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Original Article

Aerosol inhalation of Mycobacterium bovis can reduce the Th2 dominant immune response induced by ovalbumin sensitization

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Abstract: Objective: To investigate whether *M. vaccae* inhalation affects asthma via γδ T cell regulation. Methods: Forty male Balb/c mice were randomly divided into 4 groups: normal group, asthma group, control group and intervention group. The normal group was given no treatment. For the asthma group, control group and intervention group, the mice were sensitized and stimulated with ovalbumin (OVA) to establish asthma models. Mice in the asthma group were not treated. Mice in the control group were treated with γδ T cell suspension from normal mice, and those in the intervention group were treated with γδ T cell suspension from mice intervened by Mycobacterium bovis. ELISA assay was adopted for quantification of IL-4 and IFN-γ in mouse alveolar fluid (BALF), and flow cytometry for determining the percentage of IL-4 and IFN-γ from mononuclear cells of lung tissues. Results: The airway responsiveness of the asthma group was higher than that of the normal group. The degree of airway inflammation in the intervention group was lighter than that in the control group, and it was significantly alleviated compared with the asthma group ($P<0.05$). Compared with the asthma group, the level of IL-4 in the BALF of the control group and the intervention group decreased significantly, while the level of IFN-γ increased significantly ($P<0.05$). Compared with the control group, the level of IL-4 in the BALF of the intervention group was significantly lower, while the level of IFN-γ was significantly higher ($P<0.05$). In addition, the results of flow cytometry were basically consistent with the results of Elisa. Conclusion: Aerosol inhalation of Mycobacterium bovis can alleviate the Th2 dominant immune response induced by OVA sensitization and regulate the Th1/Th2 immune imbalance in patients with asthma.

Keywords: Asthma, adoptive immune γδ T cells, aerosol inhalation, Mycobacterium bovis, airway inflammation, cytokines

Introduction

The incidence of asthma around the world is about 1-18% [1]. Asthma is usually induced by allergens and immune stimuli, exercise, weather changes, or viral respiratory infections [2]. Asthma includes chronic airway inflammation, and patients often suffer cough, shortness of breath, wheezing, chest tightness, variable expiratory flow limitations, etc. Three major pathological changes that are associated with expiratory flow limitation are airway inflammation, smooth muscle dysfunction, and airway remodeling. The pathological basis for fixed airflow obstruction in patients with asthma is airway remodeling [3]. Reportedly, the number of asthma patients worldwide is still on the rise, and the resulting medical expenses are constantly increasing, which bring a huge burden to social and economic development and human health, and the incidence in children is also increasing [4]. Up to now, due to the limited understanding of the etiology and pathogenesis of asthma and the lack of effective clinical treatment measures, the prevention and control of asthma is still the primary problem in the society.

Most children suffer asthma since infancy, and asthma in 40% to 75% of children will completely disappear in adolescence or adulthood [5].

Early respiratory syncytial virus (RSV) infection in lower respiratory tract is a risk factor for allergic sensitization in children. Aoyagi et al. [6] reported that compared with the same age group, infants had lower content of IFN-γ-producing T cells in their peripheral blood due to the influence of respiratory syncytial virus and bronchiolitis. In addition, they also found the normalization of the content of γδ T cells in convalescence, which indicated that the decrease of IFN-γ in γδ T cells might play a crucial role in the development of asthma. Previous clinical work has revealed that moderate persistent asthma symptoms can be alleviated by inhaling inactivated *Mycobacterium phlei*, which improves lung function and lowers total serum IgE level in a manner that is both safe and similar to that of Seretide. Compared with the adverse reactions caused by BCG injection, aerosol inhalation of inactivated Mycobacterium phlei is safer [7].

T cells are a key component of the adaptive immune system and are essential for defending against alien organisms and self-disorders [8]. According to the composition of T cell receptor (TCR), T cells can be classified into two subsets: γδ T cells and αβ T cells [9]. γδ T cells are the third branch of adaptive immune system in jaw vertebrates beside αβ T cells and B cells. Like the other two types of lymphocytes, they express different antigen receptors and can specifically recognize ligands [10]. γδ T cells are considered to be the bridge between adaptive immunity and innate immunity. γδ T cells and their subsets play a vital role in the pathogenesis of asthma. In the previous study of our group, we found that there is a Th1/Th2 immune regulation model in γδ T cell subsets [11]. γδ T cells have been proved to affect allergen-driven airway hyperresponsiveness (AHR) and have antiviral activity, suggesting that γδ T cells are involved in the pathogenesis of asthma [12].

Accordingly, we carried out this research to study *Mycobacterium bovis* and asthma mice to find a therapeutic target for asthma, which is also the innovation of our research.

Materials and methods

Sources of instruments and reagents

Ovalbumin (OVA, Grade V) and phorbol ester (PMA) (Sigma Company, USA); Mycobacterium vaccae for aerosol injection (batch number: 20171001, Anhui Zhifei Longcom Biopharmaceutical Co., Ltd., China); aluminum hydroxide powder (analytically pure; Pierce Company, USA); Wright stain solution (Guangzhou Weijia Biotechnology Co., Ltd., China); Elisa kits for IL-4 and IFN-γ (Shanghai Yikesai biology products Co., Ltd., China); flow antibodies APC-IFN-γ and PE-IL-4 (Ecoscience Company, USA); Percp-CD4, monensin, and fixing/membrane breaking solution (BD Company, USA); mouse CD4 Cell Sorting Kit and Sorting Column MS (Miltenyi Biotec, Germany); ultrasonic atomizer WH-2000 (Guangdong Yuehua Medical Instrument Factory Co., Ltd., China); enzyme micro-plate reader (BIO Company, China); RAD imark and FACS Canto II flow cytometer (BD Company, USA).

Magnetic bead sorting (positive sorting method) of T cells

Buffer was diluted (1:10) with PBS supplemented with 0.5% BSA, 2 mM MEDTA and 0.1% sodium azide or sterile distilled water. The cell suspension was adjusted to 20×10^6 cells/mL, and 59 mL of purified anti-mouse CD16/CD32 monoclonal antibody (Novus, NBP1-43672, USA) or biotin-labeled mouse dendritic cells were added per 1×10^6 cells. The mixture was heated in a water bath (15 min). The labeled cells were washed with a buffer, centrifuged for 5 min (1500 rpm), and the precipitation was obtained by discarding the supernatant. The collected cells were centrifuged for 5 min (1500 rpm/min), the supernatant was discarded, buffer and cellular magnetic beads were added, and the precipitates were obtained after incubation for 15 min (4°C). The mixture was then added to buffer to full, centrifuged for 5 min (1500 rpm), and the supernatant was discarded to obtain a pellet. The cells passing through the MS column were placed in a suitable collection tube. After that, γδ T cells were centrifuged for 5 min (1500 rpm/min), and the supernatant was discarded to obtain precipitation. The cells were collected and finally counted.

Preparation of mononuclear cell suspension from lung tissue

RPMI1640 medium (2 mL) containing collagenase IV (2.5 $g\bullet L^{-1}$) was injected into the right

lung of the sheared mouse, and then the right lung was put into a shaking table for about 40 min digestion at a constant temperature of 37°C, during which it was evenly mixed with a Pasteur pipet every 15 min. The cell suspension and the incompletely digested lung tissue mass were gently ground and filtered via a 200-mesh filter screen, followed by 5-min centrifugation (1500 r/min) and discarding of the supernatant. It was let to stand still in the dark for 3 min after addition of red blood cell lysate, followed by 5 min centrifugation (1500 r/min). Finally, mononuclear cells of the lung were resuspended in RPMI1640 medium containing 10% fetal bovine serum after three times of washing with PBS.

Constructions of animal models

Bronchial asthma models were established and improved based on the research by Koh et al. [2]. Specifically, the bronchial asthma models were established by OVA injection and inhalation stimulation. Each mouse was given intraperitoneal injection of OVA-aluminum hydroxide suspension on the 1st, 7th and 14th days, respectively. On the 21st day, the mouse was placed in an atomizing box and let to inhale OVA phosphate buffer solution for approximate 30 min, once every other day, for a total of five times. For mice in normal group, PBS was used for injection and inhalation stimulation.

Experimental grouping

Forty male Balb/c mice were randomly assigned to four groups: normal group, asthma group, control group and intervention group, with 10 mice in each group. The asthma group, control group and intervention group were all sensitized with OVA to establish mouse models of bronchial asthma according the above method.

In addition, TCR-β-/-mice were divided into the TCR-β-/-saline group and the TCR-β-/-*Mycobacterium bovis* group. Mice in the TCR-β-/-*Mycobacterium bovis* group were treated with ultrasonic atomization inhalation of *Mycobacterium bovis* for injection (WH-2000, Guangdong Yuehua Medical Device Factory Co., Ltd.), once a day, for 5 consecutive days. Mice in the TCR-β-/-normal saline group were treated with ultrasonic atomization inhalation of 10 mL normal saline, once a day, for 5 consecutive days. After the last atomization, the spleen of each mouse was taken, and γδ T cell suspension was separated and injected into tail vein of BALB/C male mice in the control group and intervention group respectively. Namely, in the control group, mice were given aerosol inhalation of γδ T cell suspension from TCR-β-/-saline group once a day until the end of stimulation. In the intervention group, mice were given aerosol inhalation of γδ T cell suspension from TCR-β-/-*Mycobacterium bovis* group once a day until the end of stimulation. The normal group and asthma group didn't receive inhalation treatment.

Determination of airway responsiveness

After the last stimulation with OVA, the airway resistance of mice was measured by FinePointeNAM function instrument. The calibration instrument was washed with 20 uL PBS before use. The mice were put in the test cabin for 6 min and then they were inhaled 20 uL (0, 12.5, 25, 50 mg/mL) methacholine (Mch.PBS) solution for 30 s and the data within 3 min were recorded. Excitation with the next concentration was performed after recovery for 4 min.

Sample collection

Each anesthetized mouse was injected intraperitoneally with 1% pentobarbital sodium (80 mg/kg) followed by tracheal intubation. First, 0.5 mL PBS at 4°C was used for slowly lavaging the lungs, and bronchoalveolar lavage fluid (BALF) was recovered for 3 times, with a recovery rate of >85%. The BALF was collected, followed by 10-min centrifugation (1500 r/min, 4°C). The supernatant was sub-packaged in -80°C refrigerator for detection of cytokines by ELISA. The total number of cells was counted by Wright's staining smear precipitate.

HE staining

The lung tissues of mice were collected, fixed with 4% paraformaldehyde at 4°C for 24 h, dehydrated, embedded, and sliced (5 μm). Subsequently, the slices were subjected to 12-s hematoxylin staining and then washed. Scoff blue solution was put in the slices to make them blue, followed by 3-min washing with water, 3-min washing with alcohol. In the end, the slices were placed in oven. The sec-

Table 1. Adverse reactions

Group	Times of scratching mouth and nose	Shortness of breath
Normal group (n=10)	0	0
Asthma group (n=10)	42	24
Control group (n=10)	22	10
Intervention group (n=10)	8	2

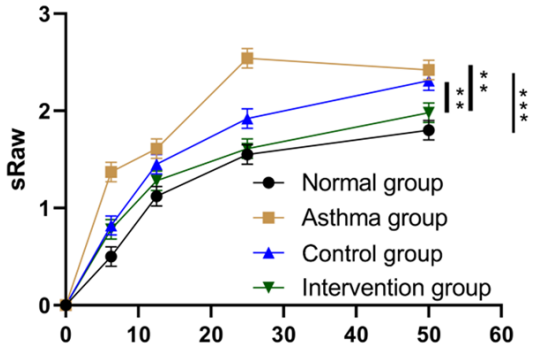

Figure 1. sRaw growth curve (n=10 for each group) One-way ANOVA was conducted and LSD-T test was used for post-hoc test. **P<0.01, ***P<0.001.

tion was recorded with BX 51 optical microscope (Olympus, Tokyo, Japan) at 200X.

ELISA detection

The ELISA method was used to detect IL-4 (China, Beijing, beyotime, PI612) and INF-γ (China, Beijing, beyotime, PI508) in bronchoalveolar lavage fluid (BALF) according to the instructions of the kit.

Flow cytometry

The concentration of mononuclear cell suspension of the lung was adjusted to 1×10^9 cells/L with RPMII640 medium, and every 1 mL cell suspension was cultured with PMA (25 μg/L) (USA, sigma, P1269), ionomycin (1 μg/L) (USA, sigma, 407951) and monensin (USA, BD, 55-4724), followed by 4-6 h incubation (5% CO_2, 37°C). The cells were acquired, and washed via PBS, followed by centrifugation and discarding of supernatant. PerCP anti-CD4 antibody (e-Bioscience Company, USA) was added, followed by 9-min incubation (4°C). After 30 min, the cells were cleaned with PBS. The fixing/membrane breaking solution was added, followed by 20-min incubation (4°C) in the dark and washing with wash-buffer. PE anti-IL-4 antibody (e-Bioscience Company, USA) and APC anti-IFN-γ (e-Bioscience Company, USA) were added, followed by 30-min incubation (4°C) in the dark, two times of washing with PBS, and discarding of the supernatant. The cells were resuspended in 2009L PBS, and finally quantified via a flow cytometry.

Statistical methods

Charts were processed by Prism 5.0 and flow cytometry data were assessed by Flowjo 7.6. SPSS 17.0 was used for statistical analyses. Measurements were expressed as $\bar{x}\pm sd$, and homogeneity of variance was tested first. One-way ANOVA followed with the LSD test was used for the comparison of measurement data that were normally distributed. Correlations between the tests were assessed using Pearson's correlation. P<0.05 was considered statistically significant.

Results

Symptoms and manifestations of asthma mice

After modeling, mice from the asthma group, control group, and intervention group displayed asthmatic reactions such as anxiety-like behavior, irritability, rapid breathing, nose and mouth scratching, a hunchback posture, belly myotonic dystrophy, gatism, and so on. After inhalation treatment, in the control group, the symptoms and nose scratches were reduced with the nebulized challenge and rapid breathing was improved compared to the asthma group. While mice in the intervention group exhibited improvements in rapid breathing compared to those in the control group (**Table 1**). None of these symptoms were noted in the normal group and no mice in any group died.

Airway reactivity

A non-invasive pulmonary instrument (FinePointeNAM) was used to test the lung function of mice in each group. After Mch stimulation (12.5, 25 and 50 mg/mL), the airway responsiveness of asthmatic mice was higher than that of normal mice. The sRaw growth values of mice from the asthma group were higher than those of mice from the control group and the intervention group (P<0.05). The sRaw growth value in the intervention group was lower than that in the control group (**Figure 1**, P<0.05).

Table 2. Comparison of the total number and classification of cells in mouse bronchoalveolar lavage fluid

Group	Total number of cells ($\times 10^6$/ml)	Cell count (%)			
		Eosinophils	Lymphocytes	Neutrophils	Monocytes
Normal group (n=10)	41.36±2.15	1.87±0.31	23.39±4.36	51.89±2.75	9.90±2.41
Asthma group (n=10)	116.32±2.13$^{\Delta}$	15.01±1.43$^{\Delta}$	31.61±2.18$^{\Delta}$	58.86±2.43	4.62±1.58
Control group (n=10)	93.41±3.74*	11.03±1.98*	29.11±2.52*	55.32±2.67	6.12±1.23
Intervention group (n=10)	72.61±1.92$^{*,\#}$	7.00±6.21$^{*,\#}$	25.35±1.63$^{*,\#}$	53.19±1.74	7.70±2.96$^{\#}$

$^{\Delta}$P<0.05, compared with normal group; *P<0.05, compared with asthma group; $^{\#}$P<0.05, compared with control group.

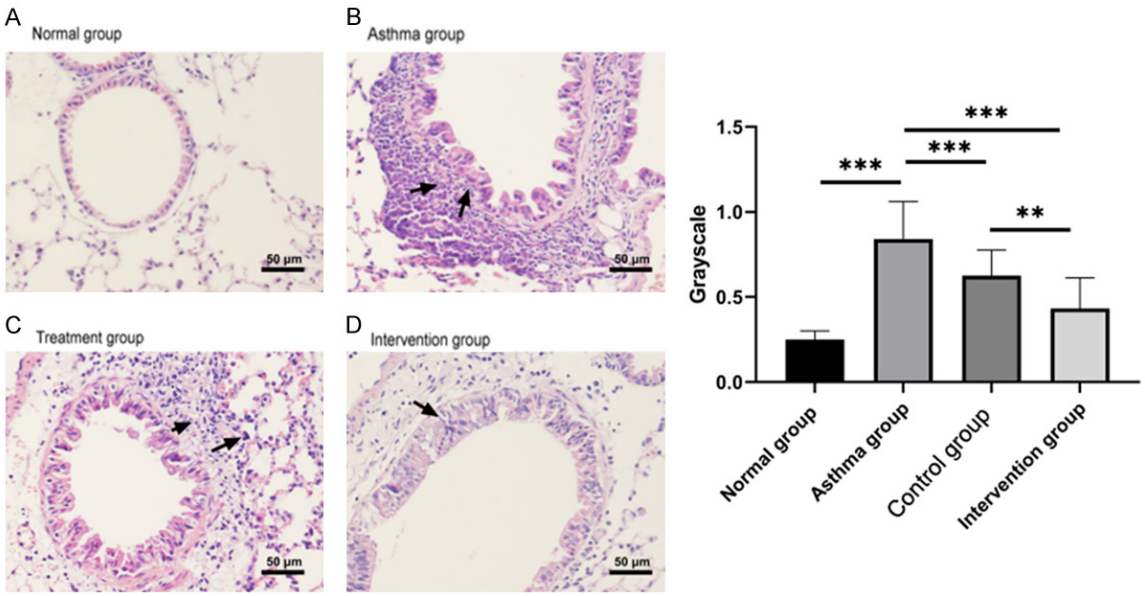

Figure 2. Histopathological manifestations of lung tissue (HE staining ×40, n=10 for each group). Note: The arrow shows cell infiltration. One-way ANOVA was conducted, and LSD-T test was used for post-test.

BALF cells counts

Compared to the normal group, BALF in the asthma group exhibited a significantly increased proportion of eosinophils, lymphocytes, and total cell numbers (P<0.05). As compared to the asthma group, BALF in the control group and intervention group had significantly decreased proportions of eosinophils, lymphocytes, and total cell numbers (P<0.05). The percentages of eosinophils, lymphocytes and neutrophils in the intervention group were lower than those in the asthma group, while the proportion of monocytes was higher (**Table 2**, P<0.05).

Pathological observation of lung tissue

Microscopically, the airway mucosa and the structure of the alveolar wall were intact, and the endothelium of the pulmonary small vessels was smooth in the mice of the normal group. There was no infiltration of inflammatory cells around the airway and blood vessels, no obvious viscous secretion, and the smooth muscle layer and basement membrane were thin. Compared with the normal group, airway mucosal edema, eosinophils and lymphocytes significantly increased in the asthma group, a large number of inflammatory cells infiltrated around the bronchi and small vessels, and the alveolar wall were found in the asthma group. In addition, compared with the asthma group, hypertrophic airway epithelial cells, goblet cells, basement membrane thickening, smooth muscle hypertrophy, infiltration of lymphocytes and eosinophils around the airway and blood vessels were lighter in the control group and those in the intervention group. Moreover, the decrease of lymphocyte and eosinophil infiltration in the intervention group was more obvious than that in the control group (**Figure 2**).

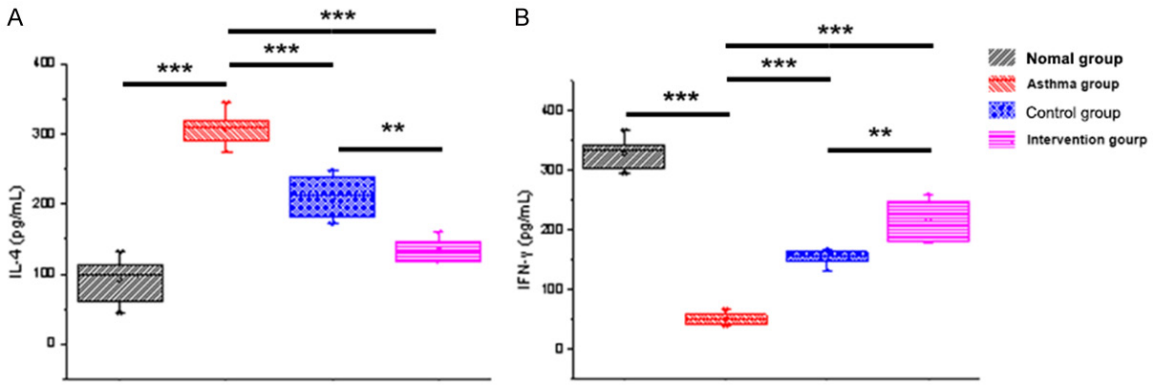

Figure 3. Changes of IL-4 and IFN-γ levels in BALF of mice. A. IL-4 expression in BALF of mice according to Elisa assay (n=10 for each group). B. IFN-γ expression in BALF of mice according to quantification by Elisa assay (n=10 for each group). ** indicates P<0.01 in inter-group comparison; *** indicates P<0.001 in inter-group comparison. One-way ANOVA was conducted, and LSD-T test was used for post-test.

Effect of Mycobacterium bovis on BALF IL-4 and INF-γ in mice

ELISA revealed significantly increased BALF IL-4 levels and decreased BALF INF-γ levels in the asthma group relative to the normal group (**Figure 3**, P<0.05). BALF IL-4 in the control group and intervention group was significantly lower than that in the asthma group, though INF-γ levels were significantly higher (**Figure 3**, P<0.05). The level of BALF IL-4 in the control group was higher than that in the intervention group, while the level of INF-γ in the control group was lower (**Figure 3**, P<0.05).

Impacts of Mycobacterium bovis on IL-4 and INF-γ secreted by γδ T cells

Compared with the normal group, the level of IFN-γ secreted by γδ T cells in the lung tissue of asthmatic mice significantly decreased and the level of IL-4 secreted by γδ T cells significantly increased. Compared with the asthma group, the level of IFN-γ secreted by γδ T cells in the control group and intervention group increased significantly, and the level of IL-4 decreased significantly (all P<0.05). The proportion of IFN-γ secreted by γδ T cells in the intervention group was higher than that in the control group, while the level of IL-4 in the intervention group was lower than that in the control group (**Figure 4**, P<0.05).

Discussion

T cells are a key component of adaptive immune responses and are essential for the fight against foreign substances and self-diseases [8-11]. γδ T cells do not express CD4 and CD8 molecules, but they are more unique antigen receptors of MHC unrestricted lymphocytes. Therefore, γδ T cells are considered to be the bridge between adaptive immune system and innate immune system, and they play a key role in human defense against infection, tumor and autoimmune diseases [12-15]. The subtypes and immunoreactivity of γδ T cells are different in different diseases [16-19].

γδ T cells and their subsets play a crucial role in the pathogenesis of asthma. Our previous work found that γδ T cell subsets have the function of Th1/Th2 immune regulation [20]. The key is that γδ T cell subsets have different regulatory effects on airway hyperresponsiveness. V γ 1 γδ T cells improve eosinophilic airway inflammation and airway hyperresponsiveness, while V γ 4 γδ T cells reduce airway hyperresponsiveness [21]. Cook and others have shown that γδ T cells affect allergen-driven airway hyperresponsiveness and have antiviral activity, suggesting that they are involved in the deterioration of asthma [5]. Glanville et al. [22] found that the increase of γδ T cells in airway and blood is related to airway obstruction and increased airway hyperresponsiveness. Liu et al. [23] further found that interleukin 17A (IL-17) induces the response of γδ T cells to acute pseudomonas aeruginosa pulmonary infection. Misiak et al. proved that lung γδ T cells are the early source of IL-17, and IL-17 can promote the production of antimicrobial peptides [24]. Our team previously found that

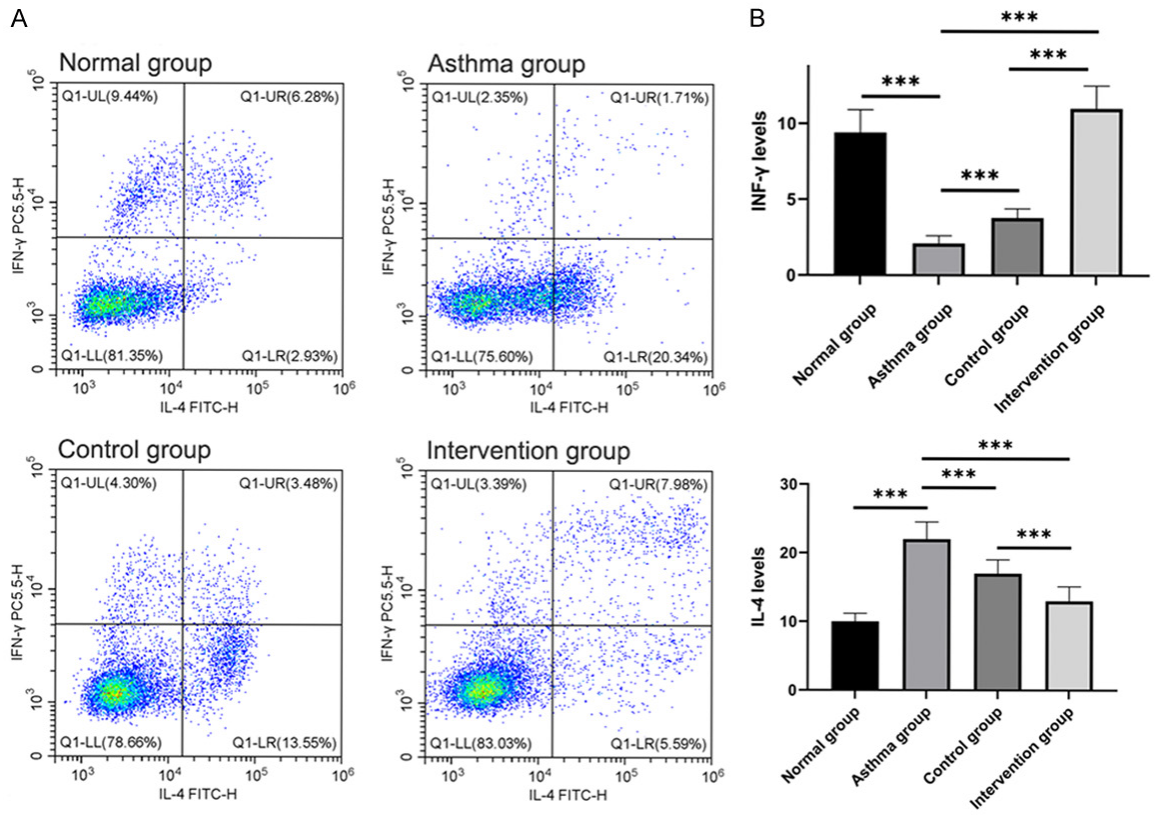

Figure 4. Detection of INF-γ and IL-4 expression in γδ T cells by Flow cytometry (FCM). A. Flow cytometry profile (n=10 for each group); B. INF-γ and IL-4 expression in mice of each group according to flow cytometry (n=10 for each group). *P<0.05, **P<0.01, ***P<0.001. One-way ANOVA was conducted, and LSD-T test was used for post-test.

the imbalance of δ T17/γδ Treg in [25] asthmatic mice may be involved in the pathogenesis of OVA-induced asthma. In this research, after Mch challenge, asthmatic mice showed higher airway hyperresponsiveness than normal control mice, and we found that the pulmonary function in the intervention group was improved after inactivated *Mycobacterium* treatment. These results suggest that atomized *Mycobacterium* vaccine may regulate airway responsiveness in asthmatic mice by interfering with γδ T cells.

We also detected the proportion of total eosinophils, lymphocytes and total cells in BALF in mice and we found that Mycobacterium aerosol interfered with γδ T cells to limit the effect of inflammation. Murdoch et al. [26] blocked the function of TCR δ antibody in asthmatic mice and found acute airway inflammation characterized by eosinophilia and aggravation of Th2 cells, suggesting airway remodeling. These results reveal that γδ T cells may be a good candidate for prevention and treatment of airway remodeling. Our previous studies have demonstrated that aerosol-inactivated *mycobacterium phlei* inhibits the secretion of Th2 cytokines by regulating transcription factors, increasing T-bet mRNA and reducing GATA-3 mRNA expression, thereby reducing airway inflammation [27]. Our current study showed that the IL-4 level in the asthma group was significantly higher than that in the normal group, and the levels of BALF IL-4 in the control group and the intervention group were lower than those in the asthma group, but the levels of IFN-γ in the control group and the intervention group were higher than those in the asthma group. We also found that the γδ T cells of the asthma group was in a tendency to differentiate into Th2 cells, while γδ T cells in the control group and intervention group was in a tendency to differentiate into Th1 cells. These results further suggest that the introduction of γδ T cells into *Mycobacterium bovis* can increase the number of γδ T1 cells by promoting the expression of T-γ (γδ). Therefore, we

speculated that Mycobacterium bovis can down-regulate GATA-3 to inhibit the secretion of IL-4, reduce the number of γδ-T2 cells and regulate the imbalance of γδ-T1/γδ-T2. This suggests that aerosol-inactivated *Mycobacterium phlei* may affect the airway hyperresponsiveness of asthmatic mice by regulating the inflammatory activity of γδ T cells.

Nevertheless, there are still some limitations. First of all, we conducted an animal model study, whether there is the same mechanism *in vitro*, and whether this mechanism can have the same effect in human needs to be further verified. Furthermore, we hope to carry out more experiments in the future research to supplement our research conclusions.

In conclusion, inhaled *Mycobacterium bovis* can reduce the Th2-dominated immune response induced by OVA sensitization and regulate the immune imbalance of Th1/Th2 in asthmatic mice. Hence, *Mycobacterium* may affect the pro-inflammatory airway response of asthmatic mice by regulating the secretion of γδ T cells and suppressing cytokines downstream.

Disclosure of conflict of interest

None.

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