Inhaled inactivated-*Mycobacterium phlei* modulates γδT cell function and alleviates airway inflammation in a mouse model of asthma

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Summary

Background: ***Mycobacterium bovis*** **Bacille Calmette-Guérin (BCG) and other mycobacterial infections suppress airway hyperresponsiveness and eosinophilic inflammation in asthma. γδT cells are important modulators of airway function and allergic inflammation. Vγ1+γδT cells increase eosinophilic airway inflammation and airway hyperresponsiveness, while Vγ4+γδT reduce airway hyperresponsiveness. The objective of this investigation was to determine the role of γδT cells and its subsets in the treatment of inhaled inactivated-*Mycobacterium phlei* for asthma.**

Methods: **OVA-sensitised mice were treated with aerosol *Mycobacterium phlei* after OVA challenge. Pathological HE staining was performed to measure lung inflammation, flow cytometric analysis was used to assess the intracellular cytokines of each γδT cell, and quantitative real-time PCR was performed to assess Vγ1 mRNA and Vγ4 mRNA expression.**

Results: **Airway inflammation was attenuated by treatment with inhaled inactivated-*Mycobacterium phlei*. IL-10+γδT cells, IFN-γ+γδT cells,Vγ4 mRNA expression were significantly increased.**

Conclusions: **Our results indicate that inhalation of *Mycobacterium phlei* can modulate γδT cell function, the proportion of different γδT cell subsets and reduce airway inflammation in asthmatic mice. *(Asian Pac J Allergy Immunol 2013;31:286-91)***

Key words: *bronchial asthma, Mycobacterium phlei, nebulised inhalation, airway inflammation, Vγ4+γδTcell, mucosal immunity*

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Introduction

Bronchial asthma is a common chronic respiratory disease and its prevalence has increasingly risen throughout the world. According to international management guidelines (GINA), treatment for this disease is inhaled corticosteroid therapy, either alone or in combination with long-acting beta-agonists. In the majority of cases, the disease can be well controlled. However, a proportion of asthma sufferers remain poorly controlled in spite of the current standard therapy. In addition, a serious concern has been increasingly raised about the side-effects of glucocorticoids, particularly in children and patients with severe asthma requiring high dose treatment.[1] Consequently, novel strategies to minimise side-effects and improve the efficacy of therapy for asthma are urgently needed. According to the "hygiene hypothesis", the reduced microbial exposure induces a shift of the Th1/Th2 balance in the immune system towards the biased Th2 response, which results in allergic disease and asthma. In recent years, studies have indicated that Treg cells are also responsible for the increased prevalence of asthma[2]. Many investigators have shown that *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) and other mycobacterial infections suppress airway hyperresponsiveness (AHR) and eosinophilic inflammation in asthma.[3,4] Recently Ahrens et al.[5] reported that BCG-induced suppression of Th2-type allergic airway inflammation appears to be associated with an up-regulation of both Treg and Th1 function.

γδT cells are most commonly known for their response to mycobacterium; they are found in greater numbers on mucosal and epithelial surfaces.

γδT cells are important modulators of airway function and allergic inflammation,[6] but their roles in asthma are not entirely clear. Our previous results[7] showed that the BCG vaccine is able to prevent the formation of a rat asthmatic model and γδT cells may also have a Th1/Th2 profile, which is important in the immunoregulation of the BCG vaccine and the pathogenesis of asthma. Moreover, different subtypes of γδT lymphocytes have different functions.[6] Animal experiments in mice have illustrated that Vγ1+γδT lymphocytes increased eosinophilic airway inflammation and AHR, and Vγ4+γδT lymphocytes can reduce AHR.[8,9] We recently reported that the inhalation of *Mycobacterium phlei* can regulate IL-4, IL-10 and IFN-γ secretion in lungs and reduce airway inflammation in asthmatic mice.[10] To evaluate whether inactivated-*Mycobacterium phlei* could modulate γδT cells function and whether Vγ1+γδT and Vγ4+γδT lymphocyte subsets could be used to treat bronchial asthma, we observed the effects of inhaled inactivated-*Mycobacterium phlei* aerosol therapy on attenuating asthmatic airway inflammation and examined the cytokine profile of γδT cells and the Vγ1 mRNA and Vγ4 mRNA expression in lungs, using an OVA-induced murine asthma model.

Methods

Experimental animals and experimental protocols

Specific pathogen-free male Balb/c mice, 4–6 weeks of age, were obtained from the Laboratory Animal Centre of Guangxi Medical University (Nanning, Guangxi, China) and were housed under standard laboratory conditions in a pathogen-free cage. They were maintained in an air-conditioned room at 23±2°C with 55±10% humidity, and fed a standard laboratory diet with ad libitum access to food and water. All animal care and experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Guangxi Medical University for Animal Experimentation, and the Guide for the Care and Use of Laboratory Animals was followed.

Male Balb/c mice were randomly categorised into three experimental groups of six mice each: the normal control group (group A), the asthma model group (group B), and the treatment group (group C). The mice of the asthma model group and the treatment group were sensitised and challenged with Ovalbumin (OVA) to make a murine asthma model. After the challenge, the treatment group inhaled the atomised solution of inactivated-*Mycobacterium phlei* for 5 days.

Establishment of a murine model of asthma

A murine model of asthma was established according to a modification of the methods proposed by North ML et al.[11] Mice were sensitised and challenged with OVA to make a murine asthma model, as described in our previous publication[10]. The normal control group received only saline (instead of OVA) at both the sensitisation and airway challenge stages. After the challenge, mice in the treatment group inhaled a solution of inactivated-*Mycobacterium phlei* (each Amp injection of 1.72μg *Mycobacterium phlei* dissolved in 10 ml saline) by nebuliser once daily for 5 days. The normal and model groups were sham-treated (10 ml saline atomisation instead). The animals were killed 24 hours after the last inactivated-*Mycobacterium phlei* treatment. Lung tissue harvest was performed. The left upper lobes were fixed with 10% formalin for HE staining, while the left lower lobes of lungs were quick-frozen by immersion in liquid nitrogen, and were then stored at -80°C until quantitative real-time PCR was performed. Right lungs were also stored at -80°C until further use for flow cytometric analysis.

Histological examination with HE staining

Lungs were isolated from the mice. Left upper lobes were fixed with 10% formalin for 24 hours, and then embedded in paraffin. Specimens were cut into 4μm sections. The microsections were stained with haematoxylin and eosin (H&E) for the examination of inflammation under a light microscope (Olympus, Tokyo, Japan) in a blind fashion.

Digestion of pulmonary tissue and Cell preparation

Single cell suspensions from lung tissues were made by mechanical disruption combined with enzymatic digestion according to the procedures provided in the paper by Lahn[6]. Firstly, the right lungs of mice were minced using sterile scalpels. Then, the tissue was incubated in phosphate-buffered saline (PBS) containing 1mg/mL collgenase IV for 90 minutes at 37°C in a sterile polypropylene tube. After incubation, lung tissue was vigorously pipetted up and down to further dissolve the remaining tissue clumps and then filtered using a 70μm cell-strainer to obtain a single-cell suspension. This pulmonary cell suspension was passed over a nylon-wool column to obtain lymphocyte-enriched cells. Filtered fluids were

centrifuged (500×g for 10 min at 4°C), and the cell pellet was washed and resuspended in 200μL of PBS. The total number of cells was counted manually using a haemocytometer chamber.

Antibodies and flow cytometric analysis for intracellular cytokines

Antibodies to study γδT cell surface markers (γδTCR) and intracellular cytokines (IL-10,IFN-γ) for flow cytometric analysis were purchased from eBioscience (San Diego, California, USA). Single cell suspensions of lung were analysed by flow cytometry. Staining with isotype control antibodies was performed in all experiments. For each sample to be analysed, 1×10^6 cells were treated with appropriate anti-mouse gamma delta TCR PE-Cy5 (eBioscience, Clone GL3) or the appropriate isotype controls, according to the manufacturer's instructions. Cells were incubated for 15 minutes in the dark at room temperature, before 100μl of fixation medium (Invitrogen, Camarillo, CA) was added; the solution was incubated for 15 minutes at room temperature. After one wash in PBS+0.1%NaN3+5%FBS, cells were centrifuged (350×g for 5min at 4°C) and resuspended. Then, 100μl of permeabilisation medium (Invitrogen, Camarillo, CA) was added along with the recommended volume of anti-mouse IL-10 PE (eBioscience, Clone JES5-16E3) and anti-mouse IFN-γ FITC (eBioscience, Clone XMG1.2), or the corresponding isotype controls, according to the manufacturer's instructions. Cells were incubated for 20 minutes in the dark at room temperature. After washing with PBS containing 0.1% NaN3 and 5% FBS, the cells were resuspended. Samples were analysed using a BD FACSCalibur (BD, California, USA). Data were processed with FCS Express software (BD Biosciences, California, USA).

Quantitative real-time PCR

To study the Vγ1 mRNA and Vγ4 mRNA expression, 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. Quantitative PCR analyses were performed with the SYBR qPCR mix(Toyobo, Osaka, Japan). Quantitative PCR detection of mouse β-actin, TCRgV1 and TCRgV4 was conducted in triplicate using the Real-Time PCR Detection System (Slan, Hongshi, Shanghai, China). Mouse β-actin was used as the housekeeping gene. The sequences of the forward and reverse primers are as follows:

mouse TCRgV1, 5'-CTTCTCTTCCAGACTTCG GCA-3'
and 5'-CCTCCTAAGGGTCGTTGATTG-3';
mouse TCRgV4, 5'-CGGCAGAAACCAAACC AAG-3'
and 5'-GCTTCGTCTTCTTCCTCCAAG-3';
mouse β-actin, 5'-CTGAGAGGGAAATCGTG CGT-3'
and 5'-CCACAGGATTCCATACCCAAGA-3'.

The following PCR reaction condition parameters were used: after an initial incubation for 1 min at 95°C, samples were subjected to 40 cycles of 15 sec at 95°C, 15 sec at 58°C and 45 sec at 72°C. Annealing temperatures of all primers was 58°C. Cycle thresholds obtained were normalized to β-actin. Relative fold changes were calculated by the comparative threshold cycle (CT) method, $2^{-\Delta\Delta CT}$.

Statistical analysis

Statistical analyses were performed using SPSS version 17.0 statistical software. The data are expressed as means±SEM (standard error of the mean). Multiple groups were compared by one-way analysis of variance (ANOVA) followed by post-testing with LSD's multiple comparison of means. A value of $p < 0.05$ was considered significant.

Results

Histological study of lung tissues (HE staining)

Histological examination of the lungs from the normal control group mice challenged with saline showed normal tissue, with no inflammatory cells (Figure.1A). In the asthma model group mice, histological staining showed a significant airway inflammation with obvious infiltration of eosinophils and lymphocytes (Figure.1B). In the treatment group, the infiltration of inflammatory cells was reduced in comparison with the asthma model mice (Figure.1C). This result was consistent with our previous study.[10]

Intracellular cytokines profile of γδT cells by flow cytometry

The percentage of IL-10+γδT cells and IFN-γ+γδT cells in the asthma model group were lower compared with those in the normal control group ($P < 0.05$). Inactivated-*Mycobacterium phlei* increased the percentage of IL-10+γδT and IFN-γ+γδT cells when compared with the asthma model group ($P < 0.01$). (Table 1).

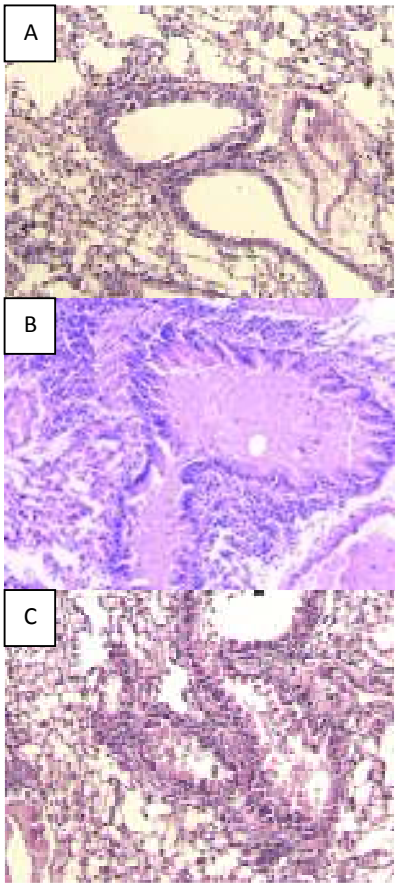

Figure 1. Histological Study of Lung Tissues
The microsections of lung samples were stained with hematoxylin-eosin(HE) to measure lung inflammation. **A:** The normal group. **B:** The model group. **C:** The treatment group.Original magnification:×200.

Detection of Vγ1 mRNA and Vγ4 mRNA levels in lung tissue by real-time PCR

The levels of Vγ1 mRNA in the lung of all groups were not detectable. The Vγ4 mRNA levels in the asthma model group were lower compared with the normal control group (P <0.05). Inactivated-*Mycobacterium phlei* increased the levels of Vγ4 mRNA in the lung of the treatment group as compared with the asthma model group (P <0.01). (Figure. 2)

Discussion

Chronic airway inflammation and AHR are characteristic features of asthma. The mechanisms underlying asthma involve a variety of inflammatory cells and cellular components (cytokines, chemokines). T lymphocytes are key regulators of the inflammatory immune response underlying the pathogenesis of asthma, while αβT cells are considered more important as they contribute to airway inflammation and AHR. γδT cells constitute 1-5% of the total blood lymphocytes but account for up to 50% of T cells in mucosal tissues[12]; they may function as a "first line of defence" of mucosal epithelium surfaces against foreign pathogens.[13] Actually, γδT cells appear to be important modulators of airway function and allergic inflammation in many human pulmonary diseases, including asthma and allergy. In different studies of asthma models, γδT lymphocytes were shown to both promote airway inflammation[14] and suppress inflammation and AHR[15] in murine models. Our previous research revealed that there are two subsets of γδT cells which can secrete either IL-4 or IFN-γ in PBMC or BALF in an asthmatic model of rats. We indicated that γδT cells are involved in the pathogenesis of asthma, presenting a Th1/Th2 immune response and the predominance of the Th2-cytokine profile.[16] In short, the influence of γδT cells on the allergic response in the airways appears to be complex.

Table 1. The percentage of IL-10+γδT cells and IFN-γ+γδT cells in lung cell suspension

Group	Number	IL-10+γδT cells	IFN-γ+γδT cells
A	6	5.39±1.09	5.49±1.97
B	6	3.25±1.17#	3.63±1.43#
C	6	9.71±2.45**	8.36±0.82**

Date expressed as mean±SD (n=6 per group). ## P <0.01 and # P <0.05 as compared to the normal control group (group A); ** P <0.01 and * P <0.05 as compared to the model group (group B).

It has been suggested that Mycobacterium is one of the most potent immunomodulatory microorganisms and that a mycobacterial vaccine could be one immunotherapy method for the treatment of allergy and asthma. Many studies have shown that immunisation with mycobacterial products have established a Th1-type immune response that suppressed Th2 cytokines, AHR and airway inflammation in murine asthma models.[17,18] In ovalbumin-sensitised mice, infection with *Mycobacterium bovis* BCG suppresses airway eosinophilia, which correlates with a reduced level of IL-5 production. IFN-γ produced during the Th1 immune response against BCG suppresses the development of local inflammatory Th2 responses in

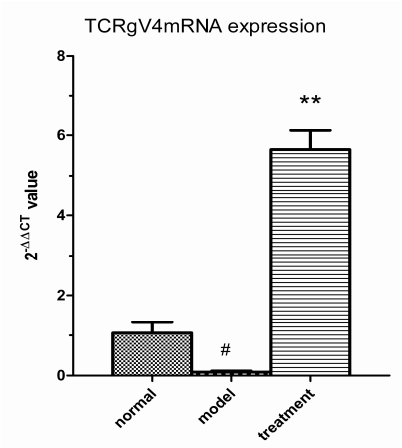

Figure 2. Vγ4 mRNA Levels in Lung Tissue by Real-Time PCR

The levels of Vγ1 mRNA in the lung of all groups were not detectable.The Vγ4 mRNA levels in the asthma model group were lower compared with the normal control group. Inactivated-Mycobacterium phlei increased the levels of Vγ4 mRNA in the lung of the treatment group as compared with the asthma model group.Date expressed as mean±SEM (n =6 per group).## P <0.01 and # P <0.05 as compared to the normal control group(groupA); ** P <0.01 and * P <0.05 as compared to the model group (groupB).

the lung.[19] Recently, Zuany-Amorim et al.[1] showed that the treatment of mice with heat-killed *Mycobacterium vaccae* reduces asthmatic manifestations, and this protection can be transferred with splenocytes; this is in contrast to the BCG studies, where neither primary nor transferred protection was associated with the induction of TH1-type responses. Subsequent studies suggested that heat-killed *Mycobacterium vaccae* suspensions enhance allergen-specific CD4+CD45RB^{Low} regulatory T cells and the inhibition of airway inflammation was mediated through IL-10 and TGF-β[4]. Taken together, BCG and other mycobacterial infections suppress AHR and eosinophilic inflammation, likely through a Th1 and regulatory Treg response.

In the present study, we observed that in OVA-challenged mice, inhaled inactivated-*Mycobacterium phlei* attenuated the infiltration of inflammatory cells in the lung. Inhaled inactivated-*Mycobacterium phlei* has anti-inflammatory properties in the mouse model. We detected the percentage of γδT cells expressing IL-10 and γδT cells expressing IFN-γ in the pulmonary cell suspension. In the asthma model group, their percentage was lower than those in the normal control group. Inactivated-*Mycobacterium phlei* increased the percentage of γδT expressing IL-10 and γδT expressing IFN-γ as compared with the asthma model group. However, γδT cells have different subtypes functionally. Isogai et al. demonstrated that CD8+γδT cells inhibit late airway responses through the release of IFN-γ[20]. γδT cells can acquire either Th1-type properties or Th2-type properties depending on the type of stimulus.[21] Kühl et al.[22] concluded that human peripheral γδT cells showed *in vitro* suppressive behaviour as well as production of both pro- and anti-inflammatory cytokines; they are not only strong IFN-γ and IL-10 producers but also very strong TGF-β producers. Our current research revealed that treatment with inhaled inactivated-*Mycobacterium phlei* attenuates airway inflammation in a mouse model of asthma by both regulating a Th1/Th2 immune response balance of γδT cells and inducing a regulatory effect of γδT cells. Indeed, γδT cells are commonly regarded as different subsets based upon the Vγ and/or Vδ chains preset in their TCR. These γδT cell subsets have different structures and differ from one another functionally. In the allergen-induced development of lung allergic responses, the γδT cell subset enhances the airway response to methacholine (MCh), as well as levels of Th2 cytokines and eosinophilic infiltrates in the lung, by the expression of Vγ1.[9] In contrast, γδT cell expressing Vγ4 strongly suppressed AHR.[6,23] Vγ1+γδT cell and Vγ4+γδT cells are two subsets that are functionally distinct and can alter AHR. We examined Vγ1mRNA and Vγ4mRNA expression in the lung. In this paper, treatment with inactivated-*Mycobacterium phlei* increased the levels of Vγ4 mRNA in the lungs of the treatment group in comparison with the asthma model group. Therefore, we proved that inactivated-*Mycobacterium phlei* can reduce airway inflammation and increase Vγ4+γδ T cell proportions, playing a negative regulatory role in the lung. Dodd et al.[24] reported that lung Vγ1+γδ T cells were very scarce. The fact that levels of Vγ1 mRNA in the lung were not detectable may be due to the too small percentage of Vγ1+γδT cells that immigrated.

We concluded that *Mycobacterium phlei* can modulate γδT cell function and the proportion of different γδT cell subsets, which is partly the underlying mechanism of its therapeutic effect in asthma. In addition, the aerosol administration of inactivated-*Mycobacterium phlei* through mucosal immunity may be a more efficacious vaccine to stimulate immunomodulation. The mechanism behind how γδT cell subsets exert their function is not fully clear. Additional studies are needed to

characterise the precise mechanism of *Mycobacterium phlei* treatment in asthma.

Conflict of interest

The authors have no conflicts of interest.

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