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Co-immunization with virus-like particle and DNA vaccines induces protection against respiratory syncytial virus infection and bronchiolitis

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Abstract

This study demonstrates that immunization with non-replicating virus-like particle (FFG VLP) containing RSV F and G glycoproteins together with RSV F DNA induced T helper type 1 antibody responses to RSV F similar to live RSV infection. Upon RSV challenge 21 weeks after immunization, FFG VLP vaccination induced protection against RSV infection as shown by clearance of lung viral loads, and the absence of eosinophil infiltrates, and did not cause lung pathology. In contrast, formalin-inactivated RSV (FI-RSV) vaccination showed significant pulmonary eosinophilia, severe mucus production, and extensive histopathology resulting in a hallmark of pulmonary pathology. Substantial lung pathology was also observed in mice with RSV re-infections. High levels of systemic and local inflammatory cytokine-secreting cells were induced in mice with FI-RSV but not with FFG VLP immunization after RSV challenge. Therefore, the results provide evidence that recombinant RSV FFG VLP vaccine can confer long-term protection against RSV without causing lung pathology.

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HSH, YMK, JSL, SEY, EJK and SMK conceived and carried out experiments as well as analyzed data. HSH, YMK, JSL, SEY, YNL, EJK, MCK, YTL, MKC, YJJ, and JYL carried out experiments. HSH, JDL, and SMK were involved in writing the paper. All authors had final approval of the submitted and published versions.

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Keywords

RSV VLPs; DNA vaccine; histopathology; RSV; protection

Introduction

Human respiratory syncytial virus (RSV) is a major cause of viral bronchiolitis in infants, young children, elderly, and immune-compromised patients. During 1960s, a formalin-inactivated RSV vaccine (FI-RSV) administered to children resulted in approximately 80% hospitalization and two deaths during next epidemic winter season, causing vaccine-related enhanced RSV disease (ERD) (Kapikian et al., 1969; Kim et al., 1969).

Despite the extensive endeavor to develop RSV vaccines, there is no licensed RSV vaccine (Graham, 2011; van Drunen Littel-van den Hurk et al., 2007). RSV fusion (F) or attachment (G) glycoprotein subunit and recombinant vectored RSV vaccines were reported to cause ERD in animal studies (Castilow et al., 2008b; Delgado et al., 2009; Hancock et al., 2001; Murphy et al., 1990). Clinical trials of live attenuated RSV vaccines demonstrated some reduction in illness upon the second infection, but safety and immunogenicity of these live RSV vaccines in infants were not highly encouraging (Graham, 2011; Wright et al., 2007). Even natural RSV infection fails to establish long-lasting immunity as reinfection is common throughout life (Bont et al., 2002; Piedra, 2003).

Virus-like particles (VLP) can be formed by the assembly of structural proteins and lipid bilayer membranes, which mimic the enveloped infectious virus in structure and morphology but lack viral genomes (Kang et al., 2012; Zeltins, 2013). Recent studies demonstrated that Newcastle disease virus (NDV) VLP expressing chimeric RSV G or F and G proteins conferred protection without ERD in mice (McGinnes et al., 2011; Murawski et al., 2010). RSV F DNA vaccines are likely to induce T helper type 1 (Th1) immune responses due to their endogenous antigen expression (Kohlmann et al., 2009; Ternette et al., 2007). In our previous study (Quan et al., 2011), recombinant F VLP and G VLP vaccines that were produced in insect cells showed capacity to raise similar RSV neutralizing titers. In this study, we hypothesized that co-immunization of RSV VLP and F DNA vaccines would induce protective immune responses to ensure virus clearance but not to cause RSV disease after RSV challenge. We have investigated immunogenicity, protection, and lung histopathology after immunization with a combined vaccine of RSV F VLP, G VLP, and RSV F DNA in comparison with FI-RSV (a failed vaccine causing disease) and live RSV infection-mediated immunity.

Methods

Cells, Virus, and RSV F DNA

Spodoptera frugiperda Sf9 cells were maintained in suspension in serum-free SF900II medium (GIBCO-BRL) (Quan et al., 2011). HEp-2 cell line was obtained from ATCC and the RSV A2 strain was originally provided by Dr. Barney Graham. The expression plasmid

encoding human codon bias-optimized RSV A2 F was kindly provided by Dr. Martin Moore (Emory University) and used as previously described (Stokes et al., 2013).

Preparation of RSV F and G VLP, RSV F DNA, and FI-RSV

VLP consisting of an influenza virus matrix (M1) core protein and RSV F (RSV F VLP) or G (RSV G VLP) glycoproteins on the VLP surface was produced using the insect cell expression system and characterized as described (Quan et al., 2011). The incorporation of RSV F and G proteins on VLP was confirmed by ELISA using RSV F and G specific monoclonal antibodies (Supplementary Fig. 4). The plasmid DNA encoding RSV F protein was propagated in *E. coli* cells and purified using endotoxin-free kits (Qiagen). The expression of RSV F DNA was confirmed by western blot of transfected 293 T cells (Supplementary Fig. 2). RSV that was grown in HEp-2 cells was inactivated with formalin (1:4000 vol/vol) for 3 days at 37°C, and then purified using ultracentrifugation (Prince et al., 2001; Quan et al., 2011). Inactivation was confirmed by an immuno-plaque assay (Quan et al., 2011). FI-RSV vaccine was adsorbed to aluminium hydroxide adjuvant (4 mg/ml) for immunization of FI-RSV vaccines.

Immunization with RSV vaccines and RSV challenge

Female BALB/c mice purchased from Charles River (5 to 10 weeks old, n=10 per group, two independent experiments were carried out with each n=5) were primed intramuscularly with a mixture (FFG VLP) of 10 µg total protein of each RSV F VLP and RSV G VLP, and 50 µg of RSV FDNA at week 0, and then boosted with half doses of mixed vaccines (100 µl) 4 weeks later (5 µg RSV F VLPs, 5 µg RSV G VLPs, 25 µg of RSV F DNA). As for the FI-RSV control group (n=10), BALB/c mice were immunized intramuscularly with 2 µg total protein FI-RSV (100 µl PBS) at week 0 (prime) and 1 µg FI-RSV at week 4 (boost). As a live virus control, a group of mice (n=10) was infected intranasally with 1×10^6 plaque forming units (PFU) of live RSV at week 0 (prime) and 0.5×10^6 PFU of live RSV at week 4 (boost, 2nd infection) with RSV A2 strain. At 21 weeks after boost immunization, mice were challenged with RSV A2 strain (2×10^6 PFU/mice). All experiments were approved and performed according to guidelines of the Animal Care and Use Committee from the Georgia State University.

RSV specific antibody ELISA

RSV specific antibody responses were determined by enzyme-linked immunosorbent assay (ELISA) (Quan et al., 2011). Briefly, RSV-F protein (100 ng/ml, BEI, NIAID, NIH) or RSV-G protein (200 ng/ml, Sino biological) was used as an ELISA coating antigen. O-phenylenediamine (Invitrogen) was used as a substrate for horseradish peroxidase (HRP) conjugates of secondary antibodies. The optical density at 450 nm was read using an ELISA reader (BioTek, EL800).

ELISpot assay

Interferon (IFN)-γ or IL-4 secreting cell spots were determined on Multi-screen 96 well plates as described previously (Song et al., 2010). Briefly, cytokine secreting cell spots (ELISpot) were developed after stimulation of lung cells (1×10^5 cells/well) or spleen cells

(5×10^5 cells/well) with ultracentrifugation-purified FI-RSV, and counted using BioSys ELISpot reader (Song et al., 2010).

RSV immunoplaque assay and RSV neutralization activity

RSV titers in virus stocks or lung tissue extracts were determined by an RSV immunoplaque assay (Quan et al., 2011). HEp-2 cell monolayers with diluted virus samples were cultured for 3 to 5 days, and fixed with ice-cold acetone-methanol (60:40) for 10 minutes. Anti-F monoclonal antibody (Millipore) and then HRP conjugated anti-mouse IgG antibodies were used. Individual plaques were developed using a DAB substrate kit (Invitrogen) (Quan et al., 2011). For RSV neutralization activity, virus-serum mixtures were added to confluent monolayers of HEp-2 cells and incubated for 1 hour. The next steps were followed as an immune plaque assay procedure as described above. The mean percent plaque reductions by sera from vaccinated mice compared with sera from naïve control sera were determined (Quan et al., 2011).

Histopathology

For histological analysis, lung samples were fixed in 10% neutral buffered formalin for 48hrs, 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) (Meyerholz et al., 2009). At least eight 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 with the severity scoring system as previously described (Meyerholz et al., 2009). A score 1 was assigned when the surrounding space is free or has few infiltrating cells, score 2 when the surrounding space contains focal aggregates of infiltrating cells or the structure is cuffed by one definite layer of infiltrating cells, and score 3 when structure is cuffed by two or more definite layers of infiltrating cells with or without focal aggregates. The goblet cell hyperplasia as a measure of mucin expression was identified in 40 randomly selected airways in additional section with PAS stain. Areas of Epithelium were annotated using magnetic lasso tool of Adobe Photoshop CS5.1 software and PAS-positive areas within the airway epithelium were identified by Adobe Photoshop CS5.1 software. The degrees of pulmonary eosinophilia were indicated by using an H&CR stain to enumerate eosinophil and expressed as numbers of eosinophil present per 400X field.

Statistical analyses

The groups were compared by one-way analysis of variance (ANOVA) and Tukey multiple comparison tests. A *P* value of <0.05 was considered significant. Data are shown as means $\pm$ standard errors of means (SEM).

Results

Combined VLP and DNA vaccines induce RSV F specific IgG2a antibodies dominantly

We have shown that both RSV F VLP and RSV G VLP can contribute to inducing RSV neutralizing antibodies and controlling lung viral loads (Quan et al., 2011). In an attempt to

enhance the effectiveness of non-replicating VLP vaccine, we tested whether inclusion of RSV F DNA would increase the immunogenicity of mixed RSV F and G VLP (FG VLP) after intramuscular immunization of mice (n=5) (Supplementary Fig. 1). Inclusion of F DNA in the FG VLP (FFG VLP) was found to increase the induction of IgG2a isotype antibodies specific for RSV F particularly after prime immunization (Supplementary Fig. 1). F DNA alone prime-boost immunization did not induce detectable levels of antibodies although the second boost resulted in significant levels of RSV F specific antibodies (Supplementary Fig. 2). Empty vector (M1VLP) immunization did not induce RSV specific antibodies (Supplementary Fig. 1). Therefore, inclusion of F DNA can be an advantage in increasing IgG2a isotype antibody responses.

To further determine the effectiveness of RSV vaccines, FFG VLP and other control groups of mice were intramuscularly immunized with a mixture of RSV F DNA, RSV F and G VLPs (FFG VLP), FI-RSV, or intranasally infected with live RSV A2 strain. Antibody responses to RSV were determined using purified F and G proteins as an ELISA coating antigen. The FI-RSV group showed high levels of serum IgG, and isotypes IgG1 and IgG2a antibodies specific for RSV F after prime immunization (Fig. 1A, B, 1C). Boost immunization with FI-RSV further increased IgG and IgG2a antibody responses (Fig. 1A, 1C). The first infection with live RSV A2 induced relatively low levels of IgG1 and IgG2a antibodies specific for RSV F although levels of IgG2a isotype antibodies were higher than those of IgG1 (Fig. 1B, C). FFG VLP prime immunization induced approximately 2-fold higher levels of IgG2a antibodies than live RSV infection (Fig. 1C). After second immunization or infection, both FFG VLP and live RSV showed a similar level of IgG, IgG1, and IgG2a antibodies specific for the RSV F protein (Fig. 1). Overall, IgG2a antibodies specific for RSV F were induced at similar levels in all 3 groups. However, RSV F specific IgG1 antibody levels were significantly higher in the FI-RSV group than those in the FFG VLP or live RSV group (Fig. 1B, C).

Interestingly, IgG, IgG1, and IgG2a antibody responses to the RSV G protein were induced at highest levels in the FI-RSV group (Fig. 1D, 1E, 1F). In contrast, RSV G specific IgG, IgG1, and IgG2a antibodies were not induced at such high levels in the live RSV group even after second infection compared to those in the FI-RSV group (Fig. 1). The FFG VLP group showed much lower levels of IgG1 and IgG2a antibodies specific for RSV G (Fig. 1E, F). These results suggest that FI-RSV immunization induced high levels of antibodies specific for both F and G proteins whereas RSV infection resulted in higher levels of antibody responses specific for RSV F but not for RSV G. It is worthy to note that FFG VLP immunization induced higher levels of antibody responses to RSV F similar to those by live RSV reinfections. This pattern of antibodies specific for RSV F or G has been observed up to 20 weeks post immunization (Fig. 1).

RSV vaccination induces neutralizing antibodies

We wanted to determine whether inclusion of F DNA in FG VLP immunization would increase RSV neutralizing activity, RSV neutralization titers of FG VLP and FFG VLP serum samples were determined after boost immunization (supplementary Fig. 3). FFG VLP immune sera showed a higher level of RSV neutralizing activity up to 160 fold serum

dilutions. These results provide an additional rationale for including F DNA in the FG VLP vaccine.

We further compared the effectiveness of FFG VLP with other immunization control groups of FI-RSV and live RSV infection in the independent setting. The FFG group showed a 50% RSV neutralizing (plaque reduction) titer of approximately 200 similar to the FI-RSV group whereas a lower neutralizing titer of 100 was observed in the live RSV group (Fig. 2A). At 21 weeks after boost vaccination, mice were challenged with RSV A2 to determine long-term protection. At day 5 post RSV challenge, titers of virus neutralizing activity were found to be further increased particularly in the group of FI-RSV (Fig. 2A). Unvaccinated naïve mice at day 5 post RSV infection did not show serum neutralizing antibody titers, which is similar to naïve serum samples (Fig. 2B).

FFG VLP RSV vaccination protects mice from severe weight losses and controls lung viral loads

Monitoring body weight changes after RSV infection of immunized mice provides an important parameter for assessing the efficacy of vaccination (Fig. 3). Upon RSV A2 challenge infection, substantial weight losses in a range of 8 – 12% were observed in unimmunized naïve mice, FI-RSV immunized mice, and live RSV re-infected mice (Fig. 3). Mice with live RSV reinfections showed moderate levels of 6 to 8% weight losses. In contrast, FFG VLP immunization of mice did not show such significant weight losses and exhibited 0–6% minimal weight changes (Fig. 3). Thus, FFG VLP immunization can prevent severe weight loss from RSV infection.

Determination of lung viral titers is an important parameter in assessing the efficacy of vaccine candidates. Higher titers of RSV were detected in lungs from unvaccinated naïve mice at day 5 post RSV challenge (Fig. 4). Unimmunized naïve mice showed highest levels of lung viral titers with an average of 3.5 log₁₀, which is similar to those reported in previous studies (McGinnes et al., 2011; Schmidt et al., 2012). Lower levels of virus titers were observed in the lungs of mice immunized with FI-RSV. The lung viral titers were significantly lower in the FFG-VLP group compared to those in the live RSV infection group ($p < 0.05$, Fig. 4). These results suggest that vaccination of mice with FFG VLP might be more effective in controlling lung viral loads than immunity of live RSV infection.

FI-RSV but not FFG VLP induces systemic cytokine responses

A safe RSV vaccine should not induce abnormal T cell immune responses upon RSV challenge. As an indicator of cellular immune responses, we determined IFN- γ and IL-4 cytokine secreting cell responses in lungs (a local organ where RSV replicates) and spleens (a systemic organ) using ELISpot (Fig. 5). We found that there was a striking difference between FI-RSV immunization and live RSV A2 reinfection in the levels of IL-4 secreting lung and spleen cell spots. Lung cells collected from FI-RSV immunized mice showed extremely high levels of IL-4 secreting cell spots regardless of antigenic stimulation (purified FI-RSV, media) (Fig. 5A). In addition, spleen cells from FI-RSV immunized mice showed more than a hundred fold higher level of IL-4 secreting cell spots than spleen cells from FFG VLP immunized mice (Fig. 5B). Live RSV reinfection induced a low level of

RSV specific IL-4 secreting spleen cells and did not show IL-4 secreting cell spots from lung samples (Fig. 5A, 5B). In contrast to FI-RSV immunization, FFG VLP did not induce IL-4 secreting cell spots in both lungs and spleens.

The FI-RSV group showed 2 to 3 fold higher levels of IFN- γ secreting cells from lung tissues at day 5 post RSV challenge even in the absence of antigenic stimulation compared to the live RSV or FFG VLP group that showed low levels of RSV specific IFN- γ secreting lung cells (Fig. 5C). Both FI-RSV and live RSV groups exhibited substantial levels of IFN- γ secreting splenic cell spots in response to RSV antigen stimulation (Fig. 5D). In contrast to FI-RSV and live RSV, IFN- γ secreting splenic-cell spots were not observed at a detectable level in the FFG VLP group (Fig. 5D). These results suggest that FFG VLP vaccination and RSV infection did not induce IFN- γ and IL-4 cytokine secreting cells in lungs and in spleens at a substantial level. Whereas, FI-RSV immunization induced high levels of IFN- γ and IL-4 cytokine secreting cells locally and systemically.

FFG VLP immunization does not induce lung disease after RSV challenge

Lung histological sections of immunized and naïve control mice were examined at day 5 post challenge with RSV A2 (2×10^6 PUF/mouse) (Fig. 6). FI-RSV immunized mice showed a massive influx of inflammatory cells around airways and alveolar septa (Fig. 6A, 6B), blood vessels (Fig. 6A, 6C), in the peribronchial and perivascular spaces (Fig. 6A, 6D) as well as cell thickening of airway linings (Fig. 6A). Thus, immunization with FI-RSV recapitulated the previously reported abnormal histology of lungs after RSV infection. Upon RSV re-infection, mice previously infected with RSV two times displayed moderate lung pathology as evidenced by a certain degree of infiltrates around airways and alveolar septa (pathology score 1.8, Fig. 6A, 6B) and severe infiltrates around blood vessels (highest pathology score of 3, Fig. 6A, 6C). The group of naïve mice that were infected with RSV A2 showed a certain degree of inflammation as indicated by the average pathology score of 1.17. Also, FFG VLP immunized mice showed the average pathology score of 0.96, which has no statistical significance compared to naïve infection control although there was a low trend of pulmonary inflammation in the FFG VLP group (Fig. 6C). In contrast, mice that were immunized with FFG VLP did not show such abnormal pathology around airways, blood vessels, and interstitial spaces (Fig. 6A, 6B, 6C, 6D).

Since abnormal cells along the airways were observed in the lung sections of FI-RSV immunized mice, we performed PAS staining to determine mucus production (Fig. 7A, 7C). Airway linings from FI-RSV immunized mice showed strong PAS staining (Fig. 7C). The group of live RSV showed a lower level of PAS positive area than the FI-RSV group but exhibited a considerable level of PAS positive staining (Fig. 7C). In contrast, FFG VLP immunized mice did not exhibit such PAS staining of airway linings after RSV challenge (Fig. 7C).

To estimate the degree of lung eosinophilia, we examined hematoxylin-Congo red (H&CR) stained lung sections (Fig. 7B, 7D). H&CR positive eosinophil accumulations in lungs were significantly greater in FI-RSV immunized mice than those in FFG VLP immunized or live RSV infected mice (Fig. 7D). H&CR positive eosinophils were detected in the group of live RSV at a lower level than that observed in the FI-RSV group (Fig. 7D). However, the live

RSV group showed higher levels of H&CR positive eosinophils than the FFG VLP group (Fig. 7D). Importantly, eosinophil staining spots were not detected in FFG VLP immunized mice at a meaningful level (Fig. 7D). In agreement with inflammatory scores on histopathological observations, mucus production and eosinophilia in FFG VLP immunized mice were significantly lower than those in FI-RSV immunized mice, and even lower than those in live RSV mice. Therefore, these results suggest that FFG VLP immunization does not induce lung disease upon RSV challenge and can be developed as a safe and effective RSV vaccine.

Discussion

There is no licensed vaccine against RSV since the tragic failure of FI-RSV. This study demonstrates that FFG VLP without adjuvants conferred protection against RSV challenge and did not cause inflammatory lung disease. Immunization of mice with FI-RSV induced severe pulmonary histopathology as shown by a high level of eosinophils. In particular, FFG VLP immunization of mice might be more effective in clearing lung viral loads and preventing RSV pulmonary disease than immunity by live RSV infection. Therefore, the results highlight a potential that safe protective immunity against RSV could be achieved by recombinant RSV VLP and F DNA vaccines.

Antibodies specific for RSV F and G proteins are known to neutralize RSV and thus to confer protection against RSV (Arbiza et al., 1992; Quan et al., 2011). Thus, it is a rational approach to include both RSV F and G in the RSV vaccine component as shown by NDV VLP with F and G (McGinnes et al., 2011). Since both F VLP and G VLP are non-replicating protein vaccines presented on VLP, we assumed that inclusion of F DNA in the VLP vaccines would favor the induction of Th1 type immune responses such as IgG2a isotype antibodies in BLAB/c mice, supplementing protective immunity. In this study, we investigated the effectiveness of the cocktail FFG VLP. F DNA itself was not highly immunogenic although a significant level of IgG2a antibodies was induced after 3 immunizations (Supplementary Fig. 2). We found that FFG VLP was more effective in inducing RSV F specific IgG2a isotype antibodies and RSV neutralizing activity than FG VLP (Supplementary Fig. 1, Supplementary Fig. 3). It will be important to determine the contributions of the cocktail FFG VLP vaccine components (F VLP, G VLP, F DNA and their possible combinations) to long-term protection against RSV or disease in comparison with live attenuated RSV vaccine candidate, which remains to be determined. It would simplify the cocktail vaccine if F and G proteins are expressed in one VLP such as NDV-based VLP with F and G (McGinnes et al., 2011), and this option should be explored further.

Natural infection is considered to induce most desirable protective immunity once the host survives. However, natural RSV infection seemed to induce RSV-associated pulmonary disease as evidenced by recurring RSV infections (Nokes et al., 2008; Scott et al., 2006). Primary RSV infection is likely to cause more severe disease in humans than RSV reinfections. In contrast, our results suggest that RSV reinfections could cause more inflammation in lungs (but not severe weight losses) than primary infection in mice. This may represent a limitation in using murine RSV models. Cell culture components in live

RSV preparations might contribute to sensitizing RSV disease, which remains to be determined. It is an interesting observation that recombinant FFG vaccine can confer protection in mice, which may be comparable to or better than live RSV infection-mediated immunity. A single intranasal inoculation with RSV was reported to induce short-lived RSV neutralizing antibodies compared to RSV FG VLP that was produced in avian cells based on NDV internal proteins in mice (McGinnes et al., 2011; Schmidt et al., 2012). Mice that were intramuscularly immunized with NDV FG VLP induced long-lasting RSV neutralizing antibodies (McGinnes et al., 2011; Schmidt et al., 2012). It is likely that intranasal inoculation with RSV may be less effective in inducing long-lasting memory B cells and plasma cells. Intramuscular immunization of mice with chimeric NDV-RSV FG VLP induced the generation of plasma cells in bone marrow but not intranasal inoculation with RSV (Schmidt et al., 2012). Comparison of FFG VLP and live RSV via the same intramuscular route will be informative in providing some insights into long-lasting protection and preventing RSV disease.

Most previous studies included the live RSV infection group as a positive control to assess the short-term protection, but its long-term effects on reinfections and inflammatory disease have not been well known. In our study, mice even with live RSV previous infections two times showed a significant level of lung disease upon RSV reinfection 21 weeks later. This might be different from observations in humans. Our results provide evidence that live RSV would not be highly effective in preventing inflammatory lung disease for a long period. Therefore, a successful RSV vaccine candidate needs to be safer and more effective in preventing RSV lung disease than inactivated RSV and natural infection. FFG VLP vaccination might provide a possible approach in developing a safe and effective RSV vaccine.

Control of lung viral loads is an important parameter in assessing vaccine efficacy since there would be a positive correlation between viral replication and clinical disease during natural or experimental infections (DeVincenzo et al., 2005; Karron et al., 1997). FI-RSV used to immunize mice in this study was quite immunogenic than expected. We tested the efficacy of inactivation of FI-RSV by a plaque-forming infectivity assay and confirmed 100% loss of infectivity in all FI-RSV preparations. The method of formalin inactivation for RSV is similar to that of inactivating influenza viruses. FI-inactivated influenza viruses such as licensed influenza vaccines are highly immunogenic and protective. The immunogenicity of experimental FI-RSV was reported to be variable. Several studies reported low immunogenicity and poor neutralizing antibodies in FI-RSV immunized animals (Murawski et al., 2010; Polack et al., 2002). In contrast, other previous studies demonstrated that FI-RSV immunized mice or cotton rats well controlled RSV lung viral loads after infection (Boelen et al., 2000; Johnson et al., 2004; Kamphuis et al., 2012; Prince et al., 2001; Waris et al., 1996). Our results in this study and other animal models on FI-RSV immunization indicate that lung viral clearance alone would not provide a protective correlate because of potential lung disease regardless of viral loads after RSV infection. A previous study demonstrated this discrepancy that severity of illness was not influenced by RSV titers in nasal secretions in some young infants who were hospitalized (Wright et al., 2002). Early studies reported that RSV specific neutralizing antibodies played a major role in clearing lung viral loads, meanwhile immune cells primed by FI-RSV or recombinant vaccinia

expressing RSV F or G were associated with ERD in animal models (Castilow et al., 2008a; Graham et al., 1991). Taken together, both lung viral clearance and pulmonary disease should be assessed in evaluating RSV candidate vaccines. In addition, non-RSV viral proteins (formalin inactivated PBS or cellular proteins) can contribute to pulmonary inflammation to a certain degree after RSV challenge of cotton rats (Shaw et al., 2013). FI-mock immunized mice showed a moderate level of histopathology upon RSV challenge infection (data not shown). We cannot exclude a possibility that severity of lung inflammation observed in mice with RSV re-infections might be partially caused by non-viral components present in the challenge RSV preparations. Thus, it is important to minimize sensitization with non-viral proteins such as using purified RSV (Shaw et al., 2013). Notably, even the purified RSV F or G protein vaccines, and recombinant vaccinia virus vaccines expressing the full-length RSV F or G protein can cause ERD in animal models (Castilow et al., 2008b; Connors et al., 1992; Hancock et al., 2001; Murphy et al., 1990). Thus, the underlying mechanisms linked to ERD should be further investigated.

The main features of ERD are the induction of T helper type 2 (Th2) responses and infiltrates of eosinophils in mice (Fischer et al., 1999). Also, both CD4 and CD8 T cells, and IFN- γ , if too strong, are known to cause ERD in mice (Castilow et al., 2008a; Fulton et al., 2010; Graham et al., 1991; Weiss et al., 2011). The mechanism of ERD in human to this date has not been defined. This study showed that, upon exposure to RSV, FI-RSV immunization exhibited main features of severe ERD including Th2 type responses (IL-4, IgG1 isotype), and pulmonary eosinophilia. FI-RSV immunization induced high levels of IL-4 and IFN- γ secreting cells locally and systemically. Mice that were immunized with FFG VLP did not display abnormality of lung histology. Excess INF- γ was demonstrated to contribute to clinical signs of systemic disease after RSV challenge (Castilow et al., 2008a), suggesting its complex dual role of inhibiting and exacerbating ERD.

In this study, it is interesting to note that immunization with FFG VLP induced a pattern of immune responses similar to that by live RSV infection. We found that live RSV infection induced antibodies more reactive to the F protein of RSV and less amounts of antibodies binding to RSV G. Meanwhile FI-RSV immunization induced high levels of antibodies binding to both F and G proteins. In line with live RSV, higher levels of F-reactive antibodies were observed in the FFG VLP group than those of G-specific antibodies. Induction of F specific antibodies at higher levels by vaccination would be desirable as effective licensed drugs are based on F specific antibodies (Palivizumab, Motavizumab, MEDI-557)(Geevarghese and Simoes, 2012; Venkatesh and Weisman, 2006). It was also reported that there was a correlation between RSV protection and levels of F specific antibodies (Kasel et al., 1987). RSV F is known to stimulate Toll-like receptor 4 (Zeng et al., 2012), which might be contributing to higher immune responses to F compared to G in the groups of live RSV and FFG VLP.

Taken together, these results in this study suggest that uniquely combined FFG VLP can induce long-lived IgG2a antibody responses specific for RSV F. Mice immunized with FFG VLP showed long term protection against RSV without causing lung disease. FFG VLP did not over-stimulate lymphocytes compared to FI-RSV in a mouse model, offering a potential safety.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Arbiza J, Taylor G, Lopez JA, Furze J, Wyld S, Whyte P, Stott EJ, Wertz G, Sullender W, Trudel M, et al. Characterization of two antigenic sites recognized by neutralizing monoclonal antibodies directed against the fusion glycoprotein of human respiratory syncytial virus. *The Journal of general virology*. 1992; 73 (Pt 9):2225–2234. [PubMed: 1383404]
- Boelen A, Andeweg A, Kwakkel J, Lokhorst W, Bestebroer T, Dormans J, Kimman T. Both immunisation with a formalin-inactivated respiratory syncytial virus (RSV) vaccine and a mock antigen vaccine induce severe lung pathology and a Th2 cytokine profile in RSV-challenged mice. *Vaccine*. 2000; 19:982–991. [PubMed: 11115725]
- Bont L, Versteegh J, Swelsen WT, Heijnen CJ, Kavelaars A, Brus F, Draaisma JM, Pekelharing-Berghuis M, van Diemen-Steenvoorde RA, Kimpfen JL. Natural reinfection with respiratory syncytial virus does not boost virus-specific T-cell immunity. *Pediatric research*. 2002; 52:363–367. [PubMed: 12193668]
- Castilow EM, Legge KL, Varga SM. Cutting edge: Eosinophils do not contribute to respiratory syncytial virus vaccine-enhanced disease. *Journal of immunology*. 2008a; 181:6692–6696.
- Castilow EM, Olson MR, Meyerholz DK, Varga SM. Differential role of gamma interferon in inhibiting pulmonary eosinophilia and exacerbating systemic disease in fusion protein-immunized mice undergoing challenge infection with respiratory syncytial virus. *Journal of virology*. 2008b; 82:2196–2207. [PubMed: 18094193]
- Connors M, Collins PL, Firestone CY, Sotnikov AV, Waitze A, Davis AR, Hung PP, Chanock RM, Murphy BR. Cotton rats previously immunized with a chimeric RSV FG glycoprotein develop enhanced pulmonary pathology when infected with RSV, a phenomenon not encountered following immunization with vaccinia–RSV recombinants or RSV. *Vaccine*. 1992; 10:475–484. [PubMed: 1609551]
- Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, Diaz L, Trento A, Chang HY, Mitzner W, Ravetch J, Melero JA, Irusta PM, Polack FP. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. *Nat Med*. 2009; 15:34–41. [PubMed: 19079256]
- DeVincenzo JP, El Saleeby CM, Bush AJ. Respiratory syncytial virus load predicts disease severity in previously healthy infants. *The Journal of infectious diseases*. 2005; 191:1861–1868. [PubMed: 15871119]
- Fischer JE, Johnson TR, Peebles RS, Graham BS. Vaccination with pertussis toxin alters the antibody response to simultaneous respiratory syncytial virus challenge. *The Journal of infectious diseases*. 1999; 180:714–719. [PubMed: 10438359]
- Fulton RB, Meyerholz DK, Varga SM. Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. *Journal of immunology*. 2010; 185:2382–2392.
- Geevarghese B, Simoes EA. Antibodies for prevention and treatment of respiratory syncytial virus infections in children. *Antivir Ther*. 2012; 17:201–211. [PubMed: 22311607]
- Graham BS. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. *Immunol Rev*. 2011; 239:149–166. [PubMed: 21198670]

- Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. *The Journal of clinical investigation*. 1991; 88:1026–1033. [PubMed: 1909350]
- Hancock GE, Heers KM, Smith JD, Scheuer CA, Ibraghimov AR, Pryharski KS. CpG containing oligodeoxynucleotides are potent adjuvants for parenteral vaccination with the fusion (F) protein of respiratory syncytial virus (RSV). *Vaccine*. 2001; 19:4874–4882. [PubMed: 11535341]
- Johnson TR, Teng MN, Collins PL, Graham BS. Respiratory syncytial virus (RSV) G glycoprotein is not necessary for vaccine-enhanced disease induced by immunization with formalin-inactivated RSV. *Journal of virology*. 2004; 78:6024–6032. [PubMed: 15141000]
- Kamphuis T, Meijerhof T, Stegmann T, Lederhofer J, Wilschut J, de Haan A. Immunogenicity and protective capacity of a virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid A in mice. *PLoS One*. 2012; 7:e36812. [PubMed: 22590614]
- Kang SM, Kim MC, Compans RW. Virus-like particles as universal influenza vaccines. *Expert Rev Vaccines*. 2012 (in Press).
- Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am J Epidemiol*. 1969; 89:405–421. [PubMed: 4305197]
- Karron RA, Wright PF, Crowe JE Jr, Clements-Mann ML, Thompson J, Makhene M, Casey R, Murphy BR. Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial virus vaccines in chimpanzees and in human adults, infants, and children. *The Journal of infectious diseases*. 1997; 176:1428–1436. [PubMed: 9395351]
- Kasel JA, Walsh EE, Frank AL, Baxter BD, Taber LH, Glezen WP. Relation of serum antibody to glycoproteins of respiratory syncytial virus with immunity to infection in children. *Viral immunology*. 1987; 1:199–205. [PubMed: 3509676]
- Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am J Epidemiol*. 1969; 89:422–434. [PubMed: 4305198]
- Kohlmann R, Schwannecke S, Tippler B, Ternette N, Temchura VV, Tenbusch M, Uberla K, Grunwald T. Protective efficacy and immunogenicity of an adenoviral vector vaccine encoding the codon-optimized F protein of respiratory syncytial virus. *Journal of virology*. 2009; 83:12601–12610. [PubMed: 19776123]
- McGinnes LW, Gravel KA, Finberg RW, Kurt-Jones EA, Massare MJ, Smith G, Schmidt MR, Morrison TG. Assembly and immunological properties of Newcastle disease virus-like particles containing the respiratory syncytial virus F and G proteins. *Journal of virology*. 2011; 85:366–377. [PubMed: 20980510]
- Meyerholz DK, Griffin MA, Castilow EM, Varga SM. Comparison of histochemical methods for murine eosinophil detection in an RSV vaccine-enhanced inflammation model. *Toxicologic pathology*. 2009; 37:249–255. [PubMed: 19181630]
- Murawski MR, McGinnes LW, Finberg RW, Kurt-Jones EA, Massare MJ, Smith G, Heaton PM, Fraire AE, Morrison TG. Newcastle disease virus-like particles containing respiratory syncytial virus G protein induced protection in BALB/c mice, with no evidence of immunopathology. *Journal of virology*. 2010; 84:1110–1123. [PubMed: 19889768]
- Murphy BR, Sotnikov AV, Lawrence LA, Banks SM, Prince GA. Enhanced pulmonary histopathology is observed in cotton rats immunized with formalin-inactivated respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV 3–6 months after immunization. *Vaccine*. 1990; 8:497–502. [PubMed: 2251875]
- Nokes DJ, Okiro EA, Ngama M, Ochola R, White LJ, Scott PD, English M, Cane PA, Medley GF. Respiratory syncytial virus infection and disease in infants and young children observed from birth in Kilifi District, Kenya. *Clin Infect Dis*. 2008; 46:50–57. [PubMed: 18171213]
- Piedra PA. Clinical experience with respiratory syncytial virus vaccines. *The Pediatric infectious disease journal*. 2003; 22:S94–99. [PubMed: 12671459]
- Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H, Lirman DD, Rabold R, Hoffman SJ, Karp CL, Kleeberger SR, Wills-Karp M, Karron RA. A role for immune complexes in enhanced

- respiratory syncytial virus disease. *The Journal of experimental medicine*. 2002; 196:859–865. [PubMed: 12235218]
- Prince GA, Curtis SJ, Yim KC, Porter DD. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. *The Journal of general virology*. 2001; 82:2881–2888. [PubMed: 11714962]
- Quan FS, Kim Y, Lee S, Yi H, Kang SM, Bozja J, Moore ML, Compans RW. Viruslike particle vaccine induces protection against respiratory syncytial virus infection in mice. *The Journal of infectious diseases*. 2011; 204:987–995. [PubMed: 21881112]
- Schmidt MR, McGinnes LW, Kenward SA, Willems KN, Woodland RT, Morrison TG. Long-term and memory immune responses in mice against Newcastle disease virus-like particles containing respiratory syncytial virus glycoprotein ectodomains. *Journal of virology*. 2012; 86:11654–11662. [PubMed: 22896618]
- Scott PD, Ochola R, Ngama M, Okiro EA, James Nokes D, Medley GF, Cane PA. Molecular analysis of respiratory syncytial virus reinfections in infants from coastal Kenya. *The Journal of infectious diseases*. 2006; 193:59–67. [PubMed: 16323133]
- Shaw CA, Galarneau JR, Bowenkamp KE, Swanson KA, Palmer GA, Palladino G, Markovits JE, Valiante NM, Dormitzer PR, Otten GR. The role of non-viral antigens in the cotton rat model of respiratory syncytial virus vaccine-enhanced disease. *Vaccine*. 2013; 31:306–312. [PubMed: 23153444]
- Song JM, Hossain J, Yoo DG, Lipatov AS, Davis CT, Quan FS, Chen LM, Hogan RJ, Donis RO, Compans RW, Kang SM. Protective immunity against H5N1 influenza virus by a single dose vaccination with virus-like particles. *Virology*. 2010; 405:165–175. [PubMed: 20580392]
- Stokes KL, Currier MG, Sakamoto K, Lee S, Collins PL, Plemper RK, Moore ML. The respiratory syncytial virus fusion protein and neutrophils mediate the airway mucin response to pathogenic respiratory syncytial virus infection. *Journal of virology*. 2013; 87:10070–10082. [PubMed: 23843644]
- Ternette N, Tippler B, Uberla K, Grunwald T. Immunogenicity and efficacy of codon optimized DNA vaccines encoding the F-protein of respiratory syncytial virus. *Vaccine*. 2007; 25:7271–7279. [PubMed: 17825960]
- van Drunen Littel-van den Hurk S, Mapletoft JW, Arsic N, Kovacs-Nolan J. Immunopathology of RSV infection: prospects for developing vaccines without this complication. *Rev Med Virol*. 2007; 17:5–34. [PubMed: 17004293]
- Venkatesh MP, Weisman LE. Prevention and treatment of respiratory syncytial virus infection in infants: an update. *Expert Rev Vaccines*. 2006; 5:261–268. [PubMed: 16608425]
- Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. *Journal of virology*. 1996; 70:2852–2860. [PubMed: 8627759]
- Weiss KA, Christiaansen AF, Fulton RB, Meyerholz DK, Varga SM. Multiple CD4+ T cell subsets produce immunomodulatory IL-10 during respiratory syncytial virus infection. *Journal of immunology*. 2011; 187:3145–3154.
- Wright PF, Gruber WC, Peters M, Reed G, Zhu Y, Robinson F, Coleman-Dockery S, Graham BS. Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. *The Journal of infectious diseases*. 2002; 185:1011–1018. [PubMed: 11930309]
- Wright PF, Karron RA, Belshe RB, Shi JR, Randolph VB, Collins PL, O'Shea AF, Gruber WC, Murphy BR. The absence of enhanced disease with wild type respiratory syncytial virus infection occurring after receipt of live, attenuated, respiratory syncytial virus vaccines. *Vaccine*. 2007; 25:7372–7378. [PubMed: 17868959]
- Zeltins A. Construction and characterization of virus-like particles: a review. *Molecular biotechnology*. 2013; 53:92–107. [PubMed: 23001867]
- Zeng R, Cui Y, Hai Y, Liu Y. Pattern recognition receptors for respiratory syncytial virus infection and design of vaccines. *Virus research*. 2012

Highlights

- FFG VLP vaccines induce RSV F specific IgG2a dominant response.
- FFG VLP confers protection against RSV without causing disease.
- FFG can be more effective in preventing RSV disease than live RSV.

Fig. 1. Antibody responses specific for RSV F or G proteins

(A) IgG antibody responses specific for the RSV F protein. (B) IgG1 isotype antibody responses specific for the RSV F protein. (C) IgG2a isotype antibody responses specific for the RSV F protein. (D) IgG antibody responses specific for the RSV G protein. (E) IgG1 isotype antibody responses specific for the RSV G protein. (F) IgG2a isotype antibody responses specific for the RSV G protein. Naïve: unimmunized mice. FI-RSV: formalin inactivated alum precipitated RSV (FI-RSV) vaccine (2 µg for prime, 1 µg for boosting). FFG-VLP: a combined vaccine of RSV F DNA, RSV F VLP and RSV G VLP (50, 10, 10 µg for prime, 25, 5, 5 µg for boost). Live-RSV: live RSV A2 strain via intranasal route (10^6 PFU for priming, 0.5×10^6 PFU for boost). Groups of mice ($n=10$ per group) were intramuscularly immunized (FI-RSV, FFG-VLP) or intranasally infected with live RSV at week 0 (prime) and 4 (boost). The numbers 4, 8, and 20 in each group indicate the 4 (primed sera), 8 (boosted sera), and 20 weeks post vaccination or infection respectively. Statistical analysis is as follows. ***: $P<0.001$, **: $P<0.01$: when compared between FI-RSV and FFG-VLP. †††: $P<0.001$, ††: $P<0.01$, †: $P<0.05$ when compared between FI-RSV and Live-RSV. Bars indicate standard errors (SE). ##: $P<0.01$, #: $P<0.05$ between FFG-VLP and Live-RSV.

Fig. 2. RSV neutralizing activities

Serum samples collected at 4 weeks after boost and at day 5 post challenge infection were tested for the inhibition of RSV plaque formation as an indicator of RSV neutralizing activity. (A) Boost immune sera tested for RSV neutralizing activity. (B) Post challenge immune sera tested for RSV neutralizing activity. Immunized mice were challenged with infectious RSV (2×10^6 PFU/mouse) at 21 weeks post boost immunization (or post the second dose of RSV infection for the Live-RSV group). Serially diluted mouse sera ($n=5$ per group) were incubated with live RSV (400 PFU/well) and percentages of plaque reduction were presented. Statistical analysis; ***, $P<0.001$, **, $P<0.01$, *, $P<0.05$ when each dilution point was compared between immune sera and naïve sera. Bars indicate standard errors (SE). Naïve inf.: unimmunized naïve mice after RSV infection. Other groups are the same as described in the legend of Fig. 1. The numbers 100, 200, 400 and 800 in each group indicate dilution folds of serum samples.

Fig. 3. Changes in body weight after RSV A2 challenge

Naïve or vaccinated BALB/c mice were intranasally infected with 2×10^6 PFU of RSV A2 strain 21 weeks after boost immunization and monitored for changes in body weight. (A) Naïve-inf.: unimmunized mice with RSV infection. (B) FI-RSV: FI-RSV immunized mice with RSV infection. (C) FFG-VLP: a combined vaccine of RSV F DNA, RSV F VLP and RSV G VLP immunized mice with RSV infection. Body weight changes are presented as percentages of weights at day 0. The data are representative of two independent experiments. *: $P < 0.05$, **: $P < 0.01$: when compared between FI-RSV and FFG-VLP.

Fig. 4. Immunized mice with RSV vaccines control lung virus loads after RSV challenge
Lungs from individual mice in a different set of experimental groups (n=5) were collected on day 5 post challenge, and lung virus loads in each mouse (PFU/g of lung tissue) were determined by an immuno plaque assay in HEp2 cells. Previously immunized or infected mice were challenged with infectious RSV (2×10^6 PFU/mouse) at 21 weeks post boost immunization. Naïve inf.: unimmunized naïve mice after RSV infection. Other groups are the same as described in the legend of Fig. 1. Statistical significance; *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$ when compared between the naïve group and RSV immunized group.

Fig. 5. Cytokine-secreting cell responses in lungs and spleens

(A) IL-4 secreting lung cell ELISpots (n=5). (B) IL-4 secreting spleen cell ELISpots (n=5). (C) IFN-γ secreting lung cell ELISpots (n=5). (D) IFN-γ secreting spleen cell ELISpots (n=5). The lung and spleen cells were prepared from corresponding tissues collected at day 5 post challenge infection (2×10^6 PFU/mouse). The numbers in the Y axis are presented as cytokine-secreting spots in million cells. Immunized mice were challenged with infectious RSV at 21 weeks post boost immunization or post the second dose of RSV infection for the live-RSV group. Naïve: Unimmunized mice with RSV infection. The vaccine groups are the same as described in the legend of Fig. 1. Each value represents the mean $\pm$ SE. Horizontal bars indicate the comparing groups for statistical analysis. *: $P < 0.05$, ***: $P < 0.001$.

Fig. 6. Pulmonary pneumonia histopathology at day 5 post RSV challenge

Lung tissues were collected from individual mice ($n=5$) at day 5 post RSV challenge (2×10^6 PFU/mouse) and prepared for histology analysis. (A, B) Staining of lung tissues in the area of airways and blood vessels. H&E stain shows peribronchiolar and perivascular pneumonia. Immunized mice were challenged with RSV at 21 weeks post boost immunization or post the second dose of RSV infection for the Live-RSV group. Scale bars for H&E indicate 100 μ m. (C–E) H&E stained tissue sections from each mouse were scored for inflammation on a scale of 0 to 3 as diagnostic criteria. (C) Inflammation scores around airways. (D) Inflammation scores around blood vessels. (E) Inflammation scores around interstitial spaces. Naïve inf.: unimmunized naïve mice after RSV infection. Naïve: uninfected naïve mouse lung tissues, all other groups (Naïve-inf., FI-RSV, FFG-VLP, Live-RSV): RSV infected mouse lung tissues. The vaccine groups are the same as described in the legend of Fig. 1. Horizontal bars indicate the comparing groups for statistical analysis. *, $P < 0.05$; *** $P < 0.001$.

Fig. 7. Periodic acid Schiff (PAS) and hematoxylin/Congo Red (H&CR) staining of lung tissues
Lung tissues were collected from individual mice (n=5) at day 5 post RSV challenge (2×10^6 PFU/mouse) and prepared for analysis of mucus production and eosinophil infiltration. (A) PAS staining to determine bronchiolar mucus production. Scale bars for PAS indicate 100 μ m. (B) H&CR staining to determine pulmonary eosinophils. Scale bars for H&CR indicate 20 μ m. Red arrows indicate the amplified location as an insert in each group (H&CR). The insets in H&CR images are details of eosinophil infiltration in lungs. (C–D) Scores for bronchiolar mucus production and pulmonary eosinophils. (C) 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. (D) Inflammation scores of H&CR staining. Pulmonary eosinophils per 40 $\times$ field counts in two different regions of each mice. Naïve inf.: unimmunized naïve mice after RSV infection. Lung tissue samples for PAS and H&CR staining were collected from the same groups as described in the legend of Fig. 6. Horizontal bars indicate significant differences between comparing groups (***: $P < 0.001$, *: $P < 0.05$).