Lipopolysaccharide Inhibits FI-RSV Vaccine-enhanced Inflammation Through Regulating Th Responses*

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Summary: Respiratory syncytial virus (RSV) infection is the primary cause of respiratory disease in infants. The formalin-inactivated RSV (FI-RSV) vaccine resulted in an enhanced respiratory disease (ERD) in infants upon natural RSV infection, which is a major obstacle for development of safe and efficacious vaccines. Excessive and uncontrolled Th immune responses could be involved in the ERD. Agonists of TLRs are used as adjuvants to guide the type of immune response induced by vaccines. We evaluated the impact of lipopolysaccharide (LPS), the agonist of TLR4, on ERD as the adjuvant of FI-RSV. The results showed that LPS remarkably inhibited FI-RSV-enhanced lung inflammation, mucus production, airway inflammatory cell infiltration, and inflammatory cytokines following RSV challenge. Interestingly, LPS inhibited both Th2 and Th17 type cytokines in lungs of FI-RSV-immunized mice following RSV challenge, without an increase in the Th1 type cytokines, suggesting a controlled immune response. In contrast, Pam3Cys and Poly(I:C), the agonist of TLR1/2 or TLR3, partly inhibited FI-RSV-enhanced lung inflammation. Pam3Cys inhibited Th17 type cytokine IL-17, but promoted both Th1 and Th2 type cytokines. Poly(I:C) inhibited Th2 and Th17 type cytokines, but promoted Th1 type cytokines. In addition, LPS promoted IgG and IgG2a antibody production, which might provide protection from RSV challenge. These results suggest that LPS inhibits ERD without impairment in antibody production and protection, and the mechanism appears to be related with regulation of Th responses induced by FI-RSV.
Key words: lipopolysaccharide; adjuvant; formalin-inactivated RSV; vaccine-enhanced respiratory disease

Respiratory syncytial virus (RSV) is the primary cause of lower respiratory disease in infants and children worldwide. Almost all children are infected at least once by 2 years of age[1]. RSV is also a significant cause of respiratory illness in high-risk adults and the elderly[2]. Despite the RSV is a important respiratory pathogen, licensed vaccines and efficacious post-infection therapies are not currently available. Safety is of the utmost importance for an RSV vaccine due to the early failed experiences with a formalin inactivated, alum-precipitated RSV vaccine (FI-RSV) that resulted in enhanced disease in infants upon natural RSV challenge[3]. The main clinical manifestations in children with enhanced respiratory disease (ERD) were severe peribronchiolitis and alveolitis[4]. Histological examination of the lungs from the deceased revealed extensive neutrophils, mononuclear cells, and lymphocytes, and increased number of eosinophils infiltration[4]. Understanding pathogenesis of vaccine-ERD and exploring how to attenuate ERD contributes to development of safe and efficacious RSV vaccines. Studies indicated that excessive Th2 and/or Th17 responses played important roles in ERD pathogenesis[5, 6].

Adjuvants are used to augment the effect of a vaccine by stimulating the immune responses, and influence the type of immune response. The ERD is likely related to alum adjuvant in the FI-RSV formulation, since alum adjuvant preferentially induces Th2-type immune response[3, 7]. Our previous study also found that unadjuvanted FI-RSV elicited significantly reduced but still problematic airway inflammation compared with FI-RSV adjuvanted with alum[3, 7]. Incorporation of Toll-like receptor (TLR) ligands into vaccine formulations represents a class of adjuvants proposed for usage in next generation

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vaccines. TLRs recognize a wide variety of agonists to activate innate immunity through MyD88-dependent and TRIF-dependent signaling pathways. The MyD88-dependent pathway is mediated by the adaptors MyD88 and TIRAP and induces proinflammatory cytokine/chemokine production by activating the transcription factors NF-κB and MAPK. The TRIF-dependent pathway is initiated by the adaptors TRAM and TRIF in the endosomes to generate IFN-β and the anti-inflammatory cytokine IL-10 through activation of the transcription factor IRF3. TLR signaling also plays an important role in the activation and regulation of the adaptive immunity by inducing cytokines and upregulating costimulatory molecules of antigen presenting cells (APC)[8]. Many agonists of TLRs exert their adjuvant function by interacting with TLRs. Lipopolysaccharide (LPS) is the major surface molecule of gram-negative bacteria, and recognized by TLR4/MD2 complex, which initiates signaling pathways mediated by the adaptor protein MyD88, leading to proinflammatory cytokine and type II IFNs (IFN-γ) production. Subsequently, TLR4 is internalized into early endosomes and triggers TRIF-dependent signaling, leading to the production of inflammatory cytokines, type I IFNs, and anti-inflammatory cytokine IL-10[9, 10]. Monophosphoryl lipid A (MPLA) is also an agonist of TLR4, and a low-toxicity derivative of LPS, but unlike LPS, MPLA is a TRIF-biased agonist of TLR4[11]. Pam3Cys is recognized by TLR1/TLR2, and activates MyD88-dependent signaling pathway, and Poly(I:C) is recognized by TLR3 to mediate TRIF-dependent signaling pathway[12]. In this study, LPS, Pam3Cys and Poly(I:C) were selected to use as the adjuvant of FI-RSV. We immunized mice with adjuvanted FI-RSV by LPS, Pam3Cys, Poly(I:C), or $Al(OH)_3$, or FI-RSV alone, and investigated the impact of the adjuvants on ERD. The results indicated that LPS inhibited ERD without impairment in antibody production and protection.

1 MATERIALS AND METHODS

1.1 Virus and Vaccines

RSV A (Long strain) was propagated in HEp-2 cells[13], and purified by a discontinuous sucrose gradient[14, 15]. FI-RSV or formalin-inactivated HEp-2 cell supernatant (FI-C) was prepared as previously described[4]. Briefly, the virus or mock was inactivated by formalin (1:4000) during a 72 h incubation at 37°C and then concentrated 25 times by ultracentrifugation. A further fourfold concentration was achieved by alum precipitation. For unadjuvanted FI-RSV or FI-C, inactivated virus or mock by formalin (1:4000) was concentrated 100 times by ultracentrifugation.

1.2 Mice and Immunization

Female C57BL/6 mice, aged 6–8 weeks, were purchased from Vital River Laboratory Animal Co. (Beijing, China), housed and manipulated according to the Care and Use of Laboratory Animals under specific pathogen-free conditions. Mice were immunized two times intramuscularly with 100 μL vaccine formulation containing 50 μL FI-RSV, FI-RSV+$Al(OH)_3$ (Invivogen, vac-alu-250, USA) or FI-C, and 50 μL PBS containing Pam3Cys (Invivogen,112208-00-1) (25 μg), Poly(I:C) (Invivogen, 31852-29-6) (25 μg), or LPS (18 μg) (Invivogen, L8880) at day 0 and 28. Mice were challenged intranasally (i.n.) with 50 μL of 6.0×10^7 pfu/mL RSV 28 days after the last immunization, and sacrificed 5 days later for protection and immune pathology studies.

1.3 Histological Analysis

The lung lobes were fixed in 4% formalin overnight, subsequently embedded in paraffin, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Sections from each mouse were scored blindly for the degree of inflammation in the peribronchial and perivascular spaces and alveolar tissue[16]. The degree of inflammation in lungs was graded as follows: 0, normal; 1, slight cell infiltration and edema, not widespread; 2, moderate levels of cell infiltration, and multifocal edema; 3, abundant cell infiltration and extensive, severe, multifocal edema. Mean scores were calculated for each mouse. Mucus was graded in a blinded fashion on a 4-point scale as follows: 0, no mucus; 1, slight mucus; 2, moderate mucus; 3, extensive mucus. Mean scores were calculated for each mouse.

1.4 Collection of Bronchoalveolar Lavage Fluid (BALF) and Leucocyte Counts

BALFs were collected and centrifuged as previously described[17]. The cellular pellets were stained using Wright-Giemsa. Lymphocytes, macrophages and neutrophils were counted according to cell morphology by light microscope. The percentage of inflammatory cells was obtained by counting 400 leucocytes.

1.5 ELISA

IgG and IgG2a titers in sera were assessed by indirect ELISA protocol[15]. Antibody titers were expressed as the reciprocal of the last sample dilution giving an absorbance of at least two-fold that of the pre-immune sample and with an optical density (OD) ≥0.15. Cytokines in BALF were measured with ELISA kits (Reybiotech, USA) with double antibody sandwich method according to the Operation instructions.

1.6 Neutralization Assays

Serum neutralizing antibody assays were undertaken as previously described[18]. Briefly, Mixtures of RSV and twofold serial dilutions of antiserum were incubated for 4 h at 4°C, then added to HEp-2 monolayers and incubated at 37°C for 2 h, before being overlaid with methyl cellulose medium. After 2–3 days at 37°C, the plates were emptied, and

the cells were fixed with 10% formalin. Neutralization titers were expressed as the reciprocal of the highest dilution that reduced positive control syncytium numbers by at least 60%.

1.7 Real-time RT-PCR

RNA was isolated from lungs with Trizol (TIANGEN-DP405-02), and reverse-transcribed to synthesis of the first strand of cDNA. Cytokines in lungs were determined by real time PCR with SYBR green (Vazyme-Q711-02) according to the Operation instructions. Gene expression was normalized to β-actin. The fold reduction of RSV N-gene expression in each vaccine group was calculated by comparison to that in FI-C-treated mice.

1.8 Statistical Analysis

Statistical analyses were performed using the software SPSS 21.0. Results were considered statistically significant if $P<0.05$.

2 RESULTS

2.1 LPS Inhibited FI-RSV-enhanced Inflammation and Mucus Production in Lungs following RSV Challenge

To test the regulatory effect of adjuvants on FI-RSV-enhanced lung inflammation, lung slices were observed by H&E and PAS staining. PBS-treated mice showed no detectable lung pathological changes following PBS challenge. Mice immunized with FI-RSV or FI-RSV+$Al(OH)_3$ exhibited severe bronchitis and pneumonia (fig. 1A), and mucus production (fig. 1B) following RSV challenge. Mice immunized with FI-RSV+Pam3Cys or FI-RSV+Poly(I:C) showed alleviative inflammation and mucus. In contrast, little inflammatory cell infiltration in the alveolar, peribronchial, and perivascular spaces, and no mucus production were observed in mice immunized with FI-RSV+LPS. The inflammation score in mice immunized with FI-RSV+Pam3Cys or FI-RSV+Poly(I:C) was significantly lower than that in mice immunized with FI-RSV or FI-RSV+$Al(OH)_3$ (fig. 1C and 1D, $P<0.05$), but was higher than that in mice vaccinated with FI-RSV+LPS. Inflammation and mucus were remarkably lowered or disappeared in mice in FI-RSV+LPS group (fig. 1C and 1D). These results indicated that LPS as adjuvant remarkably suppressed FI-RSV-enhanced inflammatory pathology and mucus production in lungs of immunized mice following RSV challenge.

2.2 LPS Suppressed FI-RSV-enhanced Airway Inflammatory Cells

To further investigate airway inflammation, we measured total leucocytes, macrophages, lymphocytes and neutrophils, respectively. Vaccine-immunized mice had significantly more total inflammatory cells, lymphocytes and neutrophils in BALF than PBS-treated mice (fig. 2A, 2C and 2D, $P<0.05$), while the percentage of macrophages in vaccine-immunized mice reduced, compared with PBS-treated mice (fig. 2B, $P<0.05$). Interestingly, the number of total inflammatory cells, lymphocytes and neutrophils was significantly lower in FI-RSV+LPS group than in other vaccine-immunized groups ($P<0.05$). The percentage of macrophages was significantly higher in FI-RSV+LPS group than in other vaccine-immunized groups ($P<0.05$). The result suggested that LPS as the adjuvant of FI-RSV attenuated the FI-RSV-enhanced airway inflammation.

2.3 LPS Inhibited FI-RSV-enhanced Airway Proinflammatory Cytokines

In the inflammatory process, activated inflammatory cells produced cytokines including IL-6, IL-1β, and TNF-α[19]. Mice in the FI-RSV+Pam3Cys group produced significantly higher concentration of IL-6 than mice in FI-RSV group (fig. 3A, $P<0.05$), and mice in FI-RSV+Poly(I:C) group showed the same level of IL-6 as mice in FI-RSV group ($P>0.05$), while the level of IL-6 in FI-RSV+LPS group was significantly lower than that in FI-RSV group (fig. 3A, $P<0.05$). Mice immunized with FI-RSV+LPS showed inhibited IL-1β, GRO-α and Eotaxin, compared with FI-RSV (fig. 3B, 3D, 3E, $P<0.05$). The concentration of TNF-α in FI-RSV+Pam3Cys group was significantly higher than that in FI-RSV groups (fig. 6C, $P<0.05$), but no difference was observed between FI-RSV+LPS group and PBS group ($P>0.05$). These results indicated that LPS suppressed all tested inflammatory cytokines following RSV challenge in immunized mice.

2.4 LPS Suppressed Th2 and Th17 Type Cytokines Following RSV Challenge

In the present study, we focused on the regulating effects of different adjuvants on Th1, Th2, and Th17 type cytokines in FI-RSV-immunized mice following RSV challenge. The levels of Th1, Th2, and Th17 type cytokines were examined by ELISA. The concentration of IFN-γ in FI-RSV+LPS group was significantly lower than that in FI-RSV+Pam3Cys and FI-RSV+Poly(I:C) groups following RSV challenge (fig. 4A, $P<0.05$), and was not significantly different from that in FI-RSV group. The Th2 type cytokines IL-4, IL-10 and IL-13 in FI-RSV+LPS group were significantly reduced, compared with those in FI-RSV group (fig. 4B, C and D, $P<0.05$). The levels of Th17 type cytokine IL-17 in FI-RSV+LPS/Pam3Cys/$Al(OH)_3$/Poly(I:C) groups were all significantly lower than that in FI-RSV group (fig. 4E, $P<0.05$). These data indicated that LPS inhibited both Th2 and Th17 type cytokines without an increase in the Th1 type cytokines, suggesting a controlled Th responses, while Pam3Cys inhibited Th17 type cytokine but increased both Th1 and Th2 type cytokines, and Poly(I:C) inhibited Th2 and Th17 type cytokines and increased Th1 type cytokines.

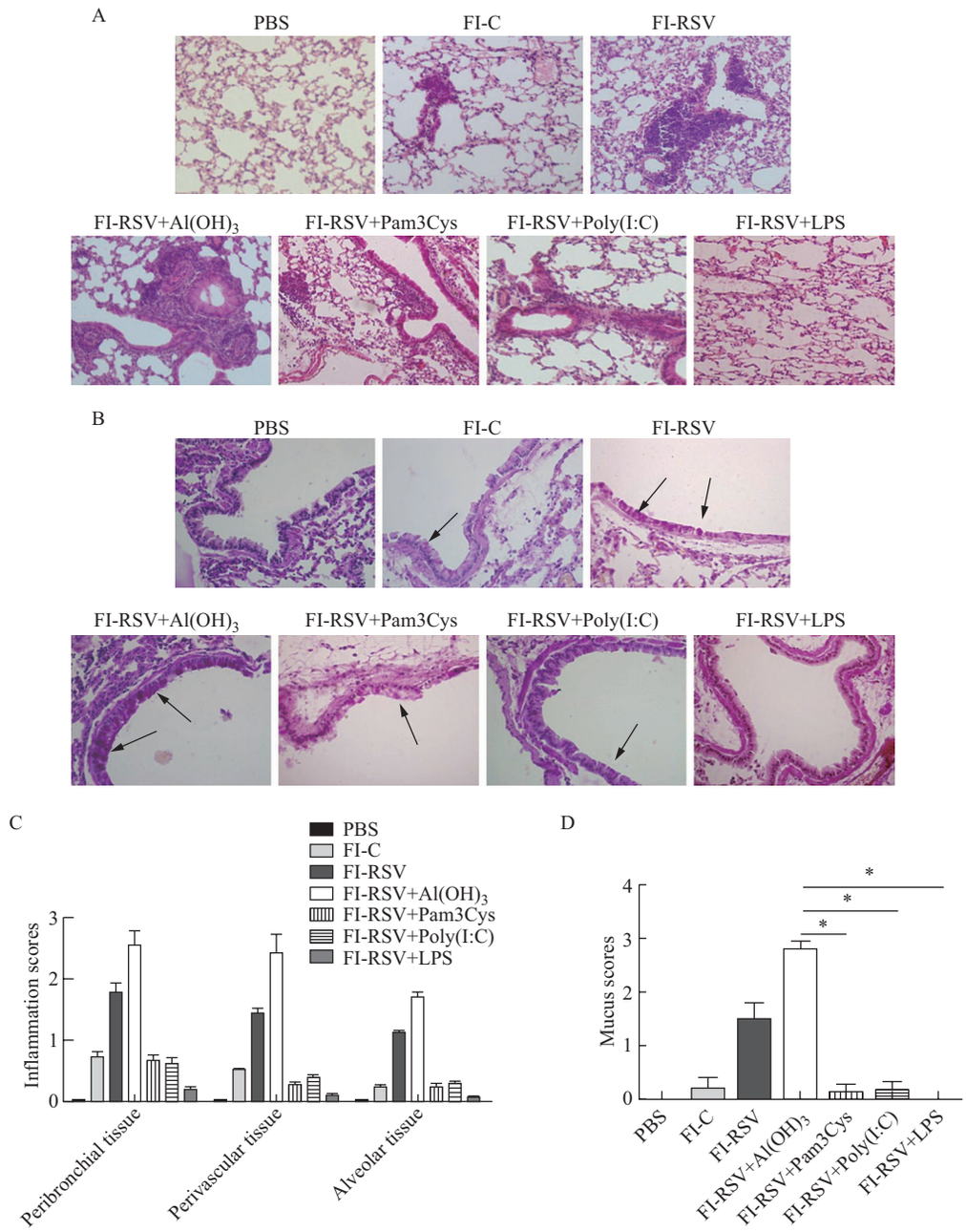

Fig. 1 LPS inhibited inflammatory cell infiltration and mucus production in lungs following RSV infection.
Mice were immunized intramuscularly two times, challenged intranasally by RSV 28 days after the last immunization, and sacrificed 5 days later for histopathology and inflammation studies. A: H&E staining shows peribronchiolar, perivascular, and interstitial pneumonia (×100); B: Periodic acid-Schiff (PAS) staining shows bronchiolar mucus production (indicated by the arrows, ×200); C: scores for pulmonary inflammation; D: scores for mucus production. Tissue sections obtained from each mouse were scored for inflammation on a 4-point-scale as described in Materials and Methods. Data are presented as mean±SD of six mice per group and are representative of two experiments. $^{*}P<0.05$ represents significant difference.

2.5 LPS Promoted Production of IgG and Opsonic IgG2a Antibodies

Since LPS inhibited FI-RSV-enhanced inflammation and Th2/Th17 responses, did LPS inhibit antibody production? We compared the IgG and IgG2a antibodies in serum between FI-RSV+LPS group and FI-RSV group. FI-RSV+LPS induced significantly higher levels of IgG and IgG2a than FI-RSV (fig. 5A and 5B, $P<0.05$). In addition, both FI-RSV+LPS and FI-RSV induced significant neutralizing antibody, compared to FI-C (fig. 5C, $P<0.05$). However, no significant difference in neutralizing antibody was observed between FI-RSV+LPS group and FI-RSV group ($P>0.05$). These result indicated that LPS

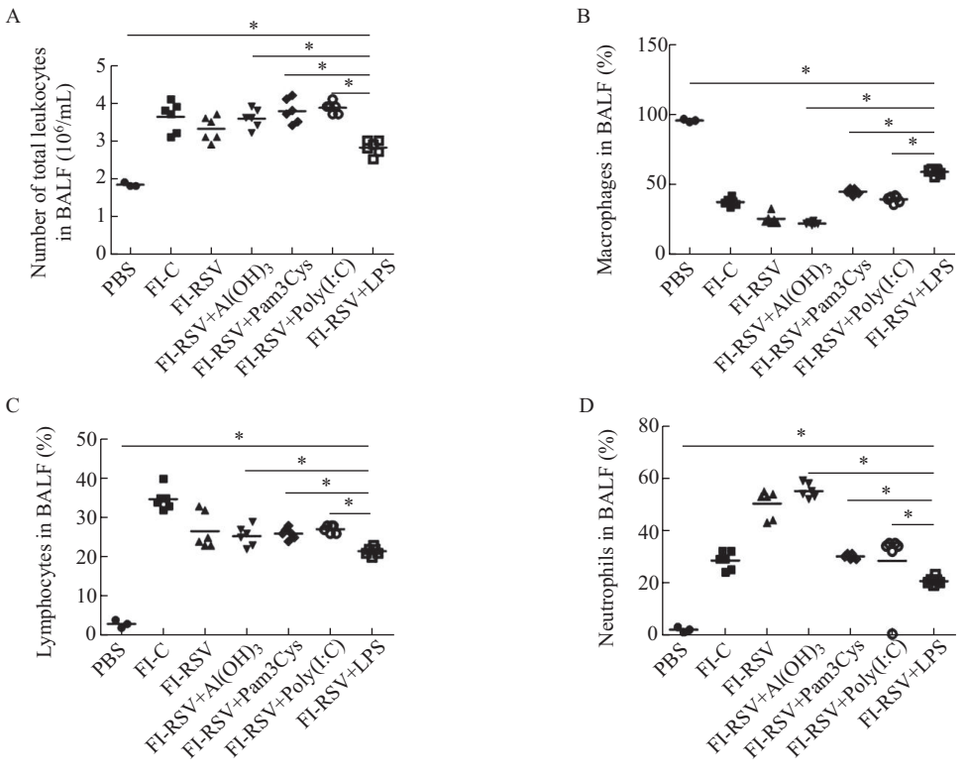

Fig. 2 LPS suppressed FI-RSV-enhanced airway inflammatory cells

BALF was collected and centrifuged. Differential and total leucocyte counts in BALF were detected by light microscopy. A: the number of total leucocytes in BALF; B to D: The percentage of inflammatory cells. Data are presented as mean±SD of six mice per group and are representative of two experiments. $^*P<0.05$ represents significant difference.

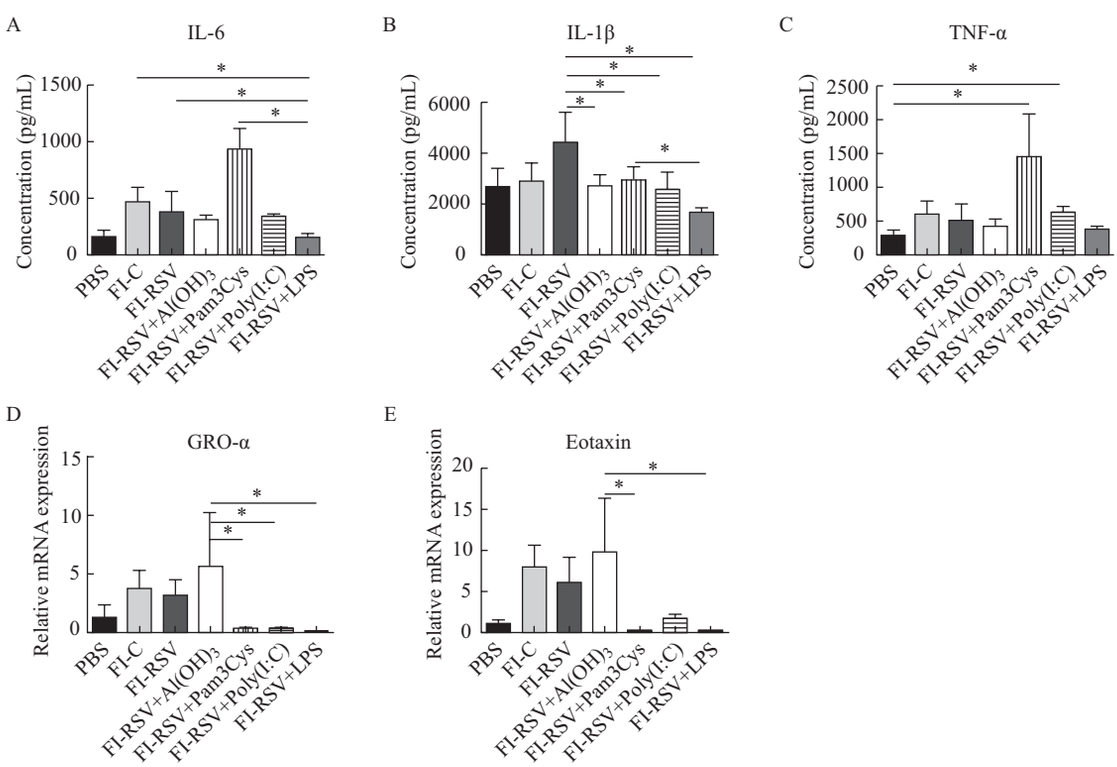

Fig. 3 LPS suppressed the inflammatory cytokines following RSV challenge

Mice were immunized intramuscularly two times, and challenged intranasally by RSV 28 days after the last immunization. The inflammatory cytokines IL-6 (A), IL-1β (B), TNF-α (C), GRO-α (D), and Eotaxin (E) in lungs were measured using ELISA and real time PCR. Data are presented as mean±SD of six mice per group and are representative of two experiments. $^*P<0.05$ represents significant difference.

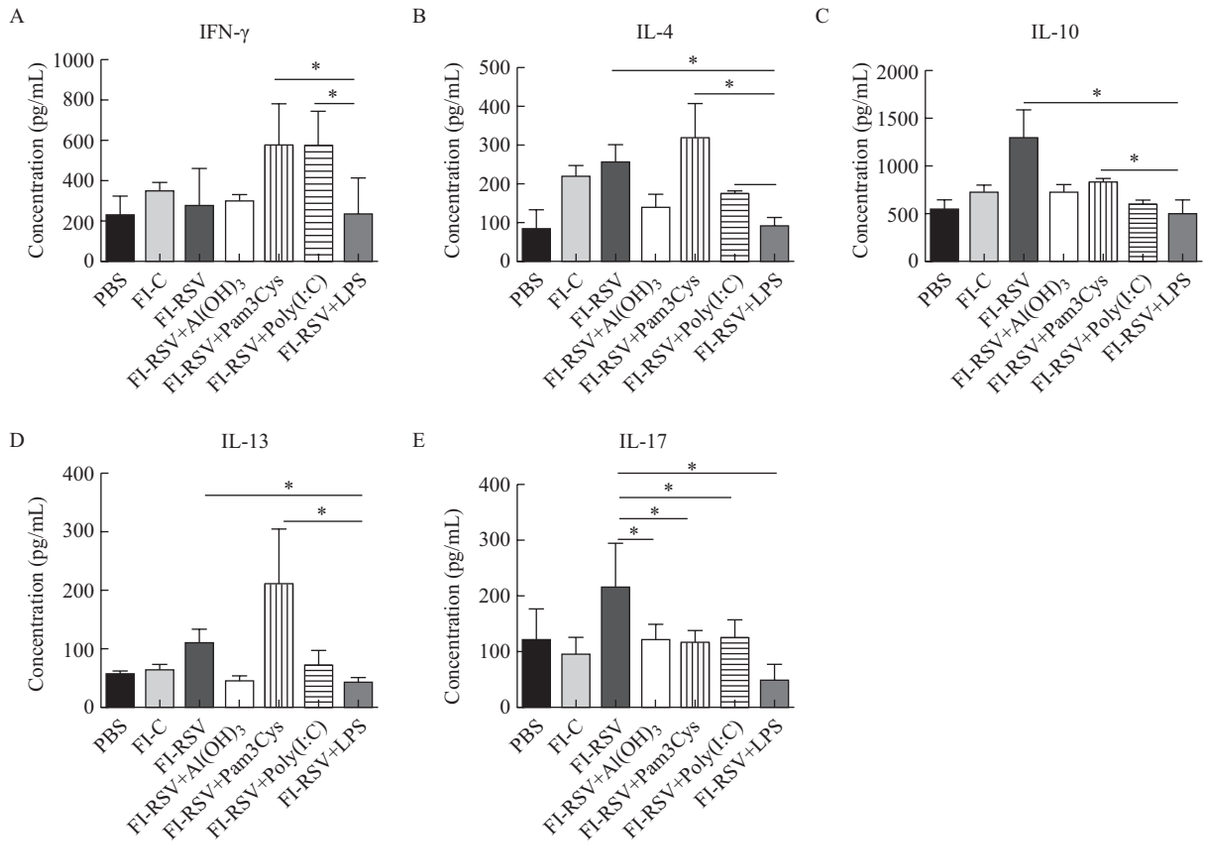

Fig. 4 LPS-immunization inhibited Th2 and Th17 responses in lungs following RSV challenge

Mice were immunized intramuscularly two times, and challenged intranasally by RSV 28 days after the last immunization. The concentrations of IFN-γ (A), IL-4 (B), IL-10 (C), IL-13 (D), and IL-17 (E) in BALF were detected by ELISA. Data are presented as mean±SD from duplicate wells of six mice per group and are representative of two experiments. $^{*}P<0.05$ represents significant difference.

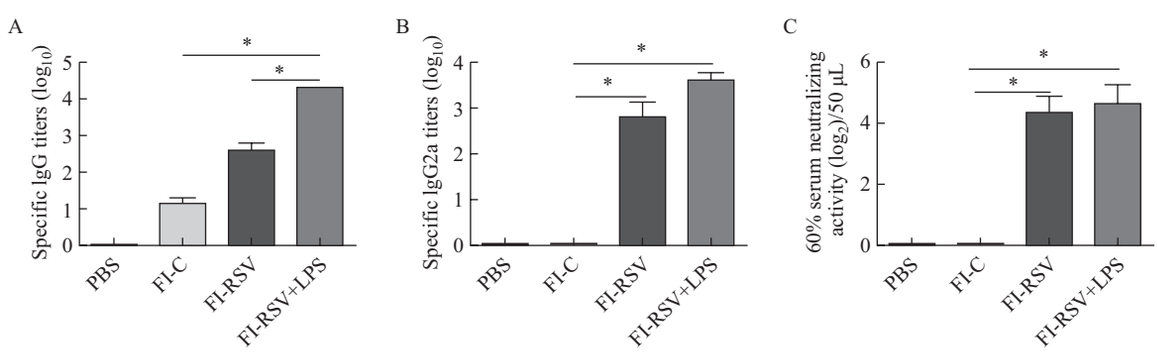

Fig. 5 LPS promoted humoral immune response induced by FI-RSV

Mice were immunized intramuscularly two times with PBS, FI-C, FI-RSV, or FI-RSV+LPS. Serum samples were collected two weeks after the last immunization. IgG (A) and IgG2a (B) antibodies were tested by ELISA. Results are expressed as geometric means of serum antibody titers. C: Serum neutralizing antibody titers against RSV obtained in a neutralization assay. Results are expressed as geometric mean titer of sera that could neutralize 60% of plaques on RSV-infected HEp-2 cells. Data are presented as mean±SD of six mice per group and are representative of two experiments. $^{*}P<0.05$ represents significant difference.

increased the production of IgG and IgG2a antibodies.

2.6 LPS Reduced RSV Load of Lung Cells in Immunized Mice

Served as the control, PBS-treated mice without RSV challenge showed no RSV-N gene expression. FI-C-treated mice showed remarkable RSV-N gene expression following RSV challenge (fig. 6). Mice immunized with FI-RSV showed significantly reduced RSV-N gene expression, compared with FI-C-treated mice ($P<0.05$). Significantly, FI-RSV+LPS reduced

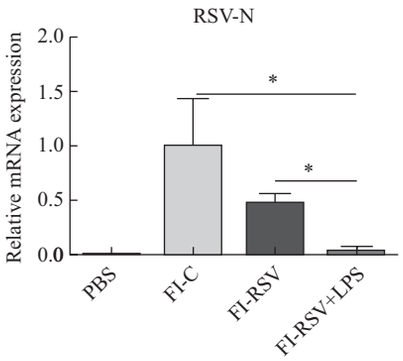

Fig. 6 Relative expression of RSV-N gene by real-time PCR
Mice were immunized intramuscularly two times as described in Materials and Methods. Mice were challenged intranasally with 50 μL 6.0×10^7 pfu/mL RSV 28 days after the final immunization. Five days later, these mice were sacrificed and lungs were removed. RSV load in lung was tested using real-time RT-PCR. The fold reduction of RSV N-gene expression in each vaccine group was calculated by comparison to that in FI-C-treated mice. Data are presented as mean±SD of six mice per group and are representative of two experiments. $^*P<0.05$ represents significant difference.

level of RSV-N gene expression, compared with FI-RSV (fig. 6, $P<0.05$). These data indicated that LPS inhibited RSV load in lung cells in FI-RSV+LPS-immunized mice.

3 DISCUSSION

LPS is a predominant, integral structural component of the outer membrane of gram-negative bacteria and one of the most potent microbial initiators of inflammation. LPS activates monocytes and macrophages to produce cytokines such as TNF-α, IL-1β, and IL-6. However, engagement of the TLR4 by LPS has been known for several decades to inhibit the function of monocytes/macrophages and dendritic cell (DC) in a process called LPS-induced tolerance[20, 21]. The effect of LPS tolerance is characterized by an abrogated release of the prototypic cytokines secreted by activated macrophages, including TNF-α, IL-1β and IL-6. Inhibitory effects of LPS were also reported when human monocytes were differentiated into DC in the presence of LPS. Those DCs were phenotypically unchanged but produced lower levels of cytokines after a second LPS stimulation[22]. In addition, this effect contributes to proliferation and function of neutrophils *in vivo*[23]. Consistently, in a current study, LPS inhibited FI-RSV-enhanced inflammatory cytokines, including IL-1β and IL-6, airway neutrophil infiltration, lung pathological changes and mucus production. Moreover, FI-RSV-enhanced Th2 and Th17 type cytokines were inhibited by LPS, which might be due to inhibition of antigen presenting function of DCs and macrophages. These results suggested a balanced and controlled immune responses induced by RSV challenge in FI-RSV+LPS-immunized mice, which will be critical for a vaccine that seeks to cause minimal harm upon natural RSV infection. Consistent with our result, an intranasal vaccine composed of LPS and enriched RSV proteins and *Neisseria meningitidis* outer membrane proteins has been demonstrated to promote balanced Th1/Th2 responses and protection against RSV without risk of enhanced pulmonary inflammation[24].

Pam3Cys is an agonist of TLR2. TLR2 activation has been reported to lead to the initiation of both Th1 and Th2 differentiation[25–27]. Mice immunized with FI-RSV+Pam3Cys following RSV challenge showed high levels of IFN-γ, IL-4 and IL-13, suggesting strong Th1 and Th2 type responses. Consistently, higher levels of proinflammatory cytokines IL-6 and TNF-α existed in airway and lung tissue of mice immunized with FI-RSV+Pam3Cys following RSV challenge. Nevertheless, FI-RSV+Pam3Cys immunization inhibited the expression of Th17 type cytokines, which could be involved in reduction of lung inflammation following RSV challenge, compared with FI-RSV. Poly(I:C), communicating with TLR3, induces type I IFNs to drive Th1 responses indirectly through DC activation[28]. Mice of FI-RSV+Poly(I:C) group showed increased Th1 cytokine IFN-γ. Poly(I:C) inhibited influx of inflammatory cells in airway and pathological changes compared with FI-RSV, suggesting that Th1 response induced by Poly(I:C) counterbalanced the Th2 response induced by FI-RSV to a certain extent.

The function of monocytes, macrophages and DCs could be inhibited in LPS tolerance[20]. However, no report showed that the function of B lymphocyte is inhibited by LPS. In this study, LPS increased the production of antibody IgG and IgG2a in serum, although LPS inhibited inflammatory cytokines and Th2 and Th17 type cytokines. High levels of IgG and IgG2a antibodies can help to kill virus-infected cells by activating complement, opsonization, and antibody-dependent cell-mediated cytotoxicity, which was consistent with reduced virus load in lungs of mice immunized with FI-RSV+LPS. In addition, LPS has no effect on neutralizing antibody induced by FI-RSV. The cause may be that the epitopes in FI-RSV that are involved in inducing neutralizing antibodies are modified by formalin, which reduces or ablates their immunogenicity[4].

In summary, the results suggest that LPS inhibited FI-RSV-enhanced inflammatory response in mice following RSV challenge without impairment in antibody production and protection, and the mechanism appears to involve regulation of Th responses induced by FI-RSV. Mechanism underlying the inhibitory effect of LPS on the vaccine-enhanced inflammation

needs further studies.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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