

Inactivated *Mycobacterium phlei* inhalation ameliorates allergic asthma through modulating the balance of CD₄⁺CD₂₅⁺ regulatory T and Th₁₇ cells in mice

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ABSTRACT

Objective(s): Th2 response is related to the aetiology of asthma, but the underlying mechanism is unclear. To address this point, the effect of nebulized inhalation of inactivated *Mycobacterium phlei* on modulation of asthmatic airway inflammation was investigated.

Materials and Methods: 24 male BALB/c mice were randomly divided into three groups: control group (Group A), asthma model group (Group B), and the medicated asthma model group (Group C). Group B and C were sensitized and challenged with ovalbumin (OVA). Group C was treated with aerosol *M. phlei* once daily before OVA challenge. Airway responsiveness in each group was assessed. All the animals were killed, and lung tissues and bronchoalveolar lavage fluid (BALF) were harvested. Inflammatory response, proportion of Th17 and CD4+CD25⁺ Treg cells, and the levels of cytokines were analyzed in lung tissue.

Results: The proportion of Th17 cells and expression level of IL17, IL23, and IL23R were increased, while Foxp3 expression was decreased in Group B. Inhaling inactivated *M. phlei* inhibited airway inflammation and improved airway hyper-responsiveness, as well as peak expiratory flow (PEF). In addition, it significantly increased Th17 proportion, Foxp3 level, and the proportion of CD4+CD25⁺ Treg cells in lung tissue in Group C.

Conclusion: Inactivated *M. phlei* was administered by atomization that suppressed airway inflammation and airway hyper responsiveness partially via modulating the balance of CD4+CD25⁺ regulatory T and Th17 cells.

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Introduction

Asthma is a chronic respiratory disease which is characterized by airway inflammation and hyper-reactivity. Although the pathogenesis of asthma remains to be determined, newly emerging CD₄⁺ Th cell subsets have been linked to general disease pathogenesis, including regulatory T cells (Treg) (1), Th₁₇ cells (2), and the Th cells which produce IL₉ (Th₉ cells) (3).

IL17 has been implicated in asthma development (4) and Th₁₇ cells are now accepted to represent a third CD₄⁺ Th subset, which has led to the resolution of some inconsistencies in the Th₁/Th₂ paradigm (5). Regulatory T cells are characterized by the expression of the transcription factor Foxp₃ (Forkhead Foxp₃) and the IL₂ receptor (CD₂₅), and are known to produce the inhibitory cytokines IL₁₀ and TGF-β (6). The Treg cells have an ability to suppress allergic inflammation and asthma manifestations upon allergen provocation in mouse model of allergic asthma. The development of Treg cells can suppress airway inflammation via

mediating the tolerance of respiratory mucosal surfaces to environmental allergens (7-9). The balanced action between Th₁₇ and Treg cells may be important for the development/prevention of inflammatory and autoimmune diseases such as asthma (10). Therefore, inducing both Th₁₇ and Treg cells using immunological tools might be a promising treatment for asthma, but technically it is difficult.

The global prevalence of asthma and allergic disease is continuously increasing, especially in population-dense countries in Africa, Latin America, and part of Asia (11, 12). In recent years, it was recognized that Th₁/Th₂ imbalance does not fully explain the aetiology of asthma, because reversing the Th₁/Th₂ imbalance does not fully control asthmatic symptoms in human. Some studies have suggested that other CD₄⁺ T cell subsets may play a role in asthma, including Th₁, Th₁₇, and regulatory T cells (Treg) (13). In terms of regulation and restriction of immune responses, researchers are particularly interested in exploring the roles of T regulatory cells and Th₁₇ cells. Extensive

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investigations have shown that *Mycobacterium bovis* bacille Calmette-Guérin (BCG) and other mycobacterial infections suppress airway hyperresponsiveness (AHR) and eosinophilic inflammation, likely through T-helper 1 (Th₁) or regulatory T cell (Treg) responses (14-19). Our previous study showed the inhalation of *Mycobacterium phlei* reduces airway inflammation in asthmatic mice via altering the balance of the Th₁/Th₂ responses (20).

This study aimed to determine whether *M. phlei* administered by atomization, affect CD₄⁺CD₂₅⁺ regulatory T cells/Th₁₇ balance in a mouse model of asthma.

Materials and Methods

Animals

Male BALB/c mice (6 weeks old, pathogen free) were obtained from Laboratory Animal Center of Guangxi Medical University. All experiments were approved by the Animal Care Committee at Guangxi Medical University (Nanning, Guangxi, China). 24 male BALB/c mice were randomly divided into 3 experimental groups: the control group (Group A), the asthma model group (Group B), and the prevention group (Group C). All mice were maintained in an air-conditioned room at 23±3 °C and 55.5±10% humidity, and fed a standard laboratory diet with *ad libitum* access to food and water.

Sensitization and airway challenge

BALB/c mice were sensitized and challenged with ovalbumin (OVA) to establish a murine model of asthma (10). In brief, Group B and Group C were sensitized by IP injection of 25 mg OVA (grade V; Sigma) emulsified in 1 mg of aluminum hydroxide (AlumImject; Pierce, Rockford, IL) in a total volume of 200 µl, on day 1 and 14. Mice were challenged (20 min) via the airways with OVA (2% in saline) for 7 days (from day 21 to day 28) using ultrasonic nebulization (AeroSonic ultrasonic nebulizer; DeVilbiss, Somerset, PA). Group A received only saline (instead of OVA).

Mycobacterial preparations, prevention protocols

Inactivated *M. phlei* (1.72 µg) was dissolved in 10 ml saline and injected (20). Group C as treated with inactivated *M. phlei*, once daily before each OVA challenge using ultrasonic nebulization, as described (20). Group A and B were treated with the same dose of saline. Twenty-four hr after the last challenge, AHR was assessed, and the lung tissues were collected for further analysis.

Airway hyper responsiveness assessment

Respiratory resistance (RL, cmH₂O.s/ml) was assessed in anesthetized and tracheotomized mice that were mechanically ventilated in response to increasing dose of methacholine inhalation, using the pulmonary function equipment from RC System for

Mouse [Buxco Research Systems 2033 Corporate Drive Wilmington, NC 28405 USA] (21).

Histological examination of murine lung tissue

For histopathological analysis, lung tissue was fixed with 10% formalin for 24 hr, after bronchoalveolar lavage fluid (BALF) collection. Lung samples were embedded in the paraffin. Microtome sections were cut in a 5 µm thickness and stained with haematoxylin and eosin (H&E) for analyzing airway inflammation and pathological changes (22).

Immunohistochemical analysis

For immunohistochemical detection of IL₁₇ and IL₂₃R in lung, the tissue sections were incubated overnight at 4 °C with the primary antibodies directed against IL₁₇ (Santa Cruz biotechnology, Inc, USA) and IL₂₃R (R&D Systems, Inc, Minneapolis, MN, USA). Image analysis was performed using a microscope (Leica, Germany). For BALF analysis, mice were anesthetized with chloral hydrate (10%, 0.04 ml/10 g body weight). The trachea was cannulated, and BALF was collected after injections of 0.5 ml saline dissolved in the phosphate buffer, into the lung. The BALF was centrifuged, and the supernatant was used to test the cytokine production. The experiments were followed a protocol as described (23).

Cytokine enzyme-linked immunosorbent assay

The serum concentration of IL₁₇ and IL₂₃ (R&D Systems, Inc.) in the supernatants of the cultured cells as measured by enzyme-linked immunosorbent assay (ELISA) using the ELISA kit (Thermo Fisher Scientific) based on the instructions of the manufacturer.

RNA extraction and RT-PCR

Total RNA from lung tissues was extracted with Trizol reagent (Invitrogen, Gaithersburg, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was generated using a cDNA reverse transcription kit (Invitrogen, Gaithersburg, USA) according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was performed by adding SYBR green I (Roche Diagnostics, Mannheim, Germany), and mouse IL₁₇, Foxp₃, and β-actin were amplified. Primer sequences were used as follow; IL₁₇ (F: GCAAAGCTGGACCACCACA and R: CACACCCACCAG-CATCTTCTC), Foxp₃ (F: TACCACTGGTTCACACGCAT-GT and R: CACCCGCACAAAGCACTTG), and β-actin (F: ATCCACGAAACTACCTTCAA and R: CACCCGCACAA-AGCACTTG), LightCycler™ (Roche Diagnostics). The relative expression levels were calculated by normalizing the IL₁₇ and Foxp₃ levels to that of β-actin mRNA.

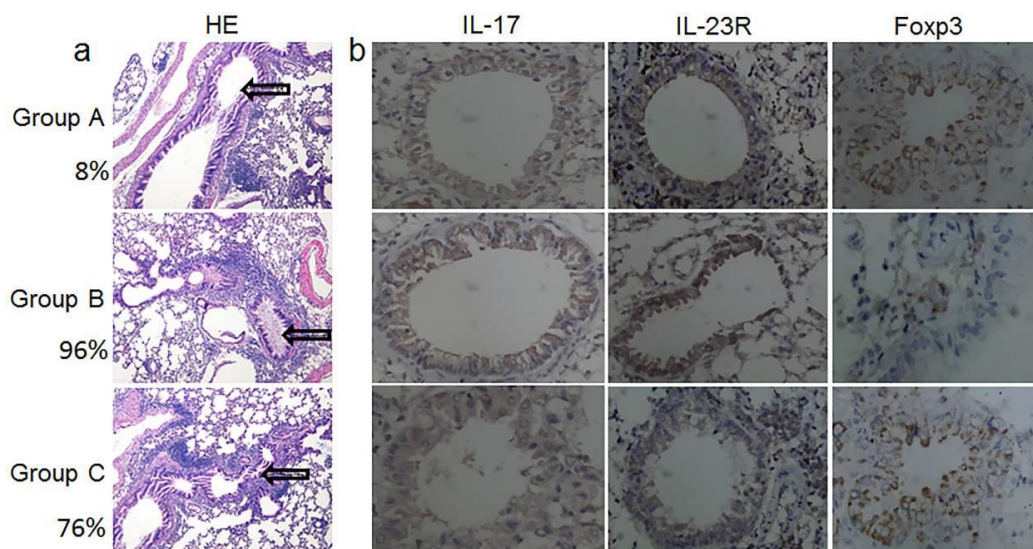


Figure 1. Histological and immunohistochemical assays of lung tissues after administration of inactivated *M. phlei* by inhalation. (a) The histopathology of lung tissues was assessed by H&E staining and examined by a light microscope. Group A showed that small airway basement membrane is complete with no significant inflammatory cell infiltration and mucosal edema; Group B showed mucosal edema, small airways and small perivascular inflammatory cell infiltration, and increased tracheal mucus secretion; Group C showed less mucosal congestion and reduction of edema, as well as small airways and small perivascular inflammatory cell infiltration, and airway mucus secretion. Black arrows indicate abnormal wall of airway. Percentage in the left of photos indicates the abnormal wall of airway in each group analyzed. 25 sections from each group were analyzed. (b) IL₁₇ and IL_{23R} levels, and promoted Foxp₃ expressions in Group C. Group A: normal control group; Group B: asthma model group; Group C: the prevention group

Detection of Th₁₇ and CD₄⁺CD₂₅⁺ Treg cells by flowcytometry

Lung tissues were removed and placed in PBS solution. Tissues were dispersed into single-cell suspensions. Peripheral blood mononuclear cells (PBMCs) were isolated by a density gradient centrifugation on Ficoll-Hypaque (24).

For cell-surface staining, CD₄⁺ (clone GK1.5), CD₂₅⁺ (clone eBio3C7), and Foxp3 (clone FJK-16S) antibodies were obtained from eBioSciences. For measurement of intracellular cytokines, T cells were stimulated with 500 ng/ml Phorbol dibutyrate (PdBU) and 500 ng/ml ionomycin in the presence of 1 mg/ml Brefeldin A (Sigma), for 2 hr. Cells were then fixed with 3.65% formaldehyde solution (Sigma) and permeabilized in a 0.1% NP40 containing buffer before analysis. IL_{17A} (clone TC11) antibody was obtained from eBioSciences. Intracellular staining and cell-surface staining were performed according to the manufacturer's instructions.

Measurement of PFP after methacoline stimulation

25 cross-section samples randomly selected from each group at complete peer trachea were used to analyze the score of airway wall inflammatory cell infiltration, as described below (25). The scoring is as follows, 0: no inflammatory cell infiltration; 1: a small amount, intermittent inflammatory cells; 2: airway wall surrounded with a thin layer of inflammatory cells (1-5 cells thin); 3: airway wall surrounded with thick layer of inflammatory cells (more than 5 cell layers).

Statistical analysis

The experimental values were presented as mean ± standard deviation. Statistical analysis was performed with the statistical software package SigmaStat (SPSS 17.0, Chicago, IL). Significant differences in expression of IL₁₇, IL_{23R}, and Foxp₃ in different groups were analyzed by using the Kruskal-Wallis test. All assays were compared using ANOVA followed by least squares difference analysis. Differences were considered statistically significant when the *P*-value < 0.05.

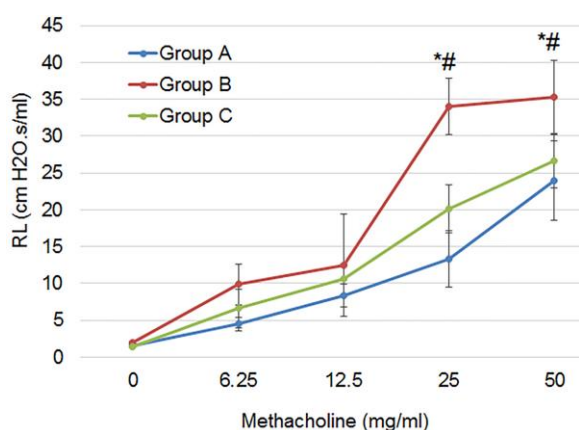
Results

Inactivated *M. phlei* administration suppressed airway inflammation

Our previous study identified that the inhalation of *M. phlei* reduces airway inflammation in asthmatic mice via altering the balance of the Th₁/Th₂ responses (20). To further monitor the effects of inactivated *M. phlei* on pathological changes in lung tissues, H&E was used to stain the lung tissues from the normal mice (Group A), asthmatic mice (Group B), and inactivated *M. phlei* inhaling asthmatic mice (Group C). In Group A, bronchial wall was smooth and complete without inflammatory cells surround the lung tissue, airway, and blood vessels. In OVA-immunized mice (Group B), OVA challenge induced infiltration of inflammatory cells. Inactivated *M. phlei* administered by inhalation led to a significant suppression of inflammatory cells recruitment into the airways. To explore pathological differences in

Table 1. PEF (ml/s) in three group ($\bar{x}\pm s$, n= 8)

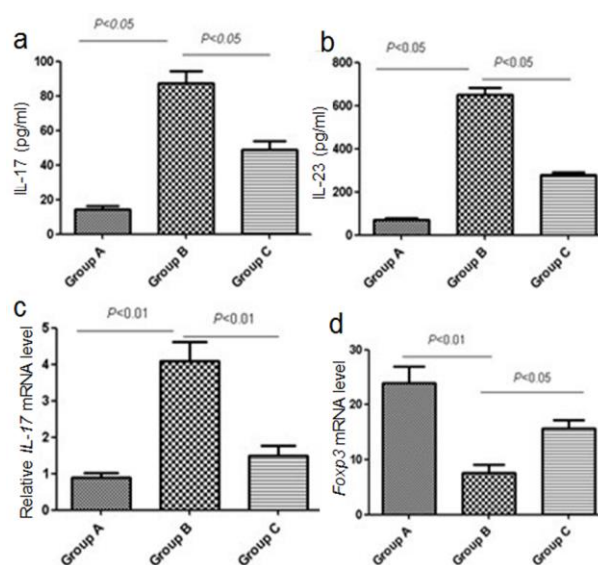
Group	Methacholine (mg/mL)				
	0	6.25	12.5	25	50
Group A	1.46±0.02	1.06±0.09	0.75±0.10	0.49±0.14	0.39±0.16
Group B	1.04±0.11 ^{*##}	0.92±0.06 ^{△##}	0.48±0.13 ^{△##}	0.45±0.13	0.31±0.08
Group C	1.42±0.03	1.12±0.08	0.93±0.17	0.65±0.16	0.47±0.12
F	54.925	7.901	12.393	2.659	2.000
P	0.000	0.006	0.001	0.111	0.178

**Figure 2.** Airways resistance to increasing concentrations of methacholine. Inhalation of inactivated *M. phlei* suppressed asthmatic airway hyper-responsiveness (AHR). Compared with Group A, * $P<0.01$. Compared with Group C, # $P<0.01$. Group A: normal control group; Group B: asthma model group; Group C: the prevention group

three groups, abnormal wall of airway was counted from each group. The results showed that OVA challenge significantly increased percentage of abnormality (96%) than normal group (8%), and inactivated *M. phlei* treatment slightly rescued it (76%). In addition, histochemical analyses revealed that inactivated *M. phlei* administration suppressed IL17 and IL23R expressions, and increased Foxp3 level, which were similar with normal mice (Figure 1).

Inactivated *Mycobacterium phlei* administration reduced airway hyper responsiveness

Airway hyper responsiveness was analyzed in three groups (Figure 1). After OVA immunization, airway hyper responsive was significantly increased, while peak expiratory flow (PEF) was decreased in Group B compared to normal mice ($P<0.01$).

**Figure 3.** Marker expression levels in BALF and lung. (a) The IL17 levels in BALF. (b) The IL23 levels in BALF. (c) The IL17 mRNA level in lung. (d) The Foxp3 mRNA level in lung. Group A: normal control group; Group B: asthma model group; Group C: the prevention group

However, inactivated *M. phlei* treatment inhibited the increase of airway sensitivity, as well as increased PEF in OVA immunization asthmatic mice model in Group C ($P<0.05$) (Figure 2). Similar with OVA challenge, Methacholine-mediated PEF levels were also lower in Group B, but inactivated *M. phlei* treatment rescued this decrease (Table 1).

Cytokines (IL17 and IL23) levels in BALF, and IL17 and Foxp3 transcript levels in lung were changed by inactivated *M. phlei* treatment

To further characterize the effects of inactivated *M. phlei* inhalation, the concentration of IL17 and IL23 in BALF was measured by ELISA, and the mRNA level of IL17 and Foxp3 was assessed by RT-PCR. As shown

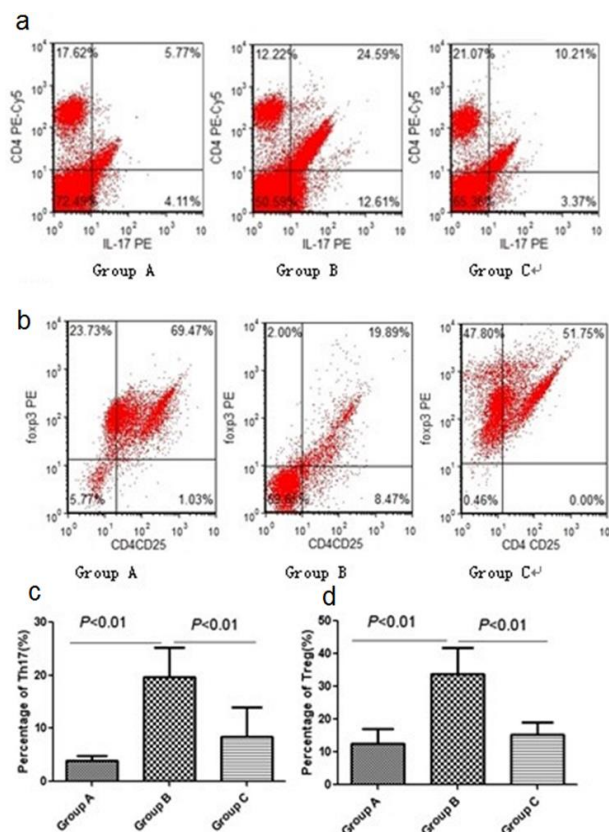


Figure 4. The proportions of Th17 and Treg cells. (a) The percentage of Th17 cells in lung by flowcytometry. (b) The percentage of Treg cells in lung by flowcytometry. (c) Comparative analysis of Th17 cells in three groups. (d) Comparative analysis of Treg cells in three groups. Group A: normal control group; Group B: asthma model group; Group C: the prevention group

in Figure 3, the concentration of IL₁₇ and IL₂₃ in BALF and the level of IL₁₇ mRNA were increased in Group B, but they were decreased by the treatment of inactivated *M. phlei* in Group C. However, there was no significant change in the Foxp3 mRNA level between the three groups.

Inactivated *M. phlei* administration increased percentage of Th₁₇ and CD₄⁺CD₂₅⁺ Treg cells in lung

Inhalation of *M. phlei* was known to change the balance of the Th₁/Th₂ responses (20). To further address its effects on other type of cells in lung, Th₁₇ and CD₄⁺CD₂₅⁺ Treg cells were monitored in normal mice (Group A), asthmatic mice (Group B), and inhaled inactivated *M. phlei* asthmatic mice model (Group C). As shown in Figure 4, the percentage of Th₁₇ cells was significantly high and CD₄⁺CD₂₅⁺ Treg cells were obviously low in Group B. However, the percentage of Th₁₇ cells was significantly low and CD₄⁺CD₂₅⁺ Treg cells were obviously high in Group C. The percentage of T cell and Th₁₇ cells in lung was also analyzed. Data showed that T/Th₁₇ cell ratio was decreased in Group B compared to Group A, and it was partially recovered in Group C (Figure 5).

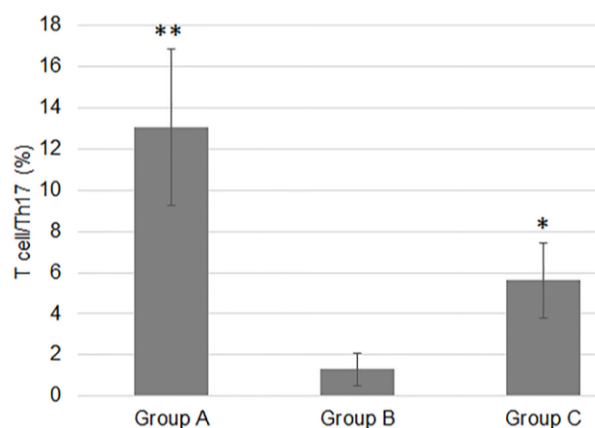


Figure 5. The ratio of regulatory T cells and Th17. Comparison of T cell and Th17 cells in lung tissues of three groups were shown as percentage. Group A: normal control group; Group B: asthma model group; Group C: the prevention group. Significant differences between Group A and Group B or Group C and Group B were shown (* $P < 0.05$, ** $P < 0.01$)

Discussion

Nonspecific immunosuppressive therapy is one of the current treatments of asthma. Immunization with *mycobacteria* or *mycobacteria* products has been reported to inhibit the development of allergic disease (16, 17, 26, 27). The most acceptable approach is the exposure to a range of mainly innocuous microorganisms, largely bacteria to trigger protective responses in the developing immune system. These effects are probably activated through innate immune receptors such as TLR₂ (28), and might affect the development of responses mediated by several cell types including basophils, natural killer cells (29), dendritic cells (30), and T-regulatory (Treg) cells (31).

Extensive studies have reported that *mycobacteria* such as BCG have suppressive effects on asthma (14-16, 18, 32-36). Many factors affect the induction of immune responses by *mycobacteria*, such as *mycobacteria* strains (37) and route of administration. Oral exposure to bacterial extracts, protects animals against the development of experimental models of asthma (30, 31). The present study demonstrated that inhalation of inactivated *M. phlei* is a way to treat asthma. OVA challenge (Group B) increased the levels of IL₁₇ and IL₂₃R, the concentration of IL₁₇ and IL₂₃, and the proportion of Th₁₇ cells, while reduced the level of Foxp3. Our results also showed that inhalation of inactivated *M. phlei* suppressed the airway inflammation and hyperreactivity, reduced the production of IL₁₇ and IL₂₃, the proportion of Th₁₇, and the expression of IL₂₃R in asthmatic mice. Inhaling inactivated *M. phlei* promoted the expression of Foxp3 and the proportion of CD₄⁺CD₂₅⁺ Treg cells in Group C.

Previous studies reported the effects of *Mycobacterium* on Th₁₇ and/or Treg cells (38-41). In which, lower expression of Th₁₇-associated cytokines

and higher expression of CD₄⁺CD₂₅⁺ Treg cells were observed in the treated mice than the untreated asthmatic mice model, which is similar with our findings in current study. However, conflicting results exist when it comes to which T cells produce IL₁₇ and are affected by BCG in asthma. Deng and colleagues (42) showed that BCG neonatal vaccination reduced IL₁₇ production in both the BALF and the lung lymphocytes, in asthmatic mice. They also showed that BCG neonatal vaccination did not reduce Th₁₇ cells. Although effective, immunotherapy by injection of BCG has the potential for systemic side effects including sclerosis, ulcer, fever, and even tuberculosis diffusion. The inhalation of certain drugs was suggested as early as 1946 (43), and now is commonly used for the patients with asthma and chronic obstructive pulmonary disease (44). Inhalation delivery of the drugs to lungs offers several substantial advantages such as simplified administration protocol, reduced drug quantity needed to achieve therapeutic effect, and increased drug concentration at the required sites (45) and is suitable to be used for children especially. Moreover, the initial interactions of microbes with epithelial cells, dendritic cells, and macrophages at the mucosal surface create potential to activate a wide range of different lymphoid types (46). In our study, inhalation of inactivated *M. phlei* successfully reduced airway inflammatory response and balanced proportion of Th₁₇ and CD₄⁺CD₂₅⁺ Treg cells, as well as the levels of cytokines in lung tissue after being immunized by OVA. Based on our findings, inhalation of inactivated *M. phlei* is an efficient approach to treat asthma.

Conclusion

The present study demonstrated that inhaled inactivated *M. phlei* is able to ameliorate allergic asthma via modulating the balance between CD₄⁺CD₂₅⁺ regulatory T cells and Th₁₇ cells in mice. Therefore, our data underlines the importance of inhaling inactivated *M. phlei* as a potential approach for the therapeutic modulation of asthma.

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