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Nebulized *Mycobacterium vaccae* protects against asthma by attenuating the imbalance of IRF4/IRF8 expression in dendritic cells

Qi-Xiang Sun, Si-Yue Xu, Lao-Dong Li, Huan Xiao, Qian-Nan Zhang, Chao-Qian Li✉

Department of Respiratory Medicine, The First Affiliated Hospital of Guangxi Medical University, Nanning, China

ABSTRACT

Objective: To assess the effects of nebulized inhaled *Mycobacterium vaccae* on allergic airway inflammation, airway hyperresponsiveness, and Th1/Th2 cell imbalance in mice with ovalbumin (OVA)-induced asthma.

Methods: Mice received OVA sensitization and challenge for establishment of the asthmatic model. For intervention, mice received *Mycobacterium vaccae* nebulization once every other day from the first day of sensitization to the day before challenge. After challenge, pulmonary histological analysis and airway responsiveness measurement were performed. In addition, Th1/Th2 cytokines and OVA-specific IgE levels in bronchoalveolar lavage fluid were measured by ELISA. Th1/Th2 subset ratios and the expression of interferon-regulatory factor 4 (IRF4), IRF8 and Toll-like receptor 4 (TLR4) in dendritic cells were evaluated by flow cytometry.

Results: Severe inflammatory infiltration and airway hyperresponsiveness were observed in OVA-induced asthmatic mice. Asthmatic mice showed higher Th2 cytokine concentration and increased percentage of Th2 cells, along with lower Th1 cytokine concentration and reduced percentage of Th1 cells compared with the normal control. Moreover, an imbalance of $IRF4^+$ and $IRF8^+$ in dendritic cells was found in asthmatic mice. Nebulized inhaled *Mycobacterium vaccae* reduced airway hyperresponsiveness and inflammation in OVA-induced asthmatic mice. In addition, nebulized inhaled *Mycobacterium vaccae* enhanced TLR4 and IRF8 expression, and alleviated the imbalance of Th1/Th2 as well as $IRF4^+$ and $IRF8^+$ in dendritic cells.

Conclusions: Nebulized inhaled *Mycobacterium vaccae* protects against asthma by alleviating the imbalance of Th1/Th2 and IRF4/IRF8 in OVA-induced asthmatic mice.

KEYWORDS: *Mycobacterium vaccae*; Asthma; Nebulization; IRF4; IRF8; TLR4

1. Introduction

Owing to the strong evidence of their effectiveness in reducing airway inflammation, inhaled corticosteroids (ICS) have long been the cornerstone treatment of asthma[1]. Long-term ICS treatment, on the other hand, may cause systemic and local side effects[2]. An estimated 5%-10% of patients with severe asthma remain uncontrolled by using conventional therapy, often requiring high-dose ICS, and/or systemic glucocorticoids[3]. Monoclonal antibodies targeting immunoglobulin E (IgE), interleukin (IL)-4, IL-13, and IL-5 have been shown to significantly reduce exacerbations in severe asthmatic patients[4]. However, access to these targeted therapeutics remains extremely limited in some areas. To address these global

Significance

Previous studies revealed that immunization with mycobacterial preparations can protect against asthma. Nebulized inhalation delivers drugs directly into the airways, thus providing a topical effect. It is unclear how nebulized inhaled *Mycobacterium vaccae* modulates airway immunity. The present study shows that nebulized inhaled *Mycobacterium vaccae* can protect against asthma by alleviating Th1/Th2 imbalance and modulating dendritic cell subsets. Nebulized inhaled *Mycobacterium vaccae* can be further explored as a potential asthma treatment.

✉To whom correspondence may be addressed. E-mail: lichaoqiangood@163.com

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health challenges, we still require more effective and affordable treatment options.

According to the hygiene hypothesis, early childhood exposure to a variety of environmental microorganisms can help to limit the development of asthma and other atopic diseases[5]. Numerous studies have reported that immunization with mycobacterial preparations can prevent or downregulate asthmatic airway hyperresponsiveness and eosinophilic airway inflammation by modulating effector T-cell responses[6–8]. Although it has been proved that live whole-cell mycobacteria, particularly Bacillus Calmette-Guérin (BCG), have a protective effect against the development of asthma, their use in medical practice may be limited due to the adverse reactions of the BCG[9,10]. Our previous studies have shown that inhaled inactivated *Mycobacterium phlei*, to a certain extent, improves asthma symptoms, and reduces the need for rescue medication and acute exacerbation of asthma[11].

Asthma is traditionally defined as an airway inflammatory disease associated with a Th2 immune response[12]. Mouse pulmonary differentiated conventional dendritic cells (cDCs) are characterized by high expression of the surface markers MHC-Ⅱ and CD11c, which are divided into two types, type 1 cDCs (cDC1s) and type 2 cDCs (cDC2s)[13,14]. In response to inhaled antigens, cDCs are essential for promoting the activation and differentiation of effector $CD4^+$ T cells[15]. Studies have demonstrated that cDC1s prime Th1 cell responses and cDC2s are the primary inducers of Th2 cells in response[16]. Furthermore, transcriptional regulation of the cDC1s and cDC2s lineage development has been shown to depend on the expression of interferon-regulatory factor 8 (IRF8) and interferon-regulatory factor 4 (IRF4), respectively[16]. Toll-like receptors (TLRs) are a member of pattern-recognition receptors expressed by DCs, macrophages, and mucosal epithelial cells and recognize pathogens to initiate immune responses[17]. IRF is the major downstream signaling molecule of the TLR4 pathway[18].

Mycobacterium vaccae (*M. vaccae*) is a non-pathogenic mycobacterium that is commonly used as a vaccine and for tuberculosis[19,20]. Nebulized inhalation delivers drugs directly into the airways, providing a topical effect while lowering the risk of systemic side effects and the medication cost[21]. The respiratory tract has a highly developed immune system connected to lymphatics and blood circulation[22]. It is unclear how nebulized inhaled *M. vaccae* modulates airway immunity. Thus, we hypothesized that nebulized inhaled *M. vaccae* could protect against asthma by attenuating Th1/Th2 imbalance as well as regulating the expression of IRF4, IRF8, and TLR4 in cDCs. In this study, we aimed to evaluate the preventive effects of nebulized inhaled *M. vaccae* against allergic airway inflammation and airway hyperresponsiveness and on the Th1/Th2 imbalance in a mouse asthma model.

2. Materials and methods

2.1. Animal model establishment

All mice were purchased from TianQin Biotechnology Company in Changsha and reared in a specific pathogen-free environment [temperature: (23±2)℃, humidity: (55.5±10.0)%, light cycle: 12/12 h], with free access to water. Male Balb/c mice (4-6 weeks old) were divided into four groups (n=6): the normal control group (NC); the OVA-induced asthma group; the group treated with nebulized inhaled *M. vaccae* in the process of OVA sensitization (Neb/OVA); and the group treated with nebulized inhaled *M. vaccae* in the process of phosphate buffered saline (PBS) sensitization (Neb). The experiments were performed using a mouse model of asthma induced by OVA as previously described[23,24]. The OVA and the Neb/OVA groups received OVA sensitization *via* intraperitoneal injection with a 200 μL mixture of 40 μg OVA (Sigma-Aldrich) and 100 μL adjuvant aluminium hydroxide (ThermoFisher Scientific) on days 0, 7, and 14. From days 21 to 25, as for the OVA challenge, the mice received 1% OVA (in PBS) nebulization in a closed chamber (20 cm×30 cm×20 cm) for 30 min per day for five consecutive days, while the NC and the Neb groups received PBS only. The Neb and the Neb/OVA groups were nebulized using 22.5 μg *M. vaccae* (Anhui Longkema Biological Pharmaceutical Co., Anhui, China) dissolved in 5 mL of PBS once every other day from the first day of sensitization to the day before challenge (day 0-20), while the NC and the OVA groups were nebulized with PBS. Figure 1 depicts a timeline of the protocol for establishing an animal model.

2.2. Measurement of airway responsiveness

The airway responsiveness to nebulized methacholine chloride (MCh, Sigma) was assessed by the Buxco Finepoint NAM System (FinePointe™ NAM system TBL4500, Buxco) in 24 h after the last challenge in mice. Based on previous experiments[25], mice were immobilized in the chamber for brief acclimatization, and then the mice initially received a challenge with nebulized PBS (as the 0 mg/mL MCh), followed by the increasing doses (6.25, 12.5, and 25 mg/mL) of nebulized MCh. Each mouse received the PBS or MCh nebulization for 30 s and the specific airway resistance (sRaw) parameters were measured continuously for 3 min after nebulization, the mice were then given 4 min to recover after the measurement of each dose. Airway responsiveness was determined by sRaw. sRaw=airway resistance×thoracic gas volume=(pressure/flow)×volume, units: cmH_2O/s.

2.3. Specimen acquisition

The mice were anesthetized using pentobarbital (60 mg/kg,

intraperitoneally). The sternum was cut off to expose the trachea, the left main bronchus was ligated for histological analysis, the lungs were lavaged with 500 μL ice-cold PBS three times, and the bronchoalveolar lavage fluid (BALF) was obtained for cell count and cytokine analysis. The left ligated lobe was fixed in formalin for histological evaluation, and all other lobes were used for flow cytometric analysis.

2.4. BALF cell count and analysis

The BALF was centrifuged at 4 ℃ by 300 ×*g* for 5 min. The supernatant was then collected and the cell pellet was resuspended with 50 μL PBS. Subsequently, 10 μL cell suspension was used for cell counting on a cell counting plate. Then 30 μL cell suspension was used for Wright's staining, and 100 inflammatory cells were classified under optical microscopy according to the previous report[26].

2.5. Pulmonary histopathological analysis

The lung tissues were immersed in 10% neutral formaldehyde for 24 h. After alcohol gradient dehydration, and xylene treatment, the tissue was embedded in paraffin. Paraffin tissue was sectioned at 5 μm thickness. Hematoxylin-eosin (H&E) staining and periodic acid Schiff (PAS) staining were conducted to assess inflammatory cell infiltration into the lung tissues and the mucus secretion of the airway epithelium. Sections were photographed using Olympus BX53/DP80 Microscope. The inflammatory cell infiltration was scored as follows: 0 (absence of inflammatory cells), 1 (rare infiltration), 2 (only in a focal area), 3 (<4 lines of cells), and 4 (≥4 lines of cells)[27]. Image-Pro Plus software was used to calculate the percentage of mucus-filled (PAS^+) cells in epithelial areas. The analysis was semi-quantitatively scored as follows: 0 (mucus-filling absent), 1 (mucus-filling<20%), 2 (mucus filling 20%-40%), 3 (mucus filling 40%-70%), and 4 (mucus filling>70%)[28]. For each mouse, five fields were scored per section in a blinded manner, and the average inflammation score and the average PAS^+ score were calculated.

2.6. Cytokine detection

Concentrations of interferon (IFN)-γ, IL-4, IL-5, and OVA-specific IgE in BALF were detected by ELISA kits (CUSABIO) according to the ELISA kits' instructions. The absorbance was quantified by using a BioTek ELX800 Microplate reader, and absorbance was measured at 450 nm.

2.7. Flow cytometric analysis

The lungs were minced to about 1 mm^3 piece and digested for 30 min on a shaker at 200 rpm and 37 ℃ with 3 mL RPMI 1640 medium containing 5 mg/mL type Ⅳ collagenase (Sigma). The digested lung tissue was then dissociated into cell suspension with the gentleMACS™ C tube and the gentleMACS™ dissociator. The cell suspension was passed through a 70-μm cell strainer. The cells were resuspended in red blood cell lysate and placed in the dark for 2-3 min. The cell suspension was washed with PBS to obtain the single-cell suspension. The cells were then stained with the anti-Mouse CD16/CD32 (Mouse BD Fc Block) before staining

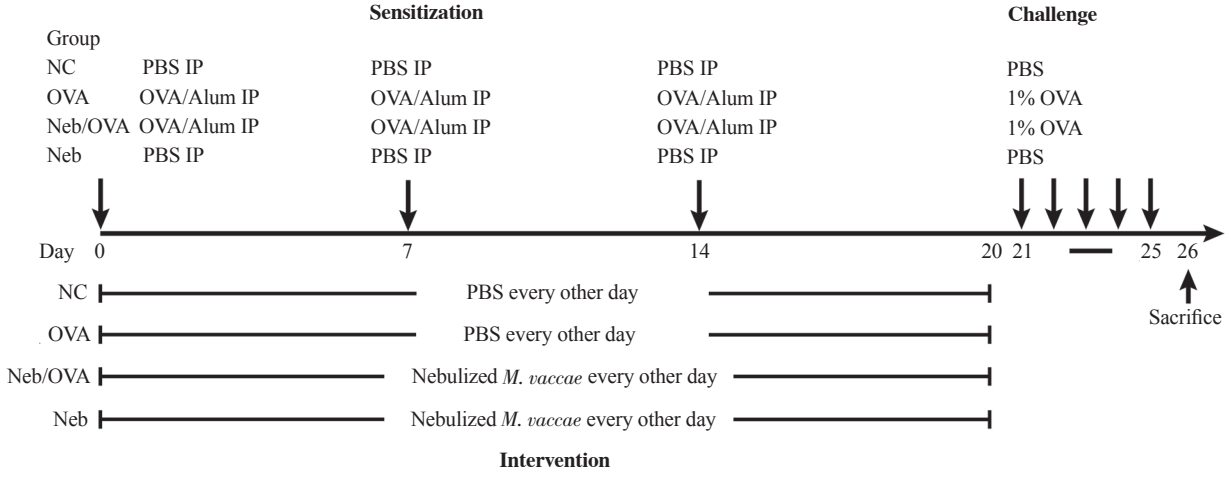

Figure 1. Timeline of the experimental protocol. The OVA and the Neb/OVA groups received OVA sensitization on days 0, 7, and 14. From days 21 to 25, the mice received 1% OVA nebulization in a closed chamber for the challenge, while the NC and Neb groups received PBS only. The Neb and the Neb/OVA groups received nebulized inhaled 22.5 μg *Mycobacterium vaccae* (*M. vaccae*) every other day during the sensitization process (days 0-20), while the NC and the OVA groups were nebulized with PBS. IP: intraperitoneal, Alum: adjuvant aluminium hydroxide, PBS: phosphate buffered saline, NC: the normal control group, OVA: the asthma model group, Neb/OVA: the group treated with nebulized inhaled *M. vaccae* in the process of OVA sensitization, Neb: the nebulized inhaled *M. vaccae* group.

with other antibodies. $CD11c^+$ cells were stained using anti-mouse antibodies: anti-CD45-PE-Cyanine7, anti-CD11c-FITC, anti-MHC-II-PerCP-eFluor710, and anti-TLR4/MD2-SB436 (eBioscience or BD Biosciences). Then cells were fixed, permeabilized with transcription factor fixation/permeabilization diluent (eBiosciences), washed, and stained using anti-IRF4-PE and anti-IRF8-APC (eBioscience or BD Biosciences) in 4℃ for 30 min. For intracellular cytokine staining in $CD4^+$ T cells, 5×10^6 single cells were incubated in a 1640 medium stimulated with phorbol myristate acetate (25 ng/mL, Sigma-Aldrich), ionomycin (1 μg/mL, Cell Signaling Technology) and Golgi Plug (Monensin, BD Biosciences) at 37℃ in 5% CO_2 for 4 h. The cells were then washed and stained for a surface marker anti-CD4-Percp (eBiosciences). After the surface staining, the cells were fixed and permeabilized with the fix/perm diluent (Cytofix/Cytoperm™, BD Pharmingen). Afterward, the cells were stained with the anti-IFN-γ-APC and anti-IL-4-PE in 4℃ for 20 min, washed using 1×Perm/Wash Buffer (BD Pharmingen), and resuspended in PBS for flow cytometric analysis. All the stained cells were detected by FACS Canto flow cytometer (BD Biosciences), and the acquired data were analyzed by Flowjo- VX software.

2.8. Statistical analysis

Data were presented as mean±standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by significant difference (LSD) analysis. The Kruskal-Wallis nonparametric test was used for data that did not have a normal distribution. *P*-values<0.05 were considered significant. Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, U.S.A.).

2.9. Ethical statement

This study was approved by the Institutional Animal Care and Ethics Committee of Guangxi Medical University (Approval No. 202005007).

3. Results

3.1. Nebulized inhaled *M. vaccae* alleviates airway hyperresponsiveness in OVA–induced asthmatic mice

We investigated the effect of nebulized inhaled *M. vaccae* on airway responsiveness in response to increasing concentrations of MCh. The sRaw of the OVA group increased significantly at doses of 12.5 and 25 mg/mL MCh compared with the NC group ($P<0.05$). Treatment with nebulized inhaled *M. vaccae* significantly reduced the sRaw in the OVA-induced asthma group ($P<0.05$). Moreover, the sRaw of the Neb group did not differ from the NC group at all doses of MCh. According to the findings, nebulized inhaled *M. vaccae* could reduce airway hyperresponsiveness in the OVA-induced asthma model (Figure 2).

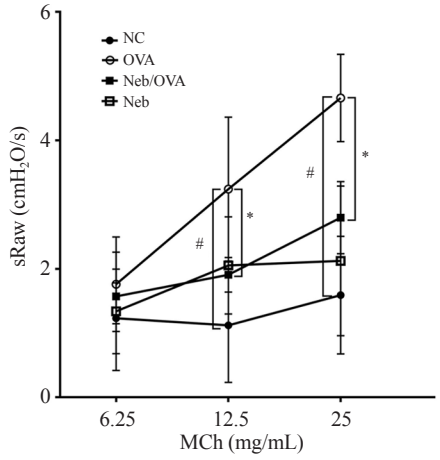

Figure 2. Nebulized inhaled *M. vaccae* alleviates airway hyperresponsiveness in the OVA-induced asthma model. Data are expressed as mean±SD (n=6) and analyzed by one-way ANOVA followed by LSD analysis. $^{\#}P<0.05$ compared with the NC group, $^{*}P<0.05$ compared with the OVA group. sRaw: specific airway resistance; MCh: methacholine chloride.

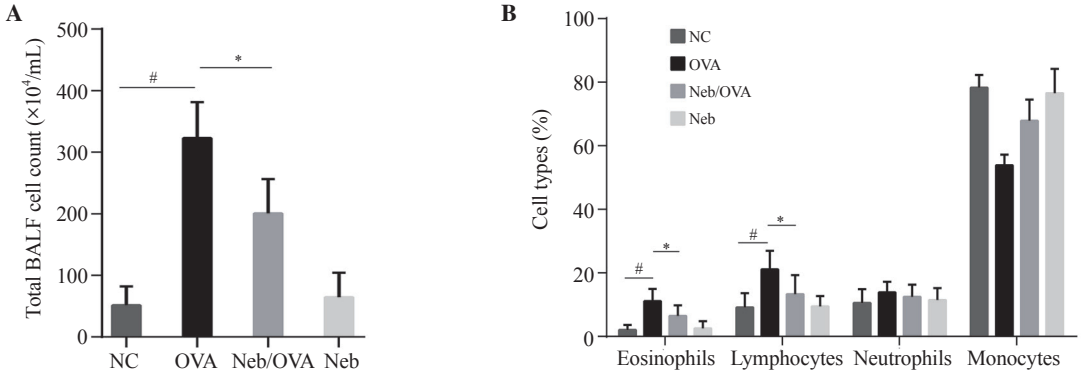

Figure 3. Nebulized inhaled *M. vaccae* reduces eosinophil and lymphocyte infiltration into the airway in OVA-induced asthmatic mice. (A) BALF inflammatory cell count. (B) Inflammatory cell classification in the BALF. Data are expressed as mean±SD (n=6) and analyzed by one-way ANOVA followed by LSD analysis. $^{\#}P<0.05$ compared with the NC group, $^{*}P<0.05$ compared with the OVA group. BALF: bronchoalveolar lavage fluid.

3.2. Nebulized inhaled M. vaccae reduces eosinophil and lymphocyte infiltration into the airway in OVA-induced asthmatic mice

BALF was collected after the last OVA challenge, and total and differential cell counts were assessed. OVA challenge induced a marked increase in total cell, eosinophils, and lymphocytes as compared with the NC group (P<0.05). Nebulized inhaled *M. vaccae* significantly decreased OVA-induced total cells, eosinophils, and lymphocytes (P<0.05). In addition, The total inflammatory cell count, eosinophils, lymphocytes, neutrophils, and monocytes of the Neb group were not significantly different from the NC group (Figure 3).

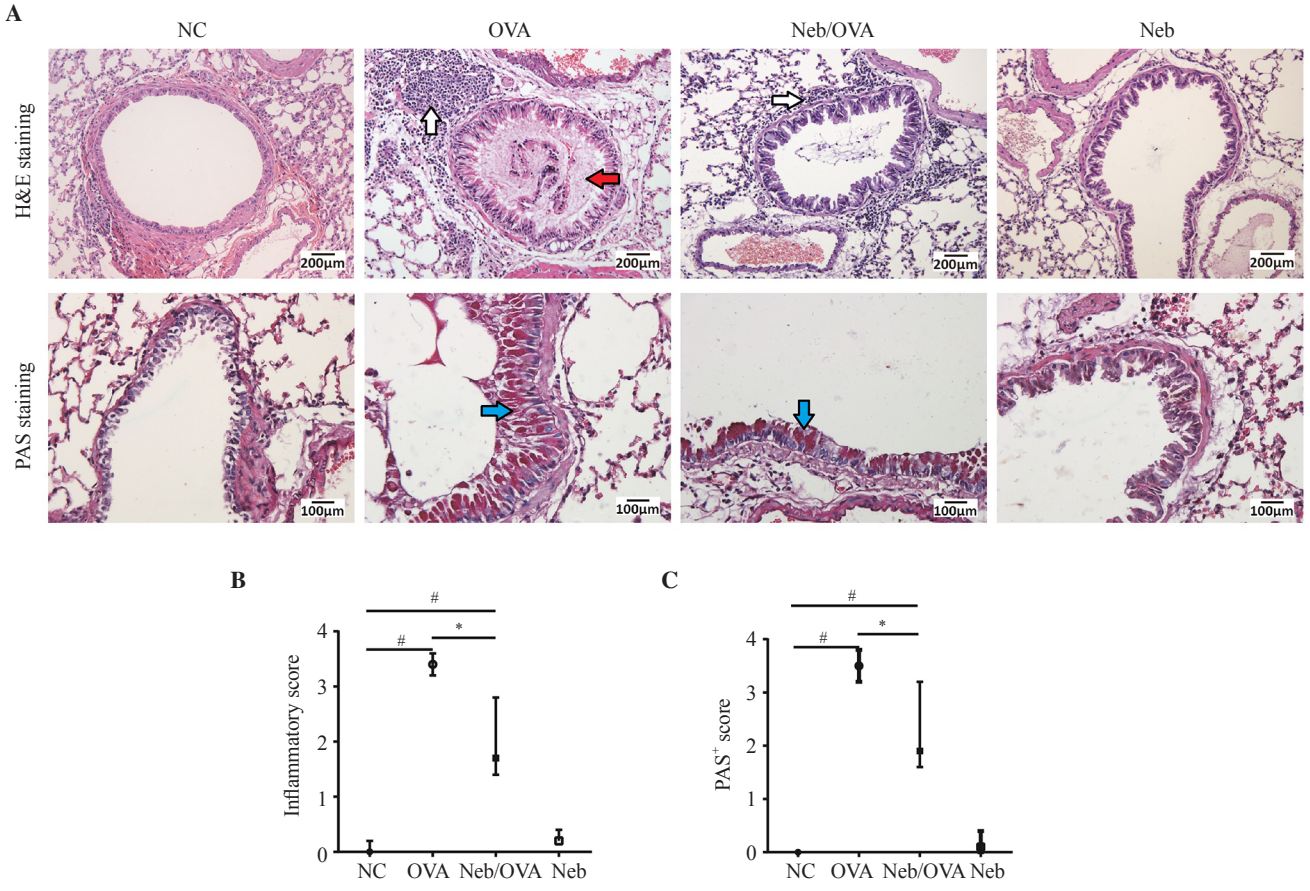

Figure 4. Nebulized inhaled *M. vaccae* alleviates OVA-induced airway inflammation. (A) Histological results of lung tissues using hematoxylin and eosin (H&E) staining (magnification: ×100) and periodic acid Schiff (PAS) staining (magnification: ×200). Inflammatory infiltration and mucus secretion are rarely seen in the NC and Neb groups. The OVA group shows inflammatory cell infiltration around the bronchi (white arrow), massive goblet cell hyperplasia (blue arrow), and abundant mucus and mucus plugs in the bronchial lumen (red arrow). Inflammatory cell infiltration and goblet cells are significantly lower in the Neb/OVA group. (B) Inflammatory score of the lung. (C) PAS score of the lung. Data are expressed as medians (n=6) and analyzed by Kruskal-Wallis nonparametric test. $^{\#}P$<0.05 compared with the NC group, $^{*}P$<0.05 compared with the OVA group.

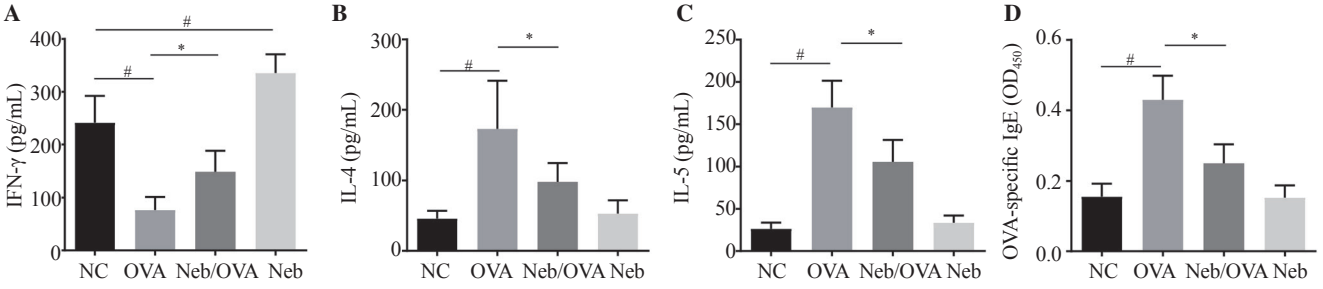

Figure 5. Nebulized inhaled *M. vaccae* alleviates the OVA-induced Th1/Th2 imbalance of BALF. IFN-γ (A), IL-4 (B), IL-5 (C), and OVA-specific IgE (D) concentrations were determined. Data are expressed as mean±SD (n=6) and analyzed by one-way ANOVA followed by LSD analysis. $^{\#}P$<0.05 compared with the NC group, $^{*}P$<0.05 compared with the OVA group.

3.3. Nebulized inhaled M. vaccae alleviates the OVA–induced airway inflammation

The inflammatory changes in H&E and PAS-stained lung tissues of mice were observed using light microscopy. Inflammatory cell infiltration in the lung tissues and mucus secretion of the airway epithelium were completely absent or rarely seen in the NC and Neb groups (Figure 4A). There were no significant differences in the inflammation and PAS scores between the Neb and NC groups (Figure 4B and 4C). Moreover, severe inflammatory cell infiltration around the bronchi, massive goblet cell hyperplasia, and abundant mucus and mucus plugs in the bronchial lumen were observed in the OVA group. Inflammatory cell infiltration and the proportion of the goblet cells were significantly lower, and the airway lumen was unobstructed in the Neb/OVA group as compared to the OVA group (Figure 4A). Furthermore, the Neb/OVA group showed higher inflammation score and PAS score when compared to the NC group (P<0.05). However, compared with the OVA group, the inflammation score, and PAS score were significantly decreased (P<0.05) (Figure 4B and 4C).

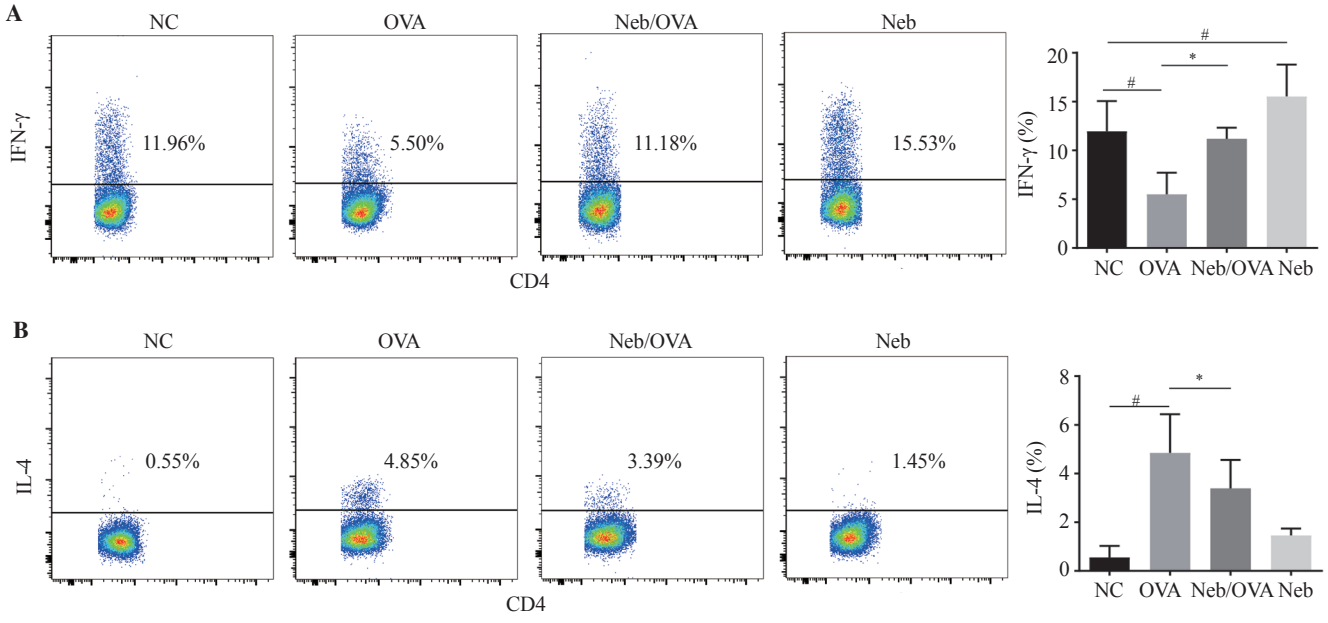

Figure 6. Nebulized inhaled *M. vaccae* alleviates the imbalance of the IFN-γ^+CD4$^+$ T cell and IL-4$^+$CD4$^+$ T cell in OVA-induced asthmatic mice. (A) The representative flow cytometric images and proportion of IFN-γ^+CD4$^+$ T cells in mouse lung. (B) The representative flow cytometric images and proportion of IL-4$^+$CD4$^+$ T cells in mouse lung. Data are expressed as mean±SD (n=6) and analyzed by one-way ANOVA followed by LSD analysis. $^{\#}P$<0.05 compared with the NC group, $^{*}P$<0.05 compared with the OVA group.

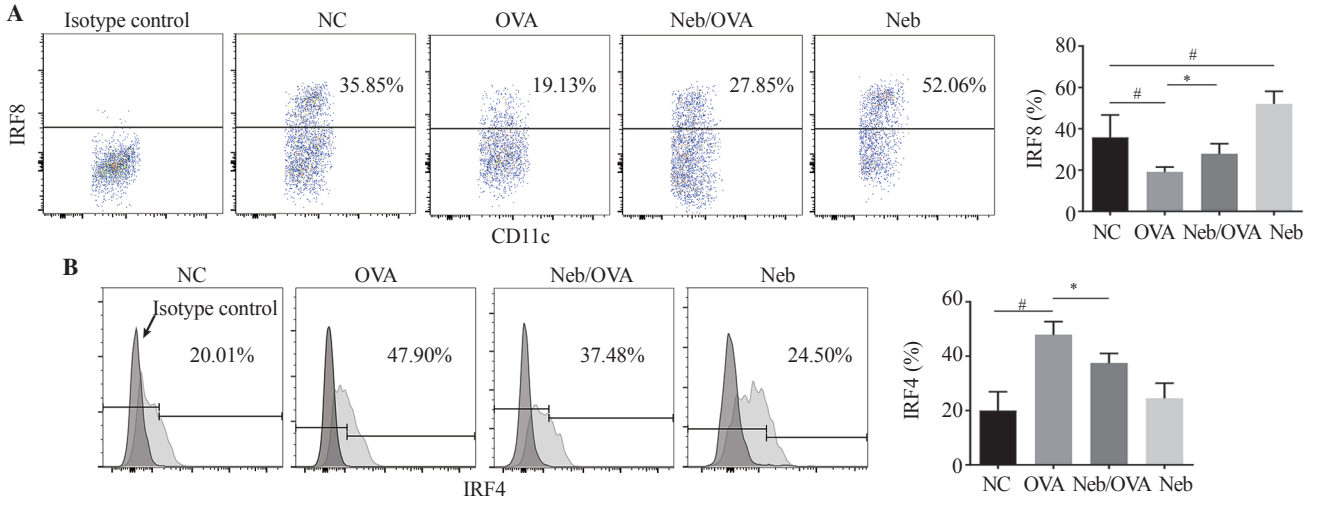

Figure 7. Nebulized inhaled *M. vaccae* relieves the imbalance of IRF4$^+$ cDCs and IRF8$^+$ cDCs in asthmatic mice. (A) The representative flow cytometric images and proportion of IRF8$^+$ cDCs. (B) The representative histograms and proportion of IRF4$^+$ cDCs in mouse lung. Data are expressed as mean±SD (n=6) and analyzed by one-way ANOVA followed by LSD analysis. $^{\#}P$<0.05 compared with the NC group, $^{*}P$<0.05 compared with the OVA group.

3.4. Nebulized inhaled *M. vaccae* reduces the OVA-induced Th1/Th2 cytokine imbalance of BALF

OVA-induced allergic asthma is characterized by an imbalance of Th1/Th2 cytokines as well as elevated OVA-specific IgE. Therefore, we measured the concentrations of IFN-γ, IL-4, IL-5, and OVA-specific IgE of BALF. Compared to the NC group, the OVA group had higher concentrations of IL-4, IL-5, and OVA-specific IgE with lower IFN-γ concentration (P<0.05). Nebulized inhaled *M. vaccae* significantly decreased IL-4, IL-5, and OVA-specific IgE concentrations while increasing IFN-γ concentration (P<0.05). Moreover, compared to the NC group, nebulized inhaled *M. vaccae* induced an increase in IFN-γ concentration in the Neb group (P<0.05). However, there were no significant differences in IL-4, IL-5, and OVA-specific IgE between the Neb group and the NC group (Figure 5).

3.5. Nebulized inhaled M. vaccae alleviates the imbalance of the IFN-γ^+CD4^+ T cell and IL-4^+CD4^+ T cell percentage in OVA-induced asthmatic mice

The OVA group showed much lower percentage of IFN-γ^+CD4$^+$ T cells than the NC group (P<0.05). Treatment with nebulized inhaled *M. vaccae* markedly increased the IFN-γ^+CD4$^+$ T cells in OVA-induced asthmatic mice (P<0.05). In comparison with the NC group, IFN-γ^+CD4$^+$ T cells were increased in the Neb group (P<0.05). In contrast, IL-4$^+$CD4$^+$ T cells were increased in the OVA group as compared with the NC group (P<0.05), which was decreased by treatment with nebulized *M. vaccae* (P<0.05) (Figure 6).

3.6. Nebulized inhaled M. vaccae enhances the TLR4 and IRF8 expression, and relieves the imbalance of IRF4$^+$ and IRF8$^+$ cDCs in OVA-induced asthmatic mice

Our findings showed that the OVA group demonstrated lower percentage of IRF8$^+$ cDCs than the NC group (P<0.05). Compared to the OVA group, the mice in the Neb/OVA group treated with nebulized inhaled *M. vaccae* demonstrated a higher percentage of IRF8$^+$ cDCs (P<0.05). The percentage of IRF8$^+$ cDCs in the Neb group that only received nebulized inhaled *M. vaccae* without sensitization was higher than that in the NC group (P<0.05) (Figure 7). In contrast, the percentage of IRF4 cDCs in the OVA group was higher than that in the NC group (P<0.05). Nebulized inhaled *M. vaccae* prominently decreased IRF4 expression in the Neb/OVA group (P<0.05). However, there was no significant difference in IRF4 cDCs between the Neb group and the NC groups (Figure 7). We further investigated the effects of nebulized inhaled *M. vaccae* on TLR4$^+$ cDCs and TLR4$^+$IRF8$^+$ cDCs. OVA increased TLR4 expression in cDCs while decreasing the TLR4$^+$IRF8$^+$cDCs in asthmatic mice when compared with the NC group (P<0.05). The asthmatic mice treated with nebulized inhaled *M. vaccae* showed increased TLR4$^+$ cDCs and TLR4$^+$IRF8$^+$ cDCs (P<0.05) (Figure 8). The mice in the Neb group demonstrated an increased percentage of TLR4$^+$ cDCs and TLR4$^+$IRF8$^+$ cDCs when compared to the NC group.

4. Discussion

Asthma pathogenesis is a pathogenic immune response to common

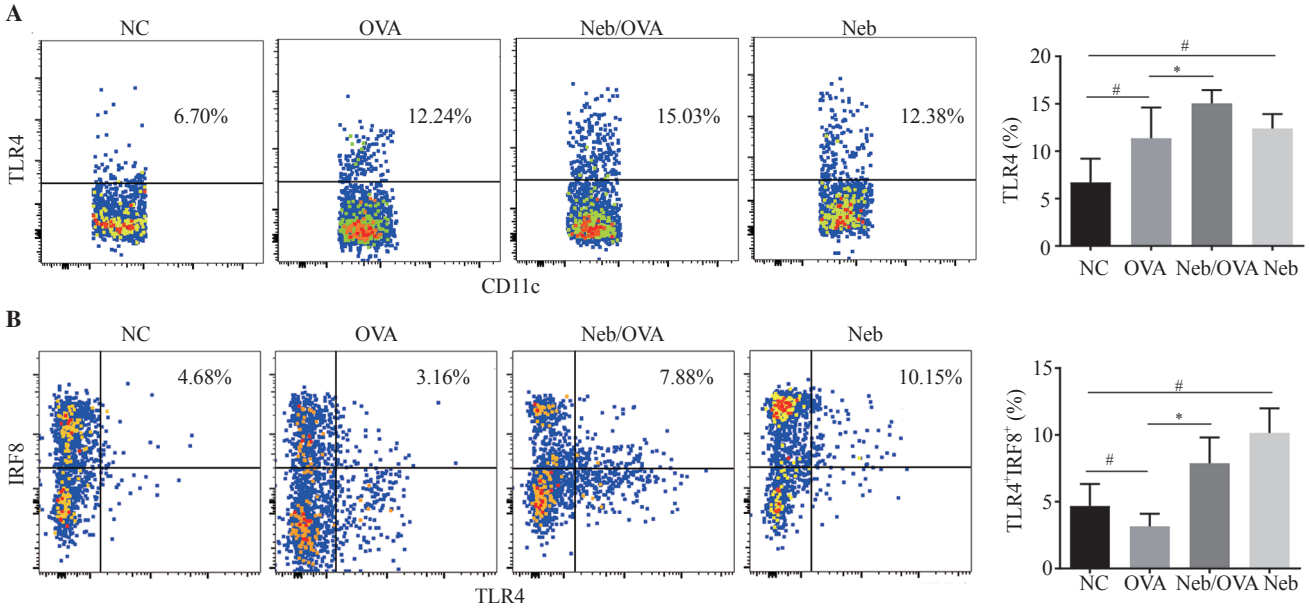

Figure 8. Nebulized inhaled *M. vaccae* upregulates the expression of TLR4 and IRF8 in cDCs of OVA-induced asthmatic mice. (A) The representative dot plots and bar graph of TLR4$^+$ cDCs. (B) The representative dot plots and bar graph of TLR4$^+$IRF8$^+$ cDCs. Data are expressed as mean±SD (n=6) and analyzed by one-way ANOVA followed by LSD analysis. $^{\#}P$<0.05 compared with the NC group, $^{*}P$<0.05 compared with the OVA group.

respiratory allergen exposure in vulnerable individuals, resulting in persistent inflammation processes in the airways. Therefore, intervention during the early developing stages of this chronic inflammatory disease would present a better strategy for managing asthma. Accumulating evidence suggests that the preventive potential of asthma is highlighted by its minimal prevalence in populations highly exposed to microbial environments[29]. Valkonen *et al*. found that high levels for *Mycobacterium* spp. were observed in farming families compared to non-farming families, possibly contributing to protecting against allergic diseases[30]. Nebulized inhalation delivers drugs directly into the airways, providing a topical effect while lowering the risk of systemic side effects and medication costs[21]. In this study, we used *M. vaccae* nebulization to simulate *Mycobacterium* exposure in the airway and regulated airway immunity in the process of sensitization of asthma mouse model, to evaluate the preventive effects of nebulized inhaled *M. vaccae* against allergic airway inflammation and airway hyperresponsiveness of an asthma mouse model. Our findings showed that nebulized inhaled *M. vaccae* reduced airway hyperresponsiveness and eosinophil and lymphocyte infiltration into the airway, alleviated airway inflammation, and decreased airway goblet cell hyperplasia and mucus secretion in OVA-induced asthmatic mice. Based on our findings, nebulized inhaled *M. vaccae* could be a potential asthma treatment.

Allergic asthma is often associated with Th1/Th2 imbalance[31]. As previously stated, Th2 cytokine overexpression is a dominant driver of allergic asthma development, Th2-related cytokines stimulate airway eosinophil infiltration and IgE production, induce mucus hypersecretion, and cause airway hyperresponsiveness and airway remodeling in asthma[32]. At the same time, IFN-γ produced by Th1 cells inhibits Th2 proliferation and activity[33]. The results showed that OVA sensitization and challenge increased IL-4, IL-5, and OVA-specific IgE expression while decreasing IFN-γ expression in asthmatic mice. Nebulized inhaled *M. vaccae* decreased OVA-specific IgE level and alleviated the OVA-induced Th1/Th2 cytokine imbalance of BALF. Additionally, the mice that only received nebulized inhaled *M. vaccae* without OVA sensitization and challenge showed an increase in IFN-γ expression. The results for Th1 and Th2 subsets were consistent with cytokine detection. The results suggested that nebulized inhaled *M. vaccae* regulated the airway immunity, induced the IFN-γ expressed in $CD4^+$ T cell, increased the Th1 subsets, and alleviated the imbalance of Th1/Th2 in asthma.

DCs are the primary antigen-presenting cells when naive T cells differentiate into Th1 or Th2 effector cells[34]. The majority of pulmonary cDCs arise from committed DC precursors in the bone marrow, which have a high antigen-presenting capacity[35]. In asthma pathophysiology, cDC2s promote Th2 differentiation when allergens enter the airways, activating cDCs *via* a complex antigen recognition system, followed by expressing MHC-Ⅱ and the costimulatory molecules CD80/CD86 on the cell surface and secreting Th2 promoting cytokines IL-33 and IL-10 in response to allergens to promote Th2 cell differentiation[35]. IRF4 directly targets the transcription of the IL-10 and IL-33 genes in DCs, as well as the differentiation and development of cDC2s[35,36]. cDC1s are critical cells that produce IL-12 *in vivo*, which have a strong ability to promote Th1 cell differentiation[34]. cDC1s, on the other hand, can mediate Th2 tolerance to respiratory allergens[37]. IRF8 as a terminal developer is required for pre-cDCs to differentiate from terminal cDC1s, and it is required to maintain the identity of cDC1s[38]. Furthermore, studies have shown that the absence of IRF8 expression significantly increases the development of IRF4-dependent DCs[38]. Our findings showed that OVA-sensitized mice had a Th2 priority response in $CD4^+$ T cells, as well as higher IRF4 expression and suppressed IRF8 expression in cDCs. The findings supported the conclusion that cDC1-regulated Th1 cell responses were inhibited, whereas the Th2 cells promoted by cDC2s were dominant in asthma. In this study, nebulized inhaled *M. vaccae* simultaneously inhibited Th2 immune response and IRF4 expression of cDCs, while enhancing Th1 immune response and IRF8 expression of cDCs in an OVA-induced asthma mouse model. In the Neb group, nebulized inhaled *M. vaccae* without OVA sensitization and challenge increased the percentage of $IRF8^+$ DCs and the $CD4^+IFN\text{-}\gamma^+$ T cells. It appears that nebulized inhaled *M. vaccae* during sensitization inhibited Th2 immune response by reducing the IRF4 expression in cDCs, while enhancing Th1 immune response by promoting IRF8 expression in cDCs.

TLRs have been developed to detect exogenous or endogenous molecules derived from microorganisms or host cells[39]. TLRs, as pattern-recognition receptors, mediate signal transduction and the maturation and activation of innate immune cells in the pathogenesis of a variety of chronic diseases[40]. TLR4 activates several signaling molecules downstream of the pathway, including nuclear factor kappa B, activator protein 1, signal transducer and activator of transcription (JAK/STAT) signaling, and IRFs, all of which play important roles in regulating the immune response[18]. IRF8 is a transcription factor that is expressed downstream in the TLR4 signaling pathway. When ligands bind to TLR4, they send signals to IRFs *via* the MyD88-dependent pathway; IRF8 interacts with TRAF6 and regulates its ubiquitination, facilitating crosstalk between IFN-γ and TLR signaling pathways[41]. This leads to increased production of proinflammatory cytokines, including IL-12 gene activation, which is required for Th1 responses[18]. Our findings showed OVA stimulation increased TLR4 expression of cDC in OVA-induced asthmatic mice, which is consistent with previous research findings[42]. Although OVA stimulation increased TLR4 expression of cDCs, the proportion of $TLR4^+IRF8^+$ cDCs in asthmatic mice was lower than that in the NC group. So far, the role of TLR4 in asthma is complex. A previous study suggested that the TLR4 and MyD88 signaling pathway is involved in promoting the development of OVA-induced asthma[43], TLR4 antagonist ameliorates combined allergic rhinitis and asthma syndrome by reducing inflammatory monocyte infiltration in a mice model[44]. On the contrary, another study showed the activation of TLR signaling

attenuates OVA-induced allergic asthma[45]. In *Streptococcus pneumoniae*-mediated suppression of OVA-induced allergic airway disease, TLR4 and MyD88 were involved in the suppression of eosinophilic and Th2 responses[43]. Thus, TLR signaling is likely required for both the development and suppression of asthma by immunoregulatory therapies. Nebulized inhaled *M. vaccae* increased the TLR4 expression and TLR4-IRF8 expression of cDCs in OVA-induced asthmatic mice. Compared to the NC group, nebulized inhaled *M. vaccae* without OVA sensitization and challenge also increased the TLR4-IRF8 expression in cDCs. $IRF8^+$ cDCs promote Th1 cell differentiation, while $IRF4^+$ cDCs enhance Th2 responses. Thereby, nebulized inhaled *M. vaccae* reduced the Th1/Th2 imbalance in OVA-induced asthmatic mice, probably by inhibiting IRF4 expression, promoting IRF8 expression and TLR4-IRF8 expression in cDCs.

To conclude, nebulized inhaled *M. vaccae* can protect against asthma by alleviating the imbalance of Th1/Th2 and IRF4/IRF8 in cDCs of OVA-induced asthmatic mice. However, our study had some limitations. First, we were unable to perform TLR4 knockdown/knockout experiments to further elucidate the effects of nebulized inhaled *M. vaccae* on TLR4 pathways of cDCs and Th1/Th2 response. Second, the dose-dependent effects of nebulized inhaled *M. vaccae*, and co-culture experiments of cDCs with T-cells and a larger sample size should be taken into account in future investigations.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

QXS contributed to the study design, experiments, data acquisition and analysis, and writing of the manuscript. CQL designed the experiments and contributed to the final version of the manuscript. SYX and QNZ contributed to animal model establishment and airway responsiveness experiments. LDL and HX contributed to the study design and data analysis.

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