# The effect of inhaled inactived *Mycobacterium phlei* as a treatment for asthma

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**Abstract.** Allergic asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR). A murine model of asthma was used to examine the antiasthmatic effect of inhaled inactived *Mycobacterium phlei* (*M. phlei*). AHR, neutrophil levels, eosinophil levels and levels of interleukin (IL)-17 and IL-23 receptor (IL-23R) were monitored. The results demonstrated that inactivated *M. phlei* alleviates the IL-17$^+$γδT cell-mediated immune response and attenuates airway inflammation and airway hyperresponsiveness in the asthmatic murine lung, partially through inhibiting the expression of IL-23R. In conclusion, inactivated *M. phlei* may be an effective antiasthmatic treatment, regulating IL-17-producing γδT (IL-17$^+$γδT) cell-mediated airway inflammation and airway hyperresponsiveness to relieve the symptoms of mice with asthma.

## Introduction

Allergic asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR) (1). The pro-inflammatory type 2 helper T (Th2) cell cytokines, interleukin (IL)-4, IL-5 and IL-13, which trigger the release of IgE from B lymphocytes and airway eosinophilia (2), may contribute to AHR in asthma (3). Asthma is most commonly associated with an aberrant Th2 cell response, but severe disease is not exclusively associated with the production of Th2 cell-associated cytokines (4). It is instead characterized by increased production of the pro-inflammatory cytokine IL-17. Previous studies have suggested that IL-17 is involved in the pathogenesis of airway diseases, including allergic asthma, and IL-17 expression has been revealed to be upregulated in the airways of mice and humans following allergen-induced airway inflammation (5-8). A neutrophilic influx is observed in the lung following IL-17 production, contributing to pulmonary diseases including asthma (9,10). Therefore, there is accumulating evidence that IL-17 is associated with allergic asthma.

γδT cells have been reported to be dominant producers of IL-17 at the site of infection during the early phase of pulmonary *Mycobacterium tuberculosis* infection (11). In addition, IL-17-producing γδT (IL-17$^+$γδT) cells are associated with certain autoimmune diseases (12). IL-17$^+$γδT cells are localized in mucosal tissues, including the lung, intestine, peritoneal cavity and reproductive organs, that are exposed to exogenous stimuli such as pathogens (13). Furthermore, several studies have reported that endogenous IL-23 induces IL-17 production by γδT cells *in vivo* and *in vitro* (14-18).

It has previously been reported that IL-17, an important pro-inflammatory cytokine, was mainly produced by γδT cells (19). γδT cells are generated from naïve T cells, and γδT cell differentiation is driven by stimuli including IL-23. IL-23- IL-23 receptor (IL-23R) signaling promotes GATA binding protein 3 (GATA-3) expression and enhances IL-17 production by γδT cells (20,21). These cells are the first immune cells found in the fetus and confer immunity to newborns prior to activation of the adaptive immune system.

The Bacillus Calmette-Guérin (BCG) vaccine, a non-specific stimulator of immune function, protects against the development of asthma in humans and mice via inhibition of Th2 immune responses, which are characteristic of asthma (22-24). The BCG vaccine is considered safe, with side-effects mainly including erythema and a papule, ulcer or scar at the immunization site. These side-effects are mild and do not require treatment. However, regional suppurative lymphadenitis and osteitis are not uncommon.

Immunotherapy is the only currently available treatment with the potential to change the natural history of allergic disease and delay allergy progression in individuals with atopic allergies (24). Mucosal immunotherapy is advantageous due to the non-injection route of administration and lower side-effect profile (25). Multiple routes for mucosal immunotherapy have been proposed and investigated, including oral, nasal, tracheal and sublingual. Atomization delivery is attractive due to the ease of administration. It has previously been observed that inhalation of inactivated *Mycobacterium phlei* (*M. phlei*) attenuates

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airway inflammation via upregulation of IL-10 and interferon (IFN)-γ secretion, which are anti-inflammatory molecules, and downregulation of IL-4 production (26). γδT cells are generated from native T cells, and γδT cell differentiation is driven by stimuli such as IL-23. IL-23-IL-23R signaling promotes GATA-3 expression and enhances IL-17 production by γδT cells (19,20). In general, γδT cells account for ~3-5% of all lymphoid cells found in the secondary lymphoid tissues and the blood. These cells are the first immune cells found in the fetus and provide immunity to newborns prior to activation of the adaptive immune system (27).

Therefore, the present study hypothesized that inactivated *M. phlei*, administrated via inhalation, would exert an antiasthmatic effect in a murine asthma model through suppression of the pro-inflammatory activity of IL-17$^+$γδT cells by downregulation of IL-23R expression.

## Materials and methods

*Animals*. Male BALB/c mice (n=30), 6-8 weeks old, weight 18-22 g, were obtained from the Laboratory Animal Center of Guangxi Medical University (Nanning, China), and housed under specific-pathogen-free conditions in a facility with an automatic 12/12 h day/night cycle and fed with a standard laboratory food and water. Mice were randomly assigned to three experimental groups (n=10 in each group): The normal control group (group A), the sensitized/*M. phlei* untreated group (group B) and the sensitized/*M. phlei* treated group (group C). Sensitization was brought about by challenge with ovalbumin to create a murine asthma model.

*Establishment of a murine model of asthma*. A murine model of asthma was established according to a modification of previous methods (26). Mice were sensitized via intraperitoneal injections of 25 μg ovalbumin (OVA) and 1 mg $Al(OH)_3$ suspended in 0.2 ml saline on days 0, 7 and 14. Following initial sensitization the mice were challenged for 20 min with 2% OVA once per day using an ultrasonic nebulizer (Model WH-2000; Guangdong Yuehua Medical Instrument Factory Co., Ltd., Guangdong, China) in a closed chamber on days 21-28. Group A mice received saline in place of OVA at the sensitization and challenge stages.

Following the challenge, the treatment group inhaled a solution of inactivated *M. phlei* (1.72 μg ampule *M. phlei* dissolved in 10 ml saline; cat. no. S20040067; Chengdu Jinxing Jiankang Pharmaceutical Co., Ltd., Chengdu, China) administered by nebulizer once per day for 5 days. The normal control group and asthma model group (groups A and B) were sham treated with 10 ml atomized saline instead. The animals were sacrificed by cervical dislocation 24 h after the final inactivated *M. phlei* treatment. Lung tissue was subsequently harvested: Left lobes were fixed with 10% formalin for hematoxylin and eosin (H&E) staining and immunohistochemistry, while right lungs were stored at -80˚C until further use for fluorescence-activated cell sorting (FACS).

*Measurement of AHR*. Total lung resistance ($R_L$), dynamic compliance (Cdyn) and peak expiratory flow (PEF) were assessed via a tracheostomy tube 3 h following the inhalation of saline or multiplied methacholine treatment as previously described, using a computerized small animal ventilator (Data Sciences International, Minneapolis, MN, USA) (28). Methacholine is used to diagnose asthma by inducing bronchoconstriction. Mice were allowed to stabilize on the ventilator for 5 min prior to measurements. Once stabilized, dose responsiveness to methacholine (6.25, 12.5, 25 and 50 mg/ml) was measured and reported as total lung resistance.

*Pulmonary histological analysis*. Lungs were harvested from the mice. Left lobes were fixed with 10% formalin for 24 h and embedded in paraffin for histopathology analysis. 4-5 μm sections were cut. The tissue sections underwent H&E staining to visualise airway inflammation changes through light microscopy (Olympus Corporation, Tokyo, Japan).

*Bronchoalveolar lavage fluid cell counting*. Bronchoalveolar lavage fluid (BALF) was isolated as previously described (2). BALF was centrifuged at 600 x g for 5 min, and the supernatant was discarded. The cell pellet was resuspended in 200 μl of RPMI-1640 medium (cat. no. 11875093; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the red blood cells were lysed using 200 μl Red Blood Cell Lysis Buffer (cat. no. R1010; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). The cells were subsequently adhered to a hemocytometer slide and counted at x100 magnification with a light microscope. The absolute cell counts per BALF sample were calculated for neutrophils and eosinophils.

*Immunohistochemistry examination of IL-17 and IL-23 receptor (IL-23R)*. For immunohistochemical detection of IL-17 and IL-23R in the airway, formalin-fixed, paraffin-embedded sections were stained with biotinylated polyclonal antibodies specific for IL-17 (cat. no. 500-P07Bt; PeproTech, Inc., Rocky Hill, NJ, USA) and IL-23R (cat. no. BAF1400; R&D Systems, Inc., Minneapolis, MN, USA). Negative control experiments were performed by omitting the primary antibodies. Sections were blocked with 3% bovine serum albumin and 0.4% Triton X-100 in TBS buffer for 30 min at room temperature, then incubated overnight at 4˚C with IL-17 antibody and IL-23R antibodies at 1:50 dilutions, with the subsequent addition of a peroxidase complex prepared according to the manufacturer's instructions. Image analysis was then performed and analysed with Lecia LAS AF software version 2.6.0 (Leica Microsystems GmbH, Wetzlar, Germany).

*Flow cytometric analysis*. The following antibodies were used for flow cytometric analysis of BALF-derived T cells: PERCP-CY5.5-conjugated IL-17 antibody (cat. no. TC11-18H10; BD Pharmingen, San Diego, CA, USA), IL-23R polyclonal antibody (cat. no. 06-1331; Merck Millipore, Darmstadt, Germany) and goat anti-rabbit IgG-PE (cat. no. sc-3739; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Intracellular cytokine detection of BALF-derived T cells was performed as previously described (29).

*Statistical analysis*. Data are expressed as the mean ± standard deviation. Statistical analysis was performed via one-way analysis of variance for multiple comparisons, followed by Fisher's Least Significant Difference test for comparisons

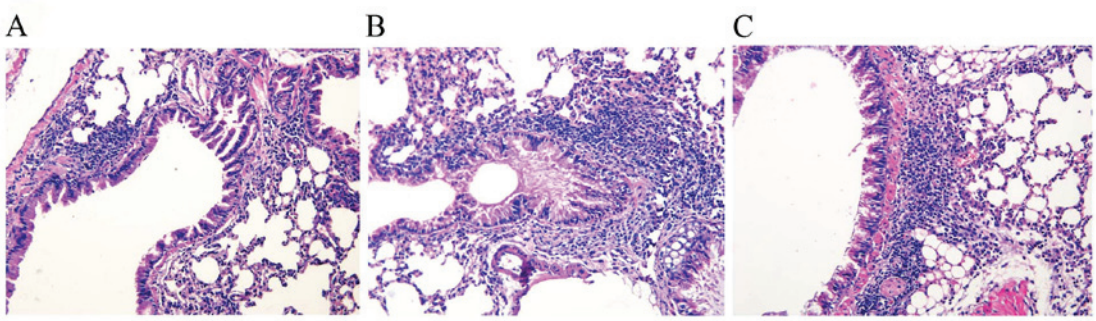


Figure 1. Effects of inactivated *Mycobacterium phlei* (*M. phlei*) on ovalbumin-induced airway inflammation. Histological examination of lung tissue by haemotoxylin and eosin staining in the (A) normal control group, (B) sensitized/*M. phlei* untreated group and (C) sensitized/*M. phlei* treated group. Original magnification, x400.

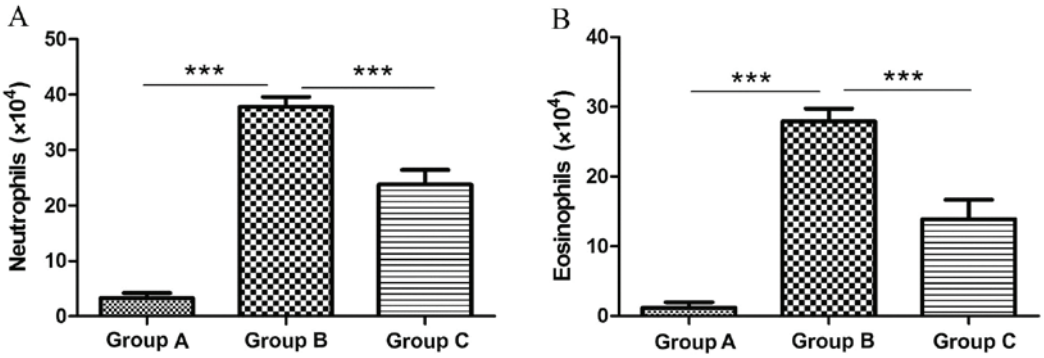


Figure 2. Effects of inactivated *Mycobacterium phlei* (*M. phlei*) on (A) neutrophil and (B) eosinophil numbers in bronchoalveolar lavage fluid. Data are presented as the mean ± standard error with comparisons indicated by lines; ***P<0.001. Group A is the normal control group, group B is the sensitized/*M. phlei* untreated group and group C is the sensitized/*M. phlei* treated group.

between groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Effects of inactivated M. phlei on the pulmonary pathology of OVA-induced asthmatic mice*. To determine the effect on the lung parenchyma following inactivated *M. phlei* treatment, formalin-fixed, paraffin-embedded whole lungs were sectioned and stained with H&E. The lung histology demonstrated increased numbers of inflammatory cells within the bronchiolar and alveolar compartments, as well cell hyperplasia, in the two sensitized groups compared with the normal control group. Predominately perivascular and peribronchiolar mixed eosinophil and lymphocyte cellular aggregates were consistently observed following OVA challenge and were not observed in the normal control group. Thickened basement membranes were present in the sensitized groups vs. the normal control group (Fig. 1A and B). The administration of inactivated *M. phlei* attenuated the infiltration of inflammatory cells in the peribronchial and perivascular areas as compared with the asthma model mice, with fewer inflammatory eosinophil and lymphocyte cellular aggregates in the sensitized/*M. phlei* treated group compared with the sensitized/*M. phlei* untreated group (Fig. 1B and C).

*Effect of inhaled inactived M. phlei on neutrophils and eosinophils in BALF*. Neutrophil numbers were significantly elevated in the sensitized/*M. phlei* untreated group ($37.8x10^4$; Fig. 2A) compared with the normal control group ($3.3x10^4$; 10.45-fold; P<0.0001; Fig. 2A). However neutrophil numbers were significantly decreased in the sensitized/*M. phlei* treated group compared with the sensitized/*M. phlei* untreated group (1.59-fold difference; P<0.0001; Fig. 2A). Eosinophil numbers were significantly increased in sensitized/*M. phlei* untreated mice ($27.9x10^4$; Fig. 2B) compared with the normal control group ($1.17x10^4$; 23.8 fold difference; P<0.0001; Fig. 2B). A 2-fold decrease in eosinophil numbers was observed in the sensitized/*M. phlei* treated group ($13.9x10^4$; Fig. 2B) compared with the sensitized/*M. phlei* untreated group (P<0.0001; Fig. 2B). The results suggest that inactived *M. phlei* may attenuate the airway inflammation of mice with asthma.

*Effect of inhaled inactived M. phlei on lung function alongside methacholine treatment in asthmatic mice*. The effect of inhaled inactived *M. phlei* on AHR to methacholine in asthmatic mice was evaluated through measuring changes in $R_L$, Cdyn and PEF.

PEF is the maximum flow rate during expiration, measured in ml/s (Fig. 3A). OVA challenge significantly decreased PEF in the sensitized/*M. phlei* untreated group compared with the normal control group at 0 mg/ml methacholine (P=0.0038; Fig. 3A), and at 12.5 mg/ml methacholine (P=0.0146; Fig. 3A). Sensitized/*M. phlei* treated mice demonstrated significantly elevated PEF compared with sensitized/*M. phlei* untreated mice at 0 mg/ml methacholine

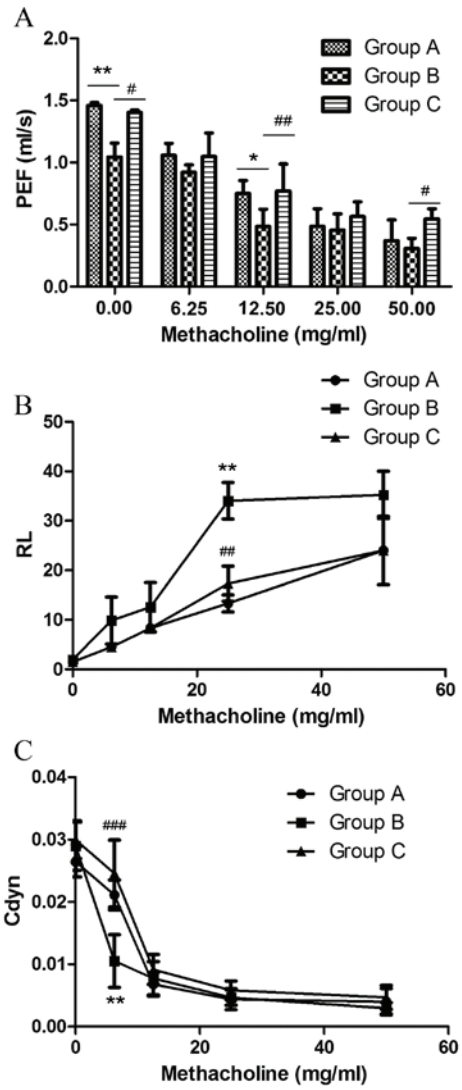


Figure 3. Effects of inhaled inactived *Mycobacterium phlei* (*M. phlei*) on airway hyperresponsiveness with methacholine treatment. PEF, RL and Cdyn are expressed as percentage change from the baseline value. (A) Effects of inhaled inactived *M. phlei* on PEF. (B) Effects of inhaled *inactived M. phlei* on $R_L$. OVA challenge significantly increased $R_L$ at all 4 methacholine doses with the maximum increase at 25 mg/ml. (C) Effects of inhaled inactived *M. phlei* on Cdyn. Data are presented as the mean ± standard deviation. Group A is the normal control group, group B is the sensitized/*M. phlei* untreated group and group C is the sensitized/*M. phlei* treated group. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. group A. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. group B. PEF, peak expiratory flow; $R_L$, total lung resistance; Cdyn, dynamic compliance.

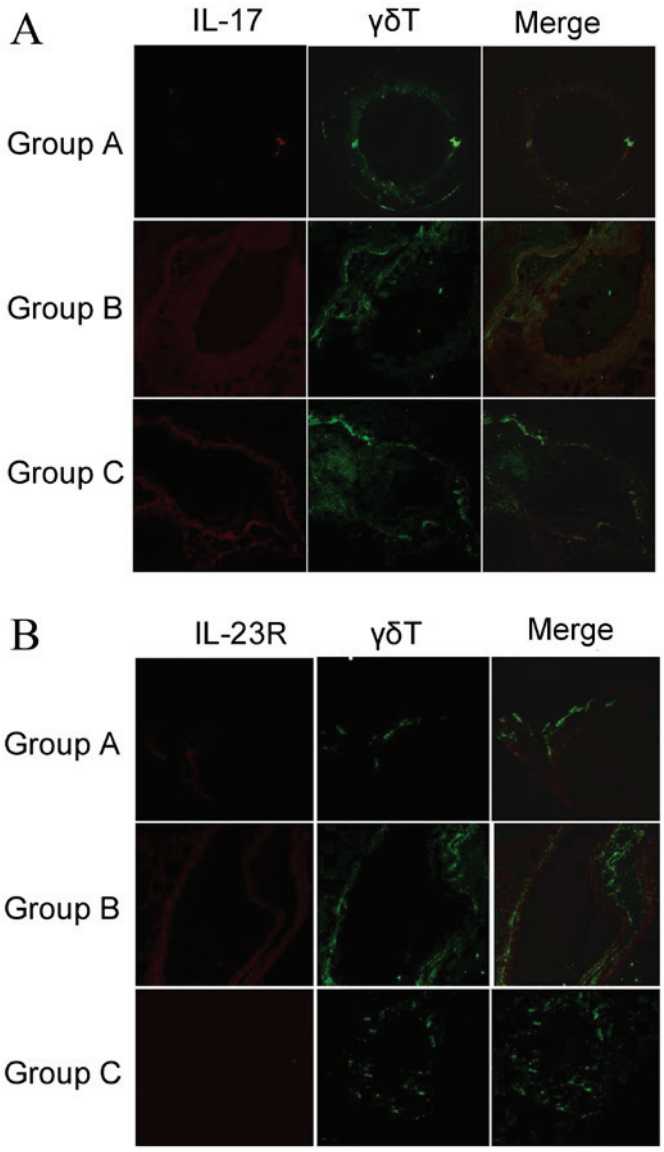


Figure 4. Effects of inhaled inactived *Mycobacterium phlei* (*M. phlei*) on inflammatory cytokine levels in lung tissues, visualized by immunofluorescence. The expression level of (A) IL-17 and (B) IL-23R in γδT cells. Group A is the normal control group, group B is the sensitized/*M. phlei* untreated group and group C is the sensitized/*M. phlei* treated group. Original magnification, x400. IL-17, interleukin-17; IL-23R, interleukin-23 receptor.

(P=0.0139; Fig. 3A), 12.5 mg/ml methacholine (P=0.00375; Fig. 3A) and 50 mg/ml methacholine (P=0.0142; Fig. 3A). No significant difference ws observed in PEF between sensitized/*M. phlei* treated and normal control groups (Fig. 3A). These results demonstrate that inhaled inactived *M. phlei* attenuates the impairment to PEF caused by methacholine in a mouse model of asthma.

OVA challenge significantly increased $R_L$ at all 4 methacholine doses tested in sensitized/*M. phlei* untreated mice, with the maximum increase at 25 mg/ml (P=0.001 vs. normal control group; P=0.06 vs. sensitized/*M. phlei* treated group; Fig. 3B). The $R_L$ of the normal control group and the sensitized/*M. phlei* treated group also increased in response to methacholine doses, but there was no significant difference between these two groups (Fig. 3B).

A dose of 6.25 mg/ml methacholine significantly decreased Cdyn in the sensitized/*M. phlei* untreated group compared with the normal control group (P=0.02; Fig. 3C) and the sensitized/*M. phlei* treated group (P<0.0001; Fig. 3C) at a dose of 6.25 mg/ml methacholine. Other methacholine doses demonstrated no significant difference among the 3 groups. There was also no significant difference between the normal control group and the sensitized/*M. phlei* treated group at any dose (Fig. 3C).

These results demonstrate that an atomized solution of inactivated *M. phlei* treatment restored these 3 aspects close to the levels recorded in healthy control mice. The atomized solution of inactivated *M. phlei* can suppress the adverse impact of methacholine, and recover pulmonary function almost to the healthy level.

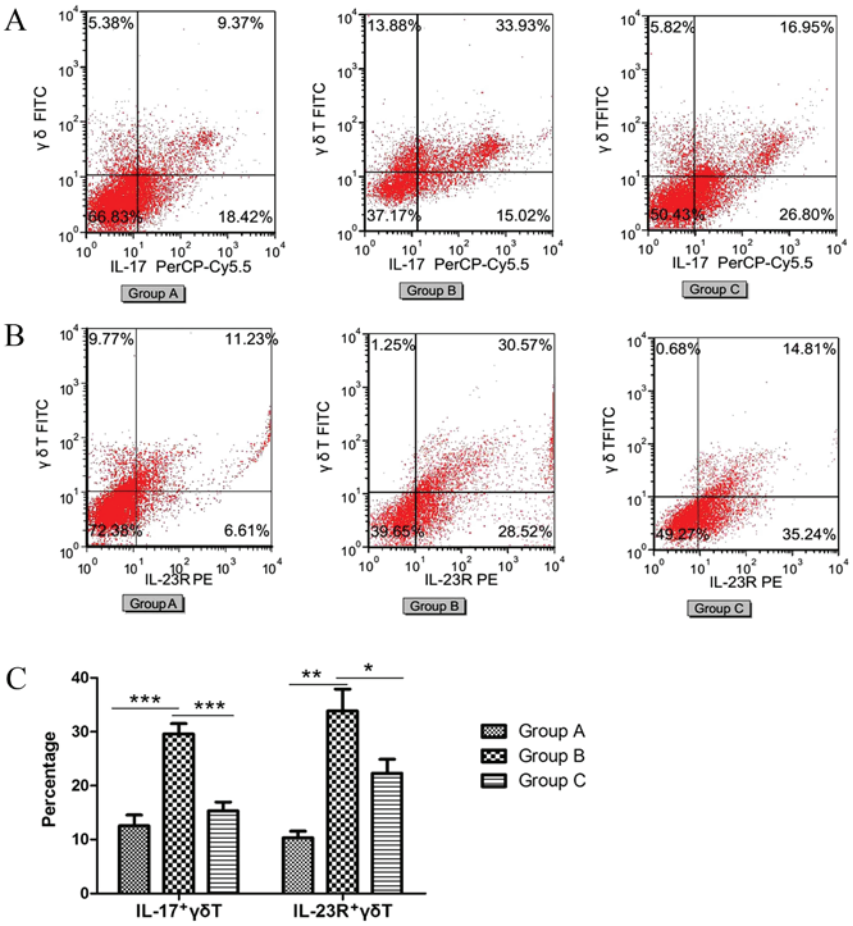


Figure 5. Effects of inhaled inactived *Mycobacterium phlei* (*M. phlei*) on the production of IL-17$^+$γδT and IL-23R$^+$γδT cells. Production of (A) IL-17$^+$γδT cells and (B) IL-23R$^+$γδT cells was assessed by fluorescence associated cell sorting analysis. (C) Quantitative analysis of the percentage of IL-17$^+$γδT or IL-23R$^+$γδT cells. Data are presented as the mean ± standard deviation and comparisons are indicated with lines: $^*$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001. Group A is the normal control group, group B is the sensitized/*M. phlei* untreated group and group C is the sensitized/*M. phlei* treated group. IL-17, interleukin-17; IL-23R, interleukin-23 receptor.

*Effects of inhaled inactived M. phlei on inflammatory cytokine levels in lung tissues, visualized with immunofluorescence*. Expression of IL-17 and IL-23R in lung tissues of the three groups was determined by immunohistochemical staining, with images acquired using laser scanning confocal microscopy as described in materials and methods. IL-17 and IL-23R expression appeared to increase in the sensitized/*M. phlei* untreated group, but decreased with administration of inactived *M. phlei* (Fig. 4). These results demonstrate that reduction of IL-17 and IL-23R may be related to the antiasthmatic effect of inactived *M. phlei* in mice with asthma.

*Effects of inhaled inactived M. phlei on the production of IL-17 or IL-23R positive γδT cells with FACS*. FACS was performed to determine the ratio of IL-17 positive γδT (IL-17$^+$γδT) cells (Fig. 5A) and IL-23R positive γδT (IL-23R$^+$γδT) cells (Fig. 5B). The percentage of IL-17$^+$γδT cells and IL-23R$^+$γδT cells significantly increased in the sensitized/*M. phlei* untreated group compared with the normal control group (P<0.0001 and P<0.0001, respectively; Fig. 5C). However, in the sensitized/*M. phlei* treated group, the percentages of IL-17$^+$γδT cells and IL-23R$^+$γδT cells were significantly decreased compared with the sensitized/*M. phlei* untreated group (P<0.0001 and P=0.015, respectively; Fig. 5C). This reduction of IL-17$^+$γδT cells and IL-23R$^+$γδT cells indicates that inflammation was attenuated and lung-function partially recovered. In addition, from the immunofluorescence detection (Fig. 4) and cell sorting (Fig. 5) results, it is possible to conclude that the antiasthmatic effect of inhaled inactived *M. phlei* is the result of the inhibition IL-17 and IL-23R expression, which decreases production of IL-17$^+$γδT cells and IL-23R$^+$γδT cells.

## Discussion

Previous studies have demonstrated that inactivated *M. phlei* nebulized therapy is effective in adults and children aged 4-12 years with moderate persistent asthma (30,31), however the detailed mechanism remains unclear. The results of the present study indicate that inhaled administration of inactivated *M. phlei* is able to alleviate allergen-induced airway inflammation in OVA-challenged mice. In addition, methacholine-associated damage is prevented in these mice by inhaled inactived *M. phlei* treatment, and pulmonary function is restored to close to the level of healthy mice. Therefore, inhaled inactived *M. phlei* may be an effective treatment for asthma.

Although it is widely accepted that the pathognomonic features of asthma are mediated mainly by Th2 cells and their associated cytokines, increasing evidence suggest IL-17, an important pro-inflammatory cytokine that is mainly produced by γδT cells, is involved in the development of asthma (32). It has been demonstrated that IL-17 is expressed in the airway of patients with asthma (7,10) and correlates with airway hyperresponsiveness (21,33,34).

The present study has clearly demonstrated that inhaled administration of inactivated *M. phlei* suppresses production of IL-17-producing γδT cells and decreased IL-23R-producing γδT cells in the lungs of treated mice (Fig. 5).

IL-23 is important for the maintenance of IL-17 production, however, pathogen products and environmental signals can also regulate IL-17-producing γδT cells, particularly *Mycobacterium*. Therefore, IL-17 production is complicated by the involvement of multiple immune mediators. Previous studies have demonstrated that combining C-C motif chemokine receptor 6 and CD44 for FACS sorting of γδT cells yielded an almost 100% pure population of IL-17-producing cells, indicating that γδT cells can be the sole source of IL-17 (21). Toll-like receptor triggering of γδT cells provides the first source of IL-17 (21). Cytokine IL-6 is responsible for the development, activation and recruitment of IL-17$^+$γδT cells (35). IL-21 may also be involved in the development of IL-17$^+$γδT cells (36). In addition, AHR-mediated environmental signals can shape the functional capacity of IL-17$^+$γδT cells (21). However, a number of mechanisms of the inhibitory effect of *M. phlei* on IL-17$^+$γδT cells remain to be identified.

In conclusion, the current study demonstrates that inactivated *M. phlei* acts as an immune regulator of the IL-17$^+$γδT-mediated response in the lung. Inactivated *M. phlei* suppresses the IL-17$^+$γδT-mediated immune response, airway inflammation and airway hyperresponsiveness in the lung, at least partially inhibiting the expression of IL-23R. Therefore, inactivated *M. phlei* may be an effective strategy for regulating IL-17$^+$γδT-mediated airway inflammation and airway hyperresponsiveness. This may, therefore, represent an effective treatment strategy for asthma.

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