

Poly(U) and CpG ameliorate the unbalanced T cell immunity and pneumonia of mice with RSV vaccine-enhanced disease

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Summary

Respiratory Syncycial Virus (RSV) is the most important pathogen responsible for children's severe lower respiratory tract infection. So far no RSV vaccine has yet been authorized for clinical use. The main impediment that blocked development of RSV vaccine is that inactivated RSV vaccine could cause RSV vaccine-enhanced disease (RVED). The mechanism of RVED remains unclear. Recently some researchers found that insufficient activation of innate immunity, including Toll-like receptors (TLRs), might be associated with the onset of RVED. Based on the above findings, this research was conducted to further study the mechanism of RVED. We first vaccinated mice with formalin-inactivated RSV vaccine (FIRSV) and then exposed them to RSV to establish a RVED mouse model. Consequently, we found that mice previously inoculated with FIRSV showed obvious weight loss and extensive pneumonia, as well as T helper 2 cells (Th2)-biased immunity and suppressed CD8⁺T cell immunity after viral exposure, suggesting that we have successfully established a RVED mouse model. Then based on this model, we further added Poly(U) (TLR7/8 agonist) and CpG (TLR9 agonist) in FIRSV to see if RVED could be ameliorated. As a result, mice inoculated with FIRSV supplemented with Poly(U) and CpG had a much relieved weight loss and pneumonia, as well as suppressed Th2-biased immunity and strengthened CD8⁺T cell function. Thus, the insufficient stimulation of TLR7/8 and (or) TLR9 might play a role in the development of RVED, which could provide evidence for using TLR agonists as vaccine adjuvants to confer a protective immune response against RSV.

Keywords: Respiratory syncytial virus (RSV), RSV vaccine-enhanced disease, Th2-biased immune response, Toll-like receptor

1. Introduction

Respiratory Syncycial Virus (RSV) is the leading cause of severe lower respiratory tract infection in infants, the elderly and immune-compromised individuals (1). As has been reported, 200,000 children under 5 years old have died of RSV infection every year (2). Nonetheless, the

deleterious impact of RSV infection on pediatric health is still difficult to avoid because no RSV vaccine has yet been authorized for clinical use (3). The development of RSV vaccine has been hampered by concerns about its safety and effectiveness, given that the first RSV vaccine clinical test in the 1960s showed that children previously inoculated with formalin-inactivated RSV vaccine (FIRSV) consequently suffered with enhanced respiratory disease after subsequent RSV exposure (4,5). Since it was only seen in vaccine recipients, the above-mentioned disease was called RSV vaccine-enhanced disease (RVED).

Current findings have revealed that RVED is characterized by T helper 2 cells (Th2)-biased immunity, non-neutralizing antibodies and lung eosinophilia

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(5,6). Some researchers demonstrated that the RSV-specific antibodies elicited by inactivated RSV vaccine, including FIRSV and ultraviolet-inactivated RSV vaccine (UVRSV), had relatively low avidity and was considered nonprotective (7,8). It is without a doubt that formalin impaired the immunogenicity of virus antigens to some extent. However, the impaired immunogens were usually sufficient to confer protection in other formalin-inactivated virus vaccines, for example, influenza virus vaccine. Furthermore, it's worth mentioning that vaccine-enhanced disease only exists in RSV, measles virus and metapneumovirus, which all belong to the paramyxoviridae family (9,10). We have no idea whether some inherent properties of the paramyxoviridae family make them more likely to cause vaccine-enhanced disease, and the mechanism of RVED has not been identified yet.

Recently some researchers found that the ineffectiveness of FIRSV was associated with its insufficient stimulation of host's innate immunity (11-13). Toll-like receptors (TLRs), which are considered to be doorkeepers in recognition of exogenous pathogens, play a critical role in induction of host's innate immunity (14). In accordance with their functions, TLRs are mainly expressed inside (e.g. TLR7/8, TLR9) or on the surface (e.g. TLR2, TLR4) of antigen presenting cells (APCs). Among the ten TLRs found in humans, TLR4 is known to be the first TLR to recognize RSV F protein, while TLR7/8 and TLR9 are considered to recognize single strand RNA (ssRNA) and alien DNA of invading pathogens (10,15). MyD88 is a downstream adaptor of most TLRs, including TLR4 and TLR7/8. Recently MyD88 has been found to be important in the maturation of protective antibodies and viral control against RSV (7,16). Moreover, with the help of TLR agonists (e.g. LPS) and TLR4 knockout mice (e.g. C3H/HeJ mice), the critical role of TLR4 in the pathogenesis of RSV-associated diseases, including RVED, has been confirmed (7,12,14,17,18). Therefore, as a supplement to the above findings, we conducted this research to further study the possible function of TLR7/8 and TLR9 in the onset of RVED.

In this study, we established a classic mouse model of RVED at first and then examined whether RVED could be ameliorated by strengthened stimulation of TLR7/8 and TLR9. Here we used two widely-accepted exogenous TLR ligands, namely Poly(U) and CpG. Poly(U) is a synthetic ssRNA which can substitute for viral RNAs to stimulate TLR7/8 (19), while CpG is a synthetic oligonucleotide which can be recognized by TLR9 (20).

2. Materials and Methods

2.1. Viruses and vaccines

RSV A2 strain was kindly offered by Prof. Shibo

Jiang (Key Lab of Medical Molecular Virology of MOE/MOH in Shanghai Medical College of Fudan University) and was propagated in HEp-2 cells. RSV stocks were collected when the cytopathic effect (CPE) appeared in over 90% of the HEp-2 cells. FIRSV was made following the general procedure used to produce the vaccine used in earlier clinical trials (3). Briefly, RSV stocks of 1×10^6 plaque-forming units (PFU)/mL were incubated with formalin (1:4,000) at 37°C and condensed (1/25 of original volume) after centrifugation at 50,000×g. Then the vaccine antigens were adsorbed onto aluminum hydroxide adjuvant at a final concentration of 4mg/mL. The mixture was then centrifuged at 3,000×g and resuspended in DMED (1/4 of original volume) and stored at 4°C. For further study, we added Poly(U) (2 µg/mL) or CpG (100 µg/mL) to FIRSV in the second part of the study. FI-HEp-2 was prepared in the same way as FIRSV but substitute RSV stocks for supernatants of lysed HEp-2 cells were used.

2.2. Immunization and viral challenge

Female 6-8 weeks old BALB/c mice were chosen to conduct the research. The animal study was approved by the institutional review board at Children's Hospital of Fudan University.

In the establishment of the RVED mouse model, mice were divided into 3 groups, namely, group FV, group VV and group BV. On day 1 and day 14, mice of group FV were intramuscularly inoculated with 50 µL FIRSV, while mice of group VV were intranasally infected with 50 µL live RSV (1×10^6 PFU/mL) and mice of group BV were intramuscularly innoculated with 50 µL FI-HEp-2. For the TLR-associated experiments in our second part, group Poly(U) and group CpG were added for a total of five groups. On day 1 and day 14, mice of group Poly(U) and group CpG were intramuscularly inoculated with FIRSV supplemented with Poly(U) and CpG respectively. On day 28, mice of all groups were challenged with live RSV (1×10^6 PFU/mL) intranasally and the daily body weights of all mice were recorded from that time. All mice were sacrificed on day 32 (4th day after challenge).

2.3. Pulmonary histopathology

The lung lobes were removed on the 4th day after challenge, fixed in 4% formalin and then embedded in paraffin. The fixed lung tissues were sliced into 4 µm slides and stained with hematoxylin and eosin (H&E). Pulmonary pathology was observed with light microscopy.

2.4. T cell immunity in splenocytes

Spleens were isolated aseptically and ground tenderly through a 75 µm nylon mesh, then erythrocytes were

lysed to get mononuclear cell suspensions. The cells were stimulated by PMA and Ionomycin at 37°C for 1 hour. Golgi-stop was added for the next 2 hours in order to withhold the secreted cytokines. Afterwards the cells were stained with fluorescent antibodies and assayed using a BD LSR Fortessa™ cell analyzer.

2.5. Quantification of RSV RNA in lungs

On the 4th day after viral challenge, lungs were isolated aseptically and ground thoroughly to get lung homogenates. Total lung RNA was extracted by TRIzol according to the manufacturer's instructions. RT-PCR was conducted with random primers, and quantitative real-time PCR (qPCR) was performed to detect levels of RSV F protein using the primers RSV-F1 (3'-CAARTCAAYATTGAGATAGAATCTA GAA-5'), RSV-F2 (3'-GCTATACAYARTATTATCATCCCACA-5') and the probe (3'-FAM-CTCCAGAATAYAGGCAT GATTCTCC-BHQ-5')

2.6. Statistical analysis

Data were analyzed with statistical software (STATA). Comparisons were made using ANOVA and *t*-test. *p* values < 0.05 were considered statistically significant.

3. Results

3.1. FIRSV recipients developed enhanced disease after RSV challenge

After RSV challenge, mice of group FV showed greater weight loss and slower rehabilitation than mice of group VV and group BV (Figure 1). Pneumonia of different severity existed in mice of all groups, but only mice of group FV suffered with extensive perivascular and peribronchiolar lymphocytic pneumonia as well as lung eosinophilia. The eosinophils dissipated in the inflammatory area are identified by arrows (Figure 2). In contrast, mice of group VV and group BV didn't show eosinophilic infiltration in lungs. Real-time PCR showed that lung RSV load of mice in group FV was much lower than that of group VV and group BV, and mice of group VV showed lower RSV load than that of group BV (Figure 3).

3.2. Mice of group FV showed aberrant T cell immune response

Flow cytometry analysis of splenocytes showed that the proportion of CD4⁺T cells in mice of group FV was highest among the three groups (Figure 4A), as were the levels of IL-4, IL-13 and IL-4/IFN- γ ratio in CD4⁺T cells of group FV (Figure 4B and Figure 4C). No statistical difference was found in the secretion of IFN- γ in CD4⁺T cells. However, the levels of TNF- α

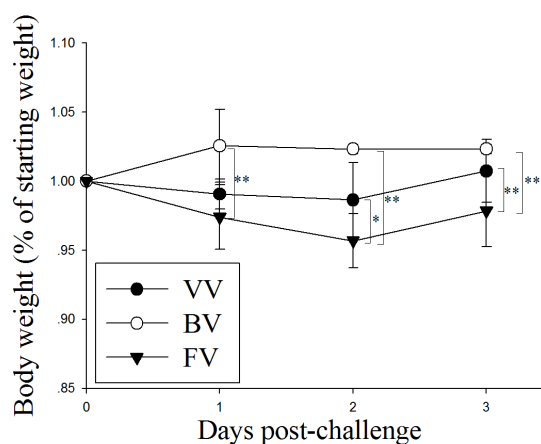


Figure 1. Body weight changes of mice in group FV, group VV and group BV after RSV challenge. On day 1 and day 14, mice of group FV and group BV were intramuscularly inoculated with 50 μ L FIRSV and 50 μ L FI-HEp-2 respectively, while mice of group VV were intranasally infected with 50 μ L live RSV (1×10^6 PFU/mL). On day 28, mice of all groups were challenged with live RSV (1×10^6 PFU/mL) intranasally and the daily body weights of all mice were recorded from that time. (* $p < 0.05$, ** $p < 0.001$)

and IFN- γ in CD8⁺T cells in mice of group FV were much lower than that of group VV. Granzyme B in CD8⁺T cells was too low to be detected with significant differences (Figure 4D).

3.3. The performances of RVED were ameliorated by the admixture of FIRSV and TLR agonists

Similar to the results we mentioned previously, mice of group FV still suffered with the largest weight loss and the most severe pneumonia after RSV exposure. However, mice of group Poly(U) and group CpG showed milder weight loss and pneumonia than that of group FV (Figure 5 and Figure 6). Lung RSV loads in mice of group FV, group Poly(U) and group CpG, although no significant differences were found among the three groups, were much lower than that of group VV and group BV (Figure 7).

3.4. The supplementation of Poly(U) and CpG in FIRSV relieved the aberrant T cell immunity of RVED

As we expected, mice of group FV had polarized Th2 immune response and dampened CD8⁺T cell function, as well as elevated IL-17 secretion. Compared with group FV, the proportions of CD4⁺T cells in mice of group Poly(U) and group CpG were much higher (Figure 8A), while the levels of IL-4 and IL-13 in CD4⁺T cells were much lower (Figure 8B). In group CpG, the secretion of IL-17 in CD4⁺T cells was inhibited compared with that of group FV (Figure 8B). IL-4/IFN- γ ratio in CD4⁺T cells in mice of group Poly(U) and group CpG were also lowered as a concomitant of decreased IL-4 (Figure 8C). The levels

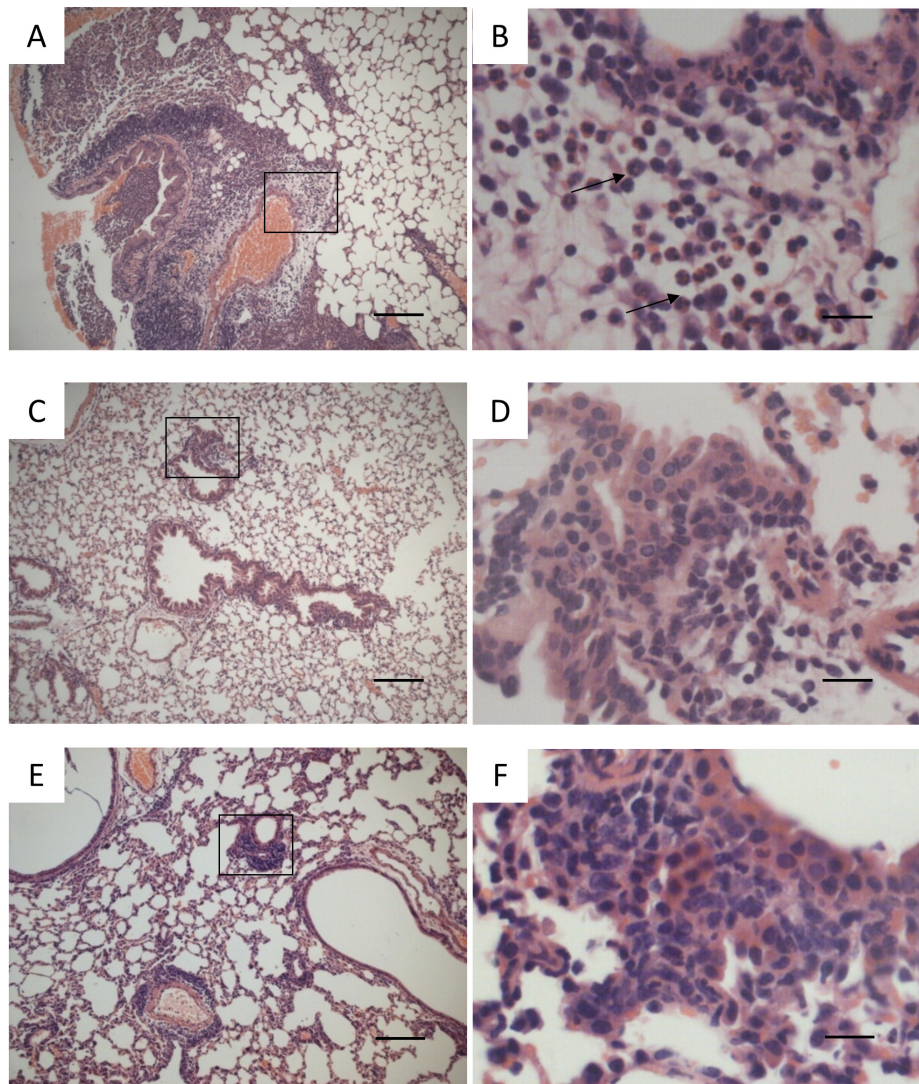


Figure 2. Pulmonary pathology of mice on the 4th day after RSV challenge. On the 4th day after challenge, lungs of mice were isolated and stained with hematoxylin and eosin (H&E). Pulmonary pathology was observed using light microscopy at an original magnification of 100 \times and 400 \times . The figure shows the H&E staining of lung tissue in mice of group FV (A), group VV (C) and group BV (E) at an original magnification of 100 \times , as well as the 400 \times magnification of the selected area in A (B), C (D) and E (F). Scale bar in A, C and E: 100 μ m. Scale bar in panel B, D and F: 25 μ m.

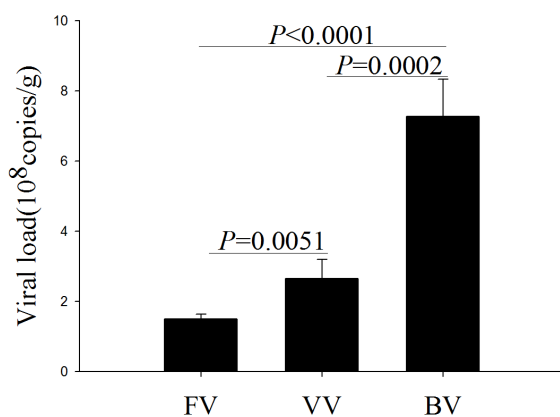


Figure 3. Lung RSV loads in mice of group FV, group VV and group BV on the 4th day after RSV challenge. On the 4th day after viral challenge, lungs of mice were isolated aseptically and ground thoroughly to obtain lung homogenates. Total lung RNA was extracted by TRIzol and then RT-qPCR was conducted to detect the level of RSV in lungs.

of both IFN- γ and TNF- α in CD8⁺T cells in mice of group CpG were upregulated compared to group FV, while for group Poly(U), only TNF- α was obviously elevated (Figure 8D).

4. Discussion

RVED is the main impediment that hampered the development of a RSV vaccine. Therefore the study of the mechanism of RVED is of paramount importance to the prevention and control of RSV infection. In this study, mice of group FV showed largest weight loss, extensive pneumonia and lung eosinophilia after subsequent viral exposure, which highly resembled the performance of children's RVED in the 1960s (21,22). In addition, mice of group FV also demonstrated Th2-biased immunity and CD8⁺T cell dysfunction compared with that of group VV, which were supposed

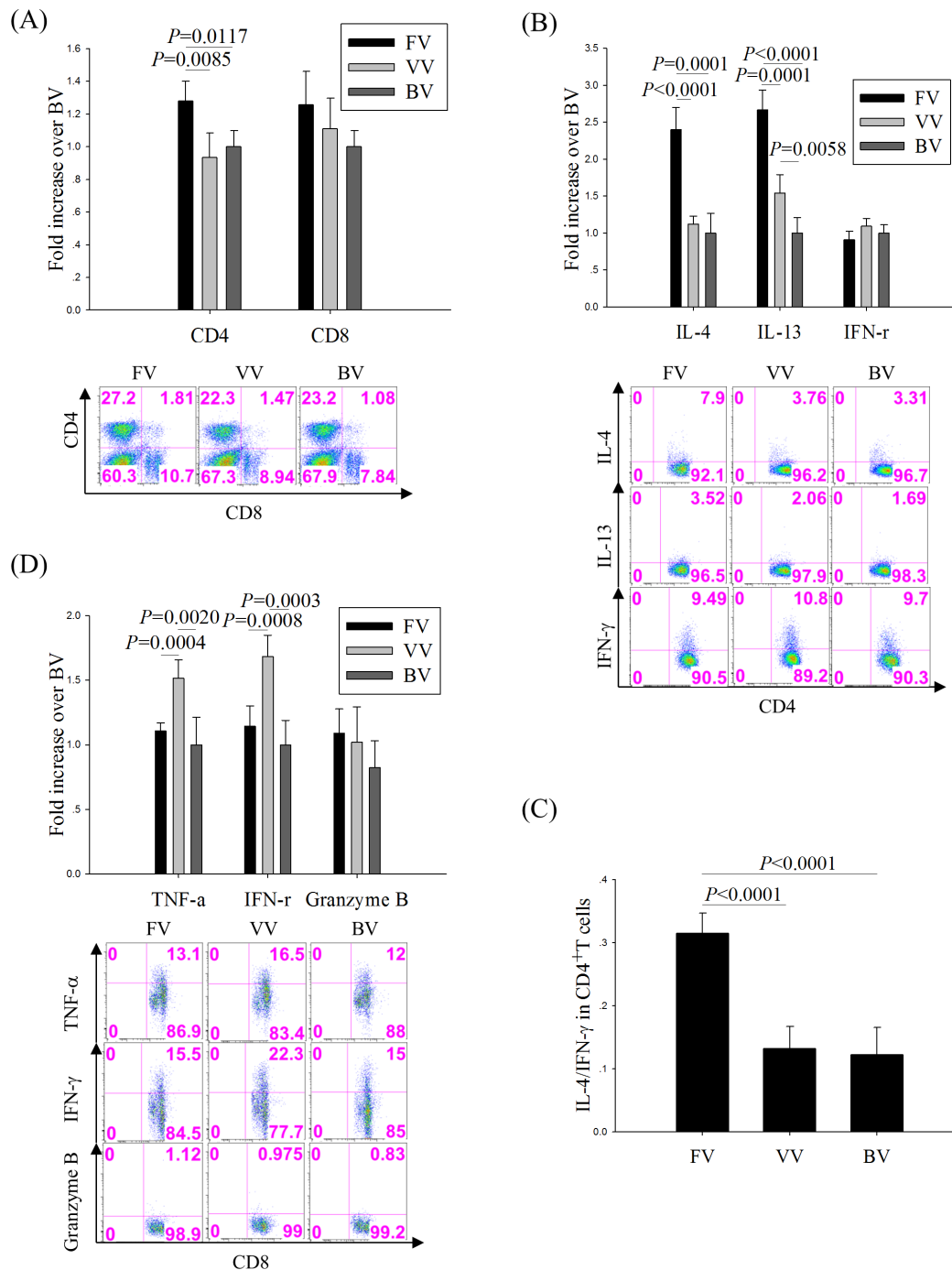


Figure 4. T cell immune responses in mice of three groups. On the 4th day after challenge, spleens of mice were isolated aseptically and ground tenderly to get a cell suspension. The cells were then stained with different fluorescent antibodies for flow cytometry analysis. **(A)** Percentage of CD4⁺T cells and CD8⁺T cells in splenocytes. **(B)** Levels of IL-4, IL-13 and IFN- γ in CD4⁺T lymphocytes. **(C)** Ratio of IL-4/IFN- γ in CD4⁺T lymphocytes. **(D)** Levels of TNF- α , IFN- γ and Granzyme B in CD8⁺T lymphocytes.

to generate relatively effective immunological memory against RSV after their primary infection. Thus we have successfully established a RVED mouse model. Furthermore, we found that FIRSV supplemented with Poly(U) and CpG could efficiently ameliorate the overall condition of RVED mice, suggesting that the insufficient activation of TLR7/8 and TLR9 might play a role in the pathogenesis of RVED.

While other researchers mainly studied the

ineffective humoral immunity of FIRSV recipients (7,23), we highlighted the aberrant cellular immunity. With the help of flow cytometry, we were able to examine the secretions of the representative cytokines in different subtypes of T cells with more sensitivity and accuracy. T helper cells (Th) could promote the proliferation and differentiation of virus-specific B lymphocytes as well as the production of specific antibodies, and thus play a critical role in the

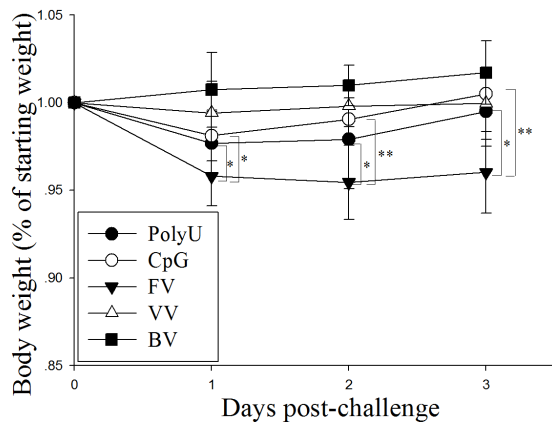


Figure 5. Body weight changes in mice of five groups after RSV challenge. On day 1 and day 14, mice of group Poly(U) and group CpG were intramuscularly inoculated with 50 μ L FIRSV supplemented with Poly(U) or CpG respectively. Mice of other groups were treated in the same way as the first part. On day 28, mice of all groups were challenged with live RSV (1×10^6 PFU/mL) intranasally and the daily body weights of all mice were recorded from that time. (* $p < 0.05$, ** $p < 0.001$)

activation of anti-virus immunity (24,25). After antigen stimulation, primary $CD4^+$ T lymphocytes differentiate into Th0 lymphocytes, which then differentiate into Th1, Th2 and Th17 lymphocytes. As one of the most important Th2 cytokines, IL-4 could inhibit Th0 lymphocytes' differentiation into Th1 lymphocytes and promote reconstruction of the respiratory tract and development of pneumonia (26). Besides, IL-17 was reported to increase airway mucus by upregulating expression of the *MUC5B* gene and cause airway hypersensitiveness (13,27). As the main source of IL-17, Th17 lymphocytes could also work synergistically with Th2 lymphocytes and inhibit the cytotoxic effect of $CD8^+$ T cells, which was consistent with the increased levels of Th2 and Th17 cytokines as well as the suppressed Th1 and $CD8^+$ T cell cytokines of RVED mice in our data (28). Therefore it's reasonable to think that Th2 cells and Th17 cells might be the main promoters in development of RVED.

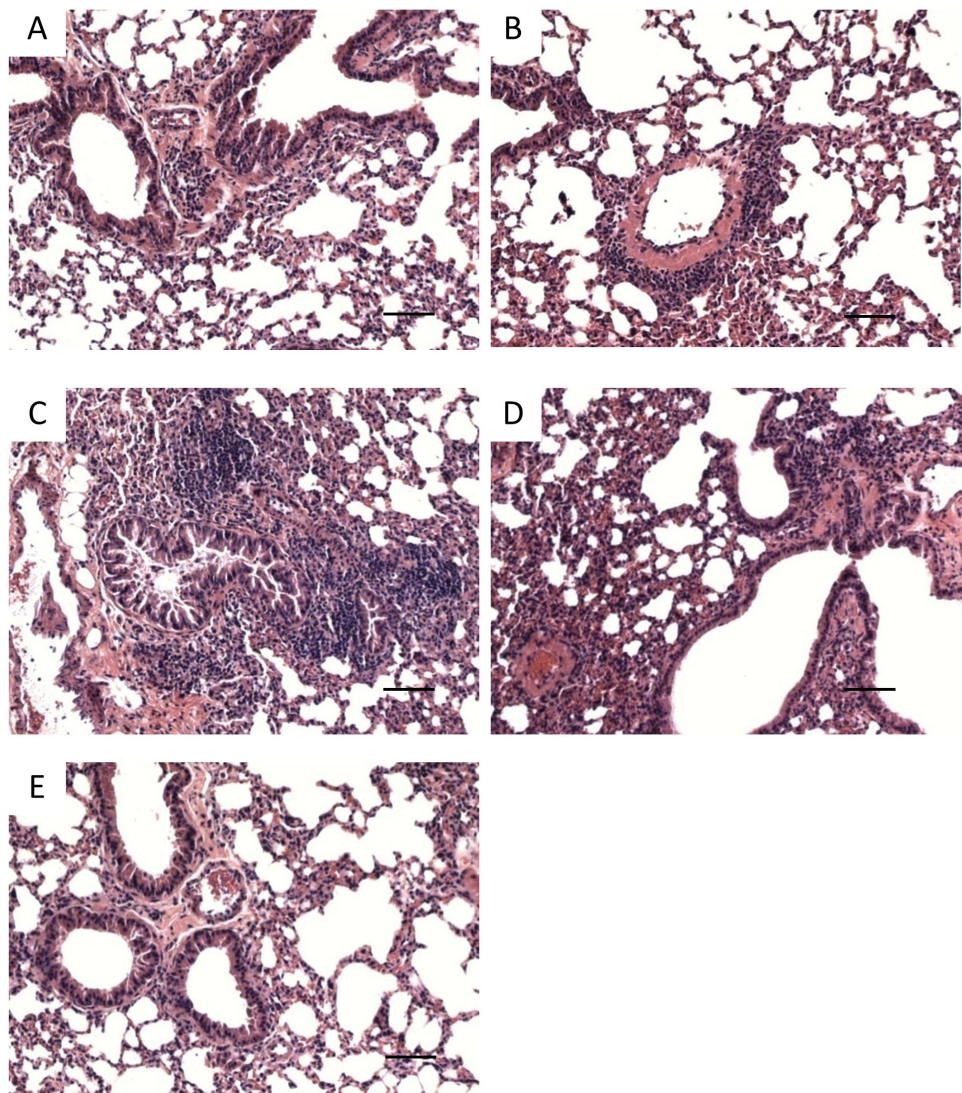


Figure 6. Pulmonary pathology of mice on the 4th day after RSV challenge. On 4th day after challenge, mice of all groups were sacrificed and their lungs were isolated and stained with (H&E). The figure shows the H&E staining photos of group Poly(U) (A), group CpG (B), group FV (C), group VV (D) and group BV (E) at an original magnification of 100 \times . Scale bar, 100 μ m.

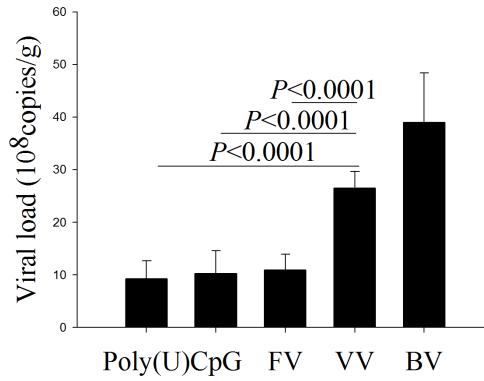


Figure 7. Lung RSV loads in mice of five groups on the 4th day after RSV challenge. On the 4th day after viral challenge, total lung RNA was extracted from lung tissue as described before and RT-qPCR was conducted to detect the level of RSV in mice of each group.

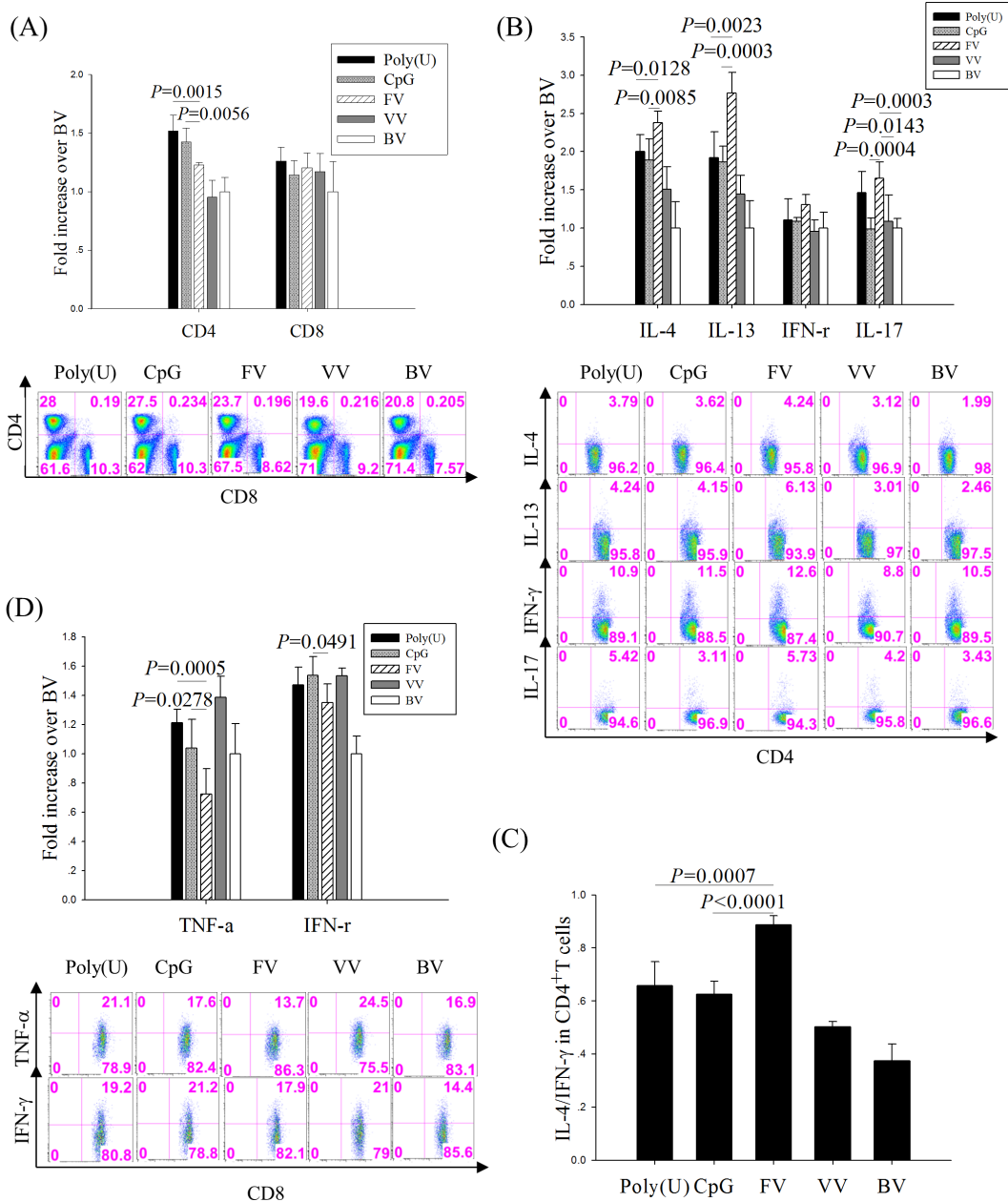


Figure 8. T cell immune responses in mice of five groups on the 4th day after RSV challenge. On the 4th day after challenge, spleens of mice were isolated for flow cytometry analysis. (A) Percentage of CD4⁺ T cells and CD8⁺ T cells in splenocytes. (B) Levels of IL-4, IL-13, IFN-γ and IL-17 in CD4⁺ T cells. (C) Ratio of IL-4/ IFN-γ in CD4⁺ T cells. (D) Levels of TNF-α and IFN-γ in CD8⁺ T cells.

Lung eosinophilia is one of the main characteristics of RVED (29). Some researchers found that deficient Th2 cytokines, especially IL-13, could significantly reduce the recruitment and activation of eosinophils (30), suggesting that eosinophilic accumulation in airway epithelium might be associated with overproduced Th2 cytokines. As the most important cells responsible for asthma and other allergic diseases, eosinophils could not only impair the airway epithelium by releasing inflammatory mediators and cause airway hyperresponsiveness (31), but also help with the RSV clearance and apoptosis of virus-infected cells by upregulating production of IFN- α/β and nitric oxide (32). In our data, RSV loads in mice of group FV were obviously lower than that of the other groups, which was beyond our expectations. Since RVED mice had been proved to develop nonprotective anti-RSV immunity (7), we tend to attribute the high RSV clearance in RVED mice to the large amount of eosinophils in their lungs. However, further studies on the relationship between eosinophilia and RSV clearance are needed to confirm our assumption.

As a well-known TLR9 agonist, CpG has been proved to promote the activation of Th1 lymphocytes and CD8⁺T lymphocytes (33). Our data demonstrated that the overall conditions of RVED mice including symptoms and aberrant T cell immunity were finely ameliorated by the admixture of FIRSV and CpG. In our study, Poly(U) had a similar impact on RVED mice as CpG except for its stronger effect on TNF- α and weaker effect on IFN- γ and IL-17. However, Johnson *et al.* found that the pneumonia and Th2 cytokines could be inhibited when FIRSV was assisted by both R848 (TLR7/8 agonist) and CpG or by CpG only, while FIRSV assisted by only R848 didn't have a similar effect, which differs from our results (34). We thought that this might be associated with the inherent properties of the two different TLR7/8 ligands. R848 is a synthetic guanine (G) analogue of low molecular weight (35), while Poly(U) is a single-stranded polyuridylic acid. Florian *et al.* compared the uridine (U)-rich ssRNA, G-rich ssRNA and GU-rich ssRNA and concluded that U-rich ssRNA and GU-rich ssRNA had a more prominent effect on TLR7/8 than G-rich ssRNA, thus U-rich ssRNA and GU-rich ssRNA were more suitable for vaccine adjuvants and immune therapy (19). Therefore, the different base sequences endowed R848 and Poly(U) with different immunostimulatory effects on TLR7/8, which could be the explanation for the discrepancy between Johnson's results and ours.

Noticeably, although Poly(U) and CpG added in FIRSV remarkably improved the overall conditions of RVED, the disease still couldn't be completely avoided in this way. As our data showed, weight loss and pneumonia in mice of group Poly(U) and group CpG were still more severe than that of group FV, and Th2 cytokines in mice of Poly(U) and CpG, although much

lower than that of group FV, were still dominant in cellular immunity. Therefore, further studies are needed to figure out whether the combination of Poly(U) and CpG or other TLR agonists as adjuvants of FIRSV could generate a more protective and powerful immune response in RVED mice.

In conclusion, we established a RVED mouse model with Th2-biased immune response and impaired CD8⁺T cell function, and then based on the mouse model, we found that Poly(U) and CpG could effectively ameliorate the unbalanced T cell immunity as well as the pneumonia in RVED mice, which supports the idea of using exogenous ligands of TLRs as vaccine adjuvants to generate a protective immune response against RSV.

Acknowledgements

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