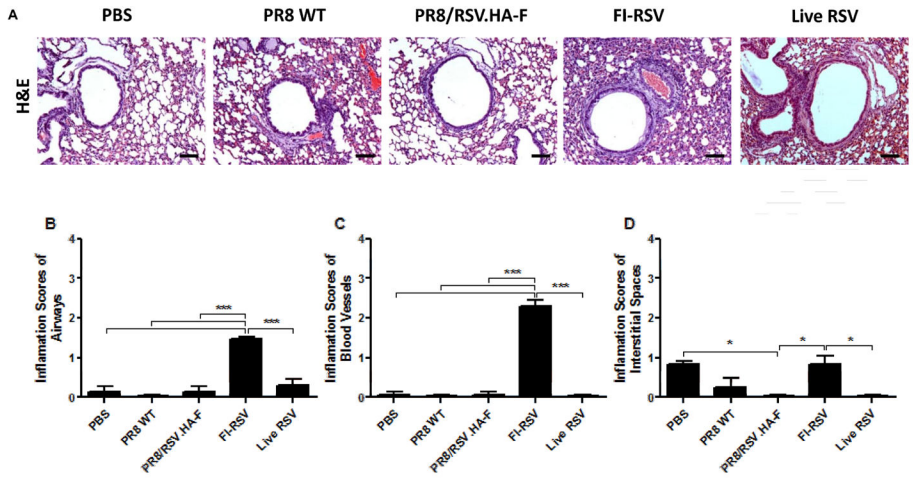

Fig. 6. Histopathological changes in lungs from mice after RSV challenge

(A) Photomicrographs of H&E stained lung tissue sections from mice at day 5 p.c.. Scale bars for H&E indicate 100 μm. (B–D) H&E stained tissue sections from each mouse were scored for inflammation on a scale of 0 to 3 as diagnostic criteria. (B) Inflammation scores around airways. (C) Inflammation scores around blood vessels. (D) Inflammation scores around interstitial spaces. Data represent mean ± SEM.

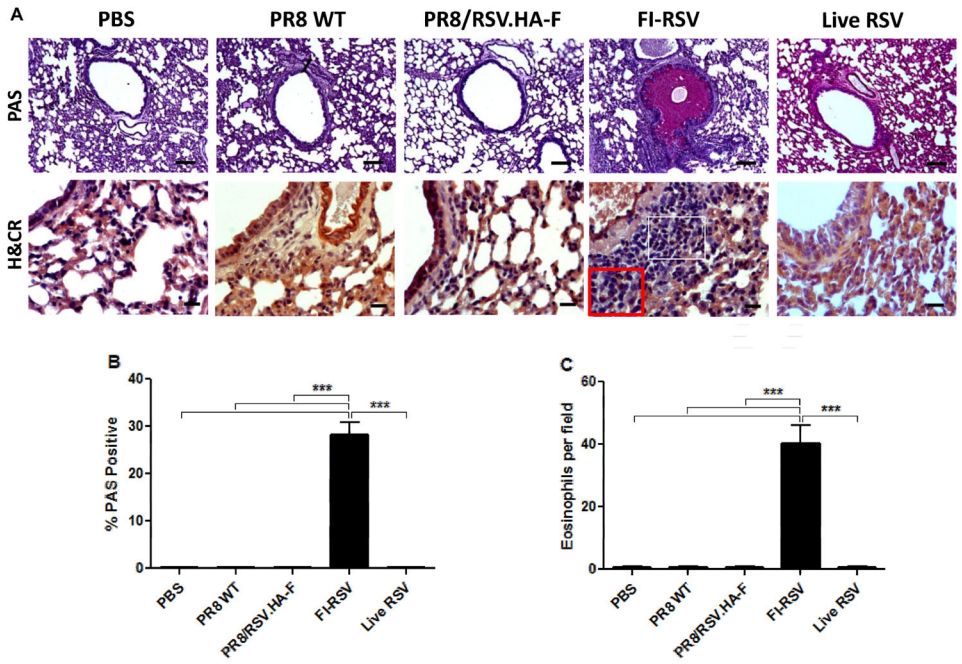

Fig. 7. PAS and H&CR staining of lungs from mice after RSV challenge

(A) Photomicrographs of PAS, and H&CR stained lung tissue sections from mice at day 5 p.c.. Scale bars for PAS indicate 100 μm and for H&CR indicate 20 μm. The insets in H&CR images are details of eosinophil infiltration in lungs. (B) Inflammation scores of PAS 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× field counts in two different regions of each mouse. Data represent mean ± SEM.