EFFECTS OF VACCINE PLATFORMS AND ADJUVANTS ON PROTECTIVE EFFICACY AGAINST HUMAN RESPIRATORY SYNCYTIAL VIRUS

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EFFECTS OF VACCINE PLATFORMS AND ADJUVANTS ON PROTECTIVE EFFICACY AGAINST HUMAN RESPIRATORY SYNCYTIAL VIRUS

by

YOURI LEE

Under the Direction of Committee Sang-Moo Kang, PhD
ABSTRACT

Human respiratory syncytial virus (RSV) is responsible for several million hospitalizations and 199,000 deaths in young children worldwide. In 1960s clinical trials, children who were vaccinated with alum-adjuvanted, formalin-inactivated whole RSV (FI-RSV) developed vaccine-enhanced respiratory disease after RSV infection, resulting in tragic vaccine failure. There is no licensed RSV vaccine. The goals of my PhD thesis studies were to better understand pulmonary inflammation in vaccinated animals and to develop safe and effective RSV vaccines.

In the first project, I investigated the vaccine efficacy of RSV fusion (F) proteins in a soluble form or on virus-like particle (F-VLP). F VLP preferentially elicited T helper type 1 (Th1) immune responses whereas alum-adjuvanted F protein induced Th2 responses. Despite lung viral clearance, F protein immune mice displayed weight loss and lung histopathology and high mucus production and eosinophils. In contrast, prime or prime-boost of F VLP prevented eosinophils’ infiltration and vaccine-associated disease. An intrinsic property of F VLP to prime Th1 type immune responses appears to prevent RSV vaccine-enhanced disease after RSV challenge. In the second project, I investigated the adjuvant effects of monophosphoryl lipid A (MPL, a TLR4 ligand) and oligodeoxynucleotide CpG (CpG, a TLR9 ligand) on vaccine-enhanced respiratory disease after F protein prime vaccination and RSV challenge in infant and adult mice. Combination CpG+MPL adjuvant in RSV F protein prime vaccination of infant and adult age mice promoted the induction of Th1 type immune responses, and lung viral clearance after challenge. Importantly, CpG+MPL adjuvant plus F protein priming of mice was effective in preventing inflammatory lung histopathology and Th2 cytokine-expressing CD4 T cell responses after RSV challenge. In the third project, I found that inactivated and detergent-split RSV vaccines exposed neutralizing epitopes at higher levels than inactivated whole virus. Split RSV vaccination of mice
induced less lung histopathology compared to FI-RSV after RSV challenge. Split RSV vaccination of mice with CpG and MPL adjuvants was found to be most effective in increasing Th1 type IgG2a antibodies, neutralizing activity, and lung viral clearance as well as modulating immune responses to prevent pulmonary histopathology after RSV vaccination and challenge.

INDEX WORDS: Respiratory Syncytial Virus, Virus like particle, Fusion (F) protein, Detergent-split RSV vaccines, Vaccine enhanced-disease, T helper type 1 and 2 CD4 T cell response, Histopathology, Adjuvants.
EFFECTS OF VACCINE PLATFORMS AND ADJUVANTS ON PROTECTIVE EFFICACY
AGAINST HUMAN RESPIRATORY Syncytial VIRUS

by

YOURI LEE

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December 2018
DEDICATION

This dissertation dedicates to my family, all my friends and professors.
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I am especially indebted to Dr. Sang-Moo Kang, who has been supportive of my career goal and who encouraged me to pursue completing the PhD degree. He gave me the golden opportunity to do this wonderful project on the topic, which also helped me in doing a lot of research and I came to learn about many new things.

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ 1

LIST OF TABLES .................................................................................................................... 7

LIST OF FIGURES .................................................................................................................. 8

LIST OF ABBREVIATIONS ..................................................................................................... 11

1 INTRODUCTION .................................................................................................................. 12

1.1 Human respiratory syncytial Virus (RSV) ..................................................................... 12

1.2 RSV vaccine-enhanced respiratory disease and vaccine adjuvants ................................. 13

1.2.1 Formalin-inactivated whole RSV vaccine (FI-RSV) enhanced respiratory disease after infection .......................................................................................................................... 13

1.2.2 RSV F soluble protein vaccines ...................................................................................... 13

1.2.3 Virus like particle (VLP) vaccines .................................................................................... 14

1.2.4 Split RSV vaccines ......................................................................................................... 15

1.3 Effects of vaccine adjuvants on RSV vaccine efficacy ...................................................... 16

1.3.1 Aluminium hydroxide (Alum) adjuvant ......................................................................... 16

1.3.2 Monophosphoryl lipid A (MPL) adjuvant ...................................................................... 17

2 EXPERIMENT ..................................................................................................................... 19

2.1 Cells, Virus and Vaccine formulation with adjuvants ......................................................... 19

2.2 Animals, Immunization and RSV challenge ....................................................................... 20

2.3 ELISA ............................................................................................................................... 21
3.1 Summary ......................................................................................................................... 25

3.2 Result ............................................................................................................................... 26

3.2.1 *RSV F VLP vaccination induces a distinct pattern of IgG isotypes compared to F proteins* .................................................................................................................................................................................. 26

3.2.2 *Mice with RSV F protein vaccination induce protection against viral replication but display disease phenotypes.* ............................................................................................................................................................................. 27

3.2.3 *RSV F protein vaccination induces histopathology upon RSV challenge.* 27

3.2.4 *Biased Th2 cytokines are elicited after F protein vaccination and RSV challenge.* 28

3.2.5 *F VLP induces transient inflammatory responses* ..................................................... 29

3.3 Discussion ......................................................................................................................... 38
CHAPTER 2. A UNIQUE COMBINATION ADJUVANT MODULATES IMMUNE RESPONSES PREVENTING VACCINE-ENHANCED PULMONARY HISTOPATHOLOGY AFTER A SINGLE DOSE VACCINATION WITH FUSION PROTEIN AND CHALLENGE WITH RESPIRATORY SYNCYTIAL VIRUS

4.1 Summary

4.2 Results

4.2.1 Combination CpG+MPL adjuvant in RSV F protein vaccination mediates the effective induction of IgG2a antibody and neutralizing activity in infant and adult mice.

4.2.2 Combination CpG+MPL adjuvant improves lung viral clearance and protection of F protein vaccine.

4.2.3 CpG+MPL adjuvant suppresses lung histopathology after F protein vaccination and RSV challenge.

4.2.4 CpG+MPL adjuvanted F protein priming of infant and adult mice effectively prevents the induction of IL-4+ CD4 T cells after RSV challenge.

4.2.5 Monocytes and Neutrophils are not infiltrated into the lungs from CpG+MPL adjuvanted F protein primed infant and adult mice after RSV challenge

4.3 Discussion

CHAPTER 3. EFFECTS OF NOVEL COMBINATION ADJUVANTS ON IMPROVING THE EFFICACY OF INACTIVATED SPLIT RESPIRATORY SYNCYTIAL VIRUS VACCINE IN MICE

5.1 Summary
5.2 Results .............................................................................................................................................. 62

5.2.1 Split RSV displays high antigenic reactivity and a unique pattern of immunogenicity in adult mice .......................................................................................................................... 62

5.2.2 Split RSV prime dose controls lung viral loads and attenuates histopathology after RSV challenge in adult mice ........................................................................................................... 63

5.2.3 CpG and MPL adjuvants in split RSV prime vaccination promotes IgG2a isotype dominant antibody responses in infant age mice .................................................................................. 64

5.2.4 Alum and combination CpG+MPL adjuvants are effective in enhancing RSV neutralizing activity and controlling lung viral loads after RSV challenge ...................... 65

5.2.5 CpG+MPL in split RSV vaccination of infant age mice prevents lung histopathology after RSV Challenge .......................................................................................................................... 66

5.2.6 CpG and MPL adjuvants in split RSV vaccination of infant age mice primes Th1 type immune responses in spleen cells ............................................................................................................. 67

5.2.7 Split RSV with CpG and MPL promotes Th1 T cells and cytokines in lung and BALF after challenge ........................................................................................................................................ 67

5.2.8 Split RSV-primed mice with CpG + MPL prevents infiltrating inflammatory immune cells into the lung after RSV challenge .................................................................................................. 68

5.3 Discussion ............................................................................................................................................. 77

6 CONCLUSION ........................................................................................................................................... 82

6.1 Protective or disease-enhancing immune responses after prime vaccination with soluble F protein or particulate F VLP and RSV challenge infection ......................... 82
6.2 Effects of adjuvants in RSV F protein vaccination on modulating immune responses 84

6.3 Comparison of whole FI-RSV and inactivated-split RSV vaccines, and adjuvant effects on split RSV vaccination in infant age mice. ............................................. 86

REFERENCES .......................................................................................................................................................... 90
LIST OF TABLES

Table 6.1 Protection or RSV disease enhancing inflammatory responses by prime or prime-boost immunization of RSV vaccines and then RSV challenge infection. ........................................ 84

Table 6.2 Adjuvants effects on RSV F protein vaccination efficacy and inflammatory disease in adult and infant age mice. ........................................................................................................ 86

Table 6.3 Comparison of whole FI-RSV and inactivated-split RSV vaccines, and adjuvant effects on split RSV vaccination in infant age mice. ......................................................................................... 88
LIST OF FIGURES

Figure 3. 1 RSV-specific IgG isotype antibody levels and neutralizing activity after prime or prime-boost vaccination with F VLP or F protein vaccine. ................................................................. 30

Figure 3. 2 Body weight changes and lung viral clearance in vaccinated BALB/C mice after RSV challenge. .................................................................................................................. 31

Figure 3. 3 Lung histopathology in prime or prime-boost vaccinated mice after RSV challenge. .................................................................................................................. 32

Figure 3. 4 Levels of mucus production in the lungs from vaccinated mice after RSV challenge. .................................................................................................................. 33

Figure 3. 5 Assessment of eosinophils in the lungs from vaccinated mice after RSV challenge. .................................................................................................................. 34

Figure 3. 6 Cytokines in the Lung tissue and BALF from prime-boost immune mice after RSV challenge. .................................................................................................................. 35

Figure 3. 7 CD4+, CD8+ T cells secreting Th1 or Th2 cytokines by intracellular cytokine staining, and .................................................................................................................. 36

Figure 3. 8 Acute responses of cytokines, chemokines, and cellular infiltrates at the site of injection with F VLP or F protein. .................................................................................................................. 37

Figure 4. 1. F protein prime vaccination of infant and adult age mice with Alum, CpG, MPL or CpG+MPL adjuvants elicits different IgG isotype antibodies. ................................................. 49

Figure 4. 2 Neutralizing titers in primed mice and lung viral clearance after challenge....... 50

Figure 4. 3. Lung histopathology and mucus production in primed infant and adult mice after RSV challenge. .................................................................................................................. 52
Figure 4.4 Eosinophilic infiltration into the lungs of adjuvanted F protein primed infant and adult mice after RSV challenge. ................................................................. 54

Figure 4.5. IL-4 and IFN-γ secreting CD4⁺ T cells in BAL and lung samples of infant and adult age primed mice after RSV challenge. ................................................................. 55

Figure 4.6. Cytokines in the BALF from the primed mice at infant and adult age after RSV challenge. ........................................................................................................... 55

Figure 4.7 Monocytes and neutrophils in the airways (BALF) of the infant and adult age primed mice after RSV challenge. ........................................................................................................... 56

Figure 5.1. Antigenic properties of whole F1-RSV and split RSV vaccines. ...................... 69

Figure 5.2. Split RSV vaccination induces higher levels of IgG2a isotype antibodies and better protection against RSV than F1-RSV vaccine in adult mice. ................................................. 70

Figure 5.3. Lung histopathology in single immunized adult mice after RSV challenge. ....... 71

Figure 5.4. RSV antigen specific IgG and isotype antibody levels in infant age mice after prime immunization. ........................................................................................................... 72

Figure 5.5. RSV neutralizing activity titers in primed-mice at an infant age and lung viral loads after RSV challenge ........................................................................................................... 72

Figure 5.6. Histopathology of Hematoxylin Eosin (H&E), Periodic acid-Schiff (PAS) staining of lung tissues in split RSV primed mice at an infant age after RSV challenge. ..................... 74

Figure 5.7. CpG+MPL adjuvant split RSV primed mice at an infant age induce IgG2a-secreting cells and IFN-γ secreting splenocytes after RSV challenge. ............................................. 74

Figure 5.8. Intracellular cytokine staining of CD4 and CD8 T cells of BALF and lung samples after RSV challenge. ........................................................................................................... 75
Figure 5. 9. Th1- and Th2- cytokine levels in lung homogenates and BALF from split RSV primed mice at an infant age after RSV challenge. ............................................................................................................. 76

Figure 5. 10. Cellular phenotypes of infiltrates into BALF and lung tissues of split RSV primed mice at an infant age after RSV infection. ............................................................................................................. 77
LIST OF ABBREVIATIONS

1. RSV: Respiratory syncytial virus
2. VLP: Virus like particle form
3. FI-RSV: Formalin-inactivated whole RSV
4. VED: vaccine enhanced disease
5. IL: Interleukin
6. Th: T helper type
7. Split: inactivated split RSV
8. MLN: mediastinal lymph nodes
9. BALF: Bronchoalveolar lavage fluid
10. Alum: Aluminum salts
11. TLR: Toll-like receptor
13. MPL: Monophosphoryl lipid A
14. mAB: Monoclonal antibody
15. H&E: Hematoxylin and eosin
16. PAS: Periodic-Acid Schiff
17. H&CR: Hematoxylin and Congo Red
18. IM: Intra muscular
19. IP: Intraperitoneal
20. PBS: Phosphate buffered saline
21. NK: Natural killer
22. DC: Dendritic cell
1 INTRODUCTION

1.1 Human respiratory syncytial Virus (RSV)

Human respiratory syncytial virus (RSV) is an enveloped virus containing a negative sense strand RNA genome and belongs to the Pneumoviridae family. RSV has three integral membrane proteins, fusion (F), glycoprotein (G), and small hydrophobic (SH). The G protein helps viral attachment to the host cell, during the processing of F protein for fusion. The SH protein forms a pentameric ion channel (1). To develop the antiviral drug against RSV infection, RSV F and G proteins are mainly targeted by neutralizing antibodies (1). RSV infection is responsible for annual outbreaks of lower respiratory tract disease in infants and elderly, resulting in global incidence of 33 million cases in children younger than 5 years old, an estimated 3.4 million hospitalizations, and up to 199,000 deaths (2). In the US, RSV infection is associated with 14,000 annual adults age 65 years and older deaths caused by RSV and 177,000 hospitalizations (3). Older adults are also highly susceptible to significant disease and mortality due to RSV infection (3). RSV is antigenically stable, but most adults are re-infected every seasonal period, suggesting that that natural immunity is not long-lasting (4, 5). There is no RSV vaccine licensed.
1.2 RSV vaccine-enhanced respiratory disease and vaccine adjuvants

1.2.1 Formalin-inactivated whole RSV vaccine (FI-RSV) enhanced respiratory disease after infection

In the 1960s clinical trials, infants and children who were vaccinated with alum-adjuvanted formulation of formalin-inactivated whole RSV (FI-RSV) developed vaccine enhanced respiratory disease upon natural infection during epidemic winter season (6). To better understand this abnormal disease of alum adjuvant FI-RSV vaccine enhancement of pulmonary histopathology has been recaptured in various animal models including mice (7, 8), cotton rats (9), cattle (10), and African green monkeys (11). Subsequent animal model studies have implicated roles for innate immune response with CD4 T cells, eosinophils and neutralizing antibodies related in enhanced RSV disease. T helper type 2 (Th2)-biased immune responses are mostly responsible for airway hyperreactivity and mucus hypersecretion. In contrast, the Th1-associated cytokine TNF-α was necessary to mediate airway obstruction and weight loss (12). However, the underlying immunological mechanisms responsible for the enhanced respiratory disease and other disease manifestations associated with FI-RSV vaccine-enhanced disease have not been fully understood yet.

1.2.2 RSV F soluble protein vaccines

Human RSV proteins are transmembrane and use to control lung viral titers as immunogenic and effective glycoprotein vaccines (13-15). RSV F glycoprotein is one of transmembrane surface proteins that are believed to be the most important vaccine antigen because of integral to infectivity of RSV. Additional studies reported that these RSV protein vaccines develop enhanced pulmonary pathology after several months of vaccination after RSV challenge
in cotton rats (7, 14). RSV F protein subunit vaccines are under clinical investigation, targeting to older populations and high-risk children or maternal immunization. Alum adjuvanted purified F protein vaccines were tested in early clinical phase I and II trials of different age groups including healthy adults, children over 12 months of age, older persons, and pregnant women (16). A phase II trial of alum-adjuvanted F protein vaccines in seropositive children showed an increase in neutralizing titers but no reduction in the incidence of RSV infections (17, 18). F protein nanoparticles-based vaccines are advanced to Phase III trials (18, 19). The pre-fusion F protein vaccine candidates are under Phase I and II clinical studies (18, 19). Although RSV F is being developed as an important subunit vaccine candidate, its vaccination is often associated with a T helper type 2 (Th2) immune marker. F protein vaccines were reported to cause enhanced lung histopathology in animal models (14, 20-23). Therefore, RSV vaccine-enhanced disease is a major concern in the development of an effective and safe RSV vaccine.

1.2.3 Virus like particle (VLP) vaccines

Virus-like particle (VLP) is non-replicating and noninfectious but can represent a morphological and structural similarity of an enveloped virus such as RSV. Newcastle disease virus (NDV) structural proteins (NP, M) were co-expressed to produce VLP vaccines containing the ectodomains of RSV F and G proteins in a chimeric fusion to the transmembrane and cytoplasmic tail of the NDV membrane proteins (24, 25). NDV VLP vaccines containing RSV F and G ectodomains provided protection against RSV by inducing long-lived RSV neutralizing antibodies without lung inflammation upon RSV challenge in mice (24, 26, 27) and cotton rats (28O). Our previous studies demonstrated the immunogenicity and efficacy of RSV F VLP produced in insect cells by co-expressing the influenza virus M1 matrix protein. RSV F VLP was
found to be immunogenic and confer protection against RSV (29, 30). F VLP vaccine is unique in conferring protection against RSV without causing lung inflammation after RSV challenge (31). It remains unknown whether prime only vaccination with F VLP would confer protection against RSV and how F protein vaccines presented in VLP or soluble platforms result in different outcomes in terms of preventing vaccine-enhanced pulmonary histopathology after RSV challenge. This study has focused on addressing immunological mechanisms underlying these important questions.

1.2.4 Split RSV vaccines

Preparation of sub-virion vaccines by ‘splitting’ inactivated influenza viruses has been most commonly used in seasonal vaccination since the dissolution of the lipid envelope allows retention of immunogenicity with reduction in reactogenicity (32). Most influenza vaccines manufactured since the 1970s have been ‘split’ preparations. An intact viral membrane is required for infectivity of enveloped viruses, and disruption of the viral envelope adds further assurance of viral inactivation. Clinical trials comparing whole-virus and split-influenza vaccines demonstrated that these split influenza vaccines retain the immunogenic properties of the viral proteins but they have lower reactogenicity than whole-virion vaccines (33-35). In contrast to many studies on whole FL-RSV, the antigenicity and immunogenicity of inactivated split RSV vaccines remain unknown. It is of high priority to develop a new RSV vaccine platform and adjuvant enhancing the vaccine efficacy and avoiding enhanced pulmonary histopathology after RSV infection. A previous study demonstrated that intranasal immunization of mice with nanoemulsion-adjuvanted, inactivated RSV vaccine provided protection against RSV challenge by promoting Th1 and Th17 responses and no evidence of Th2 mediated immunopotentiation (36).
1.3 Effects of vaccine adjuvants on RSV vaccine efficacy

Successful vaccination is the most effective measure to prevent disease against future pathogens. In general, non-replicating subunit vaccines are safer than live attenuated vaccines but typically require adjuvants for successful antigen-specific immune responses. To improve induction of immune responses in vivo is typically performed with subunit protein vaccine antigens administered together with external vaccine adjuvants, such as the most conventional aluminum salts (alum), oil-in-water emulsion (MF59), and polysaccharides-based components (Q21, delta-inulin). Vaccine adjuvants based on Toll-like receptor (TLR) ligands have been developed and used as standalone or in combinations such as alum plus TLR4 agonist (AS04). Oligodeoxynucleotides containing unmethylated cytosine-phosphate-guanosine (CpG) motifs, a TLR-9 agonist, promote the induction of Th1 immune responses to RSV F or killed RSV vaccination (22, 37, 38). However, details on pulmonary inflammation and RSV disease after RSV challenge were not investigated in CpG adjuvanted RSV vaccinated mice. Monophosphoryl lipid A (MPL) is an attenuated version of lipopolysaccharide TLR4 agonist (39) and licensed for use in human vaccines (40). We found that combination CpG and MPL adjuvants at low doses effectively promoted the efficacy of split influenza vaccines (41). However, the roles of the adjuvants in the efficacy and safety of RSV vaccination is still poorly defined.

1.3.1 Aluminium hydroxide (Alum) adjuvant

Alum adjuvant has a long history of usage and is used in human and animal with split and subunit vaccines as an adjuvant. Previous studies suggested the potency of alum adjuvant by forming T helper type 2 (Th2) antigen response in the administration sites and granting the
persistence and prolonged release of antigens (41). Inducing Th2 cytokines control the differentiation of Th2 cells and related B cells to generate Th2-associated antibodies (IgG1) and allergic immune responses. Also, alum was shown to raise proinflammatory mediators including interleukin (IL)-1β, CC-chemokine ligand 2 (CCL2; MCP1), CCL11 (eotaxin), histamine and IL-5 as well as neutrophils, eosinophils, inflammatory monocytes, myeloid dendritic cells (DCs), and plasmacytoid DCs. However, alum adjuvant would not be highly effective in the efficacy of subunit vaccines targeting intracellular viral pathogens.

1.3.2. Monophosphoryl lipid A (MPL) adjuvant

MPL, a detoxified bacterial lipopolysaccharide, is a synthetic lipid A that is a weak TLR4 agonist. MPL is licensed and has demonstrated a safe profile in two vaccines when co-administered with different RSV vaccine preparations as a vaccine adjuvant. It was previously reported that immunization of cotton rats with the original lot 100 FI-RSV, formulated with MPL, blunted both the cytokine storm and the enhanced lung pathology elicited by subsequent RSV infection, but did not inhibit viral replication in the lungs. Administration of vaccines containing MPL has resulted in high antigen-specific antibody and CD4+ T-cell responses in preclinical and clinical studies (42) (43). Also, MPL enhanced the ability of macrophages and B cells to sensitize naive T cells and to promote T cell development to Th1 and Th2 type cells (44).

1.3.3 Oligodeoxynucleotide containing unmethylated CpG motifs (CpG adjuvant)

Synthetic oligodeoxynucleotides containing unmethylated cytosine-phosphate-guanosine (CpG), a TLR-9 agonist, are known to activate Th1 immune responses to RSV F or killed RSV vaccination (22, 37, 38) but no details on CpG adjuvant effects on lung inflammation and RSV
disease after vaccination and RSV challenge were reported. Human plasmacytoid dendritic cells and B cells express TLR 9 and can be activated to enhance the production of Th1 response and proinflammatory cytokines such as IL-6 upon CpG stimulation. CpG also improves the function of professional antigen-presenting cells (APC) and boost the generation of humoral and cellular vaccine-specific immune responses as using vaccine adjuvants. The adjuvant properties of CpG are observed when administered either systemically or mucosally and persist in immunocompromised hosts. Preclinical studies indicate that CpG improve the activity of vaccines targeting infectious diseases and cancer (45, 46).

1.4 Purpose of PhD thesis study

Enhanced respiratory disease is characterized by severe pulmonary inflammation leading to bronchitis and pneumonia after RSV infection. A phenomenon of inducing enhanced respiratory disease still remains poorly understood after alum-adjuvanted FI-RSV or subunit protein vaccination and RSV infection. The purpose of my overall PhD thesis studies is to better understand the RSV-induced pulmonary inflammation in vaccinated animals, vaccine adjuvant effects on RSV vaccine efficacy, and to develop safe and effective RSV vaccines. The findings during my PhD study provide new information and insight into the desirable types of immune responses that induce neutralizing activity and control viral replication but prevent RSV vaccine enhanced pathology. RSV fusion (F) protein attached VLP (F VLP), combined CpG+MPL adjuvanted F protein and split RSV vaccines are suggested to be a protective vaccine target although its efficacy and safety concerns remain not well understood.
2 EXPERIMENT

2.1 Cells, Virus and Vaccine formulation with adjuvants.

Hep2 and Spodoptera frugiperda 9 (Sf9) insect cells were obtained from American Type Culture Collection (ATCC). Hep2 cells were grown in DMEM supplemented with 5% fetal bovine serum and penicillin/streptomycin antibiotics. The RSV A2 was provided from Dr. Martin Moore (Emory University, GA) and propagated in HEp 2 Cells (American Type Culture Collection). Sf9 insect cells were grown in serum free SF900 II medium from Gibco-BRL and used for amplifying recombinant baculoviruses (rBVs) expressing RSV F proteins and F VLP vaccine production. For RSV F VLP vaccine production, insect cells were co-infected with rBVs expressing RSV F proteins (RSV A2 strain) and influenza virus M1 matrix protein, and F VLP was purified from Sf9 culture supernatants using ultracentrifugation and characterized as previously described (29). The antigenic integrity and epitopes’ exposure of FI-RSV, split RSV, F protein, and F VLP vaccines was determined by ELISA using post-F specific (131-2a monoclonal antibody purchased from Millipore) and pre-F specific (5C4) monoclonal antibodies. The RSV A2 F glycoprotein was obtained from Biodefense and Emerging Infections Research Resources (NR-28908, BEI Resources, Manassas, VA). A recombinant form of soluble F glycoprotein with C-terminal histidine tag was expressed by transfection of 293F cells with a plasmid encoding a codon-optimized F gene and purified from the cell culture supernatant using nickel chromatography. Antigenic analysis indicates that the F protein used in this study is likely to form a post-fusion conformation. For FI-RSV preparation, RSV (A2) infected Hep2 cells were cultured by media for 3 to 5 days at 37°C. The cells and media were collected to cold chilled tubes from the RSV infected
Hep2 cell culture flask. The infected cells were sonicated and clarified by centrifugation (2000 x g, 10min, 4°C). Collected supernatants were mixed with filtrated formalin to final concentration (1:4000 vol/vol of 37% formalin) and incubated for 3 days at 37°C with stirring. Inactivated RSV was precipitated from formalin treated supernatants by ultra-centrifugation (80,000 – 100,000 x g, 1hr, 4°C). The RSV precipitates were suspended with filtered 1xPBS for storage and kept at -80°C. Also, formalin inactivated split RSV was prepared by using detergent; Triton-x 100 and purified by sucrose gradient after FI-RSV preparation. All vaccines were stored at -80°C until used in this study. Adjuvant Alum (Sigma Aldrich, St. Louis, MO), MPL (Sigma Aldrich, St. Louis, MO), or CpG (oligodeoxynucleotide 1826, 5′-TCC ATG ACG TTC CTG ACG TT-3′, DNA Technologies), was mixed with 0.1 µg F protein or 5 µg split RSV vaccine and used to vaccinate the mice.

2.2 Animals, Immunization and RSV challenge.
To investigate the RSV F VLP and RSV F protein vaccine efficacy, BALB/c mice (n=5, 6-8 weeks old males and females, Charles River Laboratories, Inc.) were intramuscularly (i.m.) primed (a single dose) with 10 µg of F VLP, 0.1 µg or 0.3 µg of F protein in the Alum (50 µg) adjuvant, and phosphate buffered saline (PBS, naïve control). Additional sets of the BALB/c groups (n=5) were prime-boost vaccinated with the same vaccines at a 3-week interval. Blood samples were collected at 3 weeks after each vaccination. Naïve control, F-VLP, F protein immunized mice were intranasally (i.n.) infected with 5 x 10⁶ PFU of RSV A2 under isofluorane anesthesia to determine the efficacy of protection and vaccine-enhanced disease 3 to 4 weeks after vaccination.

For adjuvant effect with RSV F soluble protein, BALB/c mice (n=5, Charles River Laboratories, Inc.) were intramuscularly (i.m.) primed (a single dose) with 0.1 µg of F protein only or in the presence of adjuvant Alum (50 µg), MPL (1 µg), CpG (4 µg), MPL (1 µg) + CpG (4 µg), or
phosphate buffered saline (PBS, naïve control). The infant mice were 2 weeks old and adult mice were 6 to 8 weeks old at the time of priming immunization.

For new strategy of RSV vaccines in young mice, 2 weeks old female BALB/c mice (n = 5; Charles River Laboratories, Inc.) were single immunized intramuscularly (i.m.) with 2 µg of FI-RSV or 5 µg of split RSV with/without adjuvant Alum (50 µg), CpG (1 µg) MPL (4 µg), CpG (1 µg) + MPL (4 µg) adjuvant, and phosphate buffered saline (PBS, naïve control). For immunogenicity study, blood samples were collected from individual mice at 3 weeks after vaccination. Mice were intranasally (i.n.) infected with 3.5 x 10⁵ PFU/ml of RSV A2 under isoflurane anesthesia to determine the efficacy of protection and histopathology at 4 weeks after vaccination. Lung, bronchiolar alveolar lavage fluids (BALF), mediastinal lymph nodes (MLN), and spleens were collected at 5 days after challenge. All animal experimental procedures were approved and performed by following the guidelines of Georgia State University Institutional Animal Care and Use Committee.

2.3 ELISA

Blood samples collected at 3 weeks after prime or boost were used to determine RSV F (pre-fusion F and post-fusion F form) specific IgG antibodies by enzyme-linked immunosorbent assay (ELISA). Post-fusion F protein (NR- 28908, BEI resources) or pre-fusion conformation stabilized protein with DsCav mutations was coated on 96-well plates as an ELISA antigen. The pre-fusion F protein with DsCav mutations was kindly provided by Dr. Graham (NIH). Secondary antibodies conjugated with horseradish peroxidase (Southern Biotechnology) were used to detect IgG isotype antibodies. The substrate TMB (3, 3', 5, 5'-tetramethylbenzidine, Sigma Aldrich) was treated and stopped with 1M H3PO4. Optical densities (O.D) were read by spectrophotometer reader at 450nm. The lung homogenates and bronchoalveolar lavage fluid (BALF) were used to
quantify cytokine concentrations by using cytokine ELISA kits of interleukin (IL)-4, IL-5, interferon (IFN)-γ, IL-13, and tumor necrosis factor (TNF)-α (eBioscience, SanDiego, CA).

2.4 Lung viral titers and neutralizing antibody assays.

Serum samples were collected 3 weeks after prime immunization and heat-inactivated at 56°C for 45 min. In 48-well plates, test sera (starting dilution 1:4) were serially diluted by 2-fold increments in cell culture media for a final volume of 50 μL. Each serum serial dilution was mixed with 50 μL RSV A2 (200 PFU per well), incubated for 2 h, and added to the monolayer cultures of HEp-2 cells (2.5 × 10⁴ cells in each well). Cells plus virus and cells only on wells served as controls. After 3 days of incubation at 37°C with 5% CO₂, the cell culture medium was removed, and the monolayer was fixed with chilled 4% neutral paraformaldehyde. RSV replication was visualized by immunostaining with RSV specific F-monoclonal (131-2a) and HRP-labeled secondary antibodies. The reciprocal dilutions in log2, inhibiting 50% plaque forming units of virus (IC50) was determined for each serum sample. The lower limit of detection (LLOD) of this assay is 2 log2.

Individual lung samples were collected day 5 post challenge and RSV plaque-forming units (PFU) for RSV lung viral titers determined using an immuno-plaque assay. RSV control and lung homogenates mixed with RSV were incubated on HEp-2 cells for 1h at 37°C, 5% CO₂ and removed. 3% agarose mixed with media overlay was added to each well, and cells were incubated for 2 days at 37°C. After removed overlay and fixed with 4% formalin, the plaques were detected with anti RSV F monoclonal antibody (mAb) (Millipore).
2.5 Flow cytometry

BALF were collected by flushing the lungs with 1 ml of PBS supplemented with protease inhibitors using a winged shielded catheter (1.3630 mm, BD Utah) inserted, through an incision, in the trachea of euthanized mice. Lung tissues were isolated by homogenization and used by percoll gradient (44 and 67%) centrifugation. The frosted microscope glasses were used for harvesting mediastinal lymph nodes (MLN) to suspend the cells. For intracellular cytokine staining analysis of T cell responses, lung and BAL cells were stimulated with F peptide (F51-66 CD4 and F85-96 CD8 epitopes of RSV F, 4μg/ml) prior to staining of intracellular cytokines, and then the cells were fixed and permeabilized according to the manufacturer’s instructions (BD Biosciences). Intracellular cytokine and surface makers for T cells were stained with antibodies for IFN-γ, IL-4 (eBioscience), TNF-α (BioLegend), CD3, CD4, CD8. Infiltrating innate immune cells were detected with antibodies for CD11b, CD11c, CD103, F4/80, DX5 Ly6c or Siglec F (BD Biosciences). Cellular phenotypes were analyzed with the Becton-Dickinson LSR-II/ Fortessa flow cytometer (BD, San Diego, CA) was used and acquired flow cytometry data were analyzed by using Flowjo software (Tree Star Inc.).

2.6 Lung histology and inflammation scoring

Left lungs tissues were harvested on day 5 post challenge and fixed with 10% neutral buffered formalin. They were embedded in paraffin in the dorsoventral position. Subsequently, sections of tissue blocks were obtained and stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and hematoxylin and congo red (H&CR) (47, 48). Histopathology of lung tissue slides were analyzed using a semiquantitative scale (0 to 3) by light microscope. Assigned score 0 was the surrounding space which is free or has few infiltrating cells, score 1 contains focal
aggregates of infiltrating cells or the structure is cuffed by one definite layer of infiltrating cells, score 2 is cuffed by two defined layers of infiltrating cells, and score 3 when structure is cuffed by three or more definite layers of infiltrating cells with or without focal aggregates. Randomly 20 airways were selected to quantify for PAS positive stain with mucin expression area. H&E stained slides were evaluated for inflammatory infiltration and histopathology around peribronchial and/or peribronchiolar, perivascular, and interstitial regions(47, 48). The presence of pulmonary eosinophilia was quantified by H&CR stain with 400x magnified microscope and flow cytometry by using antibodies for eosinophil phenotypic markers (CD11b+, CD11c+, SiglecF).

2.7 Intraperitoneal (IP) injection.

BALB/c mice (n=3) were injected with 200 μl of phosphate buffered saline (PBS), F VLP (10 μg), F protein (0.1 μg) with alum (50 μg) adjuvants with intraperitoneally. Collected sera were analyzed to determine the levels of cytokines and chemokines by using ELISA kits (KC, MCP-1, and RANTES; R&D system, IL-4, 5, 13, and IFN-γ; eBioscience) at 1.5, 5, and 20 hours (h) post injection. The elicited peritoneal exudate cells were obtained at 24 h post injection by PBS flushing. The cells were isolated and analyzed for cellularity of infiltrating cells by flow cytometry. Peritoneal exudates were used for detection of cytokines and chemokines.

2.8 Statistical Analysis.

Statistical differences were performed using GraphPad prim version 5 (Graph Pad software Inc, San Diego, CA). Data were analyzed for significance using one-way ANOVA with Tukey’s test for multiple comparisons or two-way ANOVA with Bonferroni posttests. The difference was considered statistically significant when the P value was less than 0.05.
3 CHAPTER 1. SOLUBLE F PROTEINS EXACERBATE PULMONARY HISTOPATHOLOGY AFTER VACCINATION UPON RESPIRATORY SYNCYTIAL VIRUS CHALLENGE BUT NOT WHEN PRESENTED ON VIRUS-LIKE PARTICLES

3.1 Summary

Respiratory syncytial virus (RSV) fusion (F) protein is suggested to be a protective vaccine target although its efficacy and safety concerns remain not well understood. We investigated immunogenicity, efficacy, and safety of F proteins in a soluble form or on virus-like particle (F-VLP). F VLP preferentially elicited IgG2a antibody and T helper type 1 (Th1) immune responses whereas F protein induced IgG1 isotype and Th2 responses. Despite lung viral clearance after prime or prime-boost and then RSV challenge, F protein immune mice displayed weight loss and lung histopathology and high mucus production and eosinophils. In contrast, prime or prime-boost vaccination of F VLP induced effective protection, prevented infiltration of eosinophils, and vaccine-enhanced disease after challenge. This study provides insight into developing an effective and safe RSV vaccine candidate.
3.2 Result

3.2.1 *RSV F VLP vaccination induces a distinct pattern of IgG isotypes compared to F proteins.*

RSV F protein is a common platform of RSV subunit vaccines that are being explored. The F VLP and soluble F protein vaccines used in this study were found to be highly reactive to post-fusion F specific monoclonal antibody (131-2a) recognizing the post fusion F conformation but low or no reactivity to pre-fusion F specific 5C4 monoclonal antibody (data not shown). We determined immunogenicity and efficacy of RSV F in a soluble protein form or in a particulate VLP after prime or prime-boost vaccination. Groups of mice were intramuscularly immunized with F-VLP (10 μg total proteins, no adjuvant), or F proteins (0.1μg or 0.3μg) with alum adjuvant. Both 0.1μg and 0.3μg dose RSV F protein immunization groups induced significantly higher levels of RSV F specific IgG1 isotype antibody than F VLP immunized group (Fig. 3.1B). In contrast, F VLP induced IgG2a isotype dominant antibody responses (Fig. 3.1C). Meanwhile, F protein groups showed low or background levels of IgG2a (Fig. 3.1C). Boost dose vaccination induced moderate increases in IgG2a and IgG1 isotype antibodies in the F VLP and F protein groups respectively. The F VLP group showed significantly higher ratios of IgG2a/IgG1 compared to those in F protein groups (Fig. 3.1D).

Neutralizing antibody is considered a major correlate of protection against RSV. F protein and F VLP prime immune sera showed similar neutralizing activities (~9 log2) which were significantly higher than those in naïve sera exhibiting the background neutralizing activity of approximately 4 log2 (Fig. 3.1E). This neutralizing assay of 50% plaque reduction titers resulted in significant increases in a range of 16- to 64-fold higher titers in immunized sera than naïve sera, which are statistically significant. Previous studies reported similar ranges of the background
neutralizing activity in sera from the naïve mice (25, 29, 30, 49-51), suggesting a limitation in this assay. Moderate increases in neutralizing titers (~10 log2) were observed after boost vaccination with F VLP or 0.3μg F protein (Fig. 3.1E).

3.2.2 Mice with RSV F protein vaccination induce protection against viral replication but display disease phenotypes.

Immunized mice were challenged with RSV (5x10^5 PFU) and body weight changes were monitored (Fig. 3.2). The naïve infection and F protein (0.3 μg) groups displayed approximately 10% loss of body weight whereas the low dose (0.1 μg) F protein group showed 5-7% body weight loss. The F-VLP group exhibited only a slight change in body weight after RSV challenge (Fig. 3.2A, B). There were no significant differences in body weight between the prime and prime-boost groups after challenge.

Lung tissues were collected from individual mouse after 5 days of challenge and viral loads were evaluated with an immunoplaque assay. The naïve infection group had highest viral loads in the lungs. Prime or prime-boost vaccinated mice showed significantly lower levels of RSV lung viral loads than naïve mice (Fig. 3.2C, D). A better control of viral loads was observed in the boost F VLP group than in the F protein groups (Fig. 3.2C, D).

3.2.3 RSV F protein vaccination induces histopathology upon RSV challenge.

To determine whether RSV F VLP or soluble F protein vaccination induces pulmonary inflammation after RSV challenge, we examined lung histology after staining with hematoxylin and eosin (H&E), and evaluated inflammation on airway, blood vessels and intestinal space in a blind scoring (Fig. 3.3A-E). The naive control (PBS) group with RSV infection exhibited moderate levels of lung inflammation around the airways, blood vessels, and interstitial spaces
(Fig. 3.3). The F protein prime or prime-boost (0.1μg, 0.3μg) groups showed the highest influx of inflammatory cells, most severe symptoms of alveolitis, and infiltrates in the airways, blood vessels, and interstitial spaces. F VLP prime only or prime-boost immune mice did not display lung histopathology after RSV challenge (Fig 3.3).

Excessive mucus production contributes to blocking the airways. Lung tissue sections were stained with Periodic acid-Schiff (PAS) to quantify mucus in the randomly selected airways epithelium. Consistent with histopathology, F protein prime or prime-boost mice showed the highest PAS-positive mucus production with an increasing trend in the high dose (0.3μg) group. Naïve mice with RSV challenge showed moderate to low levels of airway mucus production whereas mucus was not observed in F VLP immune mice (Fig. 3.4).

We examined eosinophils infiltration from lung tissue using hematoxylin and congo red (H&CR) staining. Significantly infiltrated eosinophils were detected in the airways of 0.1μg or 0.3μg F protein but not F VLP prime or prime-boost immune mice after RSV challenge (Fig. 3.5A-D). The results of eosinophilic histology were consistent with Siglec F+ eosinophils in BALF from the F protein groups but not from the F VLP group as determined by flow cytometry (Fig. 3.5E, F). Taken together, these results suggest that RSV F soluble protein immunization but not when presented on particulate VLPs causes lung histopathology upon RSV challenge.

3.2.4 Biased Th2 cytokines are elicited after F protein vaccination and RSV challenge.

To investigate the Th1 and Th2 immune responses after immunization and RSV challenge, IL-4, IL-5, IL-13 (Th2) and IFN-γ (Th1) cytokines were determined. The F protein (0.1μg, 0.3μg) groups showed high levels of Th2 cytokines IL-4, IL-5 and IL-13 in both lung and BALF samples collected at day 5 post challenge (Fig. 3.6A-C, E-G). In contrast, high IFN-γ and low Th2 cytokine
levels were detected in lung and BALF samples from the F-VLP group, suppressing the induction of Th2 type cytokines (Fig. 3.6D and H). Next, we determined the profile of IFN-γ or IL-4 secreting CD4 and CD8 T cells in response to stimulation with CD4 (F51-66) and CD8 T (F85-93) cell epitopes respectively (Fig. 3.7). Higher levels of IFN-γ secreting CD4 and CD8 T cells were observed in lungs (Fig. 3.7A-C, G) and BAL (Fig. 3.7D-F, H) samples from F VLP immune mice than those from F protein immune mice. When the ratios of IFN-γ+/IL-4+ CD4 T cells and IFN-γ+/IL-4+ CD8 T cells were compared, naïve infection and F VLP immune mice resulted in significantly higher IFN-γ ratios for both T cells, suggesting dominant Th1 responses (IFN-γ) compared to F protein immune mice (Fig. 3.7C, F, I, J). Neutrophils (CD11b+Ly6c+F4/80-) and dendritic cells (DCs, CD45+CD11c+MHCII+) were found to be recruited to higher levels in F protein immune mice compared to that in the F VLP group after RSV challenge (Fig. 3.7K, L).

### 3.2.5 F VLP induces transient inflammatory responses.

To better understand the mechanisms of inducing a distinct pattern of immune responses between F VLP and F protein vaccination, we determined innate immune responses at the site of injection. Intraperitoneal (IP) injection of F VLP transiently induced chemokines (KC; CXCL1, MCP-1) and IFN-γ in sera within 1.5 hours (h) or 5 h, which rapidly waned after 5 h (Fig. 3.8A-C). Peritoneal exudates displayed high levels of chemokines (RANTES, MCP-1) and IFN-γ at 24 h post treatment (Fig. 3.8D-F). In contrast, the levels of Th2 cytokines (IL-4, IL-5, and IL-13) were higher in peritoneal exudates after injection with F protein but not with F VLP (Fig. 3.8G-I). Flow cytometric determination of cellular phenotypes in peritoneal cavity showed that F VLP is more effective in recruiting monocytes, neutrophils, and natural killer (NK) cells at the site of injection than F protein (Fig. 3.8J-L).
Figure 3. 1 RSV-specific IgG isotype antibody levels and neutralizing activity after prime or prime-boost vaccination with F VLP or F protein vaccine.

BALB/c mice (N=5) were immunized with F VLP (10 μg) or F protein (0.1 or 0.3 μg) with alum adjuvant (50μg). Sera were collected 3 weeks after prime or prime-boost. (A-C) The IgG isotype levels were determined using F protein as antigen for antibody detection. (D) Ratios of IgG2a/IgG1 isotypes. (E) Neutralizing antibody titers. RSV neutralizing activity was determined by an immune-plaque reduction assay and titers were presented as dilution factors resulting in 50% reduction in plaque numbers. Results are representative out of two independent experiments and presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** p<0.001, ** p<0.01, * p<0.05 comparing F VLP and F protein groups. Prime; prime only immunization. Boost; prime and boost immunization, PBS; unimmunized mice, F VLP; F VLP immunized mice, Fp(0.1) or Fp(0.3); 0.1μg or 0.3μg RSV F proteins with alum adjuvant.
Figure 3. Body weight changes and lung viral clearance in vaccinated BALB/C mice after RSV challenge.

Naïve (PBS only) and vaccinated mice were i.n. challenged with RSV A2 (5 × 10⁵ PFU) at 4 weeks after prime or prime-boost immunization. (A) Body weight changes in prime immunized mice after RSV challenge. (B) Body weight changes in prime-boost immunized mice after RSV challenge. (C) Lung RSV titers after prime immunization. RSV titers were determined in individual lungs collected at 5 days after RSV challenge. (D) Lung RSV titers after prime-boost immunization. Individual lungs were collected 5 days after RSV challenge. The dotted line represents the detection limit. Results were reproducible in two independent sets of animal studies with each set n=5 and presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** P<0.001 (C, D), ** p<0.01 (D), * P<0.05 (D). Naïve R.; Naïve mice + RSV challenge, F VLP; F VLP vaccinated mice + RSV challenge, Fp (0.1); F protein 0.1μg + RSV challenge, Fp (0.3); F protein 0.3μg + RSV challenge. Independent sets of animal studies with each set n=5 and presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** P<0.001 (C, D), ** p<0.01 (D), * P<0.05 (D). Naïve R.; Naïve mice + RSV challenge, F VLP; F VLP vaccinated mice + RSV challenge, Fp (0.1); F protein 0.1μg + RSV challenge, Fp (0.3); F protein 0.3μg + RSV challenge.
Figure 3. Lung histopathology in prime or prime-boost vaccinated mice after RSV challenge.

Lung tissues collected from individual BALB/c mice (N=5) were prime or prime-boost vaccinated mice day 5 post challenge. (A, B) Photographs of H&E. Hematoxyline and Eosin (H&E) stained lung tissues were dissected to assess histopathology of peribronchiolar and alveolar pneumonia. Scale bars indicate 100 μm. (C-E) The lungs were scored using a 1-3 scoring system where 1 = minimal pathology and 3 = maximum pathology for the airways (C), interstitial spaces (D), blood vessels (E). Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** P<0.001, ** p<0.01, * p<0.05. Group labels are the same as described in the Fig. 2.
Figure 3. Levels of mucus production in the lungs from vaccinated mice after RSV challenge.

Lung tissues were collected from individual BALB/c mouse (N=5) after vaccination and RSV challenge. (A, B) Lung tissue histology after staining with Hematoxyline and Congo red (H&CR). Arrows indicate eosinophil granulocytes in the individual airways. Scale bars: 20 μm. (C, D) H&CR positive eosinophils were counted under the microscopic field and presented in percentages in the prime and prime-boost groups. (E, F) Numbers and flow profiles of gated Siglec F+ eosinophils (Siglec F+CD11b+CD11c-F4/80+CD45+) as determined by flow cytometry of BAL fluids. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** P<0.001, * p<0.05. Group labels are the same as described in the Fig. 2.
Figure 3.5 Assessment of eosinophils in the lungs from vaccinated mice after RSV challenge.

(A, B) Photographs of PAS. Periodic-Schiff (PAS) stained lung tissue were dissected to assess mucus production in the bronchiolar and alveolar area. Scale bars: 100μm. (C, D) Percentages of PAS positive airway mucus expression. The percentage of PAS positive lung section area in each airway was determined; individual airways are shown per group. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** P<0.001. Group labels are the same as described in the Fig. 2.
Figure 3.6 Cytokines in the Lung tissue and BALF from prime-boost immune mice after RSV challenge.

(A, B) Lung tissue and BALF were collected from individual mice (N=5) after RSV challenge. Levels of IL-4, IL-5, IL-13 and IFN-γ cytokines were measured in lung and BALF samples by each corresponding cytokine ELISA kits. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA and Tukey’s multiple-comparison tests in GraphPad Prism; *** p<0.001, **P<0.01, *p<0.05. Group labels are the same as described in the Fig. 2.
Figure 3. 7 CD4+, CD8+ T cells secreting Th1 or Th2 cytokines by intracellular cytokine staining, and airway infiltrating innate immune cells.

Lung and BALF cells were collected from prime-boost immunized mice at 5 days after challenge or subjected to intracellular cytokine staining. (A, B, D, E, G, H) Intracellular cytokine staining of CD4 and CD8 T cells from lungs or BALF by flow cytometry after in vitro stimulation with CD4 T cell F51−66 epitope and CD8 T cell F85−93 epitope. (C, F, I, J) Ratios of IFN-γ+/IL4+ CD4 or CD8 T cell number averages from intracellular cytokine flow cytometry of lung and BAL samples. (K) Neutrophils (CD11b+Ly6c+F4/80+) and (L) Dendritic Cells (DCs, CD45+CD11c+MHCII+) were analyzed from BALF samples. PBS: Naïve mice with RSV infection. Other groups are vaccinated mice as indicated. The results are presented as averages with standard errors and statistical significance was determined using one-way ANOVA with Tukey’s multiple comparison test in GraphPad Prism. ***, p < 0.001, **p<0.01. Other group labels are the same as described in the Fig. 2.
Figure 3.8. Acute responses of cytokines, chemokines, and cellular infiltrates at the site of injection with F VLP or F protein.

BALB/c mice (n=3) were intra-peritoneally injected with PBS, F VLP, or F protein. (A-C) Kinetics of cytokines and chemokine levels in sera from adjuvant injected mice (n=3 mice per group). ***; p<0.001 compared to naïve group (PBS only). (D-I) Cytokines and chemokines in peritoneal exudates at 24h after injection. (J) Monocytes: CD11b+Ly6c high F4/80+, (K) Neutrophils; CD11b+Ly6c+F4/80-, (L) NK cells; DX5+CD3-, data were shown as mean ± SEM. Statistical significances were calculated by 1-way ANOVA and Tukey’s multiple comparison test. ***; p<0.001 **; p<0.01, and *; p<0.05, as indicated among the groups. Naïve; unimmunized mice, F VLP; F VLP immunized mice, F protein; 0.1μg RSV F protein with alum adjuvant. Group information of D-I were same as J-L.
3.3 Discussion

In this study, we compared the pattern of immune responses to F immunogen in VLP and in a soluble protein, and their resulting efficacy and lung inflammation after RSV challenge. F VLP or F protein vaccination induced IgG2a and IgG1 isotype dominant antibody responses respectively, representing Th1 and Th2 type immune patterns. Prime or prime-boost immunization with F VLP and F protein vaccines showed comparable RSV neutralizing activity and was effective in controlling lung viral loads after RSV challenge, which is consistent with a previous study reporting moderate increases in IgG titers after boost with FI-RSV or purified F protein (14). Nonetheless, there were striking differences in pulmonary histopathology, mucus production, and eosinophilia as well as body weight loss of RSV disease between the F VLP and F protein vaccine groups.

Since RSV vaccine-enhanced disease was first observed during 1960s clinical trials of FI-RSV vaccines (6), numerous efforts have been made to develop RSV vaccines that can avoid vaccine-enhanced pulmonary histopathology. RSV F, G (attachment), and chimeric FG protein subunit vaccines were previously shown to cause vaccine-enhanced pulmonary histopathology in mouse and cotton rat animal models (14, 22, 23). Bronchiolar histopathology and pulmonary polymorphonucleocytes infiltration appeared to be evident after vaccination with Vac-F and RSV challenge in mice (52, 53) but less likely in cotton rats (54), suggesting a greater tendency for mice to develop RSV-vaccine enhanced disease. Viral-vectored RSV vaccines are likely to mimic natural infection inducing innate and adaptive responses. Modified vaccinia virus Ankara and simian adenovirus expressing RSV proteins were immunogenic and safe in healthy adults (phase I), warranting further clinical evaluation of efficacy (55). The recombinant Sendai virus, a mouse type I parainfluenza virus expressing a full-length RSV F protein, conferred protection against
RSV in a monkey model (56). RSV-reinfections appeared to induce pulmonary histopathology in mice (47) and in cotton rats (54). A recent study reported that low doses (0.3 - 0.03 µg) of post-fusion or pre-fusion F proteins primes histopathology in cotton rats after challenge despite lung viral clearance (20). F VLP and soluble F proteins used in this study were found to be expressed mostly in a post-fusion conformation (data not shown). We observed that mice prime or prime-boost immunization with RSV F protein vaccines displayed severe lung histopathology around the airways, blood vessels, and interstitial spaces as well as mucus production and eosinophil infiltration compared to naïve mice, which is correlating with weight loss disease after RSV challenge. In contrast, F VLP prime only or prime-boost vaccination could prevent pulmonary histopathology lower than that in naïve mice after RSV challenge, which are consistent with the results in cotton rats (57).

Comparative studies of F VLP and F protein vaccination might provide insight into underlying mechanisms by which RSV vaccine-enhanced disease could be avoided, which will help in developing a safe and effective RSV vaccine. F VLP raises IgG2a Th1 type dominant antibodies whereas F protein IgG1 Th2 type dominant responses. These results are consistent with cytokine patterns in lung and BALF samples where the F VLP group induced Th1 type (IFN-γ) cytokine and the F protein group elicited Th2 (IL-4, IL-5, IL-13) cytokines. Consistent with histopathology in the F protein groups, high levels of IL-13 and neutrophil infiltrates due to RSV infection were shown to be correlated with mucus production and pathology (58). CD11b+ granulocytes and plasmacytoid DCs infiltrating into the lungs after RSV challenge were suggested to contribute to RSV disease (59, 60). Lung and BAL T cell intracellular cytokine staining flow cytometry assay revealed that F VLP vaccination resulted in higher ratios of IFN-γ/IL-4 CD4 and IFN-γ/IL-4 CD8 T cells than F protein vaccination. Naïve mice with live RSV infection also
displayed similarly high ratios of IFN-γ/IL-4 CD4 and IFN-γ/IL-4 CD8 T cells although cell numbers of T cells secreting IFN-γ were lower than the F VLP group. F VLP vaccination appears to induce Th1 bias T cell responses. Induction of Th1 responses including the production of IFN-γ may modulate to suppress priming Th2 responses such as IL-4, IL-5, and IL-13 responsible for RSV pathology. The F VLP group induces higher levels of IFN-γ and CD8 T cells expressing IFN-γ compared to F protein vaccination. These humoral and cellular immunity and antiviral IFN-γ cytokines induced by F VLP vaccination might have contributed to a better control of lung viral loads in addition to neutralizing antibodies.

Previous studies explored RSV vaccine formulations with immune-modulating adjuvants and reported improved efficacy and safety of an intranasal virosomal RSV vaccine adjuvanted with monophosphoryl lipid A (61). In contrast, cotton rats immunized with the combination of F protein and oligodeoxynucleotide CpG adjuvant developed enhanced pulmonary histopathology after RSV challenge (23). Also, a squalene-based emulsion adjuvant mixed with RSV F protein vaccine was not able to avoid RSV vaccine-enhanced inflammatory disease and inclusion of glucopyranosyl lipid A (TLR4 agonist) modulated to prime IFN-γ producing T cell responses (50). RSV subunit vaccines of recombinant pre-fusion or post-fusion F proteins at low doses (<0.3 µg) were shown to prime immune responses enhancing lung histopathology regardless of a Th1- or Th2-biasing adjuvant in cotton rats after challenge (20). Mice vaccinated with Vac-G or FI-RSV and treated with a natural killer T cell activating adjuvant (α-GalCer) or live RSV vaccine plus delta-inulin adjuvant did not diminish lung histopathology after RSV challenge (62, 63). Interestingly, addition of F VLP to F protein vaccination was found to improve protective efficacy and to suppress F protein vaccine-enhanced lung histopathology in infant age mice (data not shown). It is important to determine whether F VLP with Th2 bias immune inducing adjuvant will
result in vaccine enhanced disease. Therefore, further studies are required to better understand the
detail mechanisms of RSV vaccine-enhanced disease and to develop a safe and effective RSV
vaccine.

To obtain insight into what reactions are occurring at the site of vaccine injection, we analyzed
acute cytokine, chemokine, and cellular phenotypes at day 1 after IP injection. F VLP injection
induced transiently chemokines (KC, MCP-1, and RANTES) and IFN-γ. Whereas, F protein
injection developed Th2 cytokines (IL-4, IL-5, and IL13). Monocyte chemoattractant protein-1
(MCP-1/CCL2) is known to regulate migration and infiltration of monocytes/macrophages (64).
RANTES (regulated on activation normal T cell expressed and secreted), now known to be
secreted by hematopoietic and non-hematopoietic cell types including epithelial cells, acts as a
chemoattractant for monocytes, natural killer cells, T cells, eosinophils, and dendritic cells as well
as for maintaining antiviral CD8 T cells (65). F VLP injection resulted in acutely inducing cellular
infiltrates such as monocytes, neutrophils, and natural killer (NK) cells. The levels of these innate
acute inflammatory cells appeared to be lower than those reported in previous studies on adjuvants
such as oil-in-water emulsion MF59 and combination AS04, alum + monophosphoryl lipid A
(MPL, a TLR4 ligand) (66). These intrinsic differences between F VLP and F protein in generating
inflammatory microenvironment might be contributing to developing differential quality of
immunity responsible for enhancing or preventing RSV vaccine-associated disease.
4 CHAPTER 2. A UNIQUE COMBINATION ADJUVANT MODULATES IMMUNE RESPONSES PREVENTING VACCINE-ENHANCED PULMONARY HISTOPATHOLOGY AFTER A SINGLE DOSE VACCINATION WITH FUSION PROTEIN AND CHALLENGE WITH RESPIRATORY SYNCYTIAL VIRUS

4.1 Summary

Alum adjuvanted formalin-inactivated respiratory syncytial virus (RSV) vaccination resulted in enhanced respiratory disease in young children upon natural infection, which has been a major obstacle in developing a safe and effective RSV vaccine. Here, we investigated the adjuvant effects of monophosphoryl lipid A (MPL) and oligodeoxynucleotide CpG (CpG) on vaccine-enhanced respiratory disease after fusion (F) protein prime vaccination and RSV challenge in infant and adult mouse models in comparison with alum adjuvant. Combination CpG+MPL adjuvant in RSV F protein single dose priming of infant and adult age mice was found to promote the induction of IgG2a isotype antibodies and neutralizing activity, and lung viral clearance after challenge. Importantly, CpG+MPL adjuvant F protein priming of infant and adult age mice was effective in avoiding lung histopathology, interleukin-4+ CD4 T cell responses, and cellular infiltration of monocytes and neutrophils after RSV challenge. There were differential adjuvant effects of MPL and CpG on modulating histopathology and inflammatory immune responses, depending on the ages of mice at the time of F protein priming. This study indicates that combination CpG and MPL adjuvant in RSV subunit vaccination might contribute to priming protective immune responses and preventing inflammatory RSV disease after infection.
4.2 Results

4.2.1 *Combination CpG+MPL adjuvant in RSV F protein vaccination mediates the effective induction of IgG2a antibody and neutralizing activity in infant and adult mice.*

Purified RSV A2 F glycoproteins used in this study were found to be highly reactive to post-fusion F specific 131-2a monoclonal antibody (mAb) and site II epitope recognizing Palivizumab but poorly reactive to the pre-fusion specific 5C4 mAb (Supplementary Fig. 4.1), suggesting that F proteins are mostly in a post-fusion conformation. We reasoned that F proteins would serve as a model antigen to test the adjuvant effects on the efficacy and lung inflammatory disease after subunit F vaccination and RSV challenge. Infant (2 weeks old) and adult (5~6 weeks old) mice were intramuscularly prime immunized with RSV F protein (Fp, 0.1µg) or in the presence of alum (50 µg), low dose CpG (4µg), MPL (1µg) or combination CpG (4µg)+MPL (1µg) adjuvant (Fig. 4.1). Substantial levels of RSV F specific antibodies were induced after vaccination with F proteins in the presence of adjuvants from the mice that were primed at 2-week age although higher levels of RSV F specific antibodies were observed in the mice primed at adult age (Fig. 4.1A). Combination CpG+MPL adjuvant in F protein vaccination was more effective in increasing IgG antibodies than the MPL or CpG alone F protein vaccine group, and similar to those in the Alum adjuvant group. IgG1 isotype antibodies were highly induced in the alum adjuvanted group whereas higher levels of IgG2a were induced in the CpG, MPL, and CpG+MPL groups (Fig. 4.1B, C). Adult mice induced higher levels of IgG2a isotype antibodies than those in infant age mice in response to CpG, MPL, and CpG+MPL adjuvanted F protein vaccination (Fig. 4.1C). In particular, the CpG+MPL F protein groups induced the highest levels of IgG2a isotype antibodies in primed infant and adult mice (Fig. 4.1C). Next, we determined whether adjuvants would...
promote the induction of IgG antibodies recognizing pre-fusion conformation-stabilized F protein with DsCav mutations (67). It is notable that CpG+MPL adjuvanted F protein vaccination induced IgG antibodies specific for DsCav pre-F antigen at higher levels than alum, CpG, or MPL adjuvanted F protein vaccine groups particularly after priming of mice at an infant age (Fig. 4.1D).

Adult mice showed approximately 2-fold higher levels of RSV neutralizing antibody titers than those in infant mice after prime immunization with adjuvanted F proteins (Fig. 4.2A). The CpG+MPL F protein group showed highest RSV neutralizing antibody titers among the adjuvanted F protein vaccination groups in infant mice. RSV neutralizing antibody titers were similarly high in both alum and CpG+MPL adjuvant F protein adult mice (Fig. 4.2A). These data indicate that combination CpG+MPL adjuvant in RSV F protein single vaccination effectively induces IgG2a antibodies and neutralizing activity in infant and adult mice.

4.2.2  **Combination CpG+MPL adjuvant improves lung viral clearance and protection of F protein vaccine.**

Protective efficacy of adjuvanted F proteins at 3 weeks after single vaccination was assessed by determining lung virus titers after RSV challenge. Both young age (5 weeks old) and adult (8-9 weeks old) naïve mice showed high lung viral titers at day 5 after RSV infection (104 PFU/ g lung tissues, Fig 4.2B, C). In the F protein primed groups at an infant age, the CpG+MPL group was most effective in clearing lung viral loads by 100 folds, whereas the alum, CpG, and MPL groups showed 10 – 20 folds lower RSV titers compared to the equivalent age naïve mice (Fig. 4.2B). In the adult age F protein primed groups, the CpG+MPL group was most effective in clearing lung viral loads by 100 folds, followed by the alum, CpG, and MPL groups (Fig. 4.2C). Adjuvants F protein primed mice at adult age were more effective in clearing RSV lung viral titers
than adjuvanted F protein primed mice at an infant age (Fig 4.2B, C), which is consistent with IgG antibody levels (Fig. 4.1). Overall, combination CpG+MPL adjuvant was more effective than other adjuvants in improving the protective efficacy after a single dose F protein vaccination at infant or adult ages.

4.2.3 *CpG+MPL adjuvant suppresses lung histopathology after F protein vaccination and RSV challenge.*

After RSV challenge week 3 post vaccination, alum adjuvanted F protein vaccination of mice at infant ages resulted in high PAS positive mucus production in the airway epithelium and inflammation scores in the lung histology when examined day 5 post RSV challenge (Fig. 4.3A). Also, age matching infected naïve mice displayed significant inflammation histology in the airways (scores 1.5), blood vessels (1.3) and interstitial spaces (1.0) compared to those from uninfected mice (Fig. 4.3B, C). We found lower levels of inflammation in the lung histology from CpG or MPL adjuvanted RSV F protein immunized mice after RSV challenge compared to those in F protein alone or alum adjuvanted RSV F protein vaccine mice (Fig. 4.3B, C). Similar to infant age primed mice, the Fp+Alum primed adult group displayed the highest level of PAS+ mucus production, followed by F protein and naïve mice (Fig. 4.3D). Infant age mice (Fig. 4.3A, B, C) with CpG adjuvant F protein prime was found to be more effective in suppressing inflammation in the lung histology (airways, blood vessels, interstitial spaces) than adult age mice with CpG F protein prime (Fig. 4.3D, E, F). Interestingly, MPL appeared to be more effective in suppressing lung inflammatory for adult mice (Fig. 4.3D, E, F) with F protein vaccination than CpG although there is no statistical difference between the 2 groups. Most notably, in both infant and adult (Fig. 4.3) age mice with CpG+MPL adjuvanted F protein prime vaccination, lung inflammatory
histopathology and PAS positive mucus production in the airway epithelium were not observed after RSV challenge.

We carried out H&CR staining of lung histology as a measure of eosinophilic infiltration in the airways at 5 days after RSV challenge (Fig. 4.4). Both CpG+MPL adjuvanted RSV F primed infant (Fig. 4.4A, B) and adult (Fig. 4.4E, F) age mouse groups did not show eosinophil infiltrations after RSV challenge, which is similar to naïve mice without RSV infection. In contrast, alum adjuvant in F protein vaccination exhibited high levels of eosinophilic H&CR+ staining cell spots, followed by naïve mice with RSV infection (Fig. 4.4A, B, E, F). The CpG F protein primed adult mice showed a low to moderate level of eosinophilic staining and infiltration, compared to those in the MPL F protein primed mice (Fig. 4.4E, F).

To quantitate eosinophil infiltration, BAL and lung cells from immunized mice were collected day 5 post challenge and Siglec F+ eosinophils were determined by flow cytometry staining (Fig. 4.4C, D, G, H). In consistent with the results from H&CR staining of lung histology, MPL or CpG +MPL adjuvanted RSV F immunized group showed the lowest level or no eosinophils (Fig. 4.4C, D, G, H). The F protein only or CpG adjuvant F protein primed adult mice showed a moderate level of eosinophils (Fig. 4.4G, H). In overall, the mice primed with F protein at an infant age showed lower infiltrated eosinophils in the BAL airways than the mice primed at an adult age, whereas lung eosinophils were approximately 3-fold higher in infant age Fp+Alum primed mice than those in adult age Fp+Alum primed mice, after RSV challenge (Fig. 4.4C, G). Taken together, these results suggest that CpG+MPL adjuvanted F protein vaccination suppresses inflammatory mucus production, lung histopathology, and eosinophil infiltration after RSV challenge in infant and adult age mouse models.
**4.2.4 CpG+MPL adjuvanted F protein priming of infant and adult mice effectively prevents the induction of IL-4+ CD4 T cells after RSV challenge.**

To determine whether adjuvants such as alum, CpG and MPL would modulate IFN-γ or IL-4 producing T cell responses, BAL and lung cells at 5 days after challenge week 3 post prime vaccination were analyzed by intracellular cytokine staining flow cytometry after stimulation with a known CD4 T cell epitope F51-66 (Fig. 4.5). Both groups of infant and adult age mice primed with alum adjuvanted F protein showed the highest levels of CD4 T cells secreting IL-4 cytokines in BAL and lung samples (Fig. 4.5A, B). CpG or MPL adjuvant F protein primed mice induced relatively low levels of BAL IL-4+ CD4 T cells but these Fp+CpG and Fp+MPL groups displayed 3- to 4-fold higher levels of lung IL-4+ CD4 T cells than those in FP+C+M group (Fig. 4.5A, B). The lowest cellularity of IL-4+ CD4 T cells was observed in BAL and lung samples from infant age-primed and adult age-primed mice with combination CpG+MPL adjuvant F proteins (Fig. 4.5A, B). The CD4 T cell numbers secreting cytokines were higher in adult primed mice than 2-week old infant age primed mice as presented in different Y axis scales (Fig. 4.5). The mice that were primed with CpG+MPL adjuvant F protein at 2 weeks old responded to induce BAL and lung IFN-γ+ CD4 T cells at 1.5- to 2-fold higher levels than PBS, F protein, alum, CpG, and MPL groups after RSV infection (Fig. 4.5A). The groups of CpG and CpG+MPL but not alum adjuvant F protein primed mice at an adult age induced IFN-γ+ CD4 T cells after challenge (Fig. 4.5B). These results suggest that CpG+MPL adjuvanted F protein prime vaccination effectively prevents the induction of IL-4+ CD4 T cells, while promoting the induction of IFN-γ+ CD4 T cells after RSV challenge.

The Fp+alum group of mice primed at adult age responded with higher levels of Th2 type cytokines (IL-4, IL-5, IL-13) by adjuvanted F protein compared to those in the Fp+alum group
primed at an infant age after RSV challenge as presented in different Y axis scales (Fig. 4.6A, B). The Fp+CpG, Fp+MPL, and Fp+CpG+MPL groups showed relatively low levels of Th2 type cytokines but high levels of Th1 cytokine IFN-γ, which were more prominent in the adult age primed mice than those in infant age primed mice (Fig. 4.6A, B).

4.2.5 Monocytes and Neutrophils are not infiltrated into the lungs from CpG+MPL adjuvanted F protein primed infant and adult mice after RSV challenge

We analyzed the cellular infiltrates in the airway BAL fluids day 5 post challenge by flow cytometry using phenotypic markers of monocytes (CD11b+Ly6chiF4/80+) and neutrophils (CD11b+Ly6c+F4/80−) (Fig. 4.7). Fp+alum priming of infant age mice induced highest levels of monocytes and neutrophils in BAL fluids, whereas CpG+MPL adjuvant F protein priming of infant mice avoided the cellular infiltrates of monocytes and neutrophils after RSV challenge (Fig. 4.7A). MPL adjuvant F protein priming was more effective than CpG adjuvant F protein priming in preventing the cellular infiltrates of particularly airway monocytes (Fig. 4.7). In F protein priming of adult age mice, CpG+MPL adjuvant was most effective in reducing the infiltrates into the airways and lungs compared to alum, CpG, and MPL (Fig. 4.6B). Overall, priming vaccination of adult mice induced higher cellular infiltrates than infant age primed mice approximately by 4 folds as presented in different Y axis scales (Fig. 4.7). These results suggest that CpG+MPL adjuvanted F protein priming is effective in avoiding inflammatory cellular infiltrates of monocytes and neutrophils into the lungs in infant and adult age mice after challenge.
Figure 4.1. F protein prime vaccination of infant and adult age mice with Alum, CpG, MPL or CpG+MPL adjuvants elicits different IgG isotype antibodies.

Infant (2 weeks old) or adult (5–6 weeks old) BALB/c mice (N=5) were intramuscularly primed with F protein or F protein with Alum, CpG, MPL or CpG+MPL and sera were collected 3 weeks after prime. The levels of IgG isotypes were determined using a post-fusion F antigen (BEI NR-28908, A, B, C) or pre-fusion DsCav F protein antigen (D). PBS: unimmunized mice, F protein: 0.1 µg of F protein, Fp+Alum: F protein 0.1 µg with alum 50 µg, Fp+CpG: F protein 0.1 µg with CpG 4 µg, Fp+MPL 1 µg; F protein 0.1 µg with MPL, Fp+C.+M.: F protein 0.1 µg with CpG 4 µg and MPL 1 µg combination adjuvants. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** p<0.001, ** p<0.01, * p<0.05 comparing F to F + adjuvant in mice.
Figure 4. 2 Neutralizing titers in primed mice and lung viral clearance after challenge.

(A) Neutralization titers at 3 weeks after prime vaccination of 2 weeks (2wks) old and adult mice. An immunoplaque assay was used to determine neutralizing titers and a linear line indicates detection limit. (B-C) Lung RSV titers after challenge. PBS (naïve R.) and immunized mice were intranasally (i.n.) challenged with 3.5x10⁵ PFU RSV A2. BALB/c mice (N=5) were immunized with F protein and F protein with alum, CpG, MPL or CpG+MPL. Individual lungs were collected at 5 days after RSV challenge and RSV titers were determined by an immunoplaque assay. The linear line represents the lower limit of detection (LLOD). Naïve R: Unvaccinated naïve mice with RSV infection. The group labels are the same as in the Figure 1 legend. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** p<0.0001.
A. 2wks PAS

Naive    Naive RSV    F protein    Fp+Alum

Fp+CpG    Fp+MPL    Fp+CpG+MPL

B. 2wks H&E

Naive    Naive RSV    F protein    Fp+Alum

Fp+CpG    Fp+MPL    Fp+CpG+MPL

C. Inflammation score

Airways  Blood Vessels  Interstitial spaces

Naive    Fp    Naive    Fp    Naive    Fp    Naive    Fp
Naive R.  RSV    Naive RSV  RSV    Naive R.  RSV    Naive R.  RSV
F protein    Alum    F protein    CpG    F protein    CpG    F protein    CpG
Fp+CpG    Fp+MPL    Fp+CpG+MPL    Fp+CpG    Fp+MPL    Fp+CpG+MPL
Figure 4.3. Lung histopathology and mucus production in primed infant and adult mice after RSV challenge.

Lung tissue histopathology of the mice primed at 2 weeks (2wks) old (A-C) or at 5-6 weeks old adult age (D-F) at 5 days after RSV challenge. (A, D) Periodic-Schiff (PAS) stained lung tissues from individual mice (N=5) were dissected to assess peribronchiolar, alveolar pneumonia, and mucus production. Photographs of PAS. Scale bars indicate 100 μm. The percentage of PAS stained lung section area that were PAS positive for each airway was determined; individual airways are shown per group. (B, C, E, F) Hematoxyline and Eosin (H&E) stained lung tissue were dissected to assess peribronchiolar, alveolar pneumonia. Photographs of H&E. Scale bars indicate 100 μm. (C, F) The inflammation scores of histopathology were blindly scored using a 1-3 scoring system where 1 = minimal pathology and 3 = maximum pathology for airway, interstitial spaces, blood vessels. Naive R: Unvaccinated naïve mice with RSV infection. The group labels are the same as in the Figure 1 legend. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** P<0.0001, ** p<0.005, * p<0.05.
Figure 4. Eosinophilic infiltration into the lungs of adjuvanted F protein primed infant and adult mice after RSV challenge.

The eosinophil infiltration in hematoxyline and congo red (H&CR) stained sections of individual lung tissue of the mice (N=5) primed at 2 weeks old (A) or at 5-6 weeks old adult age (E) at 5 days after RSV challenge. Scale bars indicate 400 μm. (B, F) The eosinophils per field infiltration were quantified in the airways. (B) Eosinophil granulocytes in the endometrium from infant age (2 weeks old) primed mice after challenge. (E) Eosinophil granulocytes in the endometrium in adult mice after RSV challenge. (C, D, G, H) Populations and numbers of Siglec F eosinophils gated from granulocytes (CD11b F4/80 CD45) at day 5 post challenge as determined by flow cytometry in BAL fluids and Lung tissues. Arrows: eosinophil granulocytes, individual airways were shown per group. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** p<0.0001, * p<0.05.
Figure 4. 5. IL-4 and IFN-γ secreting CD4⁺ T cells in BAL and lung samples of infant and adult age primed mice after RSV challenge.

BALF and lung cells from infant (A) and adult (B) age primed mice (n=5) were collected at 5 days after challenge. CD4⁺ T cell epitope peptide F_p51−66 was used to stimulate BALF and lung cells in vitro, which were stained with phenotypic marker and cytokine antibodies, and subsequently analyzed by Flowcytometry. The group labels are the same as in the legends of Figures 1 and 2. The results are presented as means ± SEM, and statistical significance was determined using one-way ANOVA with Tukey’s multiple comparison test performed in GraphPad Prism. ***, p < 0.0001, **p<0.005, * p<0.05.

Figure 4. 6. Cytokines in the BALF from the primed mice at infant and adult age after RSV challenge.

Airway BALF samples were collected from the primed mice (N=5) at infant age (A) and adult age (B) at 5 days after RSV challenge. IL-4, IL-5, IL-13 and IFN-γ cytokines in BALF were determined by corresponding cytokine ELISA kits. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA and Tukey’s multiple-comparison test in GraphPad Prism; *** p<0.0001, **p<0.001, *p<0.05. The Fp +/- adjuvanted vaccination group labels are the same as in the legends of Figures 1 and 2.
Airway BALF samples were collected from the primed mice (N=5) at infant age (A) and adult age (B) at 5 days after RSV challenge, stained with antibodies against monocyte and neutrophil cell type phenotypic markers, and analyzed by flow cytometry. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA and Tukey’s multiple-comparison test in GraphPad Prism; *** p<0.0001, **p<0.001, *p<0.05. The Fp +/- adjuvanted vaccination group labels are the same as in the legends of Figures 1 and 2.
4.3 Discussion

It is a challenge to develop safe non-live RSV subunit vaccines due to the potential safety concerns of enhanced inflammatory disease in RSV negative populations after RSV infection. Subunit vaccines are in general considered to be safer than live virus vaccines. RSV F protein subunit nanoparticle vaccines were shown to be safe and immunogenic in adults in clinical studies (68, 69). Nonetheless, F protein vaccines were reported to cause vaccine-enhanced lung histopathology in animal models after challenge (14, 20-23). Also, in previous studies, vaccine-associated inflammatory histopathology was not attenuated by inclusion of various adjuvants tested, including alum, squalene oil-in-water emulsion (50), TLR4 agonist glucopyranosyl lipid A (GLA) integrated into stable emulsion (SE) (20), polysaccharides-based delta-inulin (63), natural killer (NK) T cell agonist α-GalCer (62). Also, we found that RSV F protein vaccination of mice in the presence of AddaVax squalene oil-in-water emulsion adjuvant, polysaccharides fucoidan, and TLR3 agonist poly I:C adjuvants did not suppress lung histopathology after RSV challenge despite adjuvant effects on enhancing the immunogenicity of F protein vaccine (data not shown). In this study, we have investigated the adjuvant effects of MPL and CpG on immunogenicity, the efficacy of lung viral titers, and particularly on suppressing pulmonary histopathology after F protein single dose priming of infant and adult age mice and RSV challenge. It is significant to find that combined CpG+MPL adjuvanted F protein priming of infant and adult age mice was effective in avoiding lung histopathology, IL-4+ CD4 T cell responses, and cellular infiltration of monocytes and neutrophils after RSV challenge, as well as enhancing RSV neutralizing antibodies and lung viral clearance. Results in this study suggest that combined CpG and MPL adjuvanted RSV subunit vaccination might be effective in priming protective immune responses and preventing inflammatory lung pathology after RSV infection.
In the RSV F protein vaccination of mice, CpG adjuvant dose of 1 – 20 µg was shown to display moderate effects on enhancing IgG2a Th1 type antibody induction (37). Combination of alum (100 µg) + CpG (20-100 µg) adjuvants in F protein vaccination was reported to increase neutralization titers, Th1 and Th2 immune responses, and lung viral clearance (37). Triple components of adjuvant CpG (10 µg) with an innate defense regulator peptide in polyphosphazene microparticles in the RSV F protein vaccination of mice mediated the induction of IgG1 and IgG2a antibodies, resulting in controlling virus replication and no evidence of vaccine-induced pathology after challenge (22, 70). The outcomes of CpG adjuvant appear to be different in other studies. CpG in a polysaccharide adjuvant derived from delta inulin promoted the induction of predominately IgG2a antibodies to RSV whole virus vaccination of mice but exacerbated lung pathology after RSV challenge (63). Cotton rats that were immunized with F protein + CpG (20 - 100 µg) adjuvant increased the induction of neutralizing activity titers but developed enhanced pulmonary pathology consisting of alveolitis and interstitial pneumonitis after RSV challenge (23). We examined TLR agonist adjuvant effects on F protein prime vaccination of mice at infant and adult ages. CpG (4 µg) adjuvant effects on suppressing lung pathology after RSV challenge were found to be prominent in F protein primed mice at an infant age. In consistent with these studies (23, 63), CpG + F protein priming of mice at adult age resulted in substantial inflammation scores in lung histology, and infiltration of eosinophils, monocytes and neutrophils after RSV challenge despite of inducing IgG2a Th1 type immune responses.

TLR4 agonist MPL included in the alum adjuvanted FI-RSV vaccination was demonstrated to reduce the polymorphonuclear leukocyte infiltration within the alveoli and moderately attenuate peribronchiolitis in cotton rats post-challenge (71). RSV F protein
formulated with MPL (15 - 50 µg) administered to cotton rats via intranasal prime and intradermal boost demonstrated no evidence of enhanced lung pathology upon RSV infection (72). Inclusion of MPL in the Fl-RSV vaccination was found to mitigate the lung pathology with a dramatic reduction in levels of Th1- and Th2-type cytokines and chemokines (73). MPL analog TLR4 agonist (GLA) in stabilized emulsion adjuvanted F protein vaccination (GLA-SE) of BALB/c mice induced Th1-biased humoral and cellular responses including CD4 and CD8 T cells, and minimal to moderate levels of eosinophilic infiltration similar to live RSV infection control upon RSV challenge (50). In a follow up study, postfusion or prefusion F protein low dose (0.03 µg) vaccination of cotton rats even in the presence of a TLR4 agonist Th1-biasing (GLA-SE) adjuvant was reported to prime inflammatory vaccine-enhanced alveolitis although lung viral titers were below the detection limit after RSV challenge (20). In this study comparing MPL or CpG adjuvant effects on RSV F protein vaccination were similarly effective in suppressing histopathology and eosinophil infiltration at moderately low levels in infant age primed mice. In adult age priming of mice with F protein, MPL was more prominent in suppressing histopathology compared to CpG after RSV challenge although there was no statistically difference between the two adjuvants. In consistent with previous studies (20, 23, 50, 63), separate MPL or CpG adjuvant alone in F protein priming of mice at infant and adult age exhibited a certain degree of histopathology and monocyte infiltration upon RSV challenge, despite the induction of Th1 type immune responses and significant reduction in lung viral titers.

MPL as a TLR4 ligand likely triggers MyD88-dependent (TIRAP/MyD88) and TRAM/TRIF (Toll-interleukin 1 receptor domain–containing adapter)-dependent pathways, leading to activation of nuclear factor (NF)-κB and IFN-response factor 3 (IRF3) and inducing type 1 IFN innate immune responses (74, 75). Intracellular TLR9 ligand CpG interaction recruits
MyD88-dependent pathway leading to the activation of NF-κB and IRF7, eventually inducing inflammatory cytokines and type 1 IFNs (74). A previous study reported that MPL+CpG combination was more effective in inducing IL-12p70 and TNF-α in bone marrow-derived DC cultures in vitro than either one alone (66). Synergistic effects of combination TLR4 and 9 agonists on IL-1β production but not on IL-6 were demonstrated with stimulation of peripheral blood mononuclear cells isolated from healthy adults (76). The mechanisms of combination CpG+MPL adjuvant effects on RSV F protein priming remains to be determined. Inclusion of CpG+MPL in the RSV F protein single dose priming of infant and adult age mice was most prominent for avoiding lung histopathology, IL-4+CD4 T cell responses, and cellular infiltration of monocytes and neutrophils after RSV challenge, in addition to increasing protective efficacy of lung viral clearance after challenge. The levels of IFN-γ+CD4 T cells were similarly observed in F protein primed infant age mice that displayed different degrees of histopathology, suggesting that an inverse correlation between IFN-γ+ CD4 T cells and histopathology is not always predicted. In contrast, priming adult mice with alum adjuvanted F protein showed a typical pattern of high IL-4+CD4 T cells and low IFN-γ+CD4 T cells, which is correlating with inflammatory histopathology after RSV challenge.
CHAPTER 3. EFFECTS OF NOVEL COMBINATION ADJUVANTS ON IMPROVING THE EFFICACY OF INACTIVATED SPLIT RESPIRATORY SYNCYTIAL VIRUS VACCINE IN MICE

5.1 Summary

Human respiratory syncytial virus (RSV) causes severe disease of pneumonia and bronchiolitis leading to hospitalizations and mortality of over 160,000 pediatric deaths worldwide. There is no licensed vaccine on the market despite extensive effort on developing a safe and effective RSV vaccine for several decades. Alum adjuvanted formalin-inactivated whole RSV vaccine (FI-RSV) failed in clinical trials in young children due to vaccine-enhanced pulmonary disease, which is also recapitulated in animal models. In this study, we investigated the antigenicity and immunogenicity of whole FI-RSV and split RSV vaccines, as well as the adjuvant effects on improving RSV vaccine efficacy and modulating immune responses that prevent vaccine-enhanced pulmonary histopathology after vaccination and RSV infection in infant age mice. We found that inactivated and detergent-split RSV (SRSV) vaccines expose neutralizing epitopes reactive to palivizumab at higher levels than inactivated whole virus. Split RSV vaccination of mice induced more desirable immune responses towards T helper type 1 (Th1) and less lung histopathology compared to FI-RSV after RSV challenge. Next, we tested the efficacy and pulmonary histopathology after Split or FI-RSV vaccination in the presence of alum salts, MPL TLR 4 agonist, CpG TLR 9 agonist, or combination MPL+CpG adjuvants. We used 2 weeks old infant or adult BALB/c mouse models after prime vaccination and RSV challenge. Low dose combination of MPL + CpG adjuvants was found to be most effective in increasing Th1 type IgG antibodies, neutralizing activity, and lung viral clearance as well as modulating balanced immune
responses to prevent pulmonary histopathology after RSV vaccination and challenge. This study demonstrates adjuvant candidates that would improve the efficacy and safety of subunit RSV vaccines.

5.2 Results

5.2.1 Split RSV displays high antigenic reactivity and a unique pattern of immunogenicity in adult mice

RSV was inactivated using formalin and used as whole virus FI-RSV. An additional procedure of splitting FI-RSV (Split RSV) was carried out by treatment with non-ionic detergent Triton X-100 and resulting split RSV named “split”. Antigenic properties were determined using post-fusion F conformation specific monoclonal antibody (mAb) 131-2a, the site II F neutralizing epitope specific mAb palivizumab, and pre-fusion F specific 5C4 mAb (Fig. 5.1). Split displayed significantly higher levels of reactivity against 131-2a and palivizumab mAbs compared to whole virus FI-RSV (Fig. 5.1). Both FI-RSV and Split showed low reactivity against 5C4 mAb, suggesting that 5C4 mAb specific pre-fusion epitopes were not well exposed in FI-RSV and split RSV vaccine preparations.

To compare the immunogenicity, adult BALB/c mice (n=5, 8-12 weeks old) were intramuscularly immunized with Split (5 μg) and FI-RSV (5 μg) in the absence of adjuvant (Fig. 5.2). The split group showed a trend of inducing higher levels of RSV specific IgG antibodies than the FI-RSV group at 3 weeks after prime vaccination, but the differences were not significant between the two groups. Interestingly, split RSV vaccination induced lower IgG1 and higher IgG2a isotype antibody levels compared to those after FI-RSV vaccination, and these differences
are statistically significant (Fig. 5.2A-C). These results suggest that split RSV displays high antigenic reactivity and can induce a unique pattern of immunogenic properties of inducing IgG2a isotype antibodies.

5.2.2 Split RSV prime dose controls lung viral loads and attenuates histopathology after RSV challenge in adult mice

To determine the efficacy of split RSV, immunized mice were challenged with RSV at 3 weeks after prime vaccination (Fig. 5.2). The whole FI-RSV immunized group showed substantial weight loss (~8-13%) at days 3 and 4 after challenge (Fig. 5.2D). The split RSV immunized group displayed approximately 7% weight loss at day 2 after RSV challenge, similar to the unimmunized naïve mice after RSV infection. The naïve mice after RSV infection showed the highest levels of viral loads in the lung at day 5 after challenge. Split RSV prime vaccination led to lowering lung viral loads by 100, which was comparable to or more effective than FI-RSV prime reducing lung viral loads by approximately 30-50 (Fig. 5.2E).

To determine whether split RSV vaccine would protect against RSV infection and attenuate histopathology inflammation responses, histology tissues with infiltrated eosinophils and mucus-producing cells were visualized in the airway stained by Hematoxylin and eosin stain (H&E), hematoxylin and congo red (H&CR) and periodic acid-Schiff (PAS) stain (Fig. 5.3). The split RSV vaccine group showed the expression of the murine mucus in the lung as quantified (Fig. 5.3C, F). However, eosinophils and numerous PAS-positive airway epithelial cells were seen in the lung from FI-RSV immune mice compared to those in split RSV immune mice after infection with RSV (Fig. 5.3B, E). Overall, these results indicate that RSV infection in split RSV immune mice showed
much less histopathology responses, including the inflammation and mucus induction that was typically seen with FI-RSV immune mice with RSV respiratory infections.

5.2.3 **CpG and MPL adjuvants in split RSV prime vaccination promotes IgG2a isotype dominant antibody responses in infant age mice**

Young children are a more prime target for effective and safe RSV vaccination than adults. We tested whether CpG and MPL adjuvants in split RSV prime vaccination would promote desirable Th1 type (IgG2a) immune responses and the efficacy of protection preventing enhanced RSV disease in 2 weeks old infant mice after challenge. Infant age (2 weeks old) BALB/c mice were prime (single dose) immunized with whole FI-RSV (2 µg), split RSV (2 µg) alone, or split RSV adjuvanted with alum (50 µg), MPL (1 µg, TLR4 agonist), CpG (4 µg, TLR9 agonist), or MPL (1 µg) + CpG (4 µg). At 3 weeks after single dose vaccination, sera were collected and used to measure IgG and isotype antibodies specific for RSV antigens (Fig. 5.4A-C). Both FI-RSV and Split RSV prime vaccination of infant mice induced IgG antibodies specific for RSV at a comparable level. Alum and CpG+MPL adjuvant groups induced higher IgG antibody responses compared to FI-RSV or Split RSV alone (Fig. 5.4A). Higher levels of IgG1 isotype dominant antibodies were induced in the FI-RSV alone and split RSV+alum groups than those in split alone or CpG, MPL alone or combination CpG+MPL adjuvanted groups which showed relatively high levels of IgG2a isotype antibodies (Fig. 5.4B, C).

Next, we determined IgG antibodies specific for purified post-fusion F, pre-fusion F, and G130-230 fragment antigens since RSV preparations might have host cell-derived proteins. The split+alum, split+MPL, and split+CpG+MPL groups induced higher levels of IgG antibodies specific for post-fusion and pre-fusion F protein antigens (Fig. 5.4D, E). Overall, IgG antibodies
specific for post-fusion F were induced at higher levels than those for pre-fusion F. The split+Alum group induced IgG binding to $G_{130-230}$ at higher levels than other groups (Fig. 5.4E). F specific antibodies were induced at higher levels in vaccinated groups than $G_{130-230}$ antibodies.

5.2.4 *Alum and combination CpG+MPL adjuvants are effective in enhancing RSV neutralizing activity and controlling lung viral loads after RSV challenge*

Palivizumab of RSV F-specific neutralizing monoclonal antibody has been licensed as a prophylactic drug. Thus, induction of neutralizing antibodies after RSV vaccination can be a key protective immune correlate against RSV. The FI-RSV and split RSV groups showed 4- to 8-fold increases in RSV neutralizing titers in sera compared to unimmunized control at 3 weeks after single dose vaccination of infant age mice (Fig. 5.5A). The split+CpG and split+MPL groups did not display increases in neutralizing titers compared to split vaccine alone. Alum and combination CpG+MPL adjuvants in split RSV vaccination of infant age mice enhanced RSV neutralizing titers by 2- to 3-fold compared to the split alone group (Fig. 5.5A).

To determine protective efficacy at 5 weeks after single dose split RSV vaccination of infant age mice, control naïve and vaccinated mice were intranasally challenged with RSV A2 (3.5x$10^4$ PFU) (Fig. 5.5B). The groups of mice primed with whole FI-RSV or split RSV at 2 weeks old exhibited approximately 10-20 folds lower lung RSV titers than those in unvaccinated mice as determined day 5 post challenge after sacrifice of mice (Fig. 5.5B) Moderate reduction in lung RSV titers by about 7-8 folds was observed in the split+CpG and split+MPL groups compared to the split vaccine alone group. The split plus combination CpG+MPL group was the most effective in reducing lung viral titers by over 50-fold, followed by split+alum vaccination compared to the split alone group (Fig. 5.5B), which is consistent with RSV neutralization titers.
5.2.5 *CpG+MPL in split RSV vaccination of infant age mice prevents lung histopathology after RSV Challenge*

When lung histology was examined day 5 post challenge at 5 weeks after priming, the whole FI-RSV alone primed mice at infant age displayed more severe alveolitis in the airways than unimmunized mice or split RSV alone primed mice (Fig. 5.6A, B), substantial inflammation in the around blood vessels, perivascular, and interstitial spaces (Fig. 5.6A, C, D). The split RSV alone primed mice at infant age exhibited less severe histopathology than whole FI-RSV priming or naïve mouse with RSV infection (Fig. 5.6). The alum adjuvant in split RSV priming of infant mice resulted in enhancing histopathology whereas the split+CpG and split+MPL groups did not show significant histological changes compared to the split RSV alone group, displaying local infiltration of cells in the alveolar space and interstitial space (Fig. 5.6). Priming of infant mice with split RSV plus CpG+MPL resulted in most effectively preventing histopathological inflammation in the airways, blood vessels, perivascular, and interstitial spaces compared to other vaccine and control groups (Fig. 5.6).

We further analyzed the presence of PAS positive mucus production and H&CR positive eosinophilic infiltration in the histology (Fig. 5.6E, F). Split RSV alone priming of infant age mice resulted in significantly lower levels of mucus production and eosinophilic infiltration than whole FI-RSV priming (Fig. 5.6E, F). The addition of combination CpG+MPL but not alum, CpG, or MPL adjuvant to the split RSV priming of infant mice further attenuated mucus production and eosinophilic induction. Overall, these results suggest that CpG+MPL combination adjuvant in split RSV vaccination of infant age mice contributes to preventing lung histopathology after RSV Challenge.
5.2.6  *CpG and MPL adjuvants in split RSV vaccination of infant age mice primes Th1 type immune responses in spleen cells*

Spleen cells collected day 5 post challenge from RSV vaccine-primed mice were cultured to determine *in vitro* IgG antibody and cytokine production (Fig. 5.7). The FI-RSV alone and split RSV +/- alum groups showed high levels of RSV specific IgG1 antibodies in splenocyte culture supernatants, whereas the split RSV with CpG+MPL group secreted higher levels of IgG2a isotype antibodies *in vitro* (Fig. 5.7B, C). We also determined IL-4 and IFN-γ cytokine levels secreted in culture supernatants splenocyte stimulated with CD8 T cell epitopes (F85-93: KYKNAVTEL, F92-106: ELQLLMQSTPATNRR) (Fig. 5.7D, E). Spleen cells from the FI-RSV alone and Split RSV +/- alum groups produced high levels of IL-4 and low levels of IFN-γ cytokines. In contrast, spleen cells from the split+CpG, split+MPL, or split with CpG+MPL group secreted lower levels of IL-4 and higher levels of IFN-γ cytokines in culture supernatants compared to the split RSV alone group.

5.2.7  *Split RSV with CpG and MPL promotes Th1 T cells and cytokines in lung and BALF after challenge*

Cells in lung tissues and BALF collected from RSV-vaccinated mice at day 5 after challenge were *in vitro* cultured in the presence of F51-66 CD4 T cell epitope or F85-93 epitope for CD8+ T cells and analyzed by intracellular cytokine staining (Fig. 5.8). The FI-RSV alone and split RSV +/- alum groups showed relatively high numbers of BALF and lung IL-4+ CD4 T cells, TNF-α CD8 T cells and moderate levels of IFN-γ+ CD4 and CD8 T cells. Split+CpG and Split+MPL immune mice displayed low BALF IL-4+ CD4 T cells but moderate levels of both IL-4+ and IFN-γ+ CD4 T cells in lung tissue samples. In contrast, split RSV plus combination CpG+MPL immune mice
exhibited low numbers of IL-4+ CD4 T cells and high numbers of IFN-γ+ CD4 T cells in both lung tissue and BALF samples. These results suggest that CpG+MPL adjuvanted split RSV vaccination effectively prevents the induction of IL-4+ CD4 T cells and TNF-α CD8 T cells, while promoting the induction of IFN-γ+ CD4 and CD8 T cells after RSV challenge. To better understand histopathology, the pattern of Th1- and Th2-type cytokines was measured in lung homogenates by ELISA at 5 days after RSV challenge in the immunized mice (Fig. 5.9). The split RSV group showed lower levels of IL-4, IL-5, and IL-13 cytokines in lung homogenates and airway BALF than those in the FI-RSV alone or split+alum group after challenge although not all cytokines were statistically significant as indicated (Fig. 5.9A, B, C). Combination CpG+MPL in split RSV vaccination was found to be more effective in suppressing lung and BALF Th2 cytokines (IL-4, IL-5, IL-13) and promoting Th1 IFN-γ cytokine than CpG or MPL alone after RSV challenge.

5.2.8 Split RSV-primed mice with CpG + MPL prevents infiltrating inflammatory immune cells into the lung after RSV challenge

Cellular phenotypes of infiltrates into the respiratory tracts were determined 5 days after RSV challenge by flow cytometry (Fig 5.10). Lower levels of monocytes (CD11b+Ly6c<sup>high</sup>F4/80+) in BALF were observed in the groups of Split, Split+ CpG, and split with CpG+MPL than Split+alum, Split+MPL, and FI-RSV groups (Fig 10A), although their differences were not significant in lung samples (Fig 5.10F). Neutrophils (CD11b<sup>+</sup>Ly6c<sup>+</sup>F4/80<sup>−</sup>) in BALF and lung samples were detected at lower levels in the Split alone, split+CpG, and split with CpG+MPL immune mice compared to those in the split+Alum, Split+MPL, and FI-RSV group day 5 post RSV challenge (Fig. 5.10B, G). Also, eosinophils (CD11b+CD11c+SiglecF+) in BALF were
found at the lowest levels in the split with CpG+MPL immune mice, followed by the split+CpG and split+MPL groups (Fig 5.10C). The split+CpG and split+MPL did not reduce the levels of lung eosinophils compared to that in the split alone group (Fig 5.10H). Interestingly, subsets of dendritic cells (pDC; CD11c+B220+MHCII\textsuperscript{high} and CD103+DC; CD11c+MHCII+F4/80-CD11b-CD103+DCs) were found to be at the highest levels in BALF and lung samples compared with other groups (Fig. 5.10 D, E, I, J). Monocytes derived DCs play a role in regulating T cell immunity by producing Th1 or Th2 cytokines upon antigen uptake and presentation (59).

**Figure 5. 1. Antigenic properties of whole FI-RSV and split RSV vaccines.**

The antigenic properties of whole FI-RSV and split RSV vaccines were analyzed by ELISA using post-fusion conformation epitope specific monoclonal antibody (mono-F, 131-2a), site II epitope specific antibody palivizumab, and pre-fusion conformation epitope specific monoclonal antibody 5C4.
Figure 5.2. Split RSV vaccination induces higher levels of IgG2a isotype antibodies and better protection against RSV than FI-RSV vaccine in adult mice.

BALB/c mice (N=4-5 and 6-8 weeks old) were immunized with FI-RSV and Split RSV. Sera were collected 3 weeks after prime vaccination. (A-C) IgG and IgG isotype antibodies response after prime immunization. The IgG isotypes levels were detected using RSV as an ELISA antigen for antibody detection. Naive: unimmunized mice-No RSV challenge, FI-RSV: FI-RSV immunized mice, Split: Split RSV immunized mice. (D) Body weight changes in prime immunized mice after RSV challenge. (E) RSV titers at day 5 after RSV challenge. Individual lungs were collected day 5 post challenge with RSV and RSV titers were determined. Naïve R.: unimmunized mice with RSV infection, FI-RSV: FI-RSV immunized mice with RSV challenge, Split: Split RSV immunized mice with RSV challenge. Naïve and immunized mice were i.n. challenged with 3.5x10⁵ PFU RSV A2. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** p<0.001, ** p<0.01, * p<0.05 comparing FI-RSV to split + Adjuvants, Split RSV to Split RSV with adjuvants in mice.
Figure 5. 3. Lung histopathology in single immunized adult mice after RSV challenge.

Individual lung tissues were collected from immunized mice day 5 post RSV challenge (3.5x10^5 PFU/mouse) and analyzed by histology. (A) H&E staining of lung tissues. Scale bars for H&E indicate 100 μm. (B) H&CR staining to determine pulmonary eosinophil in each mouse lung airway. Scale bars for H&CR indicate 20μm. (C) PAS stained tissue sections from PAS positive area in bronchioalveolar (10 individual airways in each mouse). (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, blood vessels and interstitial spaces. (E) Pulmonary eosinophils per 40× field counts in two different regions of each mice. (F) Mucus production by PAS stained area was quantitated and represented as percentage. 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.
Figure 5. 4. RSV antigen specific IgG and isotype antibody levels in infant age mice after prime immunization.

Infant (2 weeks old) age mice (N=5) were single immunized with FI-RSV or Split RSV in the presence or absence of adjuvant (Alum, CpG, MPL or CpG+MPL adjuvants). RSV antigen specific IgG and isotype antibody levels were determined by ELISA at 3 weeks after prime immunization. (A) IgG antibodies specific for FI-RSV, (B) IgG1 isotype antibodies specific for FI-RSV, (C) IgG2a isotype antibodies specific for FI-RSV, (D) Post-fusion F protein specific IgG antibodies, (E) Pre-fusion F protein specific IgG antibodies, (F) RSV A2 G protein fragment (aa131-230) specific IgG antibodies. Naive: unimmunized mice, FI-RSV: formalin inactivated RSV (2 µg), Split: Formalin inactivated split RSV (5 µg), Split+Alum: Split RSV (5 µg) with alum (50 µg), Split+CpG: split RSV (5 µg) with CpG (4 µg), Split+MPL: Split RSV (5 µg) with MPL (1 µg), Split+C.+M.: Split RSV (5 µg) with CpG (4 µg) and MPL (1 µg) combination adjuvants. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; * p<0.05, ** p<0.01, *** p<0.001 comparing FI-RSV to split + Adjuvants, Split RSV to Split RSV with adjuvants in mice.

Figure 5. 5. RSV neutralizing activity titers in primed-mice at an infant age and lung viral loads after RSV challenge.

Infant age (2 weeks old) mice (N=5) were prime immunized and sera were collected at 3 weeks after prime vaccination. (A) Serum neutralizing antibody titers are expressed as the reciprocal of the highest serum dilutions giving 50% reduction of plaque numbers relative to the medium controls. (B) Lung RSV titers after challenge. Naïve R.: unimmunized mice with RSV infection, FI-RSV: FI-RSV immunized mice with RSV challenge, Split: Split RSV immunized mice with RSV challenge. Naive and immunized mice were i.n. challenged with 3.5x10⁵ PFU RSV A2. Split+Alum: split RSV (5 µg) with alum (50 µg), Split+CpG: split RSV (5 µg) with CpG (4 µg), Split+MPL: split RSV (5 µg) with MPL (1 µg), Split+C.+M.: split RSV (5 µg) with CpG (4 µg) and MPL (1 µg) combination adjuvants. Individual lungs were collected at 5 days after RSV challenge and RSV titers were determined by an immunoplaque assay. The dot line is the limit of detection (LOD). Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** p<0.0001, ** p<0.01, * p<0.05.
Figure 5. Histopathology of Hematoxylin Eosin (H&E), Periodic acid-Schiff (PAS) staining of lung tissues in split RSV primed mice at an infant age after RSV challenge.

Individual lung tissues were collected from prime vaccinated BALB/c mice (N=5) at 2-week old day 5 post challenge. In addition, the mice that were immunized with Fl-RSV were used for the positive control of pulmonary inflammation. (A) Photographs of H&E. Hematoxyline and Eosin (H&E) stained lung tissues were dissected to assess histopathology of peribronchiolar and alveolar pneumonia. Scale bars indicate 100 μm. (B-D) The lungs were scored using a 0-3 scoring system (0= absent, 1= minimal pathology and 3 = maximum/severe pathology) for the alveolitis (B), perivascular region (C), interstitial region (D). (E) Photographs and percentage for PAS positive pulmonary mucus production. Scale bars indicate 100 μm. (F) Photographs and score for eosinophil infiltration in alveolitis with hematoxyline and congo red (H&CR) stained lung tissue at 5 days after RSV challenge. Scale bars indicate 400μm. Arrows: eosinophil granulocytes, individual airways were shown per group. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; *** P<0.001, ** p<0.01, * p<0.05. Groups are the same as described in the Fig. 5.

Figure 5. CpG+MPL adjuvant split RSV primed mice at an infant age induce IgG2a-secreting cells and IFN-γ secreting splenocytes after RSV challenge.

(A-C) RSV specific IgG and isotype antibody secreting cell responses were determined after in vitro cultures of spleen cells from split RSV primed mice at an infant age day 5 post RSV challenge. (A) IgG, (B) IgG1 and (C) IgG2a levels were detected by ELISA using Fl-RSV as a coating antigen. (D, E) Th1 or Th2 cytokine responses were determined after in vitro cultures of spleen cells collected day 5 post RSV challenge. Spleen cells (1x10^6/ml) were stimulated with peptide F51−66 epitope for CD4 T cells or F85−93 epitope for CD8 T cells for 36hr. Culture supernatants of splenocytes were used to determine cytokines of IL-4 (D) and IFN-γ (E) by ELISA analysis. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA and Tukey’s multiple-comparison tests in GraphPad Prism; *** p<0.0001, **p<0.001, *p<0.05. Groups are the same as described in the Fig. 5.
Figure 5.8. Intracellular cytokine staining of CD4 and CD8 T cells of BALF and lung samples after RSV challenge.

BALF (A, B, C, D) and lung (E, F, G, H) cells were collected from primed mice at 2 weeks old day 5 post challenge and subjected to intracellular cytokine staining. Sorted cells were analyzed by flow cytometry after in vitro stimulation with F51–66 epitopes for CD4⁺ T cell (A, B, E, F) and F85–93 epitopes for CD8⁺ T cell (C, D, G, H). The results are presented as means ± SEM, and statistical significance was determined using one-way ANOVA with Tukey’s multiple comparison test performed in GraphPad Prism. ***, p < 0.0001, **p<0.005. Groups are the same as described in the Fig. 5.
Figure 5. Th1- and Th2- cytokine levels in lung homogenates and BALF from split RSV primed mice at an infant age after RSV challenge.

Day 5 post RSV challenge (3.5x10⁵ PFU), individual lung tissue (A, B, C, D, E, F) and BALF (G, H, I, J, K, L) samples were collected from split RSV primed mice at an infant age (N=5). IL-4, IL-5, IL-6, IL-13, IFN-γ and TNF-α cytokine levels in lung homogenates and BALF samples were determined by ELISA. Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA and Tukey’s multiple-comparison test in GraphPad Prism; *** p<0.0001, ** p<0.001, * p<0.05. Groups are the same as described in the Fig. 5.
Figure 5. Cellular phenotypes of infiltrates into BALF and lung tissues of split RSV primed mice at an infant age after RSV infection.

Phenotypes of cellular infiltrates in BALF (A, B, C, D, E) and lung (F, G, H, I, J) samples were analyzed by Flowcytometry. Monocytes: D11b+Ly6chighF4/80+ (A, F), Neutrophils: CD11b+Ly6c+F4/80- (B, G), Eosinophil: CD11b+SiglecF+ (C, H), pDC: CD11c+B220+MHCIIhigh (D, I), cDC: CD11c+MHCII+F4/80-CD11b-CD103+DCs (E, J) data were shown 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 as indicated among the groups. Groups are the same as described in the Fig. 5.

5.3 Discussion

Since the tragic failure of alum-adjuvanted whole FI-RSV vaccine in young children in 1960s, safety concerns have been raised regarding the non-replicating RSV subunit vaccines that are likely to cause enhanced respiratory disease particularly in RSV naïve infants after exposure to live RSV. Therefore, RSV subunit vaccines mostly based on purified F proteins have been targeted to the elderly or pregnant women for maternal immunization. In contrast to RSV, inactivated detergent-split influenza virus subunit vaccines are licensed for vaccination in different age populations including 6 months old age infants, children, adults, and the elderly, and most prevalently used for seasonal vaccination against influenza. We found that inactivated detergent-split RSV vaccines expose neutralizing epitopes reactive to palivizumab at higher levels than inactivated whole FI-RSV. Split RSV vaccination of adult mice induced more desirable immune responses towards Th1 type IgG antibodies and less lung histopathology compared to FI-RSV after RSV challenge. In an infant mouse model, inclusion of combination CpG+MPL adjuvants in the split RSV prime vaccination further improved the efficacy of split RSV vaccines and, more importantly, prevented pulmonary histopathology after RSV challenge. This study demonstrates a
different view on inactivated split RSV vaccination in contrast to whole FI-RSV and adjuvant effects on preventing inflammatory disease after RSV challenge in an infant mouse model.

In clinical trials of influenza virus vaccines in adults (35), inactivated whole-virus vaccines were reported to induce an increase in reactogenicity than split subvirion vaccines, which was prominently observed in naïve populations. Fevers were more frequently observed after vaccination with whole-virus than split-product vaccine especially in naïve children one to four years old, but fevers related with vaccination were less likely in primed children (34). A single dose of whole-virus vaccine was shown to be more immunogenic than split-virus vaccine in the unprimed adults. In naïve children (<10 years old), split-product vaccine induced less hemagglutination-inhibiting antibodies than whole-virus vaccine (34). Therefore, split influenza virion vaccines are less reactogenic and induce lower rates of sero-conversion compared with inactivated whole-virus vaccines in naïve hosts (77) but similarly immunogenic in primed individuals (35). Clinical effectiveness of split-virion vaccines was significantly higher than purified-protein subunit trivalent influenza vaccines in older adults (78). Long history of safety and efficacy data on split-virion influenza vaccines provides attractive rationales for developing split-RSV vaccine candidates. Split RSV was found to be more reactive to 131-2a and palivizumab mAbs, suggesting a possibility that splitting RSV might have induced conformation changes exposing post-fusion and site II neutralizing epitopes to be more reactive for antibodies. Unexpectedly, higher levels of Th1 type IgG2a were induced after prime dose of split RSV, and less weight loss and lower lung viral loads in split RSV-primed adult mice after RSV challenge compared to those in whole FI-RSV. In particular, it is highly desirable to observe that histopathology in the airways, blood vessels, and interstitial spaces as well as the levels of mucus production and H&CR+ eosinophilic spots were significantly attenuated in split RSV primed adult
mice after RSV challenge compared to those in whole FI-RSV and unimmunized naïve control with RSV infection. In a relevant previous study, nanoemulsion-based inactivated RSV intranasal vaccination of mice induced high levels of Th1/Th17 responses and protection without causing Th2 immune mediated pathology after RSV challenge (36).

Vaccine-enhanced respiratory disease was observed in naïve young children after vaccination and natural RSV infection (6). Vaccine-associated inflammatory histopathology was not attenuated by RSV vaccines in squalene oil-in-water emulsion (50) or with natural killer T cell agonist α-GalCer (62). As focused in this study, it is significant to determine the adjuvant effects on improving the efficacy of split RSV vaccines and on preventing inflammatory lung histopathology after RSV challenge in an infant mouse model. Consistent with a pattern of IgG isotype antibodies after prime vaccination of adult mice, split RSV induced a balanced IgG1 and IgG2a antibodies in contrast to whole FI-RSV inducing IgG1 dominant antibodies after prime dose in infant age mice. FI-RSV but not split RSV vaccination of infant mice in the absence of adjuvant resulted in causing substantial histopathology after challenge, which is consistent with the histology observed in adult mice. Alum adjuvant in split RSV vaccination of infant age mice enhanced the neutralizing activity titers and lung viral clearance as well as histopathology. These results are in line with the studies of alum adjuvant effects on RSV vaccination. Inclusion of alum adjuvant in the FI-RSV or F protein subunit vaccination contributed to vaccine-enhanced respiratory disease in mice after RSV challenge (59, 79).

Previous studies on adjuvant effects on RSV vaccination were reported with different outcomes. CpG adjuvant in RSV F protein vaccination was reported to exhibit moderate effects on enhancing IgG2a isotype antibodies, but detail lung histopathology was not investigated (37). Polyphosphazene microparticle formulations containing RSV F proteins, an innate defense
peptide, and CpG adjuvant were able to induce Th1 type immune responses and lung viral clearance after RSV challenge although histopathology was not reported (22, 70). Combination of CpG and delta inulin adjuvant in whole RSV vaccination induced IgG2a antibodies in mice but exacerbated lung pathology after RSV challenge (63). Combination alum+MPL in FI-RSV vaccination was shown to moderately attenuate peribronchiolitis in cotton rats post-challenge (71). Post-fusion or pre-fusion F protein in TLR4 analog in stable emulsion vaccine formulation was reported to prime inflammatory vaccine-enhanced alveolitis in cotton rats although lung viral titers were below the detection limit after RSV challenge (20). In this study, combination CpG+MPL in split RSV vaccination was relatively effective in increasing IgG and IgG2a as well as in clearing lung viral loads after challenge compared with CpG (4 μg), MPL (1 μg) alone adjuvant groups. The Split+CpG and Split+MPL groups did not significantly improve lung histopathology compared with split RSV alone vaccination after challenge. The combination CpG+MPL group was more effective in inducing IFN-γ+ CD4 and CD8 T cells in BALF and lung tissues after challenge than the CpG or MPL adjuvant groups. Whereas, IL-4 producing CD4 T cells were most effectively suppressed in both BALF and lung tissues, compared with the split alone, or CpG or MPL adjuvanted split groups. Also, IL-4, IL-5 and IL-13 cytokines were detected in BALF from the CpG+MPL group at the lowest levels of background similar to uninfected naïve mice whereas substantial levels of these Th2 cytokines were observed with the alum, CpG or MPL adjuvanted split groups.

The split plus CpG+MPL group was less likely to recruit inflammatory innate cells of monocytes, neutrophils, and eosinophils in BALF compared to MPL or eosinophils in BALF and lungs compared to CpG adjuvant after RSV challenge. Instead, CD103+ dendritic cells (DC) were recruited into BALF and lungs at higher levels after RSV challenge compared to MPL or CpG
adjuvant group. The TLR9 CpG signaling is mainly dependent on the MyD88 adaptor pathway (80). Whereas, TLR4 ligand signaling is known to involve multiple pathways including TRIF (Toll-interleukin 1 receptor domain–containing adapter inducing interferon-β) and TRAM (TRIF-related adaptor molecule) in addition to MyD88 (81, 82). Combination MPL + CpG was shown to exhibit synergistic and additive effects on producing IL-12p70 and TNF-α respectively during bone marrow-derived DC stimulation in vitro, and upregulating CD40 and CD86 markers on DCs (41). A previous study also showed that MPL+CpG in vivo treatment recruited lower levels of eosinophils and natural killer cells, and natural killer T cells compared to those by CpG or MPL (Ko, 2018 AVR). Further studies are needed to better understand the mechanisms of combination CpG+MPL adjuvant effects on attenuating lung pathology after RSV vaccination and challenge. In addition, split RSV prime immunization of infant age mice was relatively more effective in inducing IFN-γ+ CD4 and CD8 T cells, suppressing IL-4 cytokines and innate inflammatory cells in BALF than whole FI-RSV prime after RSV challenge. More studies will be carried out to better characterize the antigenic and immunogenic properties between whole FI-RSV and split RSV vaccines. This study suggests a possibility that split RSV vaccines with an appropriate selection of adjuvants would be developed into an effective and safe vaccine candidate.
6 CONCLUSION

RSV infection is responsible for annual outbreaks of lower respiratory tract disease in infants and elderly, resulting in global incidence of 33 million cases in children younger than 5 years old, an estimated 3.4 million hospitalizations, and up to 199,000 deaths (2). In the US, RSV infection is associated with 177,000 hospitalizations and 14,000 deaths in the elderly over 65 years old (3). Inactivated-whole virus FI-RSV vaccines in alum-adjuvanted formulations in the 1960s clinical trials failed due to enhancing respiratory disease after RSV season infection (6).Licensed RSV vaccines are not available despite numerous efforts to develop effective and safe RSV vaccine candidates. One of the challenges in developing RSV vaccines is to induce protective immune responses of high RSV neutralizing antibodies but to avoid respiratory disease of inflammatory pulmonary histopathology after RSV infection. My PhD studies have focused on better understanding host immune responses after RSV vaccination and virus challenge, the pattern of immune responses leading to protection or RSV-disease enhancing inflammation, and the various adjuvant effects on modulating vaccine-induced immune responses to avoid pulmonary histopathology after RSV challenge infection. Adult and infant age mouse models were used in these studies with different platforms of vaccines including RSV F protein, F VLP particulate forms produced in insect cells, or whole FI-RSV and split RSV.

6.1 Protective or disease-enhancing immune responses after prime vaccination with soluble F protein or particulate F VLP and RSV challenge infection

In the first project, I investigated the pattern of immune responses to F VLP and soluble form fusion (F) protein, and their results on inflammatory disease and efficacy of protection after
RSV challenge. F VLP vaccination of mice induced Th1 type IgG2a dominant antibody response but F protein vaccination induced IgG1 isotype dominant antibody responses, representing Th1 and Th2 type immune patterns respectively. Although prime or prime-boost immunization with F VLP and F protein vaccines showed remarkable neutralizing activity and controlling lung viral replication after RSV challenge, RSV F protein vaccine immunized mice displayed severe lung histopathology as well as mucus production and infiltration of eosinophils compared to F VLP vaccine mice which could prevent pulmonary histopathology and lower than that in naïve mice after RSV challenge.

The immunogenicity of F VLP vaccines has been focused on antigen-specific immune responses after prime immunization in comparison with prime-boost immunization. F VLP was found to be effective in inducing Th1 type IgG2a antibody responses and cytokine (IFN-γ). Whereas F protein vaccination with alum adjuvant induced Th2 cytokines (IL-4, IL-5, and IL-13) and Th2 type IgG1 isotype antibody responses in both prime and prime-boost immunized mice.

As a result, there were striking differences in pulmonary histopathology, mucus production, and eosinophilia as well as body weight loss of RSV disease between the F VLP and F protein vaccine groups of mice. These results suggest that soluble F proteins exacerbate pulmonary histopathology after vaccination upon RSV challenge but not when presented on VLP particles. An intrinsic property of F VLP to stimulate acute innate immune responses at the site of injection appears to be a mechanism responsible for the striking opposite outcomes of inducing Th1 type priming immune responses after RSV challenge, preventing RSV vaccine-enhanced disease between F protein and F VLP vaccine platforms.
Table 6.1 Protection or RSV disease enhancing inflammatory responses by prime or prime-boost immunization of RSV vaccines and then RSV challenge infection.

<table>
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<th>Vaccines</th>
<th>IgG1 or IgG2a</th>
<th>Lung viral load</th>
<th>Th1 or Th2 cytokines</th>
<th>Vaccine enhanced disease (ERD)</th>
<th>Eosinophils</th>
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Low: +/-
Moderate: +/++
High: ++++
Severe: ++++

6.2 Effects of adjuvants in RSV F protein vaccination on modulating immune responses

To development of safe and efficacy vaccines, it is still required to improve the protection conferred by RSV vaccines without causing VED. Basically, adjuvants are innate and acute immune stimulators and cell-mediated immune response to vaccine antigens on site of injection (83). In the second project, I investigated the effects of MPL and CpG adjuvants on protective immunity and on modulating host immune responses to F protein vaccination in infant and adult mouse models in comparison with alum adjuvant. Combination CpG+MPL adjuvant in RSV F protein single dose priming of infant and adult age mice was found to promote the induction of IgG2a isotype antibodies and RSV neutralizing activity, and lung viral clearance after RSV challenge infection. The adjuvant effects of CpG or MPL appear to be less effective in
promoting protective immunity and in preventing inflammatory histopathology after F protein vaccination and RSV challenge, compared to combination adjuvant. In contrast, CpG+MPL adjuvant together included in F protein priming vaccination was effective in avoiding inflammatory lung histopathology, interleukin-4+ CD4 T cell responses, and cellular infiltration of monocytes and neutrophils after RSV challenge in both infant and adult age mice. There were differential adjuvant effects of MPL and CpG on modulating histopathology and inflammatory immune responses, depending on the ages of mice at the time of F protein priming vaccination. This study indicates that combination CpG and MPL adjuvant in RSV subunit vaccination might contribute to priming protective immune responses and preventing inflammatory RSV disease after infection.
Table 6. 2 Adjuvants effects on RSV F protein vaccination efficacy and inflammatory disease in adult and infant age mice.

<table>
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<th>IgG1 or IgG2a</th>
<th>Neutralizing response</th>
<th>Lung viral Load</th>
<th>CD4 T cell response</th>
<th>Mucus</th>
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<td>IgG2a</td>
<td>High</td>
<td>LLOD to Log$_{10}^{2}$</td>
<td>IFN-γ +++</td>
<td>-</td>
</tr>
<tr>
<td>F protein</td>
<td>IgG1</td>
<td>Low</td>
<td>Log$_{10}^{3}$</td>
<td>-/+</td>
<td>Moderate</td>
</tr>
<tr>
<td>F protein +Alum</td>
<td>IgG1, IgG2a</td>
<td>High</td>
<td>Log$_{10}^{2}$</td>
<td>IL-4 +++</td>
<td>Severe</td>
</tr>
<tr>
<td>F protein +CpG</td>
<td>IgG1 ≤ IgG2a</td>
<td>Moderate/High</td>
<td>Log$_{10}^{3}$</td>
<td>IL-4+/ IFN-γ ++</td>
<td>Low/Moderate</td>
</tr>
<tr>
<td>F protein +MPL</td>
<td>IgG1 ≤ IgG2a</td>
<td>Moderate/High</td>
<td>Log$_{10}^{3}$</td>
<td>IL-4+/ IFN-γ ++</td>
<td>Low/Moderate</td>
</tr>
<tr>
<td>F protein +CpG+MPL</td>
<td>IgG2a</td>
<td>High</td>
<td>LLOD to Log$_{10}^{2}$</td>
<td>IFN-γ +++</td>
<td>-</td>
</tr>
</tbody>
</table>

**Low**: -/+  
**Moderate**: ++/++  
**High**: +++  
**Severe**: ++++  
**LLOD**: Lower limit of detection

6.3 Comparison of whole FI-RSV and inactivated-split RSV vaccines, and adjuvant effects on split RSV vaccination in infant age mice.

Licensed inactivated split influenza vaccine has been known to be safe and efficacious for human vaccination even in children over 6 months old. Similarly, RSV split vaccine might be a platform of a possible RSV vaccine. It remains unknown whether inactivated split RSV vaccination would confer protection with or without causing vaccine-enhanced disease. Studies using inactivated split RSV vaccines might reveal similarities and differences with previous FI-RSV vaccination. As a potential vaccine candidate, split RSV vaccines might provide an approach for gaining insights into protective immune correlates. It is also possible that vaccine
adjuvants would modulate immune responses to split RSV vaccination. It is an innovative approach to study the adjuvant effects on modulating immune responses to RSV split vaccination.

In the third project, I determined whether inactivated split RSV vaccines would provide protection and attenuate pulmonary histopathology after RSV challenge, in comparison with whole FI-RSV vaccines in an infant age mouse model. Also, I determined whether MPL and CpG adjuvants would improve the protective efficacy by preventing histopathology of lung inflammation after split RSV vaccination and challenge in infant (2 weeks old age) mice. I found that inactivated and detergent-split RSV vaccines expose neutralizing epitopes reactive to palivizumab, a licensed drug of monoclonal antibody, at higher levels than inactivated whole virus, FI-RSV. Split RSV vaccination of mice induced more desirable immune responses towards Th1 type IgG2a antibodies and less lung histopathology compared to FI-RSV after RSV challenge. Next, I tested the efficacy and pulmonary histopathology after a single dose of split RSV vaccination in the presence of CpG and MPL adjuvants. Among the adjuvants of alum, MPL, and CpG, split RSV prime vaccination of infant age mice with CpG+MPL adjuvant was found to be most effective in increasing Th1 type IgG2a antibodies, neutralizing activity, and lung viral clearance as well as modulating balanced immune responses to prevent pulmonary histopathology after RSV vaccination and challenge.
Table 6. 3 Comparison of whole FI-RSV and inactivated-split RSV vaccines, and adjuvant effects on split RSV vaccination in infant age mice.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>IgG1 or IgG2a</th>
<th>Neutralizing antibodies</th>
<th>Lung Virus Load</th>
<th>Histopathology (Vaccine-ERD)</th>
<th>Mucus</th>
<th>CD4 T cell response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve (No vaccine)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Naïve infection</td>
<td>-</td>
<td>-</td>
<td>Log_{10}4</td>
<td>Moderate</td>
<td>Moderate/High</td>
<td>IL-4/IFN-γ +/-</td>
</tr>
<tr>
<td>FI-RSV</td>
<td>IgG1</td>
<td>Moderate</td>
<td>Log_{10}2</td>
<td>Severe</td>
<td>Severe</td>
<td>IL-4 +++</td>
</tr>
<tr>
<td>Split</td>
<td>IgG1–IgG2a</td>
<td>Low/Moderate</td>
<td>Log_{10}3</td>
<td>Moderate</td>
<td>Moderate</td>
<td>IL-4+/ IFN-γ ++</td>
</tr>
<tr>
<td>Split +Alum</td>
<td>IgG1</td>
<td>High</td>
<td>Log_{10}2</td>
<td>Severe</td>
<td>Severe</td>
<td>IL-4++++</td>
</tr>
<tr>
<td>Split +CpG</td>
<td>IgG1≤IgG2a</td>
<td>Moderate</td>
<td>Log_{10}2</td>
<td>Low/Moderate</td>
<td>Moderate</td>
<td>IL-4+/ IFN-γ ++</td>
</tr>
<tr>
<td>Split +MPL</td>
<td>IgG1≤IgG2a</td>
<td>Moderate</td>
<td>Log_{10}2</td>
<td>Low/Moderate</td>
<td>Low</td>
<td>IL-4+/ IFN-γ ++</td>
</tr>
<tr>
<td>Split +CpG+MPL</td>
<td>IgG2a</td>
<td>High</td>
<td>LLOD</td>
<td>-</td>
<td>-</td>
<td>IFN-γ +++</td>
</tr>
</tbody>
</table>

Low: -/+  
Moderate: +/+++  
High: ++++  
Severe: ++++  
LLOD: Lower limit of detection

Cotton rats have been shown to be more susceptible to RSV infection and an appropriate model for the study of RSV pathogenesis and vaccine efficacy (84). In preliminary studies, I also investigated the immunogenicity and safety of pulmonary histopathology after split RSV vaccination and challenge in a cotton rat model. Preliminary data suggest that inactivated-split RSV vaccines would be immunogenic and effective in controlling the lung viral loads in cotton rats without causing apparent pulmonary inflammation and histopathology after RSV challenge infection. Further studies will be needed in future to determine whether inactivated-split RSV vaccines would be developed into a safe and effective RSV vaccine candidate against RSV.

In summary, my PhD studies have discovered the following findings contributing to advancing RSV vaccine field. (1) Soluble F proteins exacerbate pulmonary histopathology after vaccination upon RSV challenge but not when presented on virus-like particles. (2) A
combination of low dose MPL+CpG adjuvant displays significant effects on improving the
efficacy of soluble F subunit vaccines and preventing pulmonary histopathology after RSV
challenge in infant and adult mice. (3) Split RSV was found to be more effective in exposing
neutralizing epitopes and in conferring protection by attenuating lung histopathology compared
to whole FI-RSV. Inclusion of MPL+CpG adjuvant in split RSV vaccination resulted in
improving the protective efficacy as well as in modulating immune responses avoiding
pulmonary histopathology after RSV challenge.
REFERENCES


Respiratory syncytial virus disease in infants despite prior administration of antigenic 
inactivated vaccine. American journal of epidemiology 89:422-34.

7. Connors M, Kulkarni AB, Firestone CY, Holmes KL, Morse HC, 3rd, Sotnikov AV, 
Murphy BR. 1992. Pulmonary histopathology induced by respiratory syncytial virus (RSV) 
challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion 

Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) 
challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion 

9. Prince GA, Jenson AB, Hemming VG, Murphy BR, Walsh EE, Horswood RL, Chanock 
RM. 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats 
by prior intramuscular inoculation of formalin-inactivated virus. Journal of virology 

10. Gershwin LJ, Schelegle ES, Gunther RA, Anderson ML, Woolums AR, Larochelle DR, 
respiratory syncytial virus pathophysiology. Vaccine 16:1225-36.

11. Kakuk TJ, Soike K, Brideau RJ, Zaya RM, Cole SL, Zhang JY, Roberts ED, Wells PA, 
Wathen MW. 1993. A human respiratory syncytial virus (RSV) primate model of enhanced 
pulmonary pathology induced with a formalin-inactivated RSV vaccine but not a 


immunopathology following priming with formalin-inactivated or commercial killed bovine respiratory syncytial virus vaccine. Journal of virology 79:2024-32.


influenza vaccine is primarily controlled by Toll-like receptor signalling. PLoS Pathog 4:e1000138.


