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Baculovirus-expressed virus-like particle vaccine in combination with DNA encoding the fusion protein confers protection against respiratory syncytial virus

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Abstract

Respiratory syncytial virus (RSV) is a major viral agent causing significant morbidity and mortality in young infants and the elderly. There is no licensed vaccine against RSV and it is a high priority to develop a safe RSV vaccine. We determined the immunogenicity and protective efficacy of combined virus-like particle and DNA vaccines presenting RSV glycoproteins (Fd.VLP) in comparison with formalin inactivated RSV (FI-RSV). Immunization of mice with Fd.VLP induced higher ratios of IgG2a/IgG1 antibody responses compared to those with FI-RSV. Upon live RSV challenge, Fd.VLP and FI-RSV vaccines were similarly effective in clearing lung viral loads. However, FI-RSV immunized mice showed a substantial weight loss and high levels of T helper type 2 (Th2) cytokines as well as extensive lung histopathology and eosinophil infiltration. In contrast, Fd.VLP immunized mice did not exhibit Th2 type cytokines locally and systemically, which might contribute to preventing vaccine-associated RSV lung disease. These results indicate that virus-like particles in combination with DNA vaccines represent a potential approach for developing a safe and effective RSV vaccine.

Keywords

RSV; vaccines; VLPs; protection

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1. Introduction

Respiratory syncytial virus (RSV) is the leading cause of serious lower respiratory tract diseases, such as bronchiolitis or pneumonia in infants, patients with cardiac and pulmonary disorders, and in elderly people [1]. Vaccinated children with a formalin-inactivated RSV (FI-RSV) developed severe disease requiring hospitalization and some of them died during the follow-up epidemic season [2–4]. Previous studies suggested that failure of FI-RSV vaccine has been attributed to its induction of T helper type 2 (Th2)-biased host immune responses [5–7].

DNA vaccines are likely to induce Th1 type immune responses due to their presentation of endogenously expressed antigens to the immune systems. DNA immunization has been demonstrated to elicit both cell-mediated and humoral immune responses to a range of pathogens, including RSV [8–10]. Virus-like particle (VLP) vaccines are genetically engineered and produced in cell cultures to contain multiple copies of protein antigens similar to virus in structure and morphology. VLPs lack viral genetic material and therefore cannot replicate. Newcastle disease virus-like particles (NDV-VLP) containing respiratory syncytial virus glycoproteins that were produced by DNA transfection of avian cells were immunogenic and conferred protection [11–13]. In our previous study, we developed VLPs consisting of an influenza virus matrix (M1) protein core and RSV fusion (F) protein or RSV attachment glycoprotein (G) on the surface [14].

A combination of DNA and protein vaccines was shown to induce F-specific antibodies and cytotoxic CD8⁺ T cell responses [15]. Codon-optimized F DNA has been used as vaccine in previous studies [16, 17]. In this study, we investigated immunogenicity, protection, and RSV disease prevention of VLP vaccines containing RSV F and G proteins in combination with plasmid F DNA.

2. Materials and methods

2.1. Cells and Virus

The RSV strain A2 was originally gifted from Dr. Barney Graham. HEp2 cells were obtained from ATCC and maintained in DMEM media [14]. The expression plasmid encoding human codon bias-optimized RSV A2 F was kindly provided by Dr. Martin Moore and prepared as described [18].

2.2. Preparation of RSV vaccines

VLP vaccines containing RSV F (F VLP) or RSV G (G VLP) were prepared as previously described [14]. Briefly, Sf9 insect cells were co-infected with recombinant baculovirus expressing influenza virus M1 core protein and RSV F or RSV G, and cultured in serum-free medium. Culture supernatants containing released VLPs were harvested, cleared of cell debris, concentrated by ultrafiltration, and purified through sucrose gradient ultracentrifugation. RSV was grown in HEp-2 cells, inactivated with formalin (1:4000 vol/vol) for 3 days at 37°C, and purified using ultracentrifugation to remove cell culture supernatants and cellular proteins [19, 20]. Inactivation of FI-RSV was confirmed by an

immuno-plaque assay [14]. FI-RSV vaccine was adsorbed to aluminium hydroxide adjuvant (4 mg/ml) for immunization of FI-RSV vaccines [20].

2.3. Vaccination, sample collection, and virus challenge

For the mixed RSV VLP and DNA vaccine group (Fd.VLP), BALB/c mice (Charles River, 5 to 10 weeks old, n=10 in total per group) were intramuscularly primed with a combined vaccine containing 50 µg plasmid F DNA, 10 µg F VLP, and 10 µg G VLP at week 0 and boosted with half doses (25 µg F DNA, 5 µg F VLP, 5 µg G VLP) at week 4. The FI-RSV control group (n=10 in total per group) was intramuscularly primed (week 0) and boosted (week 4) with 2 µg and 1 µg FI-RSV respectively. Each group of immunization (n=5) and challenge experiments was duplicated. Naïve or vaccinated mice were intranasally challenged with 3×10^6 plaque-forming units (PFU) of RSV A2 strain under isoflurane anesthesia 16 weeks after boost immunization. The individual lungs, spleens, and bronchoalveolar lavage fluid (BALF) samples were removed aseptically at day 5 post-challenge, and extracts were prepared as homogenates [14]. All animal experiments were approved by the Georgia State University IACUC.

2.4. Assays for Antibody Responses, RSV neutralizing activity, and lung viral titers

RSV A2 virus-specific antibodies (IgG, IgG1, and IgG2a) were determined in sera by enzyme-linked immunosorbent assay (ELISA) using FI-RSV (4 µg/ml) [14], RSV F protein (100 ng/ml, BEI, NIAID, NIH), or RSV G protein (200 ng/ml, Sino biological Inc.) as a coating antigen. RSV-specific neutralizing antibody titers in mouse sera were measured using the red fluorescent monomeric Katushka 2 protein expressing RSV A2 strain (A2-K-line19F) as described [21, 22]. Equal volumes of the diluted sera were mixed with A2-K-line19F virus to yield approximately 100 red-fluorescent (excitation at 588 nm, emission at 635 nm) RSV infected-cell spots per well. The percentages of red-fluorescent RSV infected-cells were presented as a measure of serum neutralization activity.

RSV titers in lung samples at day 5 post-challenge were determined using an immunoplaque assay as described [14]. Serially diluted lung homogenates were added to the HEp2 cell monolayer plates and incubated 3–6 days at 37°C. After fixing with ice-cold acetone-methanol and air drying, individual plaques were visualized and counted by anti-RSV F monoclonal antibody, HRP conjugated anti-mouse IgG antibody, and 3,3'-Diaminobenzidine tetrahydrochloride substrate (Invitrogen). The viral load detection limit is estimated to be approximately 60 PFU from each mouse lung sample in this assay.

2.5. Analysis of cytokines

Ready-Set-Go cytokine kits (eBioscience) were used for detecting cytokine levels in lung homogenates following the manufacturer's recommended procedures. IL-4 secreting cell spots of splenocytes and lung cells were determined on Multi-screen 96 well plates coated with cytokine specific capture antibodies as described [23, 24]. Briefly, 0.5×10^6 splenocytes or 0.2×10^6 lung cells per well were cultured with FI-RSV, RSV-F peptide (ELQLLMQSTPPTNNR), RSV-G peptide (WAICKRIPNKKPG) (2 µg/ml) as an antigenic stimulator. The RSV-F peptide is known to be a CD8 T cell epitope [25, 26] and the RSV-G

peptide a CD4 T cell epitope (reviewed in [26]). After 36 h incubation, IL-4 secreting cell spots were counted using an ELISpot reader (BioSys).

2.6. Pulmonary histology of RSV-infected mice

Individual lungs were fixed via infusion through the trachea with 4% formalin, removed, immersed in 4% formalin for 24 hours, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) [11, 27]. Ten sections per mouse were obtained. Images were acquired using a microscope (Zeiss Axiovert 100) with an attached Canon 30D digital camera at a magnification of $\times 100$.

2.7. Flow cytometry

BAL fluids were obtained by infusing 1 mL of PBS into the lungs via the trachea using a 25-gauge catheter (Exelint International Co.) at day 5 post-challenge. BAL cells were pooled ($n=5$) and then stained with appropriate combinations of fluorochrome-conjugated antibodies (anti-CD3, CD45, CD11b, CD11c, Siglec F antibodies), and acquired by flow cytometer LSR Fortessa (BD Biosciences) and analyzed by FlowJo program (Tree Star Inc.).

2.8. Statistical analysis

A two-tailed Student's *t*-test was used, when comparing two different groups to determine the statistical significance. Significant differences among multiple groups were evaluated by 1- or 2-way ANOVA. A level of $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. A mixed vaccine Fd.VLP induces IgG2a isotype dominant antibody responses

RSV F VLP and G VLP were shown to induce protective antibody responses [14]. We determine levels of RSV specific antibodies in immune sera from mice immunized with 10 or 25 μg of VLP (Fig. 1A). Although there was a trend of dose-dependent increases in RSV specific antibody levels, these differences were marginal and not significant. Addition of F DNA would likely increase the VLP immunogenicity probably due to its endogenous antigen expression. The potential advantage of adding F DNA to the VLP (F+G) vaccine was determined by comparing their immunogenicity in inducing IgG and IgG2a isotype antibodies (Fig. 1B, C). F DNA alone (50 μg prime, 25 μg boost) was not able to induce detectable levels of RSV F-specific antibodies but its addition to VLP increased IgG2a isotype antibody responses to RSV F compared to VLP only vaccination (Fd.VLP, Fig. 1B, C). Interestingly, Fd.VLP showed higher levels of IgG2a antibodies specific for RSV F than live RSV infection (Fig. 1B, C). Thus, we have focused on evaluating the effectiveness of Fd.VLP in this study using a dose of 10 μg prime dose of VLP.

To compare the immunogenicity of Fd.VLP and FI-RSV vaccines, antibody levels were determined in sera collected from immunized mice by ELISA coated with FI-RSV at 3 weeks after prime and boost immunization (Fd.VLP, FI-RSV, Fig. 1). After prime immunization with Fd.VLP, IgG1 antibodies specific for RSV were not detected at substantial levels but IgG2a antibodies were induced at substantial levels (Fd.VLP, Fig. 1D,

E). Prime immunization with FI-RSV induced both IgG1 and IgG2a isotype antibodies at high levels (FI-RSV, Fig. 1F, G). Therefore, the ratio of IgG2a/IgG1 isotype antibodies was an average of 8 in the Fd.VLP group, which is higher than that (approximately 1.8) induced by FI-RSV immunization (Fig. 1H). Boost immunization with Fd.VLP increased the levels of both IgG1 and IgG2a isotype antibodies specific for RSV, resulting in decreasing the IgG2a/IgG1 ratios (Fig. 1H).

Next, F and G specific antibody responses were determined using purified RSV F or G protein as a coating antigen (Fig. 2). Fd.VLP immune sera showed higher levels of antibodies specific for RSV F than those for RSV G whereas FI-RSV immune sera exhibited high levels of antibodies specific for both RSV F and G (Fig. 2). IgG2a isotype antibodies to RSV F were dominantly observed in Fd.VLP immune sera (Fig. 2B) but both IgG1 and IgG2a antibodies were similarly detected in FI-RSV immune sera (Fig. 2B, C). Antibody profiles observed with purified RSV F and G were similar to those with FI-RSV when compared using different ELISA coating antigens (Fig. 1E, Fig. 2D). These results suggest that a mixed vaccine of VLP and plasmid DNA (Fd.VLP) is capable of inducing IgG2a isotype dominant antibody response specific for RSV F whereas FI-RSV relatively induces both IgG1 and IgG2a antibodies.

3.2. Fd.VLP vaccination induces RSV neutralizing activity, prevents weight loss, and controls viral replication

To assess the protective efficacy of Fd.VLP and FI-RSV vaccines, immunized mice were challenged with RSV (3×10^6 PFU/mouse) after 16 weeks of boost immunization. Weight changes were monitored. Unimmunized naïve and FI-RSV vaccinated mice showed significant weight losses up to approximately 15% in some mice after RSV challenge (Fig. 3), which are consistent with previous studies [28, 29]. In contrast, Fd.VLP immunized mice prevented severe weight losses, resulting in better clinical outcomes than those observed in the naïve and FI-RSV group after RSV challenge.

Clearance of lung viral loads is an important parameter in assessing the protective efficacy of candidate vaccines. Lung tissues were collected from individual mice at day 5 post challenge and viral titers in lung samples were determined using an immunoplaque assay. The highest RSV titers were detected in unimmunized naïve mice. Both FI-RSV and Fd.VLP immunized mice showed good control of lung viral replication as shown by low RSV titers (Fig. 4A).

To better understand the effectiveness of protection by Fd.VLP immunization, we determined RSV neutralizing titers using fluorescent A2-K-line19F (500 PFU/well) (Fig. 4B). Fd.VLP immune sera showed high RSV neutralizing activity as we observed 50% reduction in fluorescent intensity with the serum dilution of 800 (Fig. 4B). Fd.VLP and FI-RSV immune sera exhibited similar RSV neutralizing activity at the serum dilution of 400 giving approximately 70% reduction of fluorescent A2-K-line19F virus replication. Thus, neutralizing antibodies induced in mice that were immunized with Fd.VLP or FI-RSV might have contributed to controlling RSV lung viral loads (Fig. 4).

3.3. Fd.VLP vaccination does not induce inflammatory cytokines upon RSV challenge

A safe RSV vaccine should not induce abnormal host responses that might cause vaccine-enhanced disease. To determine cytokine producing cells, an IL-4 cytokine secreting ELISPOT assay was performed using lung and spleen cells that were collected from an individual mouse at day 5 post challenge (Fig. 5). Upon RSV challenge infection, lung cells from FI-RSV immunized mice showed abnormally high levels of IL-4 cytokine secreting cell spots, which was independent of antigen stimulation (Fig. 5A). In addition, spleen cells from FI-RSV immunized mice exhibited similarly high levels of IL-4 cytokine secreting cell spots in an antigen (RSV, G-peptide, or F-peptide) stimulation dependent manner (Fig. 5B). These results suggest that FI-RSV primed IL-4 secreting lymphocytes locally at the site of infection (lungs) and systemically (spleens). However, groups of naïve and Fd.VLP immunized mice did not induce lung and spleen cells secreting IL-4 cytokine upon RSV challenge (Fig. 5). Interestingly, FI-RSV immune mice produced IFN- γ secreting spots that were dependent on antigen stimulation (RSV, G-peptide, or F-peptide) in lungs and spleens at significant but lower levels than those of IL-4 at each organ (Fig. 5C, D). Fd.VLP immune mice showed only low levels of IFN- γ secreting spots in lungs and spleens in response to RSV stimulation (Fig. 5C, D).

Levels of T helper type 2 (Th2) cytokines were shown to have correlation with inflammatory lung disease after vaccination and RSV challenge. We determined cytokines IL-5, IL-6, and IL-13 in lung extracts that were prepared from naïve and immunized mice at day 5 post RSV challenge (Fig. 6). The group of mice immunized with FI-RSV showed highest levels of IL-5, IL-6, and IL-13 cytokines in lungs (Fig. 6A, B, C). Also, IL-6 was observed in naïve infected mice at a higher level than naïve uninfected mice (Fig. 6B). In contrast, levels of IL-5 and IL-13 cytokines from the groups of RSV-infected naïve and Fd.VLP immunized mice were low or similar to those of the naïve control (Fig. 6A, B, C). Importantly, a significant level of IFN- γ cytokine was detected in the Fd.VLP group (Fig. 6D). Whereas, IFN- γ cytokine was not observed at a meaningful level in the naïve or in the FI-RSV group after RSV challenge (Fig. 6D).

3.4. Fd.VLP vaccination does not cause pulmonary disease upon RSV challenge

Examination of pulmonary histopathology is important in assessing the safety of RSV candidate vaccines. Lung tissue sections in each group of mice were examined for inflammation at day 5 post challenge 16 weeks after immunization (Fig. 7). Naïve mice infected with RSV showed a low level of interstitial pneumonia as indicated by some infiltrates of inflammatory cells and thickened alveolar layers in the H&E staining of lung tissue sections (Naïve+RSV, Fig. 7A, B, C). The FI-RSV immunized mice displayed a massive influx of inflammatory cells around pulmonary airways, interstitial space, and thickened alveolar layers (FI-RSV+RSV bottom, Fig. 7A, B, C), presenting the heavily inflamed lung tissues. Thus, immunization with FI-RSV recapitulated the previously known inflammatory histology of lungs after RSV challenge. In contrast, the lungs from mice immunized with Fd.VLP did not show an obvious sign of infiltrates of inflammatory cells (Fd.VLP+RSV, Fig. 7). Therefore, these results provide evidence that Fd.VLP immunization did not cause FI-RSV like lung disease upon RSV challenge.

3.5. Fd.VLP vaccination does not induce eosinophil infiltration upon RSV challenge

Bronchoalveolar lavage (BAL) fluid samples were collected from individual mice at day 5 post RSV challenge and BAL cells were obtained for cell staining with CD45, CD11c, CD11b, and Siglec F antibodies (Fig. 8). The FI-RSV immunized group showed an extreme high level of eosinophils with CD45⁺CD11c⁻CD11b⁺SiglecF⁺ phenotypes (Fig. 8) as previously characterized [30, 31]. This eosinophil phenotypic population was observed at a low level in the BAL fluids from naïve or Fd.VLP immunized mice after RSV challenge infection (Fig. 8). Eosinophil phenotypic populations in the lung cell preparations were found to be less prominent compared to those in BAL fluids (data not shown). These results provide evidence that Fd.VLP immunization can induce protection against RSV without eosinophil infiltration upon RSV challenge in a mouse model.

4. Discussion

It has been a challenge to develop a safe and effective RSV vaccine. Both RSV G and F glycoproteins can provide appropriate antigenic targets inducing protective antibody responses that contribute to virus clearance upon RSV challenge [12, 14]. A combination of DNA and protein vaccines was reported to be a promising platform of vaccination against RSV inducing protective balanced immune responses [15, 32] but their potential to induce vaccine enhanced RSV disease (ERD) was not investigated. In this study, in comparison with FI-RSV, we investigated protective efficacy and potentiation to cause ERD after intramuscular immunization with Fd.VLP 16 weeks later. Fd.VLP was immunogenic and induced immune responses that contributed to clearing lung viral loads. Mice that were immunized with Fd.VLP did not show a severe weight loss, Th2 type inflammatory cytokines, and pulmonary histopathology. In contrast, FI-RSV vaccination caused the induction of inflammatory cytokines locally and systemically as well as severe pulmonary inflammation despite its capability to clear lung viral loads. Therefore, the results in this study provide evidence that a vaccination strategy of combining VLP and DNA vaccines could be developed to prevent RSV disease.

We found that virus clearance was not sufficient to prevent pulmonary RSV disease in FI-RSV vaccinated mice. Immunization of mice with FI-RSV induced high levels of RSV specific antibodies that were effective in clearing RSV in lungs after challenge. This result is consistent with those of previous studies demonstrating that FI-RSV immunized mice or cotton rats well controlled RSV lung viral loads after infection [29, 33–35]. FI-RSV used to immunize mice in this study seemed to be immunogenic compared to the one that was reported in other studies. Low RSV neutralizing antibodies and less efficacy of controlling lung viral loads were reported by other animal studies using FI-RSV [11, 36]. We tested the efficacy of inactivation of FI-RSV by a plaque-forming infectivity assay and confirmed 100% loss of infectivity in FI-RSV preparations used in this study. It is not clear why there are discrepancies in RSV neutralizing activity and lung viral clearance among different FI-RSV vaccine studies using animal models although ERD by FI-RSV has been consistently reported. Naïve mice infected with RSV did not show severe inflammatory disease despite higher levels of lung viral loads and significant weight loss. This and other studies suggest

that there are no cohesive correlations among lung inflammatory RSV disease, weight loss, and lung viral loads in mouse models.

Non-viral antigens can contribute to vaccine-associated pulmonary inflammation as shown in the cotton rat model [37]. In our preliminary experiments, FI-mock (no RSV components) immunization caused pulmonary inflammation to a moderate level after RSV challenge but not as severe as FI-RSV immunized mice (data not shown). It is also important to note that atypical vaccine-enhanced disease was observed in vaccines that do not contain much cellular or cell culture proteins. Immunization of animals with purified-RSV G or F protein or recombinant vaccinia virus expressing RSV G protein could cause vaccine-enhanced pulmonary inflammation after RSV challenge [38–41]. Since animal models may not represent the RSV pathogenesis in humans, it needs to be cautious in interpreting results from RSV vaccine preclinical studies.

A safe RSV vaccine should induce a balanced immune response that can control viral replication but does not inflame ERD. Pulmonary RSV disease is probably due to the induction of inappropriately primed host immune responses that include high levels of Th2 type cytokines (IL-5, IL-6, IL-13) as well as IL-4 cytokine secreting cells locally in lungs and systemically in spleens, and eosinophil infiltration into BAL fluids. In particular, FI-RSV adjuvanted with alum seems to have a strong tendency to prime inflammatory host immune responses including high levels of IL-4 cytokine producing cells, IL-13 cytokine and eosinophil infiltration in BAL fluids upon RSV challenge. Non-replicating Fd.VLP vaccination could control RSV replication in lungs without inflaming immune responses after RSV challenge.

DNA vaccines encode protein antigens that are expressed endogenously after immunization. This nature of endogenous protein expression has been suggested to avoid interference by maternal antibodies prevalent in young infants [42, 43]. Immunization with plasmid DNAs encoding an RSV F or G protein alone or in combination with plasmids encoding cytokines was able to induce IgG2a isotype antibody responses and Th1 type INF- γ or Th1/Th2 type cytokine immune responses [9, 44]. A certain level of pulmonary inflammation was reported with immunization with RSV F or RSV G plasmid DNA in spite of protection against RSV replication in lungs [9, 44]. Also, eosinophil infiltration was reported to be induced with both RSV F and RSV G plasmid DNA immunization upon RSV challenge infection [45]. In this regard, a combination of RSV VLPs and DNA can be a unique platform of candidate RSV vaccine that might overcome relatively low immunogenicity of DNA vaccines and non-replicating VLPs. However, there are concerns on complexity and regulatory entities of mixed DNA and VLP vaccines. Possible simian barriers of DNA vaccine application to humans should be considered regarding the DNA delivery and transfection efficacy. Using an effective carrier such as liposome or gold particles and intradermal delivery would be a method to circumvent the simian barrier of DNA vaccines [46, 47]. Interestingly, a previous study demonstrated that DNA mixed with baculovirus-expressed VLP was significantly effective in inducing CD8 T cell responses compared to the DNA itself [48]. Further studies should be carried out to elucidate minimum requirements of vaccine components (F VLP, G VLP, F DNA, or their minimum combinations) for protection and to reduce the complexity.

In previous studies, protection against RSV without ERD was demonstrated with NDV-based VLPs containing RSV G ectodomain or both RSV G and F ectodomains [11, 12]. NDV-based RSV G and F VLPs could induce long-term adaptive immune responses that controlled lung viral loads [13]. A self-assembled nanoparticle of the RSV F protein vaccine adjuvanted with alum was shown to provide protection without severe lung histopathology in a cotton rat model [49]. We found that Fd.VLP immunization resulted in mostly the induction of IgG2a isotype antibody (Th1 type) B cell-mediated immune responses. In contrast, soluble protein antigens induce IgG1 isotype antibody (Th2 type) B cell responses [50]. RSV vaccines that do not need adjuvant are desirable because the absence of adjuvant is less likely to prime undesirable inflaming of host immune responses after RSV challenge. Dissecting the protective roles of each vaccine components of Fd.VLP should be a future direction.

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Research highlights

- A mixed VLP and F DNA RSV vaccine (Fd.VLP) induces F-specific IgG2a antibodies.
- Fd.VLP vaccination induces RSV neutralizing activity.
- Fd.VLP vaccination confers protection against RSV without pulmonary RSV disease.
- Local and systemic Th2 type cytokine induction contributes to RSV disease.

Figure 1. RSV-specific serum antibody responses

RSV-specific antibody responses were determined in serum samples 3 weeks after prime and boost immunization (n=5) using purified FI-RSV (A, D–H) or RSV F protein (B–C) as an ELISA coating antigen. (A) FI-RSV specific serum IgG antibodies in F VLP (10 or 25 µg) or G VLP (10 or 25 µg) immunized mice. (B) RSV F protein specific serum IgG antibodies in F DNA, VLP, Fd.VLP, live RSV immune mice. (C) RSV F protein specific serum IgG2a isotype antibodies in F DNA, VLP, Fd.VLP, live RSV immune mice. (B–C) RSV F protein specific antibody responses were determined in boost serum samples 3 weeks after prime - boost intramuscular immunization of F DNA (prime; 50 µg, boost; 25 µg), VLP (a mixture of F VLP and G VLP, prime; F VLP 10 µg, G VLP 10 µg, boost; F VLP 5 µg, G VLP 5 µg), Fd.VLP (F DNA and VLP mixed vaccines, prime; F DNA 50 µg, F VLP 10 µg, G VLP 10 µg, boost; F DNA 25 µg, F VLP 5 µg, G VLP 5 µg). Live RSV: immune sera from mice 3 weeks after one time intranasal infection with live RSV A2 (50 µl of 1×10^5 PFU/mL) (n=5). (D) FI-RSV specific IgG1 isotype antibodies in Fd.VLP immunized mice. (E) FI-RSV specific IgG2a isotype antibodies in Fd.VLP immunized mice. (F) FI-RSV specific IgG1 isotype antibodies in FI-RSV immunized mice. (G) FI-RSV specific IgG2a isotype antibodies in FI-RSV immunized mice. (H) Ratios of IgG2a to IgG1 isotype antibodies specific for FI-RSV. The data are representative of two independent experiments (each N=5). *, $P < 0.05$, **, $P < 0.01$; *** $P < 0.001$ as indicated in the comparing groups by One-way ANOVA and Tukey's multiple comparison test. Naïve: Sera from unimmunized mice.

A. IgG **B. IgG2a** **C. IgG1** **D. IgG2a/IgG1** **Figure 2. RSV F or G protein specific antibody responses**

RSV-F or -G protein specific antibody responses in immune sera from Fd.VLP or FI-RSV immunized mice were determined 3 weeks after boost immunization (n=5). (A) IgG antibodies specific for purified RSV F or G protein. (B) IgG2a isotype antibodies specific for purified RSV F or G protein. (C) IgG1 isotype antibodies specific for purified RSV F or G protein. (D) Ratios of IgG2a to IgG1 isotype antibodies. Naïve: Sera from unimmunized mice.

Figure 3. Changes in body weight after RSV A2 challenge

Naïve or vaccinated BALB/c mice were intranasally infected with 3×10^6 PFU of RSV A2 strain 16 weeks after boost immunization and monitored for changes in body weight. (A) Naïve+RSV: unimmunized mice with RSV infection. (B) FI-RSV+RSV: FI-RSV immunized mice with RSV infection. (C) Fd.VLP+RSV: Fd.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. **: $P < 0.0001$, *: $P < 0.05$ by Two-way ANOVA statistics when compared between Naïve+RSV and Fd.VLP+RSV.

Figure 4. Lung virus loads after RSV A2 challenge and RSV neutralizing activities of immunized sera

(A) 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 were determined by an immunoplaque assay in HEp2 cells. The data are representative of two independent experiments. Values are the mean $\pm$ SEM. **: $p < 0.01$ when compared to the naïve group. Naïve+R: unimmunized mice with RSV infection, FI-RSV+R: FI-RSV immunized mice with RSV infection, Fd.VLP+R: Fd.VLP immunized mice with RSV infection.

(B) RSV neutralizing activity. Serum samples collected at 3 weeks after boost immunization were tested for RSV neutralizing activity using A2-K-line19F virus and percentages of fluorescent RSV infected-cells normalized to media only (100%) were presented. Statistical analysis; **: $P < 0.0001$ by Two-way ANOVA statistics when 400 each dilution point was compared between immune sera and naïve sera. Bars indicate standard errors (SE). Naïve: Sera from unimmunized mice.

Figure 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) INF- γ secreting lung cell ELISpots (n=5). (D) INF- γ secreting spleen cell ELISpots (n=5). The lung and spleen cells were prepared from corresponding tissues collected at day 5 post challenge infection (3×10^6 PFU/mouse). IL-4 or INF- γ secreting cells were determined by ELISpots after stimulation with purified FI-RSV (RSV, 2 μ g/ml), RSV-F peptide (2 μ g/ml), or RSV-G peptide (2 μ g/ml). The spots for cytokine-secreting cells from the lung or spleen were counted and expressed based on 1×10^6 cells. Values are the mean $\pm$ SEM.

** $p < 0.001$, * $p < 0.05$ when FI-RSV is compared to Naive+R or Fd.VLP+R relating to RSV F or G peptide stimulation. Naive: unimmunized mice. Naive+R: unimmunized mice with RSV infection. Fd VLP+R: Fd VLP-immunized mice with RSV infection. FI-RSV+R: FI-RSV-immunized mice with RSV infection.

A. IL-5 **B. IL-6** **C. IL-13** **D. IFN- γ** **Figure 6. Cytokine analysis of lung extracts**

The lung homogenates were prepared from lung tissues collected at day 5 post challenge infection. Cytokine levels of IL-5 (A), IL-6 (B), IL-13 (C), and IFN- γ (D) were determined using cytokine ELISA. Values are the mean $\pm$ SEM. * p <0.05; ** p <0.01; *** p <0.001 as indicated in the comparing groups by One-way ANOVA and Tukey's multiple comparison test. Naïve: unimmunized mice. Naïve+R: unimmunized mice with RSV infection. FI-RSV+R: FI-RSV-immunized mice with RSV infection. Fd.VLP+R: Fd.VLP-immunized mice with RSV infection.

Figure 7. Histology of lung sections

Lung tissues were collected at day 5 post RSV challenge and prepared for histology analysis. (A) Representative histology pictures of hematoxylin-eosin (H&E) staining of lung tissues were shown in each group at the magnification 100X (top panel) and 400X (bottom panel). H&E stain shows peribronchiolar and alveolar pneumonia. Scale bars indicate 100 μ m for 100X (top panel) and 20 μ m 400X (bottom panel). (B–C) 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 interstitial spaces. Naive+RSV: unimmunized mice with RSV infection. FI-RSV+RSV: FI-RSV-immunized mice with RSV infection. Fd.VLP+RSV: Fd.VLP-immunized mice with RSV infection. *, $P < 0.05$; *** $P < 0.001$ by One-way ANOVA and Tukey's multiple comparison test. Horizontal bars indicate comparing groups.

Figure 8. Profiles of eosinophils by flow cytometry

BAL cells were collected at day 5 post RSV challenge and stained with cell phenotype marker antibodies. The cells that were first gated by CD45⁺CD11c⁻ were displayed based on CD11b and Siglec F dot plots to show an eosinophil population (CD45⁺CD11c⁻CD11b⁺SiglecF⁺). Numbers in each plot indicate cell percentages of marked region. Naïve: naïve mice without RSV challenge. Naïve+RSV: naïve mice with RSV infection. FI-RSV+RSV: FI-RSV immunized mice with RSV infection. Fd.VLP+RSV: Fd.VLP immunized mice with RSV infection. The data shown are representative of two independent experiments.