Antiviral Research 168 (2019) 100–108

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

The efficacy of inactivated split respiratory syncytial virus as a vaccine candidate and the effects of novel combination adjuvants

Youri Lee[a], Eun-Ju Ko[a,b], Ki-Hye Kim[a], Young-Tae Lee[a], Hye Suk Hwang[a,c], Yu-Jin Jung[a], Subbiah Jeeva[a], Young-Man Kwon[a], Baik Lin Seong[d], Sang Moo Kang[a,*]

[a] Center for Inflammation, Immunity & Infection, Institute for Biomedical Sciences, Georgia State University, Atlanta, GA, USA
[b] Vaccine Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
[c] Department of Microbiology, Chonnam National University, Hwasun-gun, Jeonnam, Republic of Korea
[d] Institute of Life Science and Biotechnology, Yonsei University, Seodaemun-Gu, Seoul, Republic of Korea

ARTICLE INFO

Keywords:
RSV
Enhanced disease
FI-RSV
Split RSV
MPL
CpG
Adjuvant

ABSTRACT

Clinical trials with alum-adjuvanted formalin-inactivated human respiratory syncytial virus (FI-RSV) vaccine failed in children due to vaccine-enhanced disease upon RSV infection. In this study, we found that inactivated, detergent-split RSV vaccine (Split) displayed higher reactivity against neutralizing antibodies *in vitro* and less histopathology in primed adult mice after challenge, compared to FI-RSV. The immunogenicity and efficacy of FI-RSV and Split RSV vaccine were further determined in 2 weeks old mice after a single dose in the absence or presence of monophosphoryl lipid A (MPL) + CpG combination adjuvant. Split RSV with MPL + CpG adjuvant was effective in increasing T helper type 1 (Th1) immune responses and IgG2a isotype antibodies, neutralizing activity, and lung viral clearance as well as modulating immune responses to prevent pulmonary histopathology after RSV vaccination and challenge. This study demonstrates the efficacy of Split RSV as an effective vaccine candidate.

1. Introduction

Human respiratory syncytial virus (RSV) 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 (Nair et al., 2010, 2013). There is no RSV vaccine licensed. The 1960s clinical trials with alum-adjuvanted formulation of formalin-inactivated whole RSV (FI-RSV) failed in children due to vaccine enhanced respiratory disease upon natural infection (Kim et al., 1969). This pulmonary histopathology by alum-adjuvanted FI-RSV vaccine was recaptured in various animal models including mice (Connors et al., 1992, 1994) and cotton rats (Prince et al., 1986). RSV fusion (F) protein vaccines in alum or emulsion adjuvant formulations were also shown to cause enhanced lung histopathology in animal models after challenge (Murphy et al., 1990; Prince et al., 2003; Schneider-Ohrum et al., 2017). Alum adjuvant biasing T helper type 2 (Th2) immune responses to subunit vaccines contributes to pulmonary inflammation after RSV challenge (Graham, 2011; Kim et al., 2015).

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 (al-Mazrou et al., 1991). Most influenza vaccines manufactured since the 1970s have been 'split' preparations. 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 (Cate et al., 1977; Gross et al., 1977).

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. Oligodeoxynucleotides containing unmethylated cytosine-phosphate-guanosine (CpG), a Toll-like receptor (TLR)-9 agonist, promote the induction of Th1 immune responses to RSV F protein or killed RSV vaccination (Garlapati et al., 2012; Hancock et al., 2001; Oumouna et al., 2005). However, details on pulmonary inflammation and RSV disease after RSV challenge were not investigated after CpG adjuvanted RSV vaccination. Monophosphoryl lipid A (MPL) is an attenuated version of lipopolysaccharide TLR4 agonist (Ireton and Reed, 2013) and licensed for use in human vaccines (O'Hagan et al., 2017; Rappuoli et al., 2011).

In contrast to many studies on whole FI-RSV, the antigenicity and

* Corresponding author. Center for Inflammation, Immunity & Infection, Institute for Biomedical Sciences, Georgia State University, Atlanta, GA, 30303, USA.
E-mail address: skang24@gsu.edu (S.M. Kang).

https://doi.org/10.1016/j.antiviral.2019.05.011
Received 5 March 2019; Received in revised form 18 May 2019; Accepted 27 May 2019
Available online 28 May 2019
0166-3542/

immunogenicity of inactivated split RSV vaccines remain unknown. It would be possible that splitting FI-RSV by detergent treatment would impact on exposing epitopes, immunogenic properties, and vaccine-enhanced inflammation after RSV challenge. In this study, we investigated the antigenic properties of inactivated split RSV vaccine and pulmonary histopathology after vaccination and challenge in comparison with whole FI-RSV in mice. In addition, we determined whether CpG, MPL, and combined CpG and MPL adjuvants would promote RSV vaccine efficacy and modulate immune responses toward preventing inflammatory histopathology after prime immunization with Split RSV vaccines and challenge in an infant age mouse model in comparison with alum adjuvant. Priming of infant age mice with combined CpG + MPL adjuvanted Split RSV vaccine was effective in conferring protection by clearing lung viral loads as well as in avoiding lung histopathology.

2. Material and methods

2.1. Cells, virus and antigens

HEp-2 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS, GIBCO-BRL), 2 mM glutamine, penicillin and streptomycin (GIBCO-BRL) at 37 °C with 5% CO_2. RSV (strain A2) was kindly provided by Dr. Martin Moore (Emory University, GA) and propagated in HEp-2 cells. RSV infected Hep-2 cells were cultured for 3 days, harvested and centrifuged for 10 min at 2000 rpm in a table-top centrifuge at 4 °C. Collected RSV within supernatants was inactivated by incubating with 10% formalin (1:4000 vol/vol) for 3 days at 37 °C (Lee et al., 2017). Then, the formalin inactivated RSV (FI-RSV) was purified by ultracentrifugation for 60 min at 30,000 rpm. Splitting of FI-RSV to prepare Split RSV was carried out by treating with detergent 1% Triton-x 100 (Sigma Aldrich) in Phosphate buffered saline (PBS) for 2 hrs at 20 °C (Kon et al., 2016). The detergent was removed by dialysis cassette (10,000 MWCO, Thermo scientific) floating in PBS. Post-fusion (F) stabilized F (McLellan et al., 2011) and pre-fusion stabilized F (McLellan et al., 2013) proteins and pre-fusion specific 5C4 mAb were generously provided by Dr. Barney S. Graham (Vaccine Research Center, NIAID, NIH, Bethesda, MD, 20892, USA). RSV A2 G protein fragment (aa131-230) was described and purified as previously described (Kim et al., 2012). Palivizumab mAb was kindly provided by Dr. Frances Eun-Hyung Lee (Emory University, Atlanta, GA). D25 mAb was purchased from Creative Biolabs (Shirley, NY, USA).

2.2. Animals, immunization, RSV infection

BALB/c mice were purchased from Charles River Laboratories and bred in the breeding facility at Georgia State University. BALB/c mice at adult age (6–8 weeks old) or at infant age (2 weeks old) were one time immunized intramuscularly (n = 5 per group) with FI-RSV (5 μg or 2 μg) or Split RSV (5 μg) alone or with adjuvant, Aluminum hydroxide (Alum) (50 μg), CpG (1 μg), MPL (4 μg), combined CpG (1 μg) + MPL (4 μg) adjuvant, or PBS (naïve control). To determine serum IgG levels, blood samples were collected at 3 weeks later after prime immunization. Naïve control and immunized mice were intranasally challenged with 3.5×10^5 plaque forming units (PFU) of RSV A2 in 50 μl of PBS under isoflurane anesthesia at 5 weeks after prime vaccination to determine the efficacy of protection. At 5 days after challenge, we collected individual lungs, bronchiolar alveolar lavage fluids (BALF), mediastinal lymph nodes (MLN), spleens, and bone marrow (BM). All animal experiments were conducted with humane care under the application laws and guidelines of Georgia State University Institutional Animal Care and Use Committee (IACUC).

2.3. ELISA assay

RSV specific IgG isotype antibodies (IgG, IgG1, and IgG2a) were measured in serum samples by enzyme-linked immunosorbent assay (ELISA) as previously described (Ko et al., 2018). The sets of 96-well ELISA plates were coated with using inactivated RSV (4 μg/ml), post-fusion F, or pre-fusion F protein antigens (200 ng/ml) at 4 °C overnight. Isotype antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-goat IgG, IgG1 and IgG2a secondary antibodies (Southern Biotechnology). The developing buffer (TMB, 3,3′,5,5′-tetramethylbenzidine, Sigma Aldrich) treated and stopped with 1 M H_3PO_4. Optical densities (O.D) were read at 450 nm. The cytokine antibody concentrations were quantified and measured with levels of interleukin (IL)-4, IL-5, IL-6, interferon-γ, IL-13, and tumor necrosis factor-α (eBioscience, San Diego, CA) in lung extracts and BALF homogenates.

2.4. RSV immuno-plaque assay

RSV titers were measured in individual lung samples after 5 days post challenge and neutralizing antibody titers were determined with prime or boost immune sera. The lung samples were strained through 40-μm cell strainer (BD Biosciences). RSV plaques were determined using Immuno-plaque assay (IPA) as previously described (Lee et al., 2017). To determine RSV neutralizing titers, immune sera were heat-inactivated for 1 h at 56 °C and then serially 2-fold diluted to mix with RSV A2 (presence or absence) before to inoculating onto Hep2 cells in 48-well cell culture plates at 37 °C, 5% CO_2. After 4% formalin fixation, the plaques were detected by immunostaining with anti-F monoclonal antibody (Millipore), followed by incubation with HRP-conjugated, goat anti-mouse antibody, and then by developing with a DAB (3,3′-diaminobenzidine) HRP substrate kit (Invitrogen). Endpoint titers represent the reciprocal of the dilution that decreased the plaque count by at least 50% as determined by the Spearman-Kärber method (Cohen et al., 2007).

2.5. Flow cytometry and intracellular cytokine staining

BALFs were harvested by infusing 1 ml of PBS into the lungs via the trachea at day 5 post challenge. The cells in the lung tissues were prepared by homogenization and then by Percoll gradients (44 and 67%) centrifugation. The frosted microscope glasses were used for releasing cells from the mediastinal lymph nodes (MLN) and spleens. Lung, spleen or MLN cells were stimulated with a CD4 T cell epitope ($F_{51\text{-}66}$: GWYTSVITIELSNIKE, 4 μg/ml) or CD8 T cell epitopes ($F_{85\text{-}93}$:KYKNAVTEL, $F_{92\text{-}106}$: ELQLLMQSTPATNNR, 4 μg/ml) (Olson and Varga, 2008; Varga et al., 2000), at 37 °C for 5 h prior to staining of intracellular cytokines, and then the cells were fixed and permeabilized according to the manufacturer's instructions (BD Biosciences). Intracellular cytokines and surface phenotypic markers for T cells or eosinophils were stained with antibodies for IFN-γ, IL-4 (eBioscience), TNF-α (BioLegend), CD3, CD4, CD8, CD11a, CD11b or Siglec F (BD Biosciences). For analysis, the Becton-Dickinson LSR-II/Fortessa flow cytometer (BD, San Diego, CA) was used to distinct populations from the tissues and acquired samples were further analyzed by Flowjo software (Tree Star Inc.).

2.6. Histopathology

Lung tissues collected from mice at 5 days after RSV challenge were fixed with 10% neutral buffered-formalin. Lung tissue histology was performed by staining with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Congo red (C&R) and analyzed under light microscopy (Hwang et al., 2014; Lee et al., 2017; Meyerholz et al., 2009). The tissue slides were examined for lymphocytes and eosinophils in peribronchiolar, perivascular, interstitial, and alveolar spaces (Hwang

et al., 2014; Meyerholz et al., 2009). At least 10 sections per lung tissue from individual mouse were obtained and blind scoring was performed for histopathology analysis. Inflammation and focal aggregates of infiltrating cells in the airways of the lung were blindly examined, and measured using a severity score system defined as 0 (normal), 1 (mild inflammation, < 20% of lung affected), 2 (moderate inflammation, 20–40% of lung affected), 3 (marked inflammation, 40–60% lung affected) and 4 (severe inflammation, > 60% lung affected with tissue necrosis or damage) (Derscheid et al., 2013; Klopfleisch, 2013). The mucin expression of goblet cell hyperplasia was identified in 50 randomly selected lung airways in the PAS stained slides. Eosinophils were counted per viewing PAS-positive areas within the airway epithelium (400× magnification) and annotated using the magnetic lasso tool of Adobe Photoshop CS5.1 software as described (Lee et al., 2017).

2.7. Statistical analysis

Statistical differences were performed using GraphPad statistical software (GraphPad software Inc., San Diego, CA). Data were analyzed for significance using one-way ANOVA with Tukey's multiple comparison test for multiple comparisons. The difference was considered statistically significant when the P value was less than 0.05.

3. Results

3.1. Split RSV displays high antigenic reactivity in vitro and attenuates histopathology after vaccination and RSV challenge in adult mice

RSV was inactivated using formalin and used as whole virus FI-RSV vaccine. 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” vaccine. Antigenic properties were determined using post-fusion F specific monoclonal antibody (mAb) 131-2a, the antigenic site II specific mAb palivizumab, and pre-fusion antigenic site Ø specific mAbs (5C4 and D25) as well as RSV G specific mAb 131-2G (Supplementary Fig. S1). Split RSV vaccine displayed significantly higher levels of reactivity against 131-2a and palivizumab mAbs compared to whole virus FI-RSV (Supplementary Fig. S1). Split RSV vaccine showed low but higher levels of reactivity to 5C4 and D25 mAb than FI-RSV (Supplementary Fig. S1B, C). These results suggest that Split RSV vaccine might expose post-fusion and pre-fusion epitopes at higher levels, compared to FI-RSV, probably as a result of the detergent treatment.

The immunogenicity and efficacy of FI-RSV and Split RSV without alum adjuvant were compared in adult mice. Adult mice with Split RSV vaccination induced lower levels of IgG1 and higher levels of IgG2a isotype antibodies compared to those with FI-RSV vaccination (Fig. 1A, B, C), and these differences are significant as shown in IgG2a/IgG1 ratios (Fig. 1D). These results suggest that Split RSV can induce a unique pattern of immunogenic properties of inducing IgG2a isotype antibodies and balanced immune response.

To compare the protective efficacy, Split RSV and FI-RSV immunized mice were challenged with RSV at 3 weeks after prime vaccination. The naïve mice after RSV infection showed the highest levels of viral loads in the lung at day 5 after challenge (Fig. 1D and E). Split RSV prime vaccination led to lowering lung viral loads by 100, compared to high lung viral titers in naïve mice with RSV infection (Fig. 1E). FI-RSV prime vaccination also controlled lung viral loads (Fig. 1E).

To determine whether Split RSV vaccine would attenuate histopathology inflammation responses against RSV infection, histology tissues with infiltrated eosinophils and mucus-producing cells were visualized in the airways with H&E, H&CR, and periodic acid-Schiff (PAS) stain (Fig. 2A–F). There were significant infiltrates in the interstitial spaces from the naïve mice and the FI-RSV primed mice after RSV infection, compared to the split RSV vaccinated mice (Fig. 2A and D). Also, FI-RSV prime induced more severe inflammatory infiltrates than split RSV prime after RSV challenge as shown by H&E staining (Fig. 2A and D). The Split RSV vaccine group showed lower levels of the PAS positive mucus production in the lung than FI-RSV as quantified (Fig. 2C, F). Meanwhile, eosinophils and numerous PAS-positive airway epithelial cells were seen at higher levels in the lung from FI-RSV immune mice compared to those in Split RSV immune mice after infection with RSV (Fig. 2B, E). Overall, these results indicate that RSV challenge induced much less histopathology responses in Split RSV immune mice, including the inflammation and mucus production, whereas pulmonary histopathology was typically observed at higher levels in FI-RSV immune mice upon RSV infections. Therefore, we have further investigated the efficacy of Split RSV as a vaccine candidate.

3.2. CpG and MPL adjuvants in split RSV prime vaccination promotes IgG2a isotype dominant antibody responses in 2 weeks old mice

We tested whether CpG and MPL adjuvants in Split RSV prime vaccination in 2 weeks old mice would promote desirable Th1 type (IgG2a) immune responses and protection preventing enhanced RSV disease after challenge. BALB/c mice at 2 weeks old were prime (single dose) immunized with FI-RSV (2 μg), Split RSV (5 μg) alone, or Split RSV (5 μg) 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. 3A–C). Split RSV prime of 2-week old mice induced IgG antibodies specific for RSV at a comparable level to those with FI-RSV alone or single adjuvanted (CpG, MPL) split RSV prime. Alum and CpG + MPL adjuvant groups induced higher IgG antibody responses than Split RSV alone (Fig. 3A). 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. 3B and C). Next, we determined IgG antibodies specific for purified post-fusion F, pre-fusion F, and G fragment antigens (Fig. 3D–F). The Split + alum, Split + MPL, and Split + CpG + MPL groups induced higher levels of IgG antibodies specific for post-fusion F protein antigen than those in other groups (Fig. 3D and E). Overall, IgG antibodies specific for post-fusion F were induced at higher levels than those for pre-fusion F (Fig. 3D and E). G-specific antibodies were detected at lower levels than F-specific antibodies and the Split + Alum group induced relatively higher levels of IgG antibodies binding to RSV G protein (aa131-230) than other groups (Fig. 3F).

3.3. Split RSV vaccination induces RSV neutralizing activity and controls lung viral loads

Palivizumab is a licensed prophylactic RSV F-specific neutralizing mAb. Thus, induction of neutralizing antibodies after RSV vaccination can be a key protective immune correlate. The Split RSV groups showed 6 to 8 of log2 titers in RSV neutralizing titers in sera after single dose vaccination of 2-week old mice (Fig. 4A). Alum and combination CpG + MPL adjuvants in Split RSV vaccination of 2-week old mice enhanced RSV neutralizing titers by 2- to 3-fold compared to the FI-RSV or Split and MPL or CpG alone group (Fig. 4A).

To determine protective efficacy at 5 weeks after single dose Split RSV vaccination of 2-week old mice, control naïve and vaccinated mice were intranasally challenged with RSV A2 (3.5×10^5 PFU) (Fig. 4B). The groups of mice primed with 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. 4B). 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 CpG + MPL or alum adjuvant group was more effective in reducing lung viral titers by

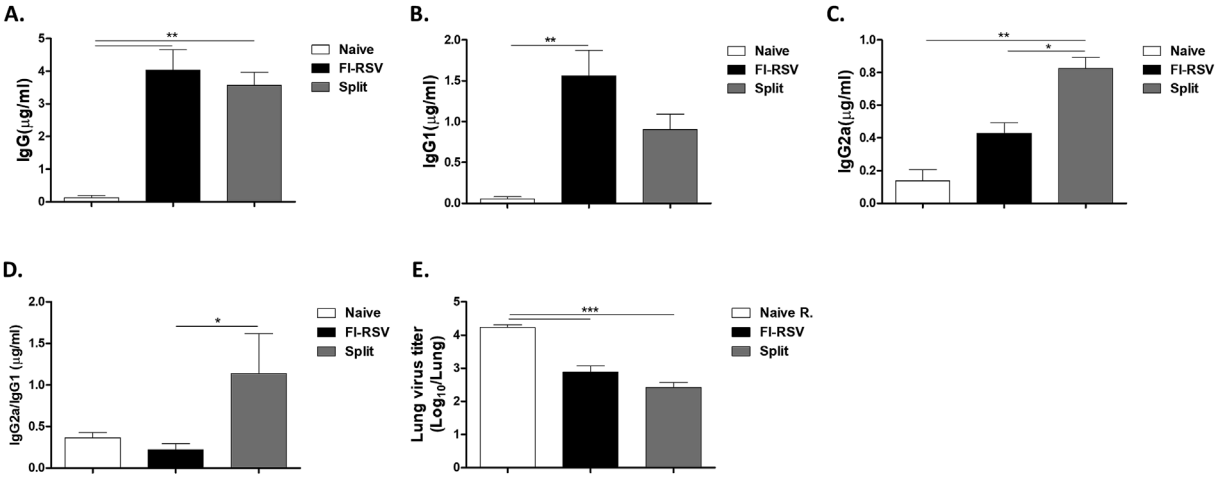

Fig. 1. Split RSV vaccination induces high levels of IgG2a isotype antibodies and effective lung viral clearance in adult mice.
Adult BALB/c (5–6 weeks old) mice (N = 5) were single immunized with FI-RSV (5 μg) and Split RSV (5 μg). (A–C) Sera were collected at 2 weeks after immunization. RSV specific IgG isotype levels were determined by ELISA using FI-RSV as a coating antigen. (D) Ratios (IgG2a/IgG1) of RSV specific IgG antibodies. (E) Lung RSV titers were determined in individual lungs after RSV challenge (RSV 3.5×10^5 PFU/mouse) at 3 weeks after prime immunization. Naive: unimmunized mice, FI-RSV: Formalin inactivated RSV (5 μg), Split: Formalin inactivated split RSV (5 μg). Results are representative out of two independent experiments and presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism; ***; $p < 0.001$, **; $p < 0.01$ and *; $p < 0.05$ comparing Naïve, FI-RSV and Split RSV groups.

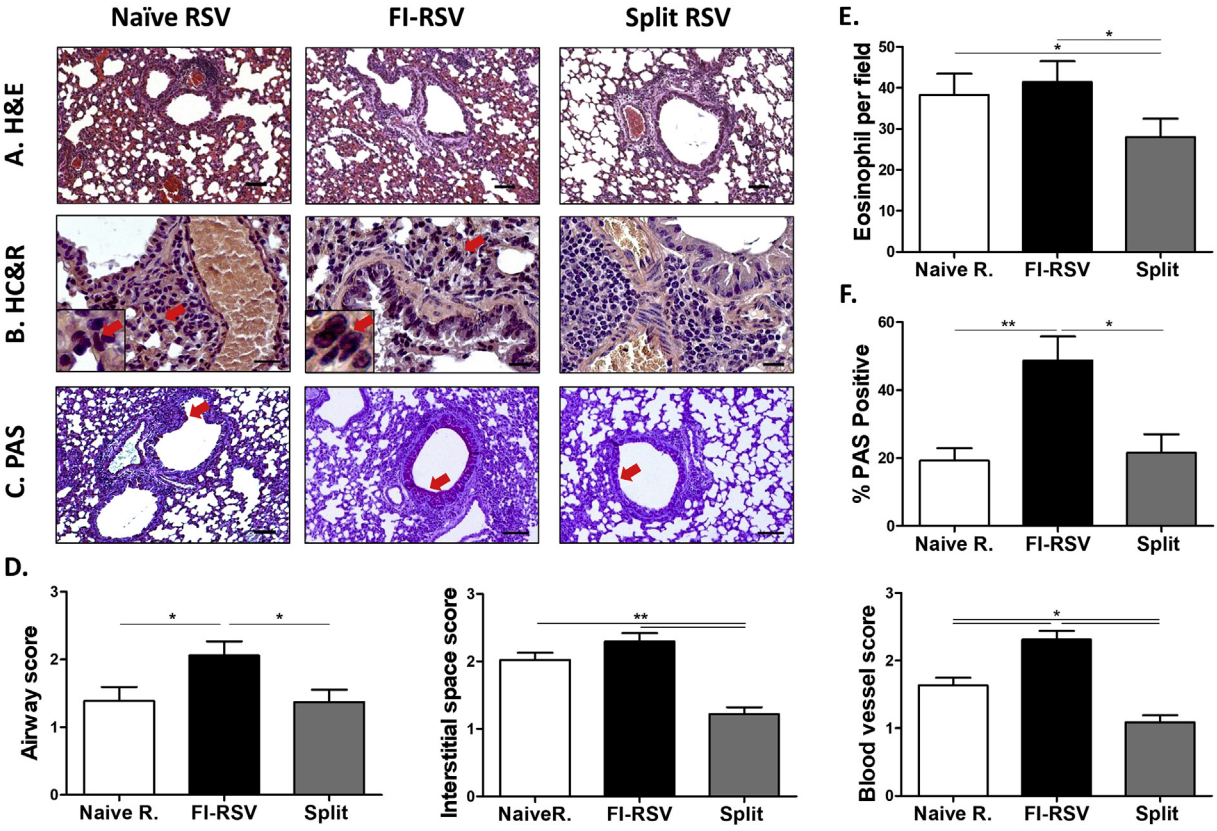

Fig. 2. Split RSV is more effective in attenuating lung histopathology than FI-RSV after RSV challenge in adult mice.
Individual lung tissues were collected from immunized mice (N = 5) day 5 post RSV challenge (3.5×10^5 PFU/mouse) and analyzed by histology. (A) H&E staining of lung tissues. Scale bars 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 20 μm. (C) PAS stained tissue sections from PAS positive area in bronchioalveolar (10 individual airways in each mouse). Scale bars for H&E indicate 400 μm. (D) H&E stained tissue sections from each mouse were scored for inflammation on a scale of 0–4 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 percentages. Groups are the same as in Fig. 1 and histology was on day 5 post RSV challenge. Results are presented as mean ± SEM. Statistical significances were calculated by 1-way ANOVA and Tukey's multiple comparison test. **; $p < 0.01$ and *; $p < 0.05$.

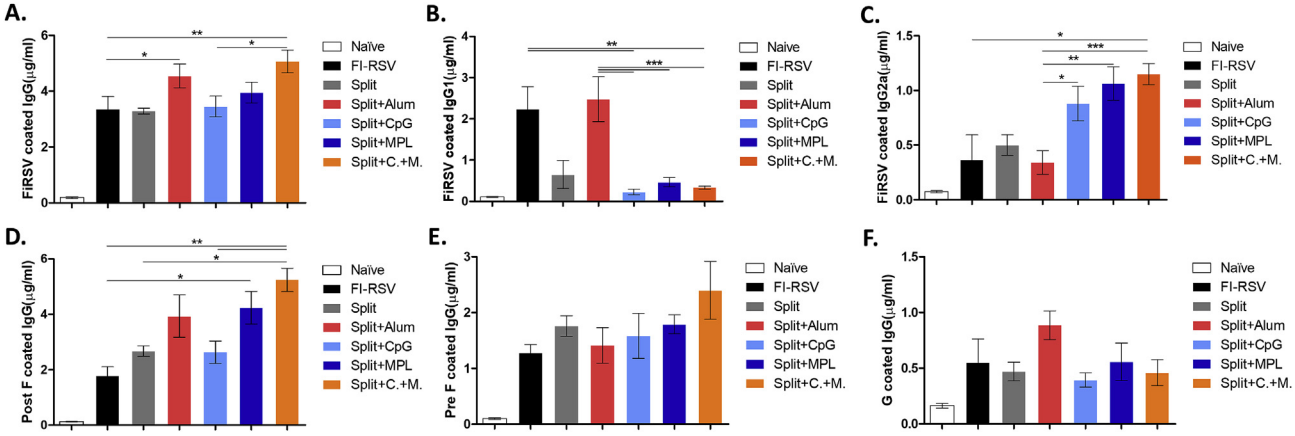

Fig. 3. RSV antigen specific IgG and isotype antibody levels in 2-week old mice after prime immunization.
Infant (2 weeks old) age mice (N = 5) were single immunized with FI-RSV (2 μg) or Split RSV (5 μg) in the presence or absence of adjuvant (Alum, CpG, MPL or CpG + MPL adjuvants). RSV antigen specific 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 (a.a 131–230) specific IgG antibodies. Naive: unimmunized mice, FI-RSV: FI-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. ***; $p < 0.0001$, **; $p < 0.001$ and *; $p < 0.05$ comparing Split RSV alone to split with adjuvants in mice.

over 50-fold, compared to the Split alone group (Fig. 4B), which is consistent with RSV neutralization titers.

3.4. CpG + MPL in split RSV vaccination of 2-week old mice prevents lung histopathology after RSV challenge

Lung histology was examined day 5 post challenge at 5 weeks after prime vaccination. FI-RSV or Split + alum primed mice at 2-week old displayed more severe alveolitis in the airways (Fig. 5A and B) and substantial inflammation in the around of blood vessels, perivascular and interstitial spaces (Fig. 5C and D) compared to unimmunized naive mice or Split RSV alone primed mice. The Split RSV alone primed mice at 2-week old exhibited less severe histopathology than FI-RSV primed or naïve mice with RSV infection (Fig. 5). Alum adjuvant in Split RSV priming of 2-week old mice resulted in enhancing histopathology whereas the Split + CpG and Split + MPL groups moderately attenuated histological inflammation around the airways, blood vessels, and interstitial spaces, compared with those in the spit RSV alone group (Fig. 5B–D). Priming of 2-week old 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. 5A–D). We further analyzed the presence of PAS positive mucus production and H&CR positive eosinophilic infiltration in the histology (Fig. 5E and F). FI-RSV priming of 2-week old mice even without alum adjuvant induced severe PAS and H&CR positive staining in histology compared to split RSV priming after RSV challenge (Fig. 5E–F). Split RSV alone priming of 2-week old mice resulted in lower levels of mucus production and eosinophilic infiltration than naïve mice with RSV infection (Fig. 5E and F). The addition of combination CpG + MPL adjuvant to the Split RSV priming of 2-week old mice further attenuated mucus production and eosinophilic induction. Addition of alum, CpG, or MPL to the split RSV prime vaccination of 2-week old mice did not reduce PAS^+ mucus production in histology after challenge (Fig. 5E). Overall, these results suggest that CpG + MPL combination adjuvant in Split RSV vaccination of 2-week old mice contributes to preventing lung histopathology after RSV Challenge.

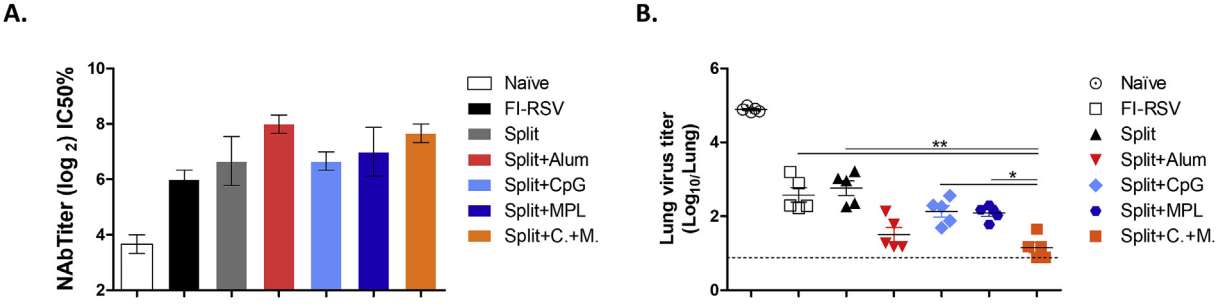

Fig. 4. RSV neutralizing activity titers in mice primed at 2 weeks old and lung viral loads after RSV challenge.
Infant age (2 weeks old) mice (N = 5) were prime immunized and sera were collected at 3 weeks after prime vaccination. (A) Serum neutralizing antibody titers are expressed as the reciprocal of the highest serum dilutions giving 50% reduction of plaque numbers relative to the medium controls. (B) Lung RSV titers at day 5 after challenge. Naïve and all immunized mouse groups were intranasally challenged with 3.5×10^5 PFU RSV A2. Naïve R.: unimmunized mice with RSV infection, FI-RSV: FI-RSV (2 μg) after RSV challenge, Split: Split RSV vaccine after challenge. 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 linear line is the limit of detection (LOD). Results are presented as mean ± SEM. Statistical significances were performed by one-way ANOVA in GraphPad Prism. **; $p < 0.001$ and *; $p < 0.05$.

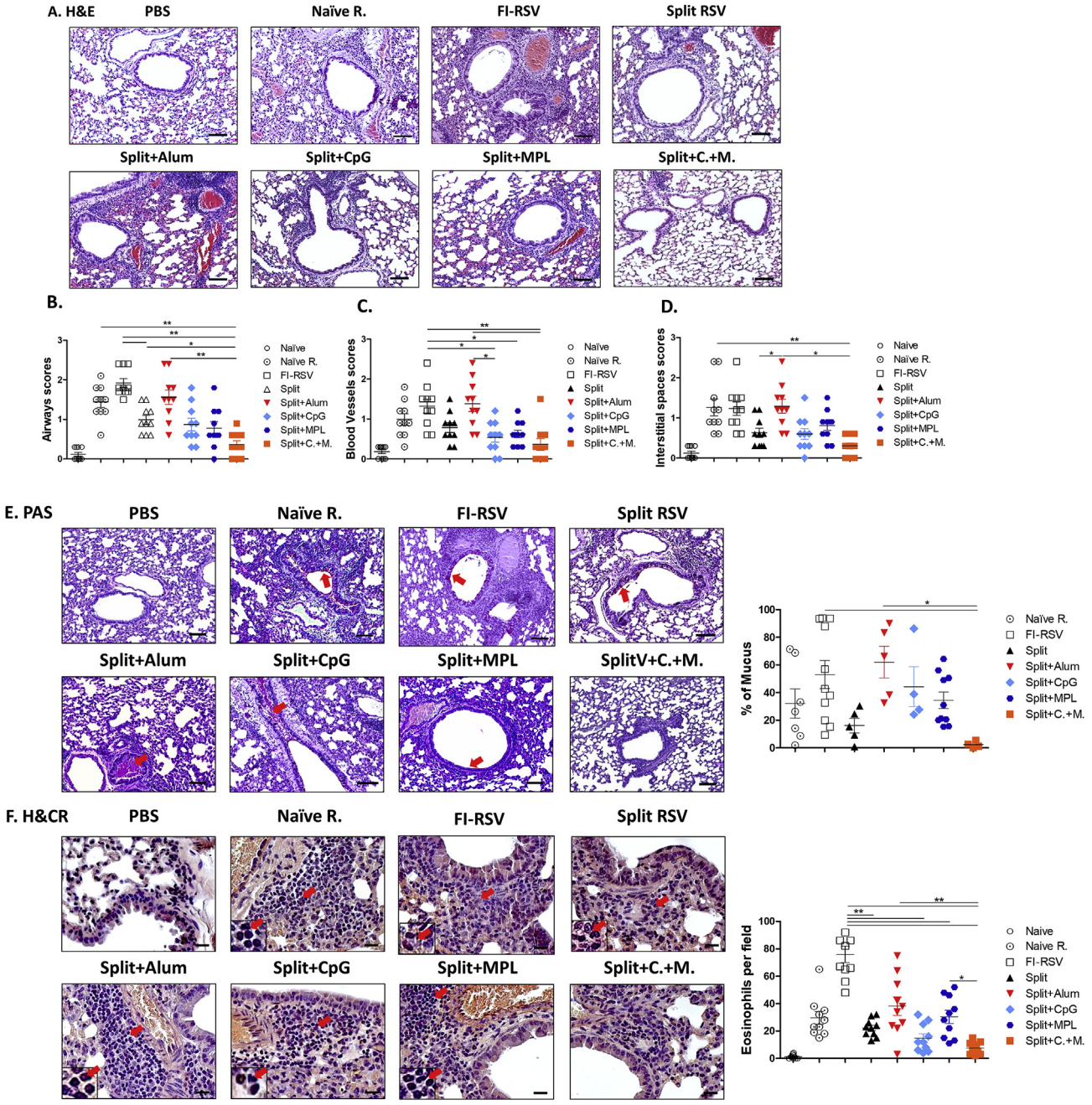

Fig. 5. Histopathology of H&E, PAS, and H&CR staining of lung tissues in Split RSV primed mice at 2 weeks old after RSV challenge. Individual lung tissues were collected from prime-vaccinated BALB/c mice (N = 5) at 2-week old day 5 post challenge. (A) Photographs of H&E. Hematoxyline and Eosin (H&E) 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 0–4 scoring system (0 = absent, 1 = minimal pathology and 4 = maximum/severe pathology) for the alveolitis. (B) H&E inflammation scores in the airways. (C) inflammation scores in the blood vessels. (D) inflammation scores in the interstitial spaces. (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 400 μm. 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. **; $p < 0.001$ and *; $p < 0.05$. Groups are the same as described in Fig. 4.

3.5. CpG + MPL adjuvanted split RSV priming of 2-week old mice promote Th1 type immune responses and prevent lung infiltrates after RSV challenge

Spleen cells from RSV vaccine-primed mice on day 5 post challenge were cultured to determine *in vitro* IgG antibody and cytokine production. The Split ± alum groups showed high levels of RSV specific IgG and IgG1 isotype antibodies in splenocyte culture supernatants, whereas splenocytes from the Split with CpG + MPL group secreted RSV specific IgG and IgG2a isotype antibodies *in vitro* at high levels (Supplementary Fig S2).

We also determined IL-4 and IFN-γ cytokine levels secreted in splenocyte culture supernatants stimulated with CD8 T cell epitopes ($F_{85\text{-}93}$, $F_{92\text{-}106}$ pooled peptides) or CD4 T cell epitope $F_{51\text{-}66}$ by intracellular cytokine staining and flow cytometry analysis (Fig. 6). Spleen cells from FI-RSV or Split RSV ± alum adjuvant priming produced high levels of IL-4 and low levels of IFN-γ cytokines. In contrast,

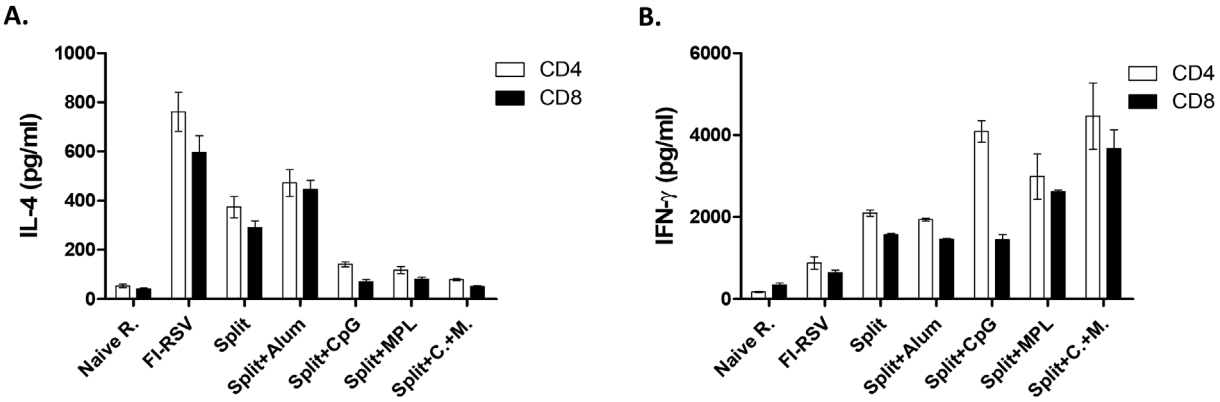

Fig. 6. CpG + MPL adjuvant Split RSV primed mice at 2 weeks old induce IFN-γ secreting splenocytes after RSV challenge.
(A, B) Th1 or Th2 cytokine responses were determined after *in vitro* cultures of spleen cells collected on day 5 post RSV challenge. Spleen cells (1×10^6/ml) were stimulated with peptide F_{51-66} epitope for CD4 T cells or $F_{85\text{-}93}$ and $F_{92\text{-}106}$ pooled epitopes for CD8 T cells for 36hr. Culture supernatants of splenocytes were used to determine cytokines of IL-4 (A) and IFN-γ (B) by ELISA analysis. Results are presented as mean ± SEM.

spleen cells from the Split + CpG, Split + MPL, or Split with CpG + MPL group secreted lower levels of IL-4 (Fig. 6A) and higher levels of IFN-γ (Fig. 6B) cytokines in culture supernatants compared to the Split RSV alone group.

Cells in lung tissues and BALF from RSV-vaccinated mice at day 5 after challenge were *in vitro* cultured in the presence of F_{51-66} CD4 T cell epitope or $F_{85\text{-}93}$ epitope for CD8 T cells and analyzed by intracellular cytokine staining. The FI-RSV and Split RSV ± alum groups showed relatively high numbers of lung IL-4$^+$ CD4 T cells, TNF-α$^+$ CD8 T cells and moderate levels of IFN-γ$^+$ CD4 and CD8 T cells (Supplementary Fig S3). 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 (Supplementary Fig S3A-B, E-F). 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 immune correlates attenuating histopathology, the pattern of Th1- and Th2-type cytokines was measured in lung homogenates by ELISA at 5 days after RSV challenge. The Split RSV group showed lower levels of IL-4, IL-5, and IL-13 cytokines in lung homogenates (Supplementary Fig S4A-F) and airway BALF (Supplementary Fig S4G-L) than those in the FI-RSV and Split + alum groups after challenge although not all cytokines were statistically significant as indicated (Supplementary Fig S4A, B, C, G, H, and J). 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 split alone or with CpG or MPL single adjuvant after RSV challenge (Supplementary Fig S4).

Cellular phenotypes of infiltrates into the respiratory tracts would provide insight for better understanding lung inflammation. Lower levels of monocytes (CD11b$^+$Ly6c^{high}F4/80$^+$) in BALF were observed in the groups of Split, Split + CpG, and Split with CpG + MPL than Split + alum, and Split + MPL groups, although their differences were not significant in lung samples (Supplementary Fig S5A). Neutrophils (CD11b$^+$Ly6c$^+$F4/80$^-$) 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 FI-RSV, Split + Alum and Split + MPL groups on day 5 post RSV challenge (Supplementary Fig S5B, G). Also, eosinophils (CD11b$^+$CD11c$^+$SiglecF$^+$) in BALF were observed at the lowest or background levels in the Split with CpG + MPL immune mice, whereas FI-RSV, Split + Alum groups displayed the highest levels of eosinophils (Supplementary Fig S5). Interestingly, subsets of dendritic cells (pDC; CD11c$^+$B220$^+$MHCII^{high} and CD103$^+$DC; CD11c$^+$MHCII$^+$F4/80$^-$CD11b$^-$CD103$^+$DCs) were found to be at the highest levels in BALF and lung samples from the Split with CpG + MPL group compared with other groups (Supplementary Fig S5D, E, I, J). Differential modulation of DC subsets and limiting neutrophils and eosinophils in the airways might play a role in attenuating pulmonary inflammation upon RSV challenge.

4. Discussion

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 for maternal immunization of pregnant women. Inactivated detergent-split influenza virus vaccines are licensed for seasonal vaccination in different age populations including 6 months old age infants, children, adults, and the elderly. We found that inactivated detergent-Split RSV vaccines expose neutralizing epitopes reactive to palivizumab at higher levels than inactivated whole FI-RSV. Split RSV prime vaccination of adult or 2-week old mice induced more balanced immune responses inducing Th1 and Th2 type IgG isotype antibodies and controlling lung viral loads with attenuated histopathology than FI-RSV after RSV challenge. Inclusion of combination CpG + MPL adjuvants in Split RSV prime vaccination improved the efficacy of Split RSV vaccines and, more importantly, prevented the induction of pulmonary histopathology after RSV challenge. This study demonstrates a different view on Split RSV vaccine in contrast to whole FI-RSV and unique adjuvant effects of combination TLR agonists on preventing inflammatory disease after RSV challenge in an infant mouse model.

In clinical trials of influenza virus vaccines in adults (Parkman et al., 1977), 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 naive children one to four years old (Gross et al., 1977). Clinical effectiveness of split-virion vaccines was significantly higher than purified-protein subunit trivalent influenza vaccines in older adults (Talbot et al., 2015). Long history of safety and efficacy data on split-virion influenza vaccines provides attractive rationales for developing Split-RSV vaccine candidates.

We do not understand how the differences regarding the split RSV vaccine antigens might occur as a result of treating whole FI-RSV with detergent Triton X-100. Splitting inactivated influenza viruses was reported to be more heterogeneous in size and morphology, and moderate increases in hemagglutinin contents per unit total split influenza vaccine compared to whole inactivated influenza virus before treatment (Kon et al., 2016). A previous study on the antigenic display of FI-RSV

reported high reactivity to site I (131-2a) and site II (Motavizumab) mAbs, demonstrating predominantly post-fusion conformation in whole FI-RSV (Killikelly et al., 2016). We found that split RSV exposed pre-fusion and post-fusion epitopes at higher levels than FI-RSV, suggesting a possibility that splitting RSV might have induced conformational changes exposing prefusion, post-fusion and site II neutralizing epitopes to be more reactive. Nonetheless, higher reactivity of split RSV with antibodies recognizing the site I epitope present in post-fusion conformation than pre-fusion epitopes (D25, 5C4), implying post-fusion conformation dominant in split RSV. Higher levels of IgG2a isotype antibodies were induced in adult mice after prime dose of Split RSV than those in whole FI-RSV. It is desirable to observe that histopathology in the airways, blood vessels, and interstitial spaces as well as the levels of mucus production and H&CR positive eosinophilic spots were significantly lower in Split RSV primed mice after RSV challenge compared to those in whole FI-RSV and unimmunized naïve control with RSV infection. Further studies will be required to better understand the mechanisms by which detergent treatment might induce conformational changes, affecting the stability, antigenic and immunogenic properties of inactivated split RSV vaccines.

Vaccine-enhanced respiratory disease was observed in naïve young children after alum-adjuvanted FI-RSV vaccination and natural RSV infection (Kim et al., 1969). Alum-adjuvanted FI-RSV vaccination induced high levels of binding antibodies with weak neutralizing activity (Murphy et al., 1986). Another aspect is that alum adjuvanted FI-RSV vaccination induced Th2-biased immune responses associated with pulmonary histopathology with airway hypersensitivitry, peribronchiolar inflammation, and cellular infiltrates (Knudson et al., 2015). Vaccine-associated inflammatory histopathology was not attenuated by RSV vaccines in squalene oil-in-water emulsion (Lambert et al., 2015) or with natural killer T cell agonist α-GalCer (Johnson et al., 2002). RSV F protein vaccines delivered as a soluble trimer and formulated with TLR4 agonist glucopyranosyl lipid A (GLA) integrated into stable emulsion (GLA-SE) were immunogenic in adults but did not protect against RSV illness in a clinical study (Falloon et al., 2017). Therefore, as focused in this study, it is significant to determine the adjuvant effects on improving the efficacy of Split RSV vaccine and on further 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 after prime dose in 2-week old mice. Split RSV vaccination of 2-week old mice even in the absence of adjuvant resulted in lower levels of histopathology after challenge than FI-RSV, which is consistent with the histopathology observed in adult mice. Alum adjuvant in Split RSV vaccination of 2-week old mice enhanced neutralizing activity titers and lung viral clearance but also exacerbated histopathology. Inclusion of alum adjuvant in the FI-RSV or F protein subunit vaccination contributed to vaccine-enhanced respiratory disease in mice after RSV challenge (Kim et al., 2015; Lee et al., 2017).

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 (Hancock et al., 2001). 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 (Garg et al., 2014; Garlapati et al., 2012). Cotton rats that were intranasally immunized with RSV F protein and CpG adjuvant developed enhanced pulmonary histopathology consisting of alveolitis and interstitial pneumonitis after RSV challenge despite of markedly reducing lung viral loads (Prince et al., 2003). Combination of CpG and delta inulin adjuvant in whole RSV vaccination induced IgG2a antibodies in mice but exacerbated lung pathology after RSV challenge (Wong et al., 2016). Post-fusion or pre-fusion F protein vaccines at low doses in TLR4 agonist analog (GLA) in stable emulsion vaccine formulation were reported to prime inflammatory vaccine-enhanced alveolitis in cotton rats although lung viral titers were below the detection limit after RSV challenge (Schneider-Ohrum et al., 2017). In a clinical study, RSV F soluble protein vaccine in GLA emulsion formulation failed to protect RSV disease in adults (Falloon et al., 2017). In this study, combination CpG + MPL adjuvant 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 reduce 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 from the CpG + MPL group, compared with the Split alone, or CpG or MPL adjuvanted Split groups. Consistently, IL-4, IL-5 and IL-13 cytokines were detected at the lowest levels in BALF from the CpG + MPL group whereas substantial levels of these Th2 cytokines were observed with the FI-RSV or 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$^+$ DCs were recruited into BALF and lungs at higher levels in the Split plus CpG + MPL group after RSV challenge compared to MPL or CpG adjuvant group. The TLR9 CpG signaling is mainly dependent on the MyD88 adaptor pathway (Kawai and Akira, 2007). 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 (Mata-Haro et al., 2007; Yamamoto et al., 2003). 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 (Ko et al., 2017). A previous study also showed that MPL + CpG *in vivo* treatment recruited lower levels of eosinophils and natural killer cells compared to those by CpG or MPL (Ko et al., 2018). Further studies are needed to better understand the mechanisms of combination CpG + MPL adjuvant effects on attenuating lung pathology after RSV vaccination and challenge.

5. Conclusion

This study reports desirable properties of inactivated split RSV vaccine, including better exposure of neutralizing epitopes, more Th1-like immune responses, and high efficacy of protection with less histopathology, compared with whole inactivated FI-RSV. Split RSV vaccination in 2-week old mice provides protection without apparent pulmonary inflammation after challenge, suggesting split RSV as a new vaccine candidate.

Acknowledgments

This work was supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases grants AI093772, AI105170, and AI134132 to SMK. The authors acknowledge the provision of RSV F purified proteins in post-fusion and pre-fusion conformation, and 5C4 monoclonal antibody from Dr. Barney S. Graham at Vaccine Research Center (NIAID, NIH, Bethesda, MD 20892, USA). The bacterial expression plasmid encoding RSV A2 G protein fragment (aa131-230) was kindly provided by Dr. MK Song and G protein fragment prepared by Drs. C Kim and JS Lee.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.05.011.

References

al-Mazrou, A., Scheifele, D.W., Soong, T., Bjornson, G., 1991. Comparison of adverse reactions to whole-virion and split-virion influenza vaccines in hospital personnel. CMAJ (Can. Med. Assoc. J.) 145, 213–218.

Cate, T.R., Couch, R.B., Kasel, J.A., Six, H.R., 1977. Clinical trials of monovalent influenza A/New Jersey/76 virus vaccines in adults: reactogenicity, antibody response, and antibody persistence. J. Infect. Dis. 136 (Suppl. l), S450–S455.

Cohen, B.J., Audet, S., Andrews, N., and, Beeler, J., 2007. Plaque reduction neutralization test for measles antibodies: description of a standardised laboratory method for use in immunogenicity studies of aerosol vaccination. Vaccine 26, 59–66.

Connors, M., Giese, N.A., Kulkarni, A.B., Firestone, C.Y., Morse 3rd, H.C., Murphy, B.R., 1994. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. J. Virol. 68, 5321–5325.

Connors, M., Kulkarni, A.B., Firestone, C.Y., Holmes, K.L., Morse 3rd, H.C., Sotnikov, A.V., Murphy, B.R., 1992. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J. Virol. 66, 7444–7451.

Derscheid, R.J., van Geelen, A., McGill, J.L., Gallup, J.M., Cihlar, T., Sacco, R.E., Ackermann, M.R., 2013. Human respiratory syncytial virus Memphis 37 grown in HEp-2 cells causes more severe disease in lambs than virus grown in Vero cells. Viruses 5, 2881–2897.

Falloon, J., Yu, J., Esser, M.T., Villafana, T., Yu, L., Dubovsky, F., Takas, T., Levin, M.J., Falsey, A.R., 2017. An adjuvanted, postfusion F protein-based vaccine did not prevent respiratory syncytial virus illness in older adults. J. Infect. Dis. 216, 1362–1370.

Garg, R., Latimer, L., Gerdts, V., Potter, A., van Drunen Littel-van den Hurk, S., 2014. Vaccination with the RSV fusion protein formulated with a combination adjuvant induces long-lasting protective immunity. J. Gen. Virol. 95, 1043–1054.

Garlapati, S., Garg, R., Brownlie, R., Latimer, L., Simko, E., Hancock, R.E., Babiuk, L.A., Gerdts, V., Potter, A., van Drunen Littel-van den Hurk, S., 2012. Enhanced immune responses and protection by vaccination with respiratory syncytial virus fusion protein formulated with CpG oligodeoxynucleotide and innate defense regulator peptide in polyphosphazene microparticles. Vaccine 30, 5206–5214.

Graham, B.S., 2011. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol. Rev. 239, 149–166.

Gross, P.A., Ennis, F.A., Gaerlan, P.F., Denson, L.J., Denning, C.R., Schiffman, D., 1977. A controlled double-blind comparison of reactogenicity, immunogenicity, and protective efficacy of whole-virus and split-product influenza vaccines in children. J. Infect. Dis. 136, 623–632.

Hancock, G.E., Heers, K.M., Smith, J.D., Scheuer, C.A., Ibraghimov, A.R., Pryharski, K.S., 2001. CpG containing oligodeoxynucleotides are potent adjuvants for parenteral vaccination with the fusion (F) protein of respiratory syncytial virus (RSV). Vaccine 19, 4874–4882.

Hwang, H.S., Kwon, Y.M., Lee, J.S., Yoo, S.E., Lee, Y.N., Ko, E.J., Kim, M.C., Cho, M.K., Lee, Y.T., Jung, Y.J., Lee, J.Y., Li, J.D., Kang, S.M., 2014. Co-immunization with virus-like particle and DNA vaccines induces protection against respiratory syncytial virus infection and bronchiolitis. Antivir. Res. 110C, 115–123.

Ireton, G.C., Reed, S.G., 2013. Adjuvants containing natural and synthetic Toll-like receptor 4 ligands. Expert Rev. Vaccines 12, 793–807.

Johnson, T.R., Hong, S., Van Kaer, L., Koezuka, Y., Graham, B.S., 2002. NK T cells contribute to expansion of CD8(+) T cells and amplification of antiviral immune responses to respiratory syncytial virus. J. Virol. 76, 4294–4303.

Kawai, T., Akira, S., 2007. TLR signaling. Semin. Immunol. 19, 24–32.

Killikelly, A.M., Kanekiyo, M., Graham, B.S., 2016. Pre-fusion F is absent on the surface of formalin-inactivated respiratory syncytial virus. Sci. Rep. 6, 34108.

Kim, H.W., Canchola, J.G., Brandt, C.D., Pyles, G., Chanock, R.M., Jensen, K., Parrott, R.H., 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89, 422–434.

Kim, K.H., Lee, Y.T., Hwang, H.S., Kwon, Y.M., Jung, Y.J., Lee, Y., Lee, J.S., Lee, Y.N., Park, S., Kang, S.M., 2015. Alum adjuvant enhances protection against respiratory syncytial virus but exacerbates pulmonary inflammation by modulating multiple innate and adaptive immune cells. PLoS One 10, e0139916.

Kim, S., Joo, D.H., Lee, J.B., Shim, B.S., Cheon, I.S., Jang, J.E., Song, H.H., Kim, K.H., Song, M.K., Chang, J., 2012. Dual role of respiratory syncytial virus glycoprotein fragment as a mucosal immunogen and chemotactic adjuvant. PLoS One 7, e32226.

Klopfleisch, R., 2013. Multiparametric and semiquantitative scoring systems for the evaluation of mouse model histopathology–a systematic review. BMC Vet. Res. 9, 123.

Knudson, C.J., Hartwig, S.M., Meyerholz, D.K., Varga, S.M., 2015. RSV vaccine-enhanced disease is orchestrated by the combined actions of distinct CD4 T cell subsets. PLoS Pathog. 11, e1004757.

Ko, E.J., Lee, Y., Lee, Y.T., Kim, Y.J., Kim, K.H., Kang, S.M., 2018. MPL and CpG combination adjuvants promote homologous and heterosubtypic cross protection of inactivated split influenza virus vaccine. Antivir. Res. 156, 107–115.

Ko, E.J., Lee, Y.T., Lee, Y., Kim, K.H., Kang, S.M., 2017. Distinct effects of monophosphoryl lipid A, oligodeoxynucleotide CpG, and combination adjuvants on modulating innate and adaptive immune responses to influenza vaccination. Immune Network 17, 326–342.

Kon, T.C., Onu, A., Berbecila, L., Lupulescu, E., Ghiorgisor, A., Kersten, G.F., Cui, Y.Q., Amorij, J.P., Van der Pol, L., 2016. Influenza vaccine manufacturing: effect of inactivation, splitting and site of manufacturing. Comparison of influenza vaccine production processes. PLoS One 11, e0150700.

Lambert, S.L., Aslam, S., Stillman, E., MacPhail, M., Nelson, C., Ro, B., Sweetwood, R., Lei, Y.M., Woo, J.C., Tang, R.S., 2015. A novel respiratory syncytial virus (RSV) F subunit vaccine adjuvanted with GLA-SE elicits robust protective TH1-type humoral and cellular immunity in rodent models. PLoS One 10, e0119509.

Lee, Y., Lee, Y.T., Ko, E.J., Kim, K.H., Hwang, H.S., Park, S., Kwon, Y.M., Kang, S.M., 2017. Soluble F proteins exacerbate pulmonary histopathology after vaccination upon respiratory syncytial virus challenge but not when presented on virus-like particles. Hum. Vaccines Immunother. 13, 2594–2605.

Mata-Haro, V., Cekic, C., Martin, M., Chilton, P.M., Casella, C.R., Mitchell, T.C., 2007. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 316, 1628–1632.

McLellan, J.S., Chen, M., Joyce, M.G., Sastry, M., Stewart-Jones, G.B., Yang, Y., Zhang, B., Chen, L., Srivatsan, S., Zheng, A., Zhou, T., Graepel, K.W., Kumar, A., Moin, S., Boyington, J.C., Chuang, G.Y., Soto, C., Baxa, U., Bakker, A.Q., Spits, H., Beaumont, T., Zheng, Z., Xia, N., Ko, S.Y., Todd, J.P., Rao, S., Graham, B.S., Kwong, P.D., 2013. Structure-based design of a fusion glycoprotein vaccine for respiratory syncytial virus. Science 342, 592–598.

McLellan, J.S., Yang, Y., Graham, B.S., Kwong, P.D., 2011. Structure of respiratory syncytial virus fusion glycoprotein in the postfusion conformation reveals preservation of neutralizing epitopes. J. Virol. 85, 7788–7796.

Meyerholz, D.K., Griffin, M.A., Castilow, E.M., Varga, S.M., 2009. Comparison of histochemical methods for murine eosinophil detection in an RSV vaccine-enhanced inflammation model. Toxicol. Pathol. 37, 249–255.

Murphy, B.R., Alling, D.W., Snyder, M.H., Walsh, E.E., Prince, G.A., Chanock, R.M., Hemming, V.G., Rodriguez, W.J., Kim, H.W., Graham, B.S., et al., 1986. Effect of age and preexisting antibody on serum antibody response of infants and children to the F and G glycoproteins during respiratory syncytial virus infection. J. Clin. Microbiol. 24, 894–898.

Murphy, B.R., Sotnikov, A.V., Lawrence, L.A., Banks, S.M., Prince, G.A., 1990. Enhanced pulmonary histopathology is observed in cotton rats immunized with formalin-inactivated respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV 3-6 months after immunization. Vaccine 8, 497–502.

Nair, H., Nokes, D.J., Gessner, B.D., Dherani, M., Madhi, S.A., Singleton, R.J., O'Brien, K.L., Roca, A., Wright, P.F., Bruce, N., Chandran, A., Theodoratou, E., Sutanto, A., Sedyaningsih, E.R., Ngama, M., Munywoki, P.K., Kartasasmita, C., Simoes, E.A., Rudan, I., Weber, M.W., Campbell, H., 2010. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet 375, 1545–1555.

Nair, H., Simoes, E.A., Rudan, I., Gessner, B.D., Azziz-Baumgartner, E., Zhang, J.S., Feikin, D.R., Mackenzie, G.A., Moisi, J.C., Roca, A., Baggett, H.C., Zaman, S.M., Singleton, R.J., Lucero, M.G., Chandran, A., Gentile, A., Cohen, C., Krishnan, A., Bhutta, Z.A., Arguedas, A., Clara, A.W., Andrade, A.L., Ope, M., Ruvinsky, R.O., Hortal, M., McCracken, J.P., Madhi, S.A., Bruce, N., Qazi, S.A., Morris, S.S., El Arifeen, S., Weber, M.W., Scott, J.A., Brooks, W.A., Breiman, R.F., Campbell, H., 2013. Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis. Lancet 381, 1380–1390.

O'Hagan, D.T., Friedland, L.R., Hanon, E., Didierlaurent, A.M., 2017. Towards an evidence based approach for the development of adjuvanted vaccines. Curr. Opin. Immunol. 47, 93–102.

Olson, M.R., Varga, S.M., 2008. Pulmonary immunity and immunopathology: lessons from respiratory syncytial virus. Expert Rev. Vaccines 7, 1239–1255.

Oumouna, M., Mapletoft, J.W., Karvonen, B.C., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2005. Formulation with CpG oligodeoxynucleotides prevents induction of pulmonary immunopathology following priming with formalin-inactivated or commercial killed bovine respiratory syncytial virus vaccine. J. Virol. 79, 2024–2032.

Parkman, P.D., Hopps, H.E., Rastogi, S.C., Meyer Jr., H.M., 1977. Summary of clinical trials of influenza virus vaccines in adults. J. Infect. Dis. 136 (Suppl. l), S722–S730.

Prince, G.A., Jenson, A.B., Hemming, V.G., Murphy, B.R., Walsh, E.E., Horswood, R.L., Chanock, R.M., 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactiva ted virus. J. Virol. 57, 721–728.

Prince, G.A., Mond, J.J., Porter, D.D., Yim, K.C., Lan, S.J., Klinman, D.M., 2003. Immunoprotective activity and safety of a respiratory syncytial virus vaccine: mucosal delivery of fusion glycoprotein with a CpG oligodeoxynucleotide adjuvant. J. Virol. 77, 13156–13160.

Rappuoli, R., Mandl, C.W., Black, S., De Gregorio, E., 2011. Vaccines for the twenty-first century society. Nat. Rev. Immunol. 11, 865–872.

Schneider-Ohrum, K., Cayatte, C., Bennett, A.S., Rajani, G.M., McTamney, P., Nacel, K., Hostetler, L., Cheng, L., Ren, K., O'Day, T., Prince, G.A., McCarthy, M.P., 2017. Immunization with low doses of recombinant postfusion or prefusion respiratory syncytial virus F primes for vaccine-enhanced disease in the cotton rat model independently of the presence of a Th1-biasing (GLA-SE) or Th2-biasing (alum) adjuvant. J. Virol. 91.

Talbot, H.K., Nian, H., Zhu, Y., Chen, Q., Williams, J.V., Griffin, M.R., 2015. Clinical effectiveness of split-virion versus subunit trivalent influenza vaccines in older adults. Clin. Infect. Dis. 60, 1170–1175.

Varga, S.M., Wissinger, E.L., Braciale, T.J., 2000. The attachment (G) glycoprotein of respiratory syncytial virus contains a single immunodominant epitope that elicits both Th1 and Th2 CD4+ T cell responses. J. Immunol. 165, 6487–6495.

Wong, T.M., Petrovsky, N., Bissel, S.J., Wiley, C.A., Ross, T.M., 2016. Delta inulin-derived adjuvants that elicit Th1 phenotype following vaccination reduces respiratory syncytial virus lung titers without a reduction in lung immunopathology. Hum. Vaccines Immunother. 1–10.

Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., Akira, S., 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat. Immunol. 4, 1144–1150.