EFFICACY AND SAFETY OF VIRUS LIKE PARTICLE VACCINES AGAINST RESPIRATORY SYNCYTIAL VIRUS IN MOUSE AND COTTON RAT MODELS

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EFFICACY AND SAFETY OF VIRUS LIKE PARTICLE VACCINES AGAINST RESPIRATORY SYNCYTIAL VIRUS IN MOUSE AND COTTON RAT MODELS

by

HYE SUK HWANG

Under the Direction of Sang-Moo Kang, Ph.D

ABSTRACT

Respiratory syncytial virus (RSV) is a major cause of infectious lower respiratory disease in infants and the elderly. Vaccine-enhanced respiratory disease (ERD) has been a major obstacle in developing a safe vaccine against respiratory syncytial virus (RSV). This study demonstrates efficacy and safety of virus like particle (VLP) vaccines containing RSV fusion (F) (F VLP), attachment (G) glycoproteins (G VLP), F+G (FG VLP), or FG VLP plus F DNA vaccine (FFG VLP) in mouse and cotton rat models.

FFG VLP vaccine was found to be effective in inducing long-lived IgG2a antibody responses specific for RSV F in mice. Mice immunized with FFG VLP showed long term protection against RSV without causing ERD, indicating vaccine safety, whereas mice with
formalin-inactivated RSV (FI-RSV) vaccination showed severe inflammatory pulmonary pathology upon RSV challenge.

In cotton rats, FFG-VLP was found to be effective in inducing B cells that are secreting RSV F specific antibodies and likely long-lived in spleens and bone marrow. In contrast to FI-RSV, FFG-VLP immunization did not prime cellular components (IL-4 secreting cells, eosinophils) responsible for RSV disease and pulmonary inflammation. Repeated live RSV infections could induce a moderate level of pulmonary inflammation, indicating that even natural infection does not induce safe immunity. F VLP and FG VLP vaccines were immunogenic and able to confer protection without causing ERD in cotton rats. Inclusion of F VLP in the G VLP vaccination could improve vaccine safety in cotton rats.

My 4th project was to determine whether F VLP priming would modulate the outcomes of immune responses suppressing ERD to subsequent FI-RSV vaccination and RSV challenge. Induction of effector CD8 T cells expressing IFN-γ in the lung as a result of F VLP priming might be responsible for suppressing pulmonary inflammation and eosinophilia as well as Th2 cytokines in the airways and lungs. An intrinsic property of F VLP to stimulate the innate immune system at the injection site appears to be contributing to modulating a Th1 pattern of immune responses.

In conclusion, these results provide evidence that FFG VLP, FG VLP and F VLP are worthwhile for further development into a safe RSV vaccine candidate.

INDEX WORDS: Respiratory Syncytial Virus, Virus like particles, Protection, histopathology, T helper type-1 immune responses
PROTECTIVE EFFICACY OF VIRUS LIKE PARTICLE VACCINES AGAINST RESPIRATORY SYNCYTIAL VIRUS IN MOUSE AND COTTON RAT MODELS

by

HYE SUK HWANG

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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Georgia State University
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December 2016
DEDICATION

This dissertation is dedicated to my mother, whose unconditional love and support provided me with the strength and endurance that I needed to achieve my goals; and to my lovely sons, Yoon and Bourne, whose innocence and happiness taught me the beauty of life and gave encouragement to live with patience; and to my husband, my permanent companion of life walking on the same journey, whose passion and faithfulness was my constant motivation in studies and showed a role-model of life for me in difficult time.
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1 CHAPTER 1. INTRODUCTION

1.1 Respiratory syncytial virus

Human respiratory syncytial virus (RSV) causes lower respiratory tract illness (LRI) in 20-30% of primary infections, and 1-3% of children are hospitalized in the USA (1, 2). Respiratory syncytial virus (RSV) causes substantial morbidity and mortality, claiming an approximated 160,000 deaths worldwide (3). RSV is a major infectious agent responsible for 70,000 to 126,000 infant hospitalizations for pneumonia or bronchiolitis every year in the USA alone. The elderly also are at risk for severe RSV disease (4) and 14,000 to 62,000 RSV-associated hospitalizations of the elderly occur annually in the USA (5). Repeated RSV infections are common and cause RSV-associated respiratory and pulmonary disease due to recurring RSV infections (6, 7). Immunity following primary exposure of RSV does not prevent secondary or subsequent infections (8), and reinfections with RSV have been recorded throughout life (9).

Respiratory syncytial virus (RSV) has a single-stranded negative-sense RNA as a non-segmented genome encoding 11 different proteins and belongs to a member of the Paramyxoviridae (10). The two major glycoproteins on the surface of RSV, the attachment glycoprotein (G) and the fusion (F) glycoprotein, mediate the initial entry phases of infection (11). RSV G attaches to the ciliated cells of the airways and helps to fusion process. RSV F activates the virion membrane to fuse with the target cell membrane and is the major target for antiviral drug development. Both RSV G and F glycoproteins are the antigens targeted by neutralizing antibodies induced by infection (11).
1.2 RSV Vaccine Enhanced RSV Disease (ERD)

In the 1960s, a clinical trial of formalin-inactivated whole RSV vaccine adsorbed to alum adjuvant (FI-RSV) led to RSV vaccine-enhanced respiratory disease (ERD), resulting in hospitalizations for 80% of vaccine recipients and two fatalities during epidemic season (2, 12). Despite the extensive endeavor to develop RSV vaccines, there is no licensed RSV vaccine (13, 14). Developing a safe and effective RSV vaccine without causing ERD has been a difficult obstacle.

RSV vaccine ERD was demonstrated to be recapitulated different animal models including mice, cotton rats, calves, and non-human primates after FI-RSV vaccination and RSV challenge infection (7-12). Key ERD features of FI-RSV are known to induce: 1) a poor RSV-specific CD8 T cell response, 2) induction of T helper type 2 (Th2) immune response, 3) eosinophil infiltrations into bronchoalveolar lavage fluids, 4) mucus production in the airways, 5) severe lung histopathology and 6) immune complex deposition (15-18). Effective therapies are not available, and there is no licensed vaccine against RSV. Inactivated, live attenuated, subunit, and viral-vectored vaccines have been tested but some of these vaccines may have safety concerns of causing ERD (2) or low efficacy (19) upon RSV infection.
Figure 1.1 Interaction of T cells and cytokines in normal and asymptomatic infections (20).

In mice, FI-RSV-mediated ERD is an aberrant cellular immune response which is characterized by pulmonary eosinophilia and an overzealous T helper type-2 (Th2) response. In cotton rats, a species more permissive to RSV infection than mice, ERD is characterized by alveolitis and enhanced peribronchiolitis (21).

1.3 Cellular immune responses contribute to protection and/or RSV vaccine ERD

RSV specific T cells limit RSV replication but also can contribute to immunopathology (22, 23). Effector CD4 helper T (Th) cells can be divided into two subsets. Th1 cells stimulate antiviral CD8 responses and produce IFN-γ. Th2 cells produce cytokines IL-4, IL-5, and IL-13 (24). The Th1/Th2 paradigm is central to RSV pathogenesis as shown in Fig. 1.1 (25). Th2 type cytokine IL-4 producing CD4 T cells was shown to induce RSV pathology, and IL-13 was known to contribute to mucus production and pulmonary obstruction (23, 26-28). FI-RSV immunization induced high levels of IL-4 and IFN-γ secreting cells locally and systemically.
After exposure to RSV, FI-RSV immunization induced all features of severe ERD including Th2 type responses (IL-4, IgG1 isotype), and pulmonary eosinophilia. Natural RSV infections can cause RSV-associated pulmonary disease as evidenced by mild to severe disease symptoms due to recurring RSV infections (29). Th2-related cytokines were detected at high levels in infants hospitalized following a severe RSV infection (30). These cytokines cause an influx of inflammatory cells, resulting in mucus production and reduced lung function. Consistent with the data from RSV-infected infants, CD4 T cell production of IL-4, IL-5, and IL-13 has all been shown to contribute to RSV-induced disease in a murine model of RSV infection (30).

1.4 RSV vaccines

RSV fusion (F) or attachment (G) glycoprotein subunit and recombinant vectored RSV vaccines were reported to cause RSV vaccine ERD in animal model studies (31-34). 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 because of difficulties in balancing appropriate immunogenicity and safety (14, 35). Even natural RSV infection fails to establish long-lasting immunity as reinfection is common throughout life (36, 37). Some RSV vaccine candidates (FI-RSV, purified-F, -G proteins, and recombinant live vectors expressing RSV F or G) are likely to have safety concerns of inducing vaccine-enhanced respiratory disease. The safety issue of vaccine-enhanced pulmonary inflammation in developing RSV vaccines should be a high priority to be addressed. Vaccination of mice with recombinant vaccinia virus (vv) expressing RSV G (vv-G) but not with vv-F was reported to induce severe pulmonary eosinophilia after RSV challenge, closely mimicking vaccine-enhanced disease as observed with FI-RSV (18, 38, 39). Both vv-G and vv-F
vaccination of mice were shown to induce significant RSV disease of weight loss and histopathology after RSV challenge (40). G-specific T cells were known to cause more severe RSV disease than F-specific T cells in mice (41). F or chimeric FG protein subunit vaccines formulated with alum were shown to induce bronchiolar and alveolar histopathology following RSV challenge in cotton rats (33, 42).

The neutralizing antibody induced by RSV infection may not be long-lived until next RSV season. For pre-mature infants at high risk for RSV infections, the passive transfer of Palivizumab licensed drug (monoclonal RSV F-specific neutralizing antibody) is recommended, but this type of prophylaxis is expensive and unavailable for most individuals (43). Palivizumab is a FDA-approved humanized monoclonal antibody drug directed against an epitope in the antigenic site of the F protein of RSV (44). Palivizumab reduced the risk of hospitalization due to RSV infection by 55% (43). The RSV F protein-based vaccine expressed in insect cells in a membrane-anchor form was developed through chromatographic purification and refolding into nanoparticles (45). RSV F nanoparticle vaccine appeared to be safe and capable of inducing RSV neutralizing antibodies in the phase I clinical trial with healthy adults 18-19 years of age and phase II with 350 healthy women (18-35 years) of childbearing age and 50 healthy pregnant women (46). Therefore, an ideal RSV vaccine should induce RSV neutralizing antibodies without causing disease even better than live RSV.

1.5 Virus like particle (VLP) vaccine

Virus-like particles (VLP) mimic the enveloped infectious virus in structure and morphology but lack viral genomes. Immunization of mice with F VLP was reported to induce
IgG2a isotype dominant antibody responses as well as RSV neutralizing activity in immune sera, which might have contributed to effective clearance of RSV challenge viruses in the lungs (47). This is likely due to the immunogenic properties of RSV proteins presented in a repetitive, particulate virus-like structure (48). Recent studies with RSV G-expressing Newcastle disease VLPs reported that RSV G or G+F glycoproteins presented on non-replicating VLP-like vaccine platforms are immunogenic, effective, and do not cause vaccine enhanced RSV disease (ERD) (49). The recombinant baculovirus (rBV) expression system in insect cells that is used for expression and production of VLP vaccines is FDA approved for human use. This rBV system has been developed for high levels of VLP expression that facilitates large scale production (48). Quan et al demonstrated the generation of RSV VLPs containing influenza M1 and RSV F proteins (47). RSV matrix (M) was shown to be more pleomorphic than influenza virus and not to be effective in assembling VLPs (50). However, the immunopathological effects of RSV VLP immunization are not well known. In addition, cellular immune components induced by VLP vaccination are poorly understood.

New castle disease (NDV) based virus like particle vaccine containing RSV G or F plus G glycoproteins, which was produced in avian cells by DNA transfection with multiple plasmids (47, 49), has been characterized in preclinical studies in a mouse animal model and it has shown sufficient immunogenicity without apparent ERD. RSV F-VLP immunization elicited IgG2a dominant RSV-specific antibody responses, viral neutralizing antibodies, and significantly decreased lung virus loads after live RSV infection (47). However, cellular immune responses and preclinical safety concerns of RSV F and G VLP vaccines have not been addressed yet.
1.6 Purpose of the study

The safety aspects of RSV VLP vaccines have not been well investigated in terms of avoiding RSV vaccine ERD. FI-RSV immunization might effectively control viral replication up to over 90% in cotton rats despite of causing severe RSV vaccine-enhanced pulmonary inflammation and pathology (51-53). Therefore, it is critically important to analyze additional parameters to assess the vaccine safety issue of RSV vaccine candidates. VLP vaccines would lead to a different pattern of immunity in terms of protection and disease. I hypothesized that RSV F VLP vaccine would induce balanced immune responses toward a Th1 pattern and prevent the induction of RSV vaccine ERD in mice and cotton rats after RSV challenge. This study investigated the efficacy and safety of virus like particle (VLP) vaccines containing RSV fusion (F) (F VLP), attachment (G) glycoproteins (G VLP), F+G (FG VLP), or FG VLP plus F DNA vaccine (FFG VLP) in mouse and cotton rat models. The aims of this study were as follows:

1) To determine whether FFG VLP would induce protection against RSV without pulmonary inflammatory disease in a mouse model.

2) To investigate the protective efficacy and safety of F VLP, G VLP, FG VLP in a cotton rat model.

3) To extend the efficacy and safety evaluation of FFG VLP in comparison with FI-RSV and live RSV as vaccine antigens in cotton rats.

4) To determine whether priming F VLP vaccination would modulate the quality of later immune responses, protection against RSV, and/or pulmonary RSV disease.

The approach of heterologous immunization to modify T helper cell responses is a novel concept to determine whether Th1 priming vaccination with VLP would reduce or prevent
vaccine-associated pulmonary RSV disease. The results and findings in my PhD studies provide informative insights into the desirable types of cellular immune responses that control viral replication but prevent RSV vaccine ERD, improving RSV vaccine safety.

2 CHAPTER 2. EXPERIMENT

2.1 Cells, Virus, and RSV F DNA

_Spodoptera frugiperda_ Sf9 cells were maintained in suspension in serum-free SF900II medium (GIBCO-BRL) (54). 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 (55).

2.2 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 (54). The incorporation of RSV F and G proteins on VLP was confirmed by ELISA using RSV F and G specific monoclonal antibodies (Figure 2.1).
Incorporation of RSV F (A) and G (B) protein on VLPs was confirmed by ELISA. The incorporation of RSV F and G proteins on VLP was confirmed by RSV F or RSV G VLP-coated ELISA plates using RSV F and G specific monoclonal antibodies.

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 (Figure 2.2).

Figure 2.1 Characterization of RSV F or G VLP.

Figure 2.2 RSV F expression and antibody responses of RSV F DNA
(A) RSV F western blot. RSV F Protein expression of RSV F DNA was confirmed in 293 T cells transfected with F DNA plasmid. F cell: F DNA transfected 293T cell lysates. F Sup: Culture supernatants from F DNA transfected 293T cells. Polyclonal goat anti-RSV antibody was used to detect the level of RSV-F expression. (B) IgG antibody responses specific for the RSV F protein in immune sera from RSV F DNA immunized mice (n=5) (50 µg for prime, 25 µg for 1st boost, 25 µg for 2nd boost). Mice were intramuscularly immunized at week 0 (prime), 4 (1st boost) and 8 (2nd boost).
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 (53, 54). Inactivation was confirmed by an immuno-plaque assay (54). FI-RSV vaccine was adsorbed to aluminium hydroxide adjuvant (4 mg/ml) for immunization of FI-RSV vaccines.

2.3 Immunization with RSV vaccines and RSV challenge in mouse

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 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 1x10^6 plaque forming units (PFU) of live RSV at week 0 (prime) and 0.5x10^6 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 x 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.

2.4 Immunization with RSV vaccines and RSV challenge in cotton rats

Cotton rats (*Sigmodon hispidus*, 4-5 weeks old) were purchased from the Harlan Laboratories (Indianapolis, IN, USA). For co-immunization with F-VLP and G-VLP vaccines and F DNA (designated as the FFG-VLP group), cotton rats (n=5) were intramuscularly (i.m.) primed with 50 μg of F plasmid DNA along with 20 μg of F-VLP and 20 μg of G-VLP at week 0 and boosted with the same doses at week 4. For VLP immunization, F VLP (20 μg), G VLP (20
μg), mixed RSV F (20 μg) and G VLP (20 μg) (designated FG VLP) were immunized at week 0 (prime) and 4 (boost). The FI-RSV group of cotton rats was i.m. primed (week 0) and boosted (week 4) with 2 μg of FI-RSV in alum formulation. For the live RSV group mimicking RSV infections, cotton rats (n = 5) were intranasally infected with $1 \times 10^6$ plaque forming units (PFU) of RSV A2 strain for prime and boost at weeks 0 and 4. To determine protective efficacy, RSV immune and naïve cotton rats were intranasally challenged with $1\times10^6$ PFU of RSVA2 strain under isoflurane anesthesia 4 weeks after boost immunization. Cotton rats were observed daily to record body weight changes. At day 5 post RSV challenge, all cotton rats were euthanized to collect lungs, spleens, bone marrow (BM), mediastinal lymph nodes (MLN) and broncho-alveolar lavage fluid (BALF) samples. All animal procedures were conducted with the approval of Institutional Animal Care and Use Committee of Georgia State University and were in full compliance with the Committee's guidelines.

2.5 **RSV specific antibody ELISA**

RSV specific antibody responses were determined by enzyme-linked immunosorbent assay (ELISA) (54). 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. The antibody responses were detected using the secondary antibodies of HRP-conjugated goat anti-rat IgG (eBioscience: cotton rat) and goat anti-mouse IgG (Southern Biotech: mouse). 3,3’,5,5’-tetramethylbenzidine (TMB, Sigma Aldrich) 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).
2.6 ELISpot assay

Interferon (IFN)-γ or IL-4 secreting cell spots were determined on Multi-screen 96 well plates as described previously (56). Briefly, cytokine secreting cell spots (ELISpot) were developed after stimulation of lung cells (1x10^5 cells/well) or spleen cells (5x10^5 cells/well) with ultracentrifugation-purified FI-RSV, and counted using BioSys ELISpot reader (56).

2.7 RSV immunoplaque assay and RSV neutralization activity

RSV titers in virus stocks or lung tissue extracts were determined by an RSV immunoplaque assay (54). HEp-2 cell monolayers with diluted virus samples were cultured for 3 to 5 days, and fixed with 10% neutral buffered formalin for 30 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) (54). 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 (54).

2.8 Histopathology

For histological analysis, lung samples were fixed in 10% neutral buffered formalin for 48 hrs, transferred to 70% ethanol, embedded paraffin blocks, 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) (57). 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 (57). 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.

2.9 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 ± standard errors of means (SEM).

3 CHAPTER 3. Co-immunization with virus-like particle and DNA vaccines induces protection against respiratory syncytial virus infection and bronchiolitis in mouse model

3.1 Summary

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.

3.2 Results

3.2.1 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 (54). 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) (Figure 3.1). Inclusion of F DNA in the FG VLP was found to increase the induction of IgG2a isotype antibodies specific for RSV F particularly after prime immunization (Figure 3.1). 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 (Figure 2.2). Empty vector (M1VLP) immunization did not induce RSV specific antibodies (Figure 3.1). Therefore, inclusion of F DNA can be an advantage in increasing IgG2a isotype antibody responses.
Figure 3.1 Antibody responses specific for RSV F protein.
IgG antibody (A), IgG1 isotype (B) and IgG2a isotype (C) responses specific for the RSV F protein. M1VLP: empty vector (without RSV F or G protein) control (10 µg for prime and boost). F-DNA: RSV F DNA vaccine (50 µg for prime, 25 µg for boost). FG-VLP: a combined vaccine of RSV F VLP and RSV G VLP (10 for prime and boost). 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). Groups of mice (n=5) were intramuscularly immunized at week 0 (prime) and 4 (boost). Antibody responses were analyzed at 4 weeks post prime and boost immunization.

To determine the effectiveness of F DNA and VLP mixed vaccines, 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 (Figure 3.2 A-C). Boost immunization with FI-RSV further increased IgG and IgG2a antibody responses (Figure 3.2 A, C). 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 (Figure 3.2 B, C). FFG VLP prime immunization induced approximately 2-fold higher levels of IgG2a antibodies than live RSV infection (Figure 3.2 C). 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 (Figure 3.2). 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 (Figure 3.2 B, C). Interestingly, IgG, IgG1, and IgG2a antibody responses to the RSV G protein were induced at highest levels in the FI-RSV group (Figure 3.2 D-F). 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 (Figure 3.2). The FFG VLP group showed much lower levels of IgG1 and IgG2a antibodies specific for RSV G (Figure 3.2E, 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 (Figure 3.2).

Figure 3.2 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 (10⁶ PFU for priming, 0.5x 10⁶ 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<0.05, #: P<0.05 between FFG-VLP and Live-RSV.

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 (Figure 3.3).

**Figure 3.3** Comparison of SV neutralizing activities between FG VLP and FFG VLP.
Serum samples were collected at 4 weeks after boost and tested for the inhibition of RSV plaque formation as an indicator of RSV neutralizing activity. Serially diluted mouse sera (n=5 per group) were incubated with live RSV (400 PFU/well) and percentages of plaque reduction were presented. Naïve: unimmunized naïve mice. The numbers 40, 80, and 160 indicate dilution folds of serum samples.

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 determined the effectiveness of FFG VLP in the independent settings of other immunization control groups with FI-RSV and live RSV infection. 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 (Figure 3.4qA). 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 (Figure 3.4A). Unvaccinated naïve mice at day 5 post RSV infection did not show serum neutralizing antibody titers, which is similar to naïve serum samples (Figure 3.4B).

![Figure 3.4 RSV neutralizing activities.](image)

Figure 3.4 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 x 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. The numbers 100, 200, 400 and 800 in each group indicate dilution folds of serum samples.

3.2.2 FFG VLP RSV vaccination protect 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 (Figure 3.5). 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 (Figure 3.5).

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 (Figure 3.5).

![Graph showing changes in body weight after RSV A2 challenge.](image)

**Figure 3.5** 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.

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 (Figure 3.6). 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 (58). 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, Figure 3.6). 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.
Figure 3.6 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 x 10^6 PFU/mouse) at 21 weeks post boost immunization. Naïve inf.: unimmunized naïve mice after RSV infection. Statistical significance; *, P < 0.05; ** : P<0.01; *** P < 0.001 when compared between the naïve group and RSV immunized group.

3.2.3 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 (Figure 3.7). 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) (Figure 3.7A). 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 (Figure 3.7B). 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 (Figure 3.7 A, B). 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 group that showed low levels of RSV specific IFN-γ secreting lung cells (Figure 3.7C). Both FI-RSV and live RSV groups exhibited substantial levels of IFN-γ secreting splenic cell spots in response to RSV antigen stimulation (Figure 3.7D). 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 (Figure 3.7 D). These results suggest that FFG VLP vaccination and RSV infection did not induce IFN-r 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.

![Figure 3.7](image.png)

**Figure 3.7** 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 x 10⁶ 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. Each value represents the mean ± SE. Horizontal bars indicate the comparing groups for statistical analysis. *: \( P < 0.05 \), **: \( P < 0.001 \).

3.2.4 **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 x 10^6 PUF/mouse) (Figure 3.8). FI-RSV immunized mice showed a massive influx of inflammatory cells around airways and alveolar septa (Figure 3.8 A, B), blood vessels (Figure 3.8 A, C), in the peribronchial and perivasculcar spaces (Figure 3.8 A, D) as well as cell thickening of airway linings (Figure 3.8 A). 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, Figure 3.8 A, B) and severe infiltrates around blood vessels (highest pathology score of 3, Figure 3.8 A, C). 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 (Figure 3.8C). In contrast, mice that were immunized with FFG VLP did not show such abnormal pathology around airways, blood vessels, and interstitial spaces (Figure 3.8 A-D).
Figure 3.8 Pulmonary pneumonia histopathology at day 5 post RSV challenge

Lung tissues were collected from individual mice (n=5) at day 5 post RSV challenge (2×10⁶ 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 µm. (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) from 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.

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.

Figure 3.9 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 µm. (B) H&CR staining to determine pulmonary eosinophilia. Scale bars for H&CR indicate 20µm. 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× 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 Figure 3.8 Horizontal bars indicate significant differences between comparing groups (***: P < 0.001, *: P<0.05).

3.3 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 (59, 60) (58-60). 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 (58, 61). 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 (58), 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 (6, 7). 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 New castle disease virus (NDV) internal proteins in mice (58, 61). Mice that were intramuscularly immunized with NDV FG VLP induced long-lasting RSV neutralizing antibodies (58, 61). 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 (61). 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 (62, 63). 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 are highly immunogenic and protective as licensed influenza vaccines. 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 (17, 49, 64). In contrast, other previous studies demonstrated that FI-RSV immunized
mice or cotton rats well controlled RSV lung viral loads after infection (53, 65-69). We found that FI-RSV was immunogenic in inducing RSV specific antibodies and also effective in clearing lung viral loads in mice. Despite effective clearance of lung viral loads, FI-RSV immunized animals showed severe pulmonary histopathology after exposure to RSV infection. Thus, our results in this study and others 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 in animal models. The substantial reduction of viral load in lung may be not a sufficient parameter to elucidate the protective efficacy of the candidate vaccines in preclinical animal studies. In those respects the virtue of the histopathological analysis should stand out among the assessments of the safety of a candidate vaccine.

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 (70). 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 (71, 72). Taken together, both lung viral clearance and pulmonary disease should be assessed in evaluating RSV candidate vaccines. A caveat is that 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 (21). 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 (21). It is also important to note that purified RSV F or G
protein vaccines, and recombinant vaccinia virus vaccines expressing the full-length RSV F or G protein can induce ERD in animal models (33, 72-75). 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 (76), and human (2, 77-79). Also, both CD4 and CD8 T cells, and IFN-γ, if too strong, are known to cause ERD in mice (34, 71, 80, 81). The mechanism of ERD in human to this date has not been defined. After exposure to RSV, FI-RSV immunization showed all 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 (34), 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) (82, 83). It was also reported that there was a correlation between RSV protection and levels of F specific antibodies (84). RSV F is known
to stimulate Toll-like receptor 4 (85), 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.

4 CHAPTER 4. Combined virus-like particle and fusion protein-encoding DNA vaccination of cotton rats induces protection against respiratory syncytial virus without causing vaccine-enhanced disease

4.1 Summary

A safe and effective vaccine against respiratory syncytial virus (RSV) should confer protection without causing vaccine-enhanced disease. Here, using a cotton rat model, we investigated the protective efficacy and safety of an RSV combination vaccine composed of F-encoding plasmid DNA and virus-like particles containing RSV fusion (F) and attachment (G) glycoproteins (FFG-VLP). Cotton rats with FFG-VLP vaccination controlled lung viral replication below the detection limit, and effectively induced neutralizing activity and antibody-secreting cell responses. In comparison with formalin inactivated RSV (FI-RSV) causing severe RSV disease after challenge, FFG-VLP vaccination did not cause weight loss, airway hyper-responsiveness, IL-4 cytokines, histopathology, and infiltrates of proinflammatory cells such as eosinophils. FFG-VLP was even more effective in preventing RSV-induced pulmonary inflammation than live RSV infections. This study provides evidence that FFG-VLP can be developed into a safe and effective RSV vaccine candidate.
4.2 Results

4.2.1 FFG-VLP immunization induces RSV binding and neutralizing antibodies in cotton rats.

In a previous study, co-immunization with F DNA and F plus G VLP (FFG-VLP) was shown to induce protective immunity against RSV without causing histopathology in a mouse model (56). In this study, we further investigated the protective efficacy and safety of FFG-VLP in cotton rats in comparison with live RSV and FI-RSV. Cotton rats were intramuscularly immunized with FFG-VLP and FI-RSV vaccines or infected with live RSV A2. Cotton rats that were primed with FFG-VLP induced RSV F-specific antibodies at 1.6~2 fold higher levels than those of live or FI-RSV immunized groups (Figure 4.1 A). RSV G-specific antibodies in FFG-VLP immune cotton rats were not detected above the levels in naïve cotton rats. The FI-RSV and live RSV groups of cotton rats induced RSV G-specific antibodies after boost vaccination or second infection but their levels were approximately 100 folds lower than those of F-specific antibodies (data not shown). Cotton rats with boost immunization further increased RSV F specific antibodies at comparable levels in FFG-VLP, FI-RSV, and live RSV groups (Figure 4.1 B). As a measure of functional antibodies, we determined neutralizing activity by RSV A2-K-line19F and showed representative figures of neutralizing activity in 512-fold (2^9) diluted sera (Figure 4.1 C). Immune sera from the FI-RSV, FFG-VLP and live RSV immunized groups showed significantly higher levels of neutralizing activity (plaque reduction) compared to naïve cotton rat sera (Figure 4.1 D, E). FFG-VLP and FI-RSV prime immune sera showed higher levels of neutralizing activity than that from the live RSV group (Figure 4.1 D). The live-RSV group showed highest levels of neutralizing activity in boost immune sera compared to the FFG-VLP or FI-RSV vaccine group (Figure 4.1 E). Thus, combination FFG-VLP vaccine was
effective in inducing RSV F specific and neutralizing antibodies after prime immunization of cotton rats.

Figure 4.1 RSV F-specific antibody and RSV neutralizing activity after vaccine immunization.
(A) Prime IgG antibodies specific for RSV F. (B) Boost IgG antibodies specific for RSV F. Serum samples were collected at 3 weeks after prime or boost administration and F protein-specific antibody levels were measured by ELISA. (C) A representative fluorescent photography of Hep2 cells infected with RSV A2-K-line19F expressing Katushka 2 fluorescent protein after incubation with immune sera. Fluorescent images were captured by an inverted fluorescence microscope in a condition with 512-fold (2^9) diluted sera. Original magnification X 200; bars indicate 50 μm. (D) Neutralizing activity of prime sera. (E) Neutralizing activity of boost sera. (C-E) For neutralizing assay, immune sera were inactivated and serially diluted. Sera (n=5 per group) were incubated with RSVA2-K-line19F (500 PFU), then were added to Hep2 cell monolayers for 2-3 days to determine percentages of plaque reduction and fluorescent images. FFG-VLP: Sera from FFG-VLP immune cotton rats, FI-RSV: Sera from FI-RSV immune cotton rats, live-RSV: Sera from live RSV re-infected cotton rats, Naïve: unimmunized control. Results (n=5) are presented as mean ± SEM. Statistical significance was performed by two-way ANOVA with Bonferroni post-test to compare replicate in Graph Pad Prism; *** p<0.001, ** p<0.01, * p<0.05; compared to indicated group or Naïve, ††† p<0.001, †† p<0.01, † p<0.05; compared to FFG-VLP.
4.2.2 **FFG-VLP vaccination confers protection without airway hyper-responsiveness and body weight loss.**

To determine protective efficacy, RSV immune cotton rats were intranasally challenged with RSV A2 strain and body weight changes were daily monitored (Figure 4.2 A). The FFG-VLP group did not show any weight loss (Figure 4.2 A). The FI-RSV and naïve-RSV groups showed substantial weight loss compared to the FFG-VLP group. The live RSV group displayed only moderate weight loss. These results suggest that FFG-VLP immunization is effective in preventing weight loss. As an indicator for severe RSV disease, RSV infection can cause significant airway obstruction and bronchoconstriction. To evaluate the pulmonary function 4 days after RSV challenge of RSV immune cotton rats, the airway resistance Penh (%) values were measured by a whole body plethysmograph. Naïve cotton rats showed the highest Penh values followed by FI-RSV immune cotton rats after RSV challenge in response to aerosolized methacholine exposure in a dose response manner (Figure 4.2 B). Meanwhile, FFG-VLP and live RSV immune cotton rats did not show an increase in Penh values, which are similar to uninfected naïve animals (Figure 4.2 B).

The lung viral clearance is a critical parameter in the assessment of protective efficacy of RSV vaccines. RSV titers were determined in individual lung lysates at 5 days after RSV challenge. The unimmunized naïve cotton rats exhibited highest lung RSV loads. RSV titers in RSV immune cotton rats (FFG-VLP, FI-RSV, RSV reinfections) were below the limit of detection (Figure 4.2 C). These results indicate that FFG-VLP vaccination of cotton rats effectively control RSV infection.
Figure 4.2 FFG-VLP vaccination clears lung viral loads without causing weight loss and AHR PenH. (A) Body weight changes after RSV infection. Immune and naïve cotton rats were intranasally challenged with RSV A2 at 4 weeks after boost immunization (n=5). (B) Airway hyper-responsiveness (AHR). Day 4 post RSV challenge, AHR to increasing concentrations of inhaled methacholine (50-250 mg/ml) was assessed by a whole body plethysmograph and enhanced pause (Penh) values were calculated (n=5). (C) Lung viral titers were determined from individual cotton rat at 5 days after challenge. Statistical significance was performed by two-way ANOVA with Bonferroni post-test to compare replicate in Graph Pad Prism; * p<0.05.

4.2.3 FFG-VLP immunization is effective in inducing RSV F protein specific antibody-secreting cells.

Memory B cell response is important in conferring long-term protection. We determined RSV F-specific IgG antibodies secreted into culture supernatants of MLN, spleen, and BM cells by ELISA (Figure 4.3 A-C). FFG-VLP immunized cotton rats showed significantly higher levels of F specific antibodies in MLN and spleen cells compared to those in the FI-RSV and live RSV groups (Figure 4.3 A, B). Also, RSV F specific IgG antibodies were detected at significantly higher levels in BM cells from FFG-VLP immune cotton rats than those from the FI-RSV group (Figure 4.3 C). These results suggest that FFG-VLP immunization of cotton rats efficiently induces F-specific antibody secreting cell responses in MLN, spleens, and BM.
**Figure 4.3** FFG-VLP is effective in inducing RSV F-specific antibody secreting cell responses. (A) F specific antibody secreting cells in MLN. (B) F specific antibody secreting cells in spleen cells. (C) F specific antibody secreting cells in BM. MLN, BM cells and splenocytes were added to the plates coated with RSV F protein (400 ng/ml) and incubated for 1 day (MLN) or 5 days (BM, Spleens). Secreted antibodies were detected by ELISA analysis. Results of each group (n=5) are presented as mean ± SEM and statistical significance was performed by one-way ANOVA with Tukey’s multiple comparisons post-test in Graph Pad Prism; *** p<0.001, ** p<0.01, * p<0.05.

4.2.4 **FFG-VLP induces a low level of F-specific cytokine secreting cellular responses**

To determine cytokine secreting cellular immune responses, IL-4 and IFN-γ cytokines were determined in culture supernatants of lung and spleen cells with RSV F peptides at day 5 post challenge (Figure 4.4). From spleen cell culture, the live RSV and FI-RSV groups showed more RSV F-specific IFN-γ cytokines than those of the FFG-VLP group (Figure 4.4 A). FI-RSV immune cotton rats exhibited highest levels of IL-4 cytokines from spleen and lung cell cultures with RSV F peptide stimulation (Figure 4.4 B, C). FFG-VLP immune cotton rats did not show IL-4 cytokine production in spleen and lung cell cultures whereas a moderate level of IL-4 cytokine was detected in spleen cell cultures from live RSV immune cotton rats (Figure 4.4 B). These results indicate that FFG-VLP immunization of cotton rats induced a lower level of IFN-γ cytokine than live RSV and FI-RSV whereas FI- RSV immunization induced highest levels of IL-4 and IFN-γ secreting cells.
Figure 4.4 Cotton rats with FFG-VLP vaccination induce lower cellular responses secreting IFN-γ cytokine. (A) IFN-γ secreting splenocytes. (B) IL-4 secreting splenocytes. (C) IL-4 secreting lung cells. Spleen and lung cells were collected from immunized or naïve cotton rats (n=5 per group) at day 5 after challenge and secreted cytokines were determined by cytokine ELISA. The lung (C) and spleen (A, B) cells were cultured by stimulating RSV F peptide on the plates for 72 hours then the levels of IFN-γ and IL-4 were determined from the supernatants. Results are presented as mean ± SEM and statistical significance was performed by one-way ANOVA with Tukey’s multiple comparisons post-test in Graph Pad Prism; *** p<0.001.

4.2.5 FFG-VLP does not cause pulmonary pathology and eosinophilia

Cotton rats have been served as a good model for assessing ERD (51). To determine pulmonary histopathology, lung tissues stained with histological hematoxylin and eosin (H&E) were examined at day 5 post RSV challenge and evaluated for peribronchiolitis, perivasculitis and interstitial pneumonitis as well as histopathological scores (Figure 4.5 A, C-E). Live RSV re-infected cotton rats and naïve cotton rats with primary RSV infection showed a persistent progression of pneumonia as determined by increasing histopathological scores [1.2, 1, 0.75], compared with the un-infected group. The FI-RSV group showed the highest influx of inflammatory cells around the airways and alveolar septa, blood vessels, and interstitial spaces in a range of 2-3 max inflammation scores as well as cell thickening of the airway linings (Figure 4.5 A, C-E). In contrast, the lowest histopathology scores of 0.68, 0.28, and 0.58 were observed
in cotton rats immunized with FFG-VLP around the airways, blood vessels, and interstitial spaces respectively (Figure 4.5 A, C-E).

Over secretion of airway mucus is a major symptom of severe RSV disease resulting in the airway obstruction. To determine the mucus production, lung tissue sections stained with Periodic acid-Schiff (PAS) were prepared and used to measure PAS positive area to quantify PAS staining in the airway epithelium (Figure 4.5 B). The Fl-RSV group exhibited ≥ 10% PAS positivity in 10 randomly selected airways in each rat. In contrast, the FFG-VLP and naïve groups showed less than 2% of PAS positivity of the airway linings after RSV challenge (Figure 4.5 B, F). The live RSV group displayed few spots but no substantial increases in PAS.

**Figure 4.5** FFG-VLP immune cotton rats do not show pulmonary histopathology. (A) H&E histology. H&E stain shows peribronchiolar, perivascular, and alveolar pneumonia. (B) Mucin staining PAS histology. Lung tissues were collected from individual cotton rats at day 5 after RSV A2 challenge and tissue section were stained with H&E (A) and PAS (B) to assess pulmonary histopathologic changes. Scale bars indicate 100 µm. (C-E) Inflammation scoring. Inflammation response on H&E stained tissue section were scored in airway, blood vessels, and interstitial spaces on a scale of 0 to 3 according to diagnostic criteria. (F) Percentages of PAS positive mucus production. Bronchiolar mucus expression was stained with PAS (B) and scored (F) as percentages of positive from 10 individual airways of each cotton rat. Results (n=5 per group) are presented as mean ± SEM and statistical significance was performed by one-way ANOVA with Tukey’s multiple comparisons post-test in Graph Pad Prism; *** p<0.001, ** p<0.01, * p<0.05.
In addition to histological inflammation, we measured eosinophilic infiltrates in the lung sections and BALF 5 days after RSV challenge. While few eosinophils were observed in the live-RSV and FFG-VLP groups following challenge, significant levels of eosinophil infiltrates were observed in the FI-RSV group (Figure 4.6 A-D). H&CR positive eosinophils were detected in the lung histology sections from unimmunized naïve cotton rats with RSV infection at substantial levels (Figure 4.6 A, C).

We also examined morphologically eosinophil-like cells in the airways of BAL cells using cytospin analysis (Figure 4.6 B, D). The numbers of eosinophil-like cells infiltrating into the airways were detected at the highest level from the FI-RSV group (Figure 4.6 B, D). The naïve, FFG-VLP, and live RSV groups did not show any prominent infiltrates of eosinophil-like cells in the airways upon RSV infection (Figure 4.6 D). Therefore, these results suggest that FFG-VLP immunization does not cause pulmonary histopathology and eosinophilia upon RSV challenge.
Figure 4.6 FFG-VLP vaccination does not cause eosinophilia after RSV challenge of cotton rats. (A) H&CR stained histology. (B) Cytospin of BAL cells. Lung tissues slides (A) stained with hematoxylin & congo red and bronchoalveolar lavage (BAL) cells stained with Diff-Quick (B) were analyzed for lung eosinophilia. Scale bars indicate 100 µm for lung and 20 µm for BAL cytospin. (C) Pulmonary eosinophils per 40× field were counted in two different regions of each cotton rats. (D) Differential counts of approximately 300 total cells were performed to determine the absolute numbers of eosinophils. Results (n=5 per group) are presented as mean ± SEM and statistical significance was performed by one-way ANOVA with Tukey’s multiple comparisons post-test in Graph Pad Prism; *** p<0.001.

4.2.6 FFG-VLP immunization modulates cellular infiltration into the lungs upon RSV infection

We determined whether FFG-VLP immunization would reduce infiltrating cells into the lungs and airways upon challenge. FI-RSV immunization of cotton rats resulted in the highest levels of cellular infiltrates into the BAL airways (Fig. 7A) and lungs (Fig. 7B), whereas the FFG and live RSV groups showed similarly low cellular infiltrates in the airways and lung tissues after challenge (Fig. 7A, B).
Figure 4.7 Cotton rats with FFG-VLP do not show cellular infiltrates in BAL and lung cells after RSV challenge. (A) Total cellularity in BALF. (B) Total cellularity in the lung. Cellularity was presented from the results of total BAL and lung cell numbers per cotton rat. Results are presented as mean ± SEM and statistical significance was performed by one-way ANOVA with Tukey’s multiple comparisons post-test in Graph Pad Prism; *** p<0.001.

To better understand infiltrating cell populations in the BAL airways, we analyzed the side-scattering (SSC) and forward-scattering (FSC) properties of cells (Figure 4.8 A), by following a similar gating strategy reported in mice (86, 87). Three distinct cellular populations were found after SSC and FSC gating of cotton rat BAL airway cells, and designated as the gate 1 cells (small lymphocyte-like cells), the large SSC gate 2 cells (monocyte, eosinophil, neutrophil-like cells), and the large SSC and FSC gate 3 cells (dendritic, macrophage-like cells) respectively. The FFG-VLP group showed the airway cellular profiles closely to naïve cotton rats. While bronchoalveolar airway cells exhibited low percentages of lymphocyte-like gate 1 cells in all groups (Gate 1; Figure 4.8 A), substantial cellularity of lymphocytes were detected in the FI-RSV group due to the high total cell numbers (Figure 4.8 B). FI-RSV immunized cotton rats showed the highest cellular infiltrates of granular cell type gate 2 cells in the airways (Gate 2, Figure 4.8 C) while the FFG-VLP and live RSV groups infiltrated low levels of the gate 2 cells (Figure 4.8 C). The live RSV group showed large size gate 3 cells in the BAL airways at
high percentages (Gate 3, Figure 4.8 A) and high cellularity, a similar level as observed in the FI-RSV group (Gate 3, Figure 4.8 D). But, the FFG-VLP group displayed a low level of the gate 3 large cell populations (Figure 4.8 D). A similar profile of granular cellular infiltrations was observed in the lungs of immune cotton rats (data not shown). Therefore there results suggest that FFG-VLP immunization does not induce abnormal infiltration of inflammatory innate and lymphocyte immune cells into the airways and lungs.

Figure 4.8 Flow cytometric analysis of BAL subpopulations from cotton rats after RSV challenge. (A) Flow cytometry profiles of BAL cells based on forward (size) and side (granularity) scattering in BAL cells. Percentages of cell populations of regional gates 1, 2, and 3 in BAL. (B) Cellularity of the gate 1 (lymphocytes). (B) Cellularity of the gate 2 (monocytes, neutrophils, eosinophils). (C) Cellularity of the gate 3 (Dendritic, granular/myeloid and macrophage cells). (B-D) Cellularity was presented from the results of total BAL cell numbers per cotton rats multiplied by percentages of each population. Gate 1: lymphocytes, Gate 2: monocytes, neutrophils, eosinophils, Gate 3: Dendritic, granular/myeloid and macrophage cells. Results are presented as mean ± SEM and statistical significance was performed by one-way ANOVA with Tukey’s multiple comparisons post-test in Graph Pad Prism; *** p<0.001, ** p<0.01.
4.3 Discussion

Cott on rats are more susceptible to RSV disease than mice and used to predict clinical outcomes of the RSV prophylactic antibody products (51, 88, 89). Thus, it is important to test the efficacy and safety of RSV vaccine candidates in cotton rats. In previous studies, FFG-VLP vaccines were shown to be immunogenic and protective in a mouse model (56, 86). Here, we investigated the efficacy and safety of FFG-VLP in a cotton rat model in comparison with FI-RSV and live RSV. FFG-VLP combination vaccine was more effective in inducing RSV F specific antibodies after prime immunization compared to live RSV and FI-RSV. Single immunization with FFG-VLP might be sufficient for clearing lung viral loads. After boost doses, live RSV reinfection induced the highest level of RSV neutralizing activity. As an indicator of protective efficacy, RSV immune cotton rat groups (FFG-VLP, live RSV, FI-RSV) controlled lung viral replication after RSV challenge. It was demonstrated that RSV G specific immune responses to the central G domain recombinant protein (BBG2Na) could effectively control lung viral titers of RSV in mice (90). RSV G specific antibody responses were reported to be significantly lower by 10 to 100 folds than RSV F specific antibodies in mice with either individual F and G VLP vaccination (47, 91) or VLPs co-presenting both F and G (58). The FFG VLP cotton rat group did not show RSV G specific antibody responses and both FI-RSV and live RSV immune cotton rats also induced low levels of anti-RSV G antibodies by approximately 100 folds compared to those specific for RSV F proteins (data not shown). It appears that cotton rats are less responsive to low immunogenic vaccine antigens than mice and that RSV G has low immunogenic properties for raising antibodies. Despite low immunogenicity of RSV G, RSV G VLP vaccination was shown to be more effective in clearing lung viral titers of RSV than RSV F VLP (47, 91). Inclusion of RSV F DNA in the RSV VLP vaccination showed an added effect on
inducing Th1 immune responses but F DNA alone was low immunogenic, requiring high doses of plasmid DNA (56). Thus, use of combination FFG VLPs would have some merits in the RSV vaccination.

In preclinical studies, most RSV vaccine candidates were shown to confer protection against RSV replication in the lungs. Since the tragic failure of FI-RSV vaccine trials in young children (78), it has been challenging to develop safe RSV vaccines. Purified F protein vaccines could confer lung viral control, but resulted in vaccine-enhanced bronchiolar and alveolar histopathology following RSV challenge (33). RSV G is prone to induce G-specific CD4 T cell responses contributing to lung inflammation and eosinophilia in mice after RSV challenge (92). Combination of RSV F and G VLP vaccines was found to have additive effects on inducing protective immunity without causing vaccine-enhanced disease after infection of mice (91). Thus, we have focused on the safety issue of vaccine-enhanced disease by FFG-VLP immunization in comparison with FI-RSV and live RSV reinfections in cotton rats, which is a major challenge in developing RSV vaccines. To assess the safety of FFG-VLP as a vaccine candidate, we determined several clinical features. The FFG-VLP cotton rat group did not show any sign of weight loss whereas FI-RSV immune cotton rats displayed moderate weight loss. Naïve cotton rats also showed a similar weight loss as the FI-RSV group. Severe RSV infection of young infants causes airway obstruction as represented by AHR, resulting in hospitalizations and often needing the mechanical oxygen support (93). The development of AHR to RSV infection is not well known in cotton rat models. Upon RSV infection, naïve cotton rats exhibited the highest Penh values indicating increased AHR. The FI-RSV group also displayed similarly increased Penh of AHR indicating RSV disease. FFG-VLP immunization prevented any increase in Penh, which is similar to uninfected naïve cotton rats. Thus, the results in this study provide
evidence that FFG-VLP does not induce abnormal host immune responses potentially inducing AHR in cotton rats. It is possible that high mucus production and inflammation around the airways might contribute to AHR, which is correlated with severe histopathology observed in the FI-RSV group. Primary infection and the 3rd infection of cotton rats with live RSV showed similarly moderate levels of lung histopathology. However, primary infection of cotton rats with RSV caused significant AHR suggesting that high RSV lung viral loads might be responsible for RSV-induced AHR lung disease.

RSV is an inflammatory disease. Lung histopathology serves as an important disease parameter in evaluating RSV pathogenesis as well as RSV vaccine safety. A moderate level of RSV pathology was reported using cotton rat models (94). In line with these studies, naïve cotton rats showed a substantial level of inflammation as evidenced by an increase in lymphocyte populations probably around the airways. FI-RSV immune cotton rats displayed most severe lung inflammation in all disease parameters including histopathology, inflammation scores around the airways, blood vessels, and interstitial spaces. In addition, FI-RSV vaccination induced highest cellularity including lymphocytes, monocytes, eosinophils, and dendritic cells and macrophages after RSV challenge. Live RSV-reinfected cotton rats showed histopathology similar to the naïve group with RSV infection. FFG-VLP immunization of cotton rats did not cause pulmonary inflammation and histopathology, suggesting that FFG-VLP can be developed as a safe RSV vaccine candidate. This is significant since non-replicating subunit vaccines in alum formulation were shown to cause ERD comparable to FI-RSV (33, 95, 96). Consistent with these results in this study, 50 µg or 150 µg of NDV VLPs containing RSV G and pre-fusion F were shown to be effective in inducing neutralizing antibodies, in clearing lung viral loads, and in preventing pulmonary inflammation after challenge of vaccinated cotton rats (97). Cotton rats
vaccinated with RSV F nanoparticles induced palivizumab-competitive RSV neutralizing antibodies and protection against lung viral replication without overt lung inflammation (45, 98). Presenting RSV F proteins on particulate forms appears to be effective in conferring protection against RSV without ERD.

The underlying cellular mechanisms for inducing vaccine-enhanced disease largely remain unknown in cotton rats probably due to the lack of immunological reagents. Induction of abnormal T cell priming by RSV vaccination was demonstrated to be responsible for RSV disease (33, 95, 99). IFN-γ producing T cells were shown to contribute to RSV protection as well as disease (34). In this study, live RSV and FI-RSV immune cotton rats showed IFN-γ producing T cell responses at significantly higher levels than the cotton rats with FFG-VLP immunization upon RSV challenge, which is consistent with high IFN-γ production in cotton rats after FI-RSV vaccination and RSV challenge in a previous study (21). In particular, FI-RSV immune cotton rats showed highest levels of IL-4 producing cellular responses in spleens and lungs whereas live RSV infections but not FFG-VLP vaccination induced moderate levels of IL-4 splenocytes in cotton rats. FFG-VLP immunization could induce RSV neutralizing antibodies for lung viral clearance without involving significant cellular immune components contributing to RSV disease of weight loss and pulmonary inflammation. The findings in this study suggest that combination of several cellular factors including eosinophils, macrophages, neutrophils, and IL-4 producing T cells locally in lungs and systemically in spleens in addition to high levels of IFN-γ might be contributing to RSV vaccine-enhanced disease and inflammation.

In summary, FFG-VLP immunization could induce protection against RSV infection without RSV disease symptoms in cotton rats. FFG-VLP was found to be effective in inducing B cells that are secreting RSV F specific antibodies and likely long-lived in spleens and bone
marrow. Most importantly, FFG-VLP immunization did not prime cellular components (IL-4 secreting cells, eosinophils) responsible for RSV disease and pulmonary inflammation. Meanwhile, FI-RSV immunization primed RSV disease-contributing cellular responses. Live RSV infections could induce a moderate level of pulmonary inflammation and were not highly effective in inducing RSV specific antibody secreting cells compared to FFG-VLP. These results provide evidence that FFG-VLP warrants to be further developed into a safe RSV vaccine candidate.

5 CHAPTER 5. VIRUS-LIKE PARTICLE VACCINES CONTAINING F OR F AND G PROTEINS CONFER PROTECTION AGAINST RESPIRATORY SYNCYTIAL VIRUS WITHOUT PULMONARY INFLAMMATION IN COTTON RATS

5.1 SUMMARY

Vaccine-enhanced disease has been a major obstacle in developing a safe vaccine against respiratory syncytial virus (RSV). This study demonstrates the immunogenicity, efficacy, and safety of virus-like particle (VLP) vaccines containing RSV F (F VLP), G (G VLP), or F and G proteins (FG VLP) in cotton rats. RSV specific antibodies were effectively induced by vaccination of cotton rats with F VLP or FG VLP vaccines. After challenge, lung RSV clearance was observed with RSV F, G, FG VLP, and formalin inactivated RSV (FI-RSV) vaccines. Upon RSV infection, cotton rats with RSV VLP vaccines were protected against airway hyper-responsiveness and weight loss, which are different from FI-RSV vaccination exhibiting vaccine-enhanced disease of airway obstruction, weight loss, and severe histopathology with eosinophilia and mucus production. FG VLP and F VLP vaccines did not cause pulmonary inflammation whereas G VLP induced moderate lung inflammation with eosinophilia and mucus production. In particular, F VLP and FG VLP vaccines were found to be effective in inducing antibody
secreting cell responses in bone marrow and lymphoid organs as well as avoiding the induction of T helper type 2 cytokines. These results provide further evidence to develop a safe RSV vaccine based on VLP platforms.

5.2 Results

5.2.1 F VLP or F VLP plus G VLP immunization induces RSV F specific antibodies in cotton rats.

In this study using cotton rats as a relevant animal model, we investigated the protective efficacy of F VLP, G VLP, and mixed F VLP and G VLP (FG VLP) vaccines in comparison with FI-RSV. Cotton rats were intramuscularly immunized with F VLP, G VLP, and FG VLP without adjuvant, or FI-RSV with alum adjuvant. RSV F-protein specific antibodies were determined in sera collected at 3 weeks after prime and boost immunization. Cotton rats with F or FG VLP vaccination induced significant levels of RSV F-specific IgG antibodies after prime (Figure 5.1A), which were further increased after boost (Figure 5.1B). The FG VLP group showed higher levels of F-specific antibodies than the F VLP group whereas the G VLP group did not induce F specific antibodies after prime and boost. IgG levels in the FG VLP group were higher than those of the FI-RSV group after prime (Figure 5.1A) and became to be similar as those of the FI-RSV groups after boost (Figure 5.1 B). FI-RSV, FG VLP and F VLP induced considerable amounts of RSV specific antibodies in prime and boost immune sera. G VLP immunization in cotton rat induced low levels of RSV specific antibodies (Figure 5.1 C, D). RSV G specific antibodies were induced at low levels in FI-RSV, FG VLP, F VLP and G VLP boost immune cotton rats but not detected in prime immune sera (Figure 5.1 E, F).
FG VLP is effective in inducing RSV F protein-specific antibodies in cotton rats. (A) Prime IgG antibodies specific for RSV F protein. (B) Boost IgG antibodies specific for RSV F protein. (C) Prime IgG antibodies specific for RSV. (D) Boost IgG antibodies specific for RSV. (E) Prime IgG antibodies specific for RSV G protein. (F) Boost IgG antibodies specific for RSV G protein. Cotton rats (n=5 per group) were immunized i.m. with F VLP, G VLP, mixed F VLP and G VLP (FG VLP), FI-RSV (FI-RSV), and PBS (Naïve) on days 0 (prime) and 28 (boost). Serum samples were collected at 3 weeks after prime or boost immunization and RSV F protein-specific antibody levels were measured by ELISA.

To gain a functional neutralizing activity of immune sera, we determined neutralizing activity of antibodies against A2-K-line19F RSV (Figure 5.2). Immune sera from the FI-RSV, FG VLP, F VLP and G VLP immunized groups showed significantly higher levels of neutralizing activity compared to the naïve group. Combination FG or F only VLP vaccines was effective in inducing RSV F specific and neutralizing antibodies after immunization of cotton rats.
Figure 5.2 F VLP or FG VLP immunization induces neutralizing activity against RSV in cotton rats. Neutralizing activity of boost sera. For neutralizing assay, immune sera were inactivated and serially diluted. Sera were incubated with RSV expressing the red fluorescent monomeric Katushka 2 protein (A2-K-line19F) (500 PFU), then were added to Hep2 cell monolayers for 2-3 days to determine percentages of plaque reduction. Results are presented as mean ± SEM.

5.2.2 RSV VLP vaccination confers protection without airway resistance and body weight loss.

To determine protective efficacy, RSV VLP immune cotton rats were intranasally challenged with RSV A2 strain and body weight changes were daily monitored (Figure 5.3 A). The F, G, or FG VLP group did show less than 2% of weight loss after RSV challenge (Figure 5.3 A). The FI-RSV and naïve cotton rat groups showed 5% and 4% of weight loss respectively after RSV challenge. These results suggest that RSV VLP immunization is effective in preventing weight loss against RSV infection.

The airway obstruction and bronchoconstriction can be an indicator for severe pulmonary disease due to RSV infection. The airway resistance Penh (%) values were measured day 4 post challenge using plethysmography. RSV infected naïve and FI-RSV immunized cotton rats displayed highest Penh values in response to aerosolized methacholine challenge in a dose
responsive manner (Figure 5.3 B). Meanwhile, F, G, or FG VLP immunized cotton rats did not show an increase in Penh values, which are similar to uninfected naïve animals (Figure 5.3 B).

RSV titers were determined in individual lung extracts at 5 days after RSV challenge by an immuno-plaque assay (Figure 5.3 C). The unimmunized naïve cotton rats exhibited high lung viral loads with average titers of 4.5 log10 at day 5 post-challenge. FI-RSV, F and FG VLP immune cotton rats did not show viral titers above the detection limit (1.7 log10). The G VLP group exhibited low viral titers close to the limit of detection. Thus, insect cell-derived VLP vaccination can effectively control RSV replication without airway resistance of hyper-responsiveness and weight loss in cotton rats.

![Figure 5.3 RSV VLP vaccination prevents weight loss, AHR and clears lung viral loads.](image)

(A) Body weight changes after RSV infection. Cotton rats were challenged i.n. with RSV A2 (1X10⁶ PFU) on day 56. (B) Airway hyper responsiveness (AHR). At 4 days post RSV challenge, AHR to increasing concentrations of methacholine (0, 50, 250 mg/ml) was assessed by whole body plethysmography and Penh values were calculated. (C) RSV titers. Lung viral titers were determined from individual cotton rat with lung lysate at 5 days after RSV challenge. Statistical significance was performed by two-way ANOVA with Bonferroni post-test to compare replicate mean values in Graph Pad Prism; ** p<0.01, * p<0.05; compared to Naïve, †† p<0.01; compared to Naïve-Inf.

5.2.3 FG VLP vaccination induces B cells capable of secreting F-specific IgG antibodies

To determine antibody secreting cell responses in vitro, BM, MLN, and spleen were cultured and RSV F protein-specific IgG antibodies determined by ELISA (Figure 5.4 A-C). FG VLP or F VLP immunized cotton rats induced higher levels of F specific antibody secreting cell
responses in BM, MLN, and spleens than those from the FI-RSV, G VLP or naïve group with infection (Figure 5.4). To determine mucosal antibody response, RSV F specific IgG antibodies were determined in bronchoalveolar lavage fluids (BALF) and lung extract samples collected at day 5 post challenge (Figure 5.4 D, E). Significantly higher levels of RSV F specific IgG antibodies were induced in the BALF (Figure 5.4 D) and lungs (Figure 5.4 E) from the FG VLP or F VLP group compared to those in FI-RSV, G VLP immune or naïve cotton rats. These results suggest that FG or F VLP immunization of cotton rats efficiently induces mucosal antibody responses as well as antibody secreting cells and long-lived B cells that can differentiate into F-specific antibody secreting cells in MLN, spleens, and BM.

Figure 5.4 FG VLP or F VLP vaccination is effective in inducing RSV F-specific antibody secreting cell and mucosal RSV F-specific antibodies. (A) BM cells secreting F specific IgG antibodies. (B) MLN cells secreting F specific IgG antibodies. (C) Spleen cells secreting F specific IgG antibodies. Cells from BM, spleens, and MLN were incubated in the culture plates coated with RSV F protein (400 ng/ml) for 2 days. Secreted antibodies were detected by ELISA analysis. RSV F-protein specific mucosal IgG antibody responses in BALF (D) and lung extracts (E) were determined by ELISA. Results are presented as mean ± SEM and statistical significance was performed by one-way ANOVA with Tukey’s multiple comparisons post-test in Graph Pad Prism; *** p<0.001, ** p<0.01, * p<0.05.
5.2.4 **FG VLP vaccination does not cause pulmonary histopathology and eosinophilia after challenge**

To determine pulmonary histopathology, lung tissues at day 5 post RSV challenge were stained and evaluated for peribronchiolitis, perivasculitis, interstitial pneumonitis, and histopathological scores (Figure 5.5 A, B-D). The FI-RSV group showed highest influx of inflammatory cells around the airways and alveolar septa (B, 2.3), blood vessels (C, 1.8), and interstitial spaces (D, 1.9) as well as cell thickening of airway linings (Figure 5.5 A, B-D). RSV infection of naïve cotton rats also caused moderate levels of pulmonary inflammation in the airways (1.2), blood vessels (0.7), and interstitial spaces (0.6). The lowest histopathology scores around the airways, blood vessels, and interstitial spaces were observed with FG VLP or F VLP vaccination (Figure 5.5 A, B-D). The G VLP group displayed a certain degree of inflammation around the airways, a higher level than the FG or F VLP group.

In addition to histological inflammation, we measured eosinophilic infiltrates in the lung sections by H&CR staining. The FG and F VLP groups did not have eosinophil infiltrations similar to naïve uninfected cotton rats (Figure 5.5 A, E). It is notable that G VLP immune cotton rats showed a higher level of eosinophils than that of FG VLP or F VLP immune animals.

To determine mucus production, lung tissue sections stained with PAS were used to quantify PAS positive area in the airway epithelium (Figure 5.5 A, F). The FI-RSV group exhibited high PAS positivity in 10 randomly selected airways in each rat. In contrast, the FG VLP group showed lowest PAS positivity similar to naïve uninfected cotton rats. G VLP immune cotton rats exhibited substantial levels of mucus production as indicated by PAS positivity. Overall these results suggest that FG VLP and F VLP can prevent RSV vaccine-
enhanced pulmonary inflammation whereas G VLP can cause a certain level of lung inflammation upon RSV infection.

**Figure 5.5** FG VLP does not cause pulmonary histopathology in cotton rats after RSV challenge. (A) Representative histology pictures with H&E, PAS, and H&CR. Lung tissues were collected from individual cotton rats at day 5 after RSV A2 challenge and tissue section stained with H&E, PAS and H&CR to assess pulmonary histopathologic changes. H&E stain shows peribronchiolar, perivascular, and alveolar pneumonia. (B-D) Histopathology scores. Inflammation response on H&E stained tissue section were scored in the airways (B), blood vessels (C), and interstitial spaces (D) on a scale of 0 to 3 according to diagnostic criteria. (E) Eosinophils. Pulmonary eosinophils per 40X field were counted in two different regions of each cotton rats. (F) Mucus producing PAS positive area (%). Bronchiolar mucus expression was stained with PAS (A, F) and scored as percentages of
positive from 5 individual airways of each cotton rat. Scale bars indicate 100 µm in H&E and PAS staining and 20 µm in H&CR staining. Results are presented as mean ± SEM and statistical significance was performed by one-way ANOVA with Tukey's multiple comparisons post-test in Graph Pad Prism; *** p<0.001, ** p<0.01, * p<0.05.

RT-PCR was applied to determine cytokine and chemokine mRNA expression levels in lung tissue samples collected from FG VLP vaccinated cotton rats at day 5 post challenge (Figure 5.6). Th1 IFN-γ mRNA levels were observed at the highest levels in the FG VLP group (Figure 5.6 B). Naïve cotton rats also showed a moderate level of IFN-γ mRNA upon RSV infection. In contrast, Th2 type IL-4 mRNA levels were highest in the FI-RSV group (Figure 5.6 C). It is notable that FI-RSV immune cotton rats induced the highest level of tumor necrosis factor (TNF)-α mRNA expression after RSV challenge whereas the FG VLP group showed a background level of TNF-α mRNA (Figure 5.6 D). The chemokine interferon-γ inducible protein-10 (IP-10) mRNA expression was detected at the highest level in naïve cotton rats with RSV infection (Figure 5.6 E). Interestingly, the naïve group showed high levels of IL-6 and IL-10 mRNA expression but these cytokine mRNAs were not detected in other groups (Figure 5.6 F, G), suggesting a correlation with high viral loads in cotton rats after RSV infection. These results suggest that FG VLP vaccination modulates the induction of Th1 and Th2 cytokines, and chemokines possibly relating to the prevention of RSV vaccine-enhanced disease.

![Image of RT-PCR results](image-url)
**Figure 5.6** FG VLP vaccination differentially modulates cytokine and chemokine gene expression in the lungs after RSV challenge.

(A) RT-PCR bands of cytokine and chemokine gene expression. Cotton rats were immunized and challenged as described in the Figure 2. (B-G) Values representing expression for a particular gene as indicated were individually quantified by densitometry; mean and SD are shown. IFN, interferon; IL, interleukin; IP-10, IFN-inducible protein-10; TNF-a, tumor necrosis factor-a. Statistical significance was performed by one-way ANOVA with Tukey’s multiple comparisons post-test in Graph Pad Prism; *** p<0.001, ** p<0.01, * p<0.05, n.d.; non detected.

### 5.2.5 FG VLP vaccines differentially modulates infiltration of granulocytes and lymphocytes into the lungs upon RSV challenge

To better understand infiltrating cell populations in the lungs, we analyzed the side-scattering (SSC) and forward-scattering (FSC) profiles of cells (Figure 5.7 A), by following a similar gating strategy reported (100, 101). SSC and FSC gating of cotton rat lung cells displayed three distinct cellular populations: the gate 1 cells (small lymphocyte like cells), the large SSC gate 2 cells (monocyte, eosinophil, neutrophil like cells), and the large SSC and FSC gate 3 cells (dendritic, macrophage-like cells) respectively. The FI-RSV group showed the large granular size gate 2 cells in the lungs at the highest percentages and cellularity, which are 5 to 10 fold higher than those in other groups (naïve, naïve-inf, FG, F, G VLP) (Figure 5.7 B-E).
FG VLP group displayed a similar pattern of cellular distributions as observed in uninfected naïve cotton rats (Figure 5.7). F or G VLP immune cotton rats showed a relatively low cellularity in the small size lymphocyte-like gate 1 cells compared to other groups (Figure 5.7 C). Therefore these results suggest that RSV F, G, or FG VLP immunization of cotton rats does not induce abnormal infiltration of inflammatory innate and lymphocyte immune cells into the lungs.

Figure 5.7 Flow cytometric analysis of cellular infiltrates into the lungs after RSV challenge of cotton rats.
(A) Flow cytometry profiles of lung cells based on forward (size, FSC) and side (granularity, SSC) scattering in lung cells. Percentages of cell populations of regional gates 1, 2, and 3 in lung cells. (B) Cellularity of the gate 1 (lymphocytes). (B) Cellularity of the gate 2 (monocytes, neutrophils, eosinophils). (C) Cellularity of the gate 3 (Dendritic, granular/myeloid and macrophage cells). (D-E) Cellularity was presented from the results of total lung cell numbers per cotton rats multiplied by percentages of each population. Gate 1: Small lymphocyte-like cells, Gate 2: Large monocytes, neutrophils, and eosinophils-like cells, Gate 3: Dendritic, granular/myeloid and macrophage-like cells. Results are presented as mean ± SEM and statistical significance was performed by one-way ANOVA with Tukey’s multiple comparisons post-test in Graph Pad Prism; *** p<0.001, ** p<0.001, * p<0.05.

5.3 Discussion
Cotton rats are considered an appropriate animal model that can reliably predict the clinical outcomes (88, 89). Recently, the RSV F nanoparticles produced in insect cells were reported to be safe in healthy individuals and young women of childbearing age in phase I and II clinical trials (102, 103). Protection was reported without causing RSV disease by immunization...
of mice with mixed F VLP and G VLP (FG VLP) vaccines with DNA plasmids expressing F proteins as evidenced by clearing lung viral loads and preventing pulmonary inflammation (104). However, the efficacy and safety of RSV F, G, or F+G VLP vaccine platforms have not been studied in cotton rats, which is the main focus in this study. We have investigated the immunogenicity, RSV disease assessment (weight loss, AHR), efficacy, cytokine patterns to indicate the type of immune response upon RSV infection, and vaccine-associated safety of F, G, and FG VLP vaccines in cotton rats. F, G, and FG VLP vaccines did not cause RSV disease of weight loss and AHR in addition to conferring protection against RSV replication. Mixed FG VLP was found to be more immunogenic in inducing RSV F specific antibody responses and less PAS positive mucus production compared to other RSV vaccines.

RSV is an enveloped virus. Therefore, enveloped non-replicating VLPs would provide an attractive approach to mimic the virus. Chimeric NDV-RSV VLPs in avian cells by transiently transfecting DNA plasmids expressing multiple NDV proteins and chimeric RSV G plus F proteins were not effective in producing F alone VLPs (58). In the insect cell expression system, full length RSV F or G proteins were effectively incorporated into VLPs (47). In cotton rats, this study demonstrated that F VLP was more immunogenic and effective in clearing lung viral titers than G VLP although both F VLP and G VLP did not cause weight loss and AHR lung disease after RSV challenge. FI-RSV immune and naïve cotton rats showed high AHR representing RSV disease mimicking the RSV pathogenesis in humans. Cotton rats with G VLP presented a tendency of causing a certain degree of lung inflammation around the airways, blood vessels, and interstitial spaces as well as eosinophils and mucus production compared to F VLP or FG VLP but much less than FI-RSV. Cotton rats with F VLP did not show RSV disease after challenge,
which is consistent with the results in mice (105). Interestingly, cotton rats with FG VLP significantly suppressed RSV disease of eosinophila and mucus production, compared to G VLP alone and even better than F VLP. This result of additive effects on preventing RSV disease in cotton rats by FG VLP is similarly observed in a mouse model (91).

FI-RSV immune or naïve cotton rats exhibited RSV disease such as weight loss and AHR after RSV infection. Cotton rats with FI-RSV showed high levels of IL-4 and inflammatory TNF-α cytokines compared to FG VLP immune cotton rats. Mice primed with vv-G vaccines induced high levels of both Th2 IL-4 and Th1 IFN-γ cytokines at high levels while vv-F primed mice showing high levels of IFN-γ producing cells (40). IFN-γ has a dual role of RSV protection and RSV disease with high IFN-γ T cell responses (34). Th2 cytokines such as IL-4 have been implicated in the development of lung immunopathology whereas TNF-α inflammatory cytokine was shown to be responsible for weight loss in mice (106). Naïve cotton rats with RSV infection induced higher levels of IP-10 and IL-6 cytokines which were also shown to be associated with acute RSV infection (107, 108). Thus, high levels of Th2, Th1, and inflammatory cytokines as well as chemokine IP-10 might be involved in causing RSV disease, which are modulated to low levels by FG VLP vaccination preventing RSV disease.

In conclusion, F VLP and FG VLP vaccines were immunogenic and able to confer protection without causing RSV disease and pulmonary inflammation in cotton rats. Importantly, inclusion of F VLP in the G VLP vaccination of cotton rats could prevent eosinophilia and mucus production after RSV challenge. This study provides evidence that F VLP and FG VLP can be developed into safe RSV vaccine candidates.
6 CHAPTER 6. Priming with virus-like particle vaccine modulates immune responses preventing vaccine-enhanced disease of respiratory syncytial virus

6.1 Summary

Formalin inactivated respiratory syncytial virus (FI-RSV) vaccination causes vaccine-enhanced respiratory disease (ERD) upon exposure to RSV. Virus-like particle vaccines presenting RSV F fusion protein (F VLP) are known to increase T helper type-1 (Th1) antibody responses and not to cause ERD. We hypothesized that priming with F VLP would modulate immune responses preventing ERD upon subsequent exposure to ERD-causing FI-RSV. In this study, we found that F VLP priming and FI-RSV boosting of mice prevented FI-RSV vaccine-enhanced lung inflammation and eosinophilia upon RSV challenge. F VLP priming redirected pulmonary T cells toward effector CD8 T cells producing Th1 cytokines and significantly suppressed pulmonary Th2 cytokines. F VLP exhibited a unique profile of stimulating innate immune responses. This study suggests that RSV F VLP subunit vaccine priming would modulate both innate and adaptive immune responses to subsequent exposure to FI-RSV, resulting in suppression of Th2 immune-mediated pulmonary inflammation and eosinophilia.

6.2 Results

6.2.1 Experimental design

BALB/c mice (n=5; Charles River Laboratories, Inc., Wilmington, MA) aged 6 to 8 weeks were used for vaccination. Mice were intramuscularly (i.m.) immunized at a 4-week
interval; FI-RSV (15 ug) prime – FI-RSV (2 ug) boost for homologous protocol and F VLP prime – FI-RSV boost for heterologous protocol.

6.2.2  F VLP priming modulates RSV specific IgG isotype antibodies.

In this study, we tested a hypothesis whether priming with F VLP would dictate immune responses to subsequently exposed FI-RSV toward Th1 patterns resulting in prevention of FI-RSV vaccine-ERD. Groups of mice were i.m. primed with FI-RSV (2 µg) or F VLP (10 µg) and RSV F specific IgG and isotype (IgG1 and IgG2a) antibodies determined in sera 3 weeks later (Figure 6.1). Total IgG antibodies specific for RSV F were induced at a similar level in both FI-RSV and F VLP groups (Figure 6.1 A). However, when IgG isotypes were determined, priming with FI-RSV induced IgG1 isotype dominant antibodies, which indicates Th2 type biased immune responses. Whereas F VLP prime immunization elicited IgG2a isotype dominant response, suggesting that F VLP primes immune responses toward a Th1 pattern (Figure 6.1 C, D).

We determined whether F VLP-primed mice would maintain a pattern of IgG2a dominance upon subsequent exposure to FI-RSV. As expected, FI-RSV prime and boost immunizations induced higher levels of IgG1 isotype antibodies specific for RSV F (Figure 6.1 B, C, D). Importantly, F VLP primed mice induced higher levels of IgG2a than IgG1 isotype antibodies even after subsequent FI-RSV exposure (F VLP/FI-RSV). Therefore, these results suggest that F VLP priming can dictate the type of IgG isotypes upon the subsequent FI-RSV vaccination.
Figure 6.1 Antibody responses specific for RSV F proteins. (A) IgG, (B) IgG1 and (C) IgG2a isotypes antibody responses specific for the RSV F protein. (D) Ratios of IgG2a to IgG1 isotype antibodies. FI-RSV: formalin inactivated alum precipitated RSV (FI-RSV) vaccine (2 μg). F VLP: RSV F VLP (10 μg). FI-RSV/ FI-RSV: homologous immunization with FI-RSV (2 μg for prime and boost). F VLP/FI-RSV: heterologous immunization prime with F VLP followed by FI-RSV boost (10 μg for prime of F VLP and 2 μg for boost of FI-RSV). Groups of mice (n=5 per group) were intramuscularly immunized at week 0 (prime) and 4 (boost). Results are presented as mean ± SEM. Statistical significances were calculated by 1-way ANOVA and Tukey’s multiple comparison test. *, p<0.05; **, p<0.01; and ***, p<0.001.

6.2.3 F VLP prime and FI-RSV boost immune mice are protected against RSV

To determine the protective efficacy of heterologous F VLP prime and FI-RSV boost vaccination, mice were challenged with RSV A2 at 10 weeks after boost (Figure 6.2). FI-RSV prime and boost immune mice displayed approximately 5% of weight loss at day 2 post-challenge and then slowly recovered body weight close to a normal level. The heterologous F VLP/FI-RSV group did not exhibit weight loss after RSV challenge. Lung viral loads were determined from individual lung lysates at 5 days after RSV challenge. Naïve mice exhibited high lung viral titers of RSV approaching to a level of 4 of log10. Both prime boost immune
mice (FI-RSV/FI-RSV, F VLP/FI-RSV) controlled RSV lung viral loads to a level below the detection limit (Figure 6.2 B). These results suggest that heterologous F VLP prime and FI-RSV boost immune mice are protected against weight loss and RSV replication.

**Figure 6.2** Body weight changes and lung viral load after RSV A2 challenge.

Naïve and all immunized mice were intranasally infected with 1X10⁶ PFU of RSV A2 strain 15 weeks after boost immunization. (A) Each mouse were monitored body weight changes. (B) Lungs from individual mice were collected on day 5 post challenge and lung viral load (PFU/g of lung tissue) were determined by immunoplaque assay in HEp2 cells. Naïve-inf.: unimmunized naïve mice after RSV. Other groups are the same as described in the legend of Fig. 1. Results are presented as mean ± SEM. Statistical significances were calculated by 1-way ANOVA and Tukey’s multiple comparison test. *; p<0.05, **; p<0.01, and ***; p<0.001. Groups are the same as described in the legend of Figure 6.1.

In functional neutralizing antibody assay, immune sera from the F VLP prime and FI-RSV boost or FI-RSV prime and boost immune mice showed significantly higher levels of neutralizing activity compared to naive sera. Thus, the F VLP prime immunization was effective in inducing RSV neutralizing antibodies after FI-RSV boost immunization of mice (Figure 6.3).
Figure 6.3 Neutralizing activity after boost sera.
For neutralizing assay, immune sera were inactivated and serially diluted. Sera were incubated with RSV expressing the red fluorescent monomeric Katushka 2 protein (A2-K-line19F) (500 PFU), then were added to Hep2 cell monolayers for 2-3 days to determine percentages of plaque reduction. Results are presented as mean ± SEM. *; p<0.05, **; p<0.01, and ***; p<0.001 compared to indicated among the groups.

6.2.4 F VLP / FI-RSV immune mice do not develop pulmonary inflammation and eosinophilia

A challenging difficulty in the development of RSV vaccine candidates is the safety aspect of vaccines. To determine pulmonary inflammation and histopathology of mice after challenge, we examined lung tissues after staining with H&E, and evaluated peribronchiolitis, perivasculitis and interstitial pneumonitis (Figure 6.4 A-D). As expected, the FI-RSV primed group showed the highest influx of inflammatory cells around the airways, blood vessels, and interstitial spaces as well as cell thickening of the airway linings (Figure 6.4 A). In contrast, severity of histopathology pulmonary inflammation was not observed in the hetero prime boost F
VLP/FI-RSV group, which is similar to or lower than that in naïve mice with RSV infection (Figure 6.4 A, C-E).

**Figure 6.4** Heterologous immunization effectively reduces pulmonary pneumonia histopathology.

Lung tissues were collected from individual mice at day 5 post RSV challenge (10⁶ PFU/mouse) and prepared for histology analysis. (A) H&E staining of lung tissues. 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. Inflammation scores around airways (B), blood vessels (C) and interstitial spaces (D). Results are presented as mean ± SEM. Statistical significances were calculated by 1-way ANOVA and Tukey’s multiple comparison test. *; p<0.05, **; p<0.01, and ***; p<0.001.

PAS staining of lung tissue sections was used to quantify mucus production in the airway epithelium (Figure 6.5 A, C). The FI-RSV group exhibited ≥ 6% PAS positivity in 10 randomly selected airways in each mouse. In contrast, the heterologous F VLP/FI-RSV groups showed less than 2% of PAS positivity of the airway linings after RSV challenge (Figure 6.5 A, C).

We measured eosinophilic infiltrates in two different methods by histological staining with H&CR in the lung sections (Figure 6.5 B) and by phenotypic determination using flow cytometry (Figure 6.5 E). The significant eosinophilic infiltrates were observed in FI-RSV
primed mice (Fig. 4 B, D). CD45\(^+\)CD11c\(^-\)CD11b\(^+\)SiglecF\(^+\) phenotypic eosinophils were also quantified at the highest level in FI-RSV/FI-RSV mice (Figure 6.5 E). In correlation with eosinophilic infiltration, eotaxin chemokine levels were highest in FI-RSV primed mice (Figure 6.5 F). In contrast, F VLP primed mice did not display such high levels of PAS positive mucus production, H&CR stained eosinophils, eosinophilic marker expressing cells, and eotaxin chemokine in the lung (Figure 6.5). Similarly, RSV-infected naïve mice did not show lung histopathology (Figure 6.5). Taken together, these results provide evidence that F VLP priming significantly diminishes FI-RSV vaccine-enhanced pulmonary inflammation and eosinophilia in mice upon exposure to RSV.

Figure 6.5 Heterologous immunization prevents mucus production and eosinophilia in lung tissues.
(A) Periodic Acid Schiff (PAS) staining to determine mucus production. (C) PAS-positive area in airways (10 individual airways in each mouse) was quantitated and represented as percentage. (B) H&CR staining to determine pulmonary eosinophilia. Scale bars for PAS and H&CR indicate 100 and 20µm respectively. The insets in H&CR images are details of eosinophil infiltration in lungs. (D) Pulmonary eosinophils per 40x field counts in two different regions of each mice. (E) Eosinophils (CD11b+SiglecF+) in CD45+CD11c− large cell gates of lung cells by flow
cytometry. (F) Eotaxin production in BALF and lung homogenates was determined by ELISA. Results are presented as mean ± SEM. Statistical significances were calculated by 1-way ANOVA and Tukey’s multiple comparison test. *: p<0.05, **: p<0.01, and ***: p<0.001.

6.2.5 F VLP priming redirects pulmonary effector CD4 and CD8 T cells

To better understand how F priming prevents severe FI-RSV vaccine-enhanced pulmonary inflammation, we determined cellular responses in the lungs at day 5 post challenge. The cellularity of lung CD4 T cells and B220+ plasmacytoid dendritic cells (pDCs) was lower in the F VLP/FI-RSV group than that of the FI-RSV/FI-RSV group (data now shown). In intracellular cytokine staining assay using an RSV F peptide, F VLP priming of mice resulted in 2- to 10-fold higher levels of lung CD8 T cells that express IFNγ+IL-4−, IFNγ+TNFα−, IFNγ+TNFα+, and IFNγ+TNFα+ compared to those in FI-RSV primed mice (Figure 6.6 A, B). These results were consistent with 10 fold higher levels of IFN-γ+ expressing cytokine ELISpots in response to CD8 T epitope (F92-106) stimulation in F VLP primed mice than those in FI-RSV primed mice after FI-RSV boost and RSV challenge, as measured by (data not shown). In contrast, lung cells from FI-RSV/FI-RSV mice showed significantly lower levels of CD8 T cells producing IFNγ+IL-4−, IFNγ+TNFα−, and IFNγ+TNFα+ compared to those in F VLP primed mice (Figure 6.6 A, B). Meanwhile, lung cells from FI-RSV/FI-RSV mice showed the highest levels of CD4 T cells producing IFNγ IL-4+, IFNγ TNF-α+, IFNγ+IL-4−, and IFNγ+TNFα− (Figure 6.6 C, D). Whereas F VLP/FI-RSV mouse lung cells displayed moderate to low levels of CD4+ T cells expressing IFNγ+IL-4−, IFNγ IL-4+, IFNγ TNF-α+, and IFNγ+TNFα− (Figure 6.6 C, D). Therefore, these results suggest that F VLP priming significantly redirects pulmonary T cells toward effector CD8 T cells expressing Th1 type cytokines. It is also likely that FI-RSV vaccination recruits high levels of lung CD4 T cells expressing Th1 and Th2 cytokines possibly contributing to FI-RSV vaccine-ERD.
Figure 6.6 Heterologous immunization elicits high levels of IFN-γ secreting CD8+ T cells.

BAL cells were isolated from mice (n=5 per group) at day 5 post challenge, then the cells were stimulated with RSV F92-106 peptide to investigate the levels of CD4+ or CD8+ T cell secreting intracellular cytokines by flow cytometry. 

(A) The numbers of CD8+ T cells secreting IFN-γ (TNF-α IFN-γ) or TNF-α (TNF-α IFN-γ). (B) The numbers of CD8+ T cells secreting IL-4 (IL-4 IFN-γ) or IFN-γ (IL-4 IFN-γ). (C) The numbers of CD4+ T cells secreting IFN-γ (TNF-α IFN-γ) or TNF-α (TNF-α IFN-γ). (D) The numbers of CD4+ T cells secreting IL-4 (IL-4 IFN-γ) or IFN-γ (IL-4 IFN-γ). Results are presented as mean ± SEM. Statistical significances were calculated by 1-way ANOVA and Tukey’s multiple comparison test. *; p<0.05, **; p<0.01, and ***; p<0.001 comparing of IL-4 IFN-γ or TNF-α IFN-γ between indicated groups, †; p<0.05, ††; p<0.01, †††; p<0.001, comparing of TNF-α IFN-γ or IL-4 IFN-γ between indicated groups.

6.2.6 F VLP priming suppresses pulmonary Th2 cytokines by FI-RSV boost

Th2 cytokines are associated with FI-RSV vaccine-ERD. We determined whether F VLP priming would suppress the induction of Th2 cytokines in the airway BAL fluids (BALFs) and in the lung from F VLP primed and FI-RSV boost immune mice after RSV challenge (Figure 6.7). Significantly lower levels of IL-4, IL-5, and IL-13 Th2 cytokines by approximately 2-, 10-, and 5 fold respectively were detected in the lung extracts from F VLP/FI-RSV mice at day 5 post
challenge compared to those from FI-RSV/FI-RSV mice (Figure 6.7 A, B, C). Similarly, IL-4, IL-5, and IL-13 cytokines in BALFs were observed at 3 to 6 fold lower levels in the F VLP/FI-RSV group than those in the FI-RSV prime boost mice (Figure 6.7 D, E, F). Naïve mice with RSV infection showed a pattern of cytokines similar to F VLP primed mice (Figure 6.7).

![Graphs A, B, C, D, E, F showing cytokine levels](image)

**Figure 6.7** Th2 cytokines upon RSV infection significantly decreases in lung lysates and BALF of heterologous immunized mice.

BALF and lung lysate cell were collected from immunized or naïve mice (n=5 per group) at day 5 post challenge of RSV A2 and cytokines production were determined by ELISA. (A, D) The levels of IL-4 in lung and BLAF. (B, E) The levels of IL-5 in lung and BLAF. (C, F) The levels of IL-13 in lung and BLAF. Results are presented as mean ± SEM. Statistical significances were calculated by 1-way ANOVA and Tukey’s multiple comparison test. *; p<0.05, **; p<0.01, and ***; p<0.001.

### 6.2.7 F VLP recruits multiple innate immune cells and macrophage cells different from FI-RSV.

To gain mechanistic insight whereby F VLP priming modulates immune responses toward Th1 patterns upon subsequent exposure to FI-RSV, we determined acute innate cytokine
and cellular responses to vaccines (Figure 6.8, 6.9). We analyzed different cytokines and chemokines in peritoneal exudate at 24 hour after i.p. injection (Figure 6.8). F VLP-treated mice induced cytokines (IL-6, IFN-γ) and chemokines (MCP-1, RANTES, IP-10) at 24 h in peritoneal exudates, which were at low or below the detection limit in FI-RSV treated mice (Figure 6.8 B). These results suggest that transient induction of inflammatory cytokines and chemokines by F VLP priming might be contributing to shaping Th1 type adaptive immune responses.

**Figure 6.8 F VLP induces acute production of cytokines**

Balb/c mice (n=5) were intraperitoneally injected with PBS, F VLP, or FI-RSV. Cytokines in peritoneal exudates at 24 h after injection. Interleukin 6 (IL-6) (A), Interferon gamma (IFN-γ) (B), Monocyte chemoattractant protein 1 (MCP-1) (C), RANTES (regulated on activation, normal T cell expressed and secreted chemokine) (D), Interferon gamma-induced protein 10 (IP-10) (E). Results are presented as mean ± SEM. Statistical significances were calculated by 2-way ANOVA with Bonferroni posttest. *; p<0.05, **; p<0.01, and ***; p<0.001 as indicated among the groups (B). nd; not detected.
6.3 Discussion

FI-RSV vaccine-ERD observed in clinical trials has been recapitulated in different animal models. In addition, vaccination with purified RSV F or G proteins, live recombinant vaccinia virus vectors (vacv) expressing RSV-G (vacvG), or FI-RSV with G deletion was shown to cause vaccine-ERD upon RSV challenge (32, 33). Priming of Th2 type cellular immune responses has been considered a main parameter responsible for causing ERD upon RSV challenge (109-112). We tested a hypothesis that proper priming would correct undesirable immune responses responsible for ERD by FI-RSV vaccine. A pattern of IgG2a isotype dominant responses by F VLP priming was found to be maintained after boost vaccination with FI-RSV. In contrast, F VLP priming prevented causing severe pulmonary inflammation and eosinophilia due to FI-RSV prime boost vaccination after RSV challenge. Findings in the present study are highly significant, providing convincing evidence that priming with F VLP was able to shift the immune responses to subsequent FI-RSV exposure and RSV challenge.

The effector functions of CD4 T cells represent many different cytokines, helping the generation of CD8 cytotoxic cells and directing antibody production by B cells. Also, CD4 T cells have been shown to play a major role in the RSV-induced immunopathology. RSV F VLP prime FI-RSV boost immune mice showed significantly less cellularity of CD4 T cells and effector CD4 T cells expressing IL-4, TNFα, and IFN-γ cytokines in the lung after RSV challenge compared to those in FI-RSV prime boost mice. F VLP priming of mice resulted in significantly lower levels of Th2 cytokines in the lung and airway BAL fluids compared to FI-RSV priming upon RSV challenge. Reductions in effector CD4 T cells appeared to have a correlation with ameliorating pulmonary inflammation and eosinophilia as a result of F VLP priming. In line with these results, mice that were previously immunized with vacvG showed
robust responses of RSV G specific CD4 T cells in the lung between 5 to 7 days after RSV challenge (92). Despite lung viral clearance, vacvG immune mice developed pulmonary eosinophilia and mononuclear cell infiltration (92), which is similar to vaccine-ERD in FI-RSV vaccination. Antibody-mediated depletion of CD4 T cells in FI-RSV immune mice significantly reduced immunopathology in the lung (95) whereas CD4 T cell depletion in vacvG immune mice did not affect viral clearance (18). Taken together with this and other studies, RSV specific effector CD4 T cells are likely involved in causing vaccine-ERD.

CD8 T cells are also known to play a role in suppressing RSV vaccine-ERD. We found that F VLP prime FI-RSV boost significantly shifted lung effector T cells to CD8 T cells producing IFN-γ, which resulted in preventing pulmonary inflammation and eosinophilia in F VLP/FI-RSV mice compared to FI-RSV /FI-RSV mice after RSV challenge. Consistent with results in this study, depletion of CD8 T cells or genetic deficiency of IFN-γ in mice previously immunized with vaccinia virus expressing F (vacvF) gave rise to lung eosinophilia after RSV challenge (34, 113). These previous and current studies are consistent with the notion that CD8 T cells producing IFN-γ are important for inhibiting vaccine-ERD.

Lung viral clearance representing a main criterion of protection against RSV is primarily mediated by RSV specific antibodies. Lung viral titers were not detected in FI-RSV immune mice at day 5 post challenge in this study. This is consistent with previous studies demonstrating that FI-RSV immunized mice or cotton rats significantly lowered RSV lung viral loads after infection (53, 65-67, 114). Also, mice immunized with live viral vectors vacvG or vacvF showed viral clearance in the lung (18, 40, 115). Similarly RSV subunit protein vaccines were shown to be capable of inducing RSV specific antibodies effectively controlling lung viral loads in
immune animals (89, 96, 102). Pups born to FI-RSV immune mothers were sufficiently protected against RSV by reducing viral loads in the lung (56). Thus, protection against RSV lung replication does not prevent vaccine-ERD in animal models and it is important to assess the safety of vaccines independent of lung viral clearance.

To gain mechanistic insight into how F VLP priming can modulate immune responses to subsequent FI-RSV exposure, we examined acute innate responses in mice after i.p. injection with F VLP or alum-adjuvanted FI-RSV, mimicking the priming condition. F VLP was found to be effective in generating inflammatory microenvironment locally and transiently by producing cytokines (IL-6 and IFN-γ) and chemokines (RANTES, IP-10, MCP-1 and KC) compared to FI-RSV in alum adjuvant. F VLP treatment recruited many different types of immune cells including NK, eosinophils, and different subsets of macrophages as well as T cells, which is in consistent with the pattern of soluble cytokines and chemokines. Meanwhile, FI-RSV with alum treatment did not recruited innate immune cells.

7    CHAPTER 7. CONCLUSION

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. Upon RSV challenge 21 weeks after immunization of mice, 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. Therefore, the results provide evidence that recombinant RSV FFG VLP vaccine can confer long-term protection against RSV without causing lung pathology.
Here, using a cotton rat model, we investigated the protective efficacy and safety of an RSV combination vaccine composed of F-encoding plasmid DNA and virus-like particles containing RSV fusion (F) and attachment (G) glycoproteins (FFG-VLP). Cotton rats with FFG-VLP vaccination controlled lung viral replication below the detection limit, and effectively induced neutralizing activity and antibody-secreting cell responses. In comparison with formalin inactivated RSV (FI-RSV) causing severe RSV disease after challenge, FFG-VLP vaccination did not cause weight loss, airway hyper-responsiveness, IL-4 cytokines, histopathology, and infiltrates of proinflammatory cells such as eosinophils. FFG-VLP was even more effective in preventing RSV-induced pulmonary inflammation than live RSV infections. This study provides evidence that FFG-VLP can be developed into a safe and effective RSV vaccine candidate.

This study demonstrates the immunogenicity, efficacy, and safety of virus-like particle (VLP) vaccines containing RSV F (F VLP), G (G VLP), or F and G proteins (FG VLP) in cotton rats. RSV specific antibodies were effectively induced by vaccination of cotton rats with F VLP or FG VLP vaccines. After challenge, lung RSV clearance was observed with RSV F, G, FG VLP, and formalin inactivated RSV (FI-RSV) vaccines. Upon RSV infection, cotton rats with RSV VLP vaccines were protected against airway hyper-responsiveness and weight loss, which are different from FI-RSV vaccination exhibiting vaccine-enhanced disease of airway obstruction, weight loss, and severe histopathology with eosinophilia and mucus production. FG VLP and F VLP vaccines did not cause pulmonary inflammation whereas G VLP induced moderate lung inflammation with eosinophilia and mucus production. In particular, F VLP and FG VLP vaccines were found to be effective in inducing antibody secreting cell responses in bone marrow and lymphoid organs as well as avoiding the induction of T helper type 2
cytokines. These results provide further evidence to develop a safe RSV vaccine based on VLP platforms.

Cotton rat is considered as a more appropriate animal model than mice for preclinical studies on RSV vaccine efficacy and safety, pathogenesis, because cotton rats appear to better mirror the histopathology of the fatal infant case. Nonetheless, the cotton rat model has disadvantages for studying cellular immune response because the cotton rat specific immunological reagents are not sufficiently available. Meanwhile, the mouse model has advantages in terms of the availability of mouse specific immunological reagents and analytical methods.

In summary, a specific pattern of immune responses and protection by different RSV vaccines is described (Table 7.1). FFG, FG, and F VLP RSV vaccines induced Th1 humoral immune responses (IgG1 < IgG2a in mice), RSV neutralizing activity, well controlled RSV lung viral titers supporting the evidence of protection against RSV, and suppressed Th2 type cytokine secretions in mice and cotton rats. RSV G VLP alone induced moderate Th1 type immune responses (IgG2a/IgG1 ratios ~2 in mice), was less immunogenic in mice and cotton rats compared to F VLP vaccines, and more or less effective in lung RSV clearance. FI-RSV vaccination of mice induced strong Th2 type humoral immune responses (IgG1 > IgG2a in mice) with high levels of Th2 type IL-4 cytokine secreting CD4 T cells in mice and showed well control of lung viral titers, suggesting sufficient protection against RSV replication in the lung. Inoculation of live RSV intranasally induced a moderate degree of Th1 type humoral responses (IgG2a ≥ IgG1), RSV neutralizing antibodies, effectively controlled lung viral replication, and both CD4 and CD8 T cell responses (Table 7.1). Therefore, different types of RSV vaccines
were able to control RSV replication in the lung after subsequent RSV challenge infection regardless of Th1 and/or Th2 immune responses.

**Table 7.1** Pattern of immune responses and protection by different RSV vaccines.

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Antibody</th>
<th>IgG2a / IgG1 Ratio</th>
<th>Neutralizing activity</th>
<th>Lung viral titration</th>
<th>T cell response</th>
</tr>
</thead>
<tbody>
<tr>
<td>No vaccine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>high</td>
<td>-</td>
</tr>
<tr>
<td>Live-RSV</td>
<td>IgG1 ≤ IgG2a</td>
<td>≥ 1</td>
<td>++</td>
<td>low</td>
<td>CD4 ≥ CD8</td>
</tr>
<tr>
<td>FI-RSV</td>
<td>IgG1 &gt; IgG2a</td>
<td>≤ 1</td>
<td>++++</td>
<td>low</td>
<td>CD4 &gt; CD8</td>
</tr>
<tr>
<td>FFG VLP</td>
<td>IgG1 &lt; IgG2a</td>
<td>≥ 5</td>
<td>++++</td>
<td>low</td>
<td>CD4 &lt; CD8</td>
</tr>
<tr>
<td>FG VLP</td>
<td>IgG1 &lt; IgG2a</td>
<td>≥ 5</td>
<td>++++</td>
<td>low</td>
<td>CD4 &lt; CD8</td>
</tr>
<tr>
<td>F VLP (47)</td>
<td>IgG1 &lt; IgG2a</td>
<td>≥ 2</td>
<td>++++</td>
<td>low</td>
<td>CD4 &lt; CD8 (105)</td>
</tr>
<tr>
<td>G VLP (47)</td>
<td>IgG1 ≤ IgG2a</td>
<td>≥ 2</td>
<td>++++</td>
<td>medium-low</td>
<td>-</td>
</tr>
</tbody>
</table>

The safe and effective vaccine against respiratory syncytial virus (RSV) should confer protection without causing vaccine-enhanced disease. Assessment of RSV vaccine-induced respiratory disease has been a main goal in this study. After RSV challenge infection, RSV vaccine induced pulmonary inflammation by different RSV vaccines was summarized in Table 7.2. FFG, FG, and F VLP RSV vaccines are likely to induce Th1 type IFN-γ cytokine and low or no RSV vaccine-induced disease as evidenced of the lack of pulmonary histopathology around the airway, blood vessels, and interstitial spaces in both mouse and cotton rat models (Table 7.2). RSV G VLP vaccine induces moderate inflammation in the airways and eosinophilia in histopathology in mice and cotton rats whereas inclusion of F VLP in G VLP vaccination (FG VLP) can avoid RSV vaccine induced pulmonary inflammation in mice and cotton rats (Table 7.2). In contrast, formalin-inactivated RSV (FI-RSV) vaccination induced Th2 type inflammatory cytokines (IL-5, IL-6, IL-13) in respiratory tracks, significant pulmonary
eosinophilia, severe mucus production, and extensive histopathology resulting in a hallmark of pulmonary pathology. High levels of systemic and local inflammatory cytokine-secreting cells were induced with FI-RSV after RSV challenge in both mouse and cotton rat models (Table 7.2). Substantial lung pathology around the airway as well as eosinophilia were observed in mice and cotton rats with RSV re-infections (Table 7.2 and Fig. 7.1).

**Table 7.2** RSV pulmonary inflammation by different RSV vaccines

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Inflammatory cytokines</th>
<th>ERD</th>
<th>Eosinophilia</th>
<th>Mucus Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>No vaccine</td>
<td>-</td>
<td>medium</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>Live-RSV</td>
<td>-</td>
<td>medium</td>
<td>medium</td>
<td>low</td>
</tr>
<tr>
<td>FI-RSV IL-5, IL-6, IL-13</td>
<td>high</td>
<td>high</td>
<td>medium</td>
<td>high</td>
</tr>
<tr>
<td>FFG VLP IFN-γ</td>
<td>low</td>
<td>low</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>FG VLP IFN-γ</td>
<td>low</td>
<td>low</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>F VLP IFN-γ</td>
<td>low</td>
<td>low</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>G VLP</td>
<td>-</td>
<td>medium</td>
<td>low</td>
<td>low</td>
</tr>
</tbody>
</table>

F VLP is known to increase T helper type-1 (Th1) antibody responses and not to cause ERD. This study was hypothesized that priming with F VLP would modulate immune responses preventing ERD upon subsequent exposure to ERD-causing FI-RSV. F VLP priming and FI-RSV boosting of mice prevented FI-RSV vaccine-enhanced lung inflammation and eosinophilia upon RSV challenge. F VLP priming redirected pulmonary T cells toward effector CD8 T cells producing Th1 cytokines and significantly suppressed pulmonary Th2 cytokines. The transient induction of inflammatory cytokines and chemokines by F VLP priming might be contributing to shaping Th1 type adaptive immune responses.
In conclusion, VLP vaccination is effective in suppressing Th2 immune-mediated pulmonary inflammation and eosinophilia. Further studies on RSV F VLP vaccine-mediated protection against RSV disease will provide insights into developing into a safe and effective RSV vaccine.
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