Long-term modulatory effect of *Mycobacterium vaccae* treatment on histopathologic changes in a murine model of asthma

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Background: Mycobacteria are being investigated for modulation of inflammation in asthma and atopic disorders by eliciting particularly strong protective T_H1 immune responses.

Objective: To investigate the long-term effects of intratracheally administered *Mycobacterium vaccae* on an experimental murine model of asthma.

Methods: BALB/c mice were placed in 4 groups: long-term *M vaccae*, *M vaccae*, asthma, and control groups. All groups but controls were sensitized intraperitoneally and challenged intratracheally with ovalbumin. The long-term *M vaccae* and *M vaccae* groups were treated with *M vaccae* intratracheally simultaneously during challenges. Finally, mice in the long-term *M vaccae* group were rechallenged with ovalbumin nebulization 24 days later. Evaluations of lung histopathologic findings and serum cytokine levels were performed.

Results: Comparison of the long-term *M vaccae* group with the asthma model group revealed that the number of hyperplasic goblet cells in small and large airways (small airway: $P < .05$; large airways: $P < .01$) and thickness of basement membrane in large airways were significantly less in the long-term *M vaccae* group. Furthermore, numbers of hyperplasic goblet cells in small airways ($P < .05$) and basement membrane in the large airway ($P < .05$), as well as inflammation in small airways ($P < .01$), were significantly less in the *M vaccae* group when compared with the asthma model group. Interferon-γ secretion from splenocytes of the *M vaccae* group was significantly higher than the asthma model and long-term *M vaccae* groups.

Conclusion: Intratracheal administration of *M vaccae* exerted a long-lasting ameliorating effect on airway histopathologic features of a murine asthma model.

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INTRODUCTION

The prevalence of asthma and allergic diseases has been increasing drastically during the last decades, especially in western societies.[1] One attractive explanation was offered by the hygiene hypothesis, which had shown that the relative lack of exposure to infectious agents early in life could potentiate the development of allergic diseases.[2] Research in the last decade has highlighted the key role played by T_H2 cells in orchestrating the chronic inflammation in allergic diseases. One mechanism for this inverse association between infectious agents and atopic diseases is thought to be through the balance between T_H1 and T_H2 immune mechanisms.[3–4]

Mycobacteria elicit particularly strong protective T_H1 immune responses. Shirakawa et al[5] were among the first to provide epidemiologic evidence for the inverse association between exposure to mycobacteria and subsequent development of atopy. Multiple studies in animal models of allergic inflammation have demonstrated that treatments with either live or killed mycobacteria are responsible of inhibiting allergen-induced lung inflammatory responses.[6–9]

Mycobacterium vaccae had been previously shown to have a potential effect in that respect by skewing T-cell responses away from T_H2.[6–8] However, more recent studies indicated that the immunological process behind the inhibitory effects of *M vaccae* is exerted by an additional non-T_H1/T_H2 regulatory mechanism. In studies by Zuany-Amorim et al,[10,11] the block of the allergic inflammation by a mechanism independent of interferon-γ (IFN-γ) and induction of allergen-specific $CD4^+$ T cells expressing low levels of CD45RB ($CD4^+$ $CD45^+$ RB^{low}) regulatory T cells with an inhibitory effect on airway lung inflammation was demonstrated. Furthermore, subcutaneous *M vaccae* treatment was demonstrated to induce a long-term antigen-specific protection. In the current study, we investigated whether intratracheally administered *M vaccae* would demonstrate a long-term ameliorating effect on lung histopathologic features and cytokine responses.

METHODS

Animals

Female BALB/c mice (5 to 6 weeks old) were obtained from the Turkish Scientific and Technical Research Institute (TUBITAK, Kocaeli, Turkey) and were raised and maintained in a pathogen-free environment at the Marmara University Research Centre

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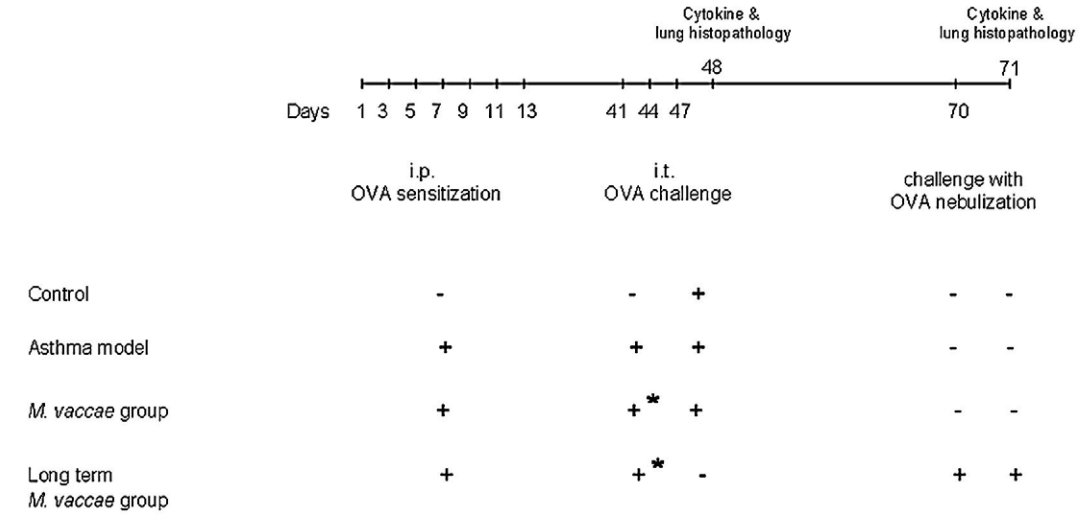

Figure 1. Study design. *Intratracheal *Mycobacterium vaccae* treatment. i.p. indicate intraperitoneal; i.t., intratracheal; and OVA, ovalbumin.

laboratory in accordance with the guidelines of the Animal Ethics Committee.

Study Groups

Mice were divided into 4 groups: a long-term *M vaccae* group (n = 10), a *M vaccae* group (n = 13), an experimental asthma model group (n = 13), and a control group (n = 11). The study design is shown in Figure 1.

Study Design

Mice in the long-term *M vaccae*, *M vaccae,* and asthma model groups were sensitized with ovalbumin 7 times on days 1 to 13 via intraperitoneal injections (Fig 1). Twenty-eight days later they were challenged with ovalbumin (days 41 to 47) by the intratracheal route. On the same days of ovalbumin administration, the long-term *M vaccae* and the *M vaccae* mice were immunized intratracheally with 10^7 CFU of *M vaccae* in 10 μL of saline. The experimental asthma model group and controls did not receive any *M vaccae* administration. Two days after the last intratracheal instillation all mice except for those in the long-term *M vaccae* group were killed. To evaluate the long-term effect of *M vaccae*, mice in the long-term *M vaccae* group were challenged with ovalbumin once more 3 weeks later before killing. Lungs and splenocytes were taken out for histopathologic evaluation and cytokine determination.

Preparation of Heat-Killed M vaccae *Suspension*

The *M vaccae* (ATCC 29678 strain) was supplied by North Carolina State University. *M vaccae* was cultured and the vials autoclaved at 120°C for 25 minutes. For intratracheal administration, suspensions of 1×10^7 CFU/mL were prepared and frozen at −20°C until needed.

Sensitization and Antigen Challenge

To establish the asthma model, all groups but controls were sensitized with 10 μg/100 μL of ovalbumin (Sigma A-5503; Sigma, St Louis, MO) intraperitoneally 7 times on each alternate day starting on day 1. Starting at 28 days after the last intraperitoneal injection, they were challenged with 20 μg of ovalbumin in 10 μL of saline 3 times 2 days apart by intratracheal instillation on days 41, 44, and 47.[12] Mice in the long-term *M vaccae* group were rechallenged with 1% ovalbumin nebulization for 20 minutes on day 70 to investigate whether the effect of *M vaccae* immunization could interfere with subsequent rechallenge in an established asthma model.

Treatment With M vaccae

On the same days with intratracheal ovalbumin instillation (days 41, 44, and 47), mice in the long-term *M vaccae* group and *M vaccae* group were treated with 10 μL of 1×10^7 CFU/mL of *M vaccae* intratracheally 3 times simultaneously. Mice in the asthma model and control groups did not receive any *M vaccae*.

Cell Culture and Cytokine Analysis

Single cell suspensions from the spleens of mice were obtained and cultured in RPMI-1640 medium supplemented with sodium bicarbonate (23.8 mL/L), 10% fetal calf serum, 1% streptomycin-penicillin, and L-glutamine. Splenocytes were suspended in ammonium chloride (8.29 g/mL), potassium bicarbonate (1 g/L), and EDTA (37 mg/L) for 10 to 15 minutes to lyse erythrocytes. Then, they were washed and resuspended in RPMI-1640 culture medium. Phytohemagglutinin (Sigma), ovalbumin, or *M vaccae* was added as antigen (5 μg/mL, 40 μg/mL, or 1×10^9 CFU/mL, respectively). Control wells contained no antigen. Cultures were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide. Supernatants were collected at 24 hours for interleukin-10 (IL-10) and IFN-γ and at 48 hours for IL-5 and stored at −20°C until analyses.

Cytokine Assays

Levels of IL-5, IL-10, and IFN-γ in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (mouse IL-10, IL-5, and IFN-γ; Endogen Inc, Rockford, IL). The assays were performed according to the instructions of the manufacturer. The absorbance values of the solutions in each well were recorded by using an ELISA plate reader at 450 nm.

Histopathologic Analysis

Forty-eight hours after the last ovalbumin instillation (intratracheally or nebulization), mice in all groups were killed and lungs were fixed for histopathologic evaluation and inflated by intratracheal instillation of buffered formol solution. Then, lungs were removed and placed in buffered solution. Subsequently, lungs were embedded into paraffin for preparation of histopathological sections. Sectioned paraffin-embedded tissues (3 to 5 μm) were stained with periodic acid–Schiff for the evaluation of hyperplasic goblet cells and with hematoxylin-eosin to analyze the thickness of smooth muscle, epithelium, and basement membrane. All histologic analyses were performed with MS Basic Image Analyser Software (Microsystem, Computerized Microscope Systems Co Ltd, Istanbul, Turkey), which was adapted on an Olympus BH2-RFCA model microscope (Olympus Optical Co Ltd, Tokyo, Japan). The airways were classified as small (<500 μm) and large (>1,000 μm) according to their circumferences. Measurements were made on airways cut in transverse section and free of branching.[9] Measurements of thicknesses of epithelium, basement membrane, subepithelial smooth muscle layers, number of hyperplasic goblet cells, and severity of inflammation on the airways were recorded. Semiquantitative analyses were achieved by scoring for inflammation severity according to the number of clusters of inflammatory cells in the peribronchial and perivascular areas (0 if no clusters of inflammatory cells, 1 for 1 to 3 clusters, and 2 for >3 clusters). All the histopathologic evaluations were performed 3 times for each parameter by 2 investigators (D.Y. and O.Y.) who were blinded to interventions. The mean of the measurements of each parameter was calculated for each observer. Then, the mean number of the sum of the 2 observers was calculated for statistical analyses.

Statistical Analyses

For statistical analyses, the GraphPad Instat Data (version 3.05, GraphPad Software Inc, San Diego, CA) program was used. The Dunnett multiple comparisons test was used to compare all 4 groups. $P < .05$ was considered statistically significant.

RESULTS

Airway Histopathologic Findings

Establishment of the asthma model. When compared with the control group, the experimental asthma model group had significantly higher numbers of goblet cells and increased thicknesses of epithelium in small and large airways, basement membrane in large airways, and subepithelial smooth muscle layers in small airways. These results revealed that the asthma model was successfully established (Figure 2). Table 1 presents the median and range values of all histopathologic parameters evaluated for all groups.

Long-term effects of intratracheal M vaccae *treatment.* Comparison of the long-term *M vaccae* group with the asthma model group revealed that the number of hyperplasic goblet cells in the small and large airways (small airway: $P < .05$; large airways: $P < .01$) (Figure 3) and thickness of basement membrane in large airways were significantly less in the long-term *M vaccae* group (Figure 4). All histopathologic parameters evaluated in the small and large airways except basement membrane thickness in the small airways and inflammation in the large airways of the long-term *M vaccae* group were not significantly different from controls (Table 1) (Figures 3 through 6).

Short-term effect of intratracheal M vaccae *treatment.* The numbers of hyperplasic goblet cells in the small airways ($P < .05$) (Figure 3) and basement membrane in the large airways ($P < .05$) (Figure 4), as well as inflammation in the small airways ($P < .01$) were significantly less in the *M vaccae* group when compared with the asthma model group (Table 1). All parameters except for basement membrane and epithelial thickness in the large airways (both $P < .05$) were not significantly different between the *M vaccae* and control groups (Table 1).

Long-term vs short-term effect of intratracheal M vaccae *treatment.* Comparison of the long-term *M vaccae* and *M vaccae* groups revealed no significant difference based on airway histopathologic parameters.

Cytokine Levels

Compared with the asthma model group, the *M vaccae* group revealed significantly higher phytohemagglutinin-induced IFN-γ levels ($P < .05$), whereas the long-term *M vaccae* group demonstrated significantly higher spontaneous and ovalbumin-induced IL-5 levels ($P < .01$ and $P < .01$, respectively). Comparison with controls demonstrated signifi-

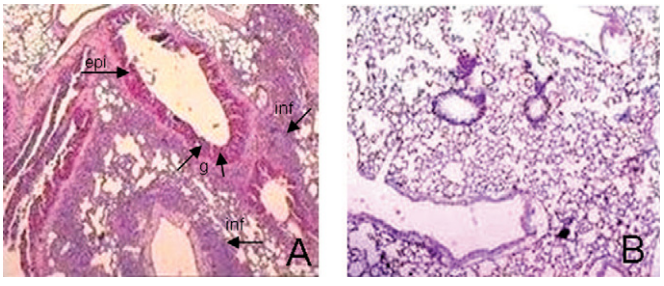

Figure 2. Histologic views of airways. A, In the periodic acid–Schiff–stained examinations of airway sections of mice in the murine asthma model group, numerous goblet cells (g) and increased thickness of epithelial layer (epi) were seen. Also marked peribronchial and perivascular inflammations (inf) were observed. B, Normal appearance of all evaluated parameters were found in sections from control group mice.

Table 1. Evaluated Histopathologic Parameters in All 3 Sized Airways*

Parameters	Long-term *Mycobacterium vaccae* group (n = 10)	*M vaccae* group (n = 13)	Asthma model group (n = 13)	Control group (n = 11)
Small airways				
Basement membrane thickness, μm	1.1 (0.8–2.0)	1.2 (0.5–2.0)†	1.8 (1.3–2.5)†‡	0.7 (0.3–1.1)‡
Epithelial thickness, μm	11.8 (6.0–26.3)§	14.8 (9.5–45.0)	23.0 (17.0–36.0)‡	11.0 (9.0–14.0)‡§
Smooth muscle thickness, μm	4.0 (3.0–5.0)§	4.5 (1.9–7.0)	5.0 (4.0–6.0)‡	3.0 (1.4–4.0)‡§
Goblet cell count, No. per every 500 cells	0 (0–2.0)§‖	0 (0–64.0)†	30.0 (4.0–128.0)†‡‖	0 (0–0)‡§
Inflammation score	1.0 (1.0–1.0)	1.5 (0–6.0)†	5.0 (3.0–7.0)†‡	0 (0–0)‡
Large airways				
Basement membrane thickness, μm	1.2 (0.9–1.5)§‖	1.5 (0.9–2.0)†¶	2.0 (1.3–2.3)†‡‖	0.8 (0.7–1.5)‡§¶
Epithelial thickness, μm	17.5 (11.3–21.8)§‖	21.0 (10.0–50.0)¶	28.0 (18.0–34.0)‡‖	12.0 (9.9–19.0)‡§¶
Smooth muscle thickness, μm	4.0 (1.9–5.0)§	5.5 (2.7–9.0)	6.0 (4.0–7.0)‡	3.0 (0–0)‡§
Goblet cell number, No. per every 500 cells	0 (0–22.0)§‖	17.0 (0–220.0)†	106.0 (35.0–216.0)†‡‖	0 (0–0)‡§
Inflammation score	2.0 (0–8.0)§	2.0 (0–6.0)†	6.0 (3.0–7.0)†‡	0 (0–0)‡§

* Data are presented as median (range).
† Comparison of *M vaccae* group and asthma model groups ($P < .05$).
‡ Comparison of control and asthma model groups ($P < .05$).
§ Comparison of long-term *M vaccae* group and controls ($P < .05$).
‖ Comparison of long-term *M vaccae* group and asthma model groups ($P < .05$).
¶ Comparison of *M vaccae* group and control groups ($P < .05$).

cantly higher spontaneous IL-5 secretion in the long-term *M vaccae* group ($P < .01$). Finally, phytohemagglutinin-induced IFN-γ levels were significantly higher in the *M vaccae* group compared with the long-term *M vaccae* group (data not shown).

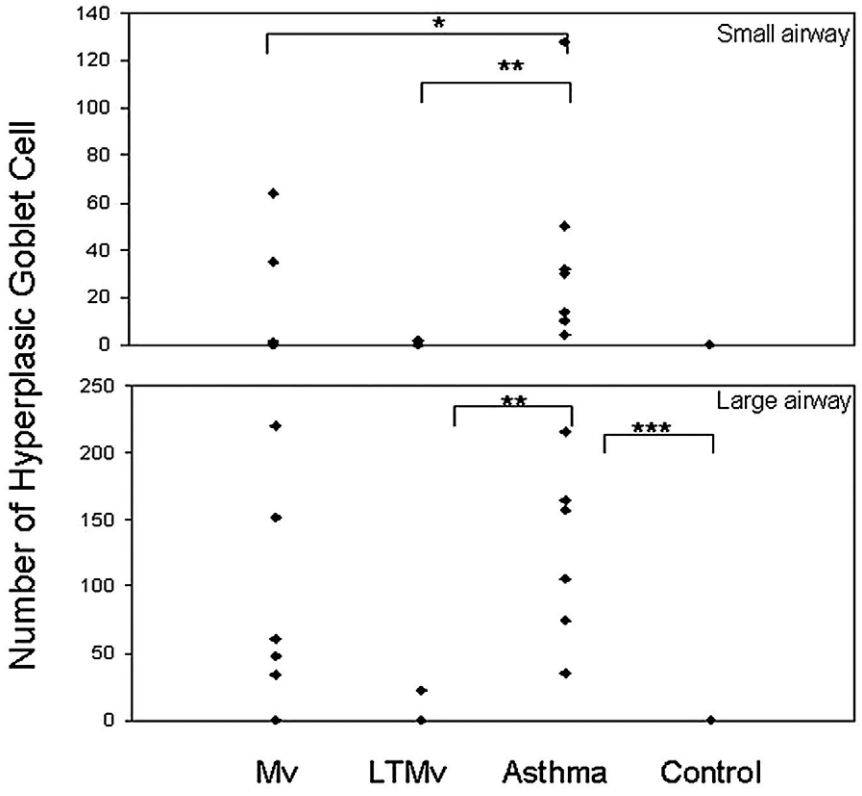

Figure 3. Effect of *Mycobacterium vaccae* treatment on hyperplasic goblet cell numbers. *Comparison of *M vaccae* vs asthma model groups (small airways: $P < .05$). **Comparison of long-term *M vaccae* vs asthma model groups (small airways: $P < .05$; large airways: $P < .01$). ***Comparison of asthma model vs control groups (small airways: $P < .05$; large airways: $P < .01$. Mv indicates *M vaccae*; LTMv, long-term *M vaccae*.

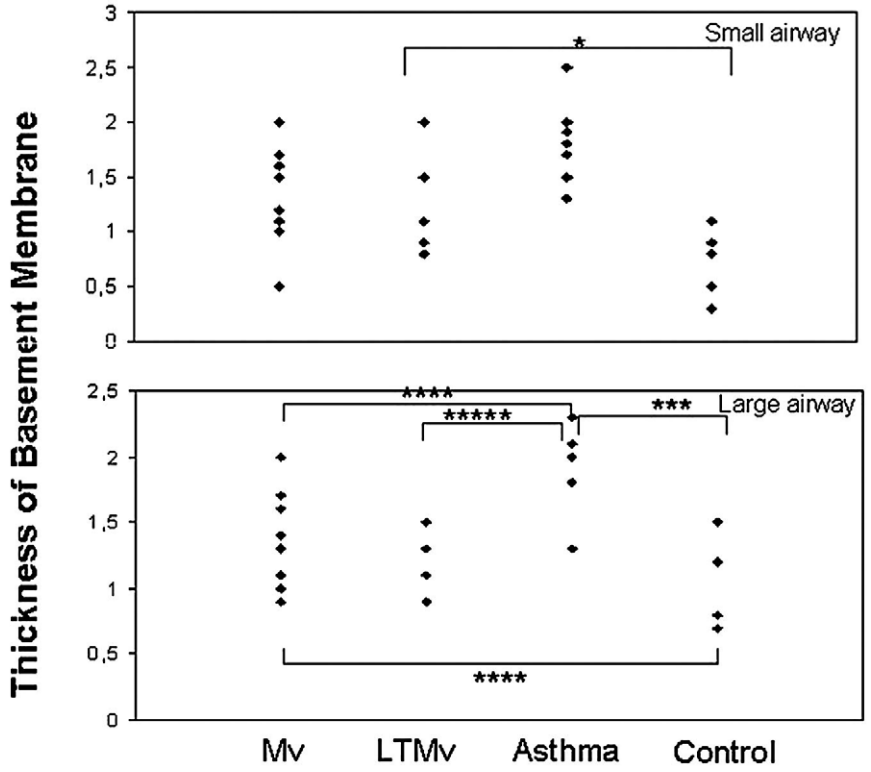

Figure 4. Effect of *Mycobacterium vaccae* administration on the thickness of basement membrane in lung histopathologic analysis in the asthma model. Histopathologic evaluation was determined by periodic acid–Schiff and hematoxylin-eosin staining, and thickness of the basement membrane was measured with an Image Analyser. *Comparison of long-term *M vaccae* group vs control group (small airways: $P < .05$). ***Comparison of asthma model vs control groups (large airways: $P < .01$). ****Comparison of *M vaccae* vs asthma model (large airways: $P < .05$). *****Comparison of long-term *M vaccae* vs asthma model (large airways: $P < .01$). Mv indicates *M vaccae*; LTMv, long-term *M vaccae*.

DISCUSSION

The present study examined the long-term protective effect of intratracheal *M vaccae* treatment on airway histopathologic parameters and cytokine levels in an already established murine experimental model of asthma. Mice in the long-term *M vaccae* group demonstrated a significantly lower number of hyperplasic goblet cells in the small and large airways when compared with the mice in the asthma model group. Meanwhile, when compared with controls, basement membrane thickness in large airways, smooth muscle thickness and number of goblet cells in both the small and large airways, and inflammation in the small airways were identical. Furthermore, comparison of the long-term *M vaccae* group with the *M vaccae* group revealed no significant differences. On the other hand, cytokine analyses did not provide any further information. These results provide evidence for the prevention of histopathologic features of the asthma model in mice by intratracheal treatment with heat-killed *M vaccae* and give information on the long-lasting effect.

To date experimental evidence is sufficient to support the role of heat-killed *M vaccae* in inhibiting allergen-induced airway inflammation.[6,9–11] In those studies, the short-term beneficial effects of systemic *M vaccae* administration were emphasized. Previously, Hopfenspirger et al[13] compared the efficacy of 2 different routes of delivery of mycobacterial antigens on numerous allergic parameters in ovalbumin presensitized mice. Their results provided evidence that intranasal delivery of mycobacterial antigens was more effective in attenuating allergic airway inflammation and associated changes in pulmonary functions in an allergen presensitized state. To further evaluate the impact of different routes of delivery, we evaluated administration of *M vaccae* intratracheally in a mouse model of asthma. We also tested whether that efficacy was still persistent 24 days after immunization.

In accordance with those findings, in the current study intratracheal treatment with heat-killed *M vaccae* administered 28 days after sensitization along with intratracheal allergen challenge was able to partly diminish histopathologic changes of airways that persisted 24 days after the treatment.

We acknowledge the limitation of the study design that extra asthma model and control groups could be included for comparison of evaluated parameters on day 71. Results of the long-term *M vaccae* group indicate that the established ameliorating effect on day 48 detected in the *M vaccae* group persisted for a longer duration when compared with the asthma model and control groups killed on day 48. Taken

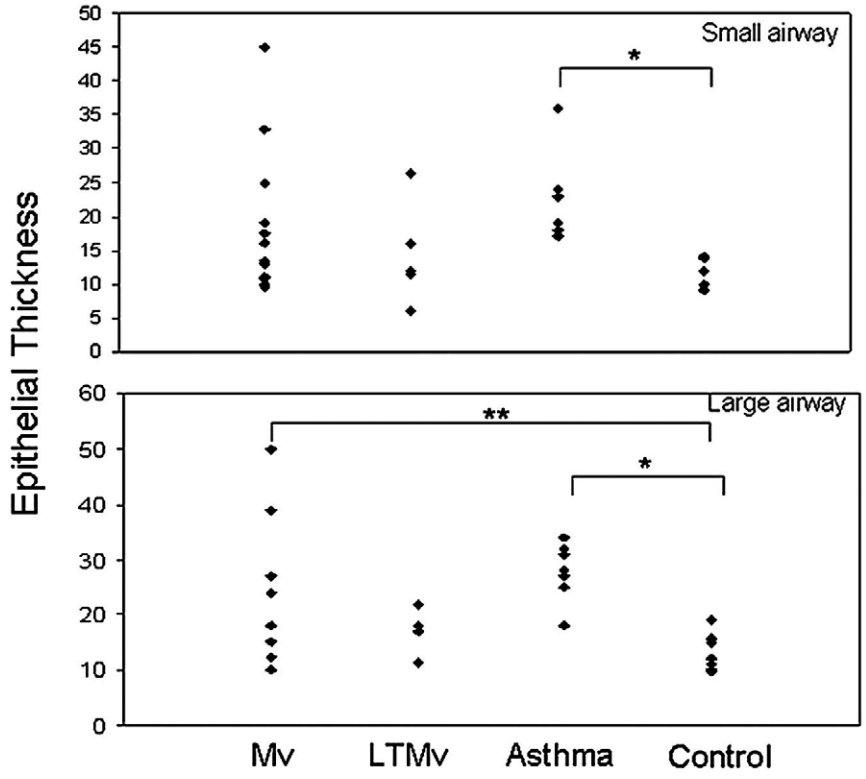

Figure 5. Effect of *Mycobacterium vaccae* treatment on epithelial thickness of all sized airways. *Comparison of asthma model vs control groups (small airways: $P < .05$ and $P < .01$; large airways: $P < .01$). **Comparison of *M vaccae* vs control (large airways: $P < .05$). Mv indicates *M vaccae*; LTMv, long-term *M vaccae*.

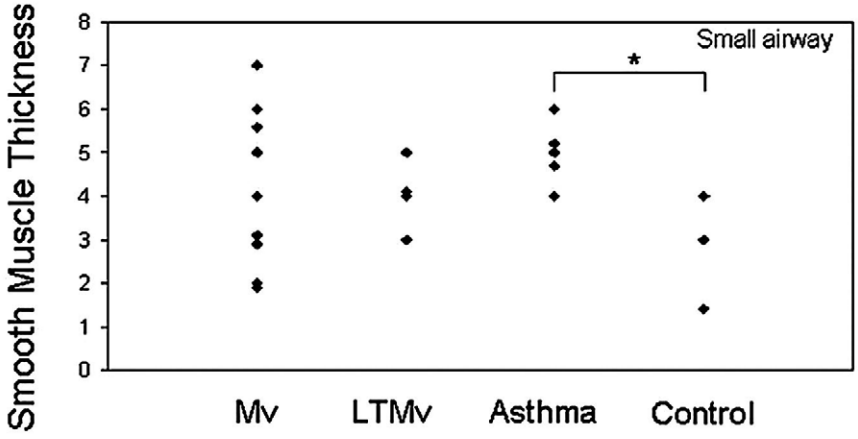

Figure 6. Effect of *Mycobacterium vaccae* treatment on smooth muscle thickness of all sized airways. *Comparison of asthma model vs control groups (small airways: $P < .05$). **Comparison of *M vaccae* vs control groups. Mv indicates *M vaccae*; LTMv, long-term *M vaccae*.

together, these results highlight the long-lasting ameliorating effect of intratracheal *M vaccae* treatment on airway histopathological parameters on a subsequent antigen challenge.

Previously, Zuany-Amorim et al[11] demonstrated that heat-killed *M vaccae* administered subcutaneously gives rise to allergen-specific $CD4^+$ CD45 RB^{low} regulatory T cells, which confer protection against airway inflammation. In their study, that specific inhibition was shown to be mediated through IL-10 and transforming growth factor β.[11] On the other hand, our cytokine results did not provide any informative data on the mechanism of intratracheal *M vaccae* administration. This difference may be explained by involvement of distinct im-

munological mechanisms by different routes of application. In conclusion, findings of our study support the suggestion that local mucosal routes for mycobacterial vaccine delivery may have therapeutic potential with long-lasting efficacy in murine models of allergic pulmonary inflammation.

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