Treatment with *Mycobacterium vaccae* ameliorates airway histopathology in a murine model of asthma

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ABSTRACT

The objective of this study was to evaluate the effect of intratracheal (i.t.) or subcutaneous (s.c.) Mycobacterium vaccae *treatment on lung histopathology and cytokine responses in a murine model of asthma. BALB/c mice were divided into four groups. To establish an asthma model, Groups I, II and III received intraperitoneal (i.p.) ovalbumin (OVA) and were challenged with i.t. OVA three times (days 41–47). On the same days, mice in Groups I and II were treated with i.t. and s.c.* Mycobacterium vaccae, *respectively. Mice in Group IV served as controls. On day 49, lungs were taken out for histopathological evaluation. Cytokine levels were determined in splenocyte culture supernatants by ELISA. The thickness of basement membrane and hyperplasic goblet cells in small airways were found to be significantly more in Group III than Group I. Furthermore, smooth muscle and epithelial thickness in small and large airways and hyperplasic goblet cell numbers in all sized airways of this treatment group were not significantly different from controls. Epithelial thickness in medium and large airways, hyperplasic goblet cells in all sized airways, and basement membrane in small and large airways were not significantly different in Group II when compared to controls. OVA-stimulated IL-5 levels was significantly higher in Group I when compared to Group III. OVA-stimulated IL-5 and spontaneous IL-5 levels were significantly higher in Group II than Group III. We demonstrate that subcutaneous and intratracheal* Mycobacterium vaccae *administered along with allergen has an ameliorating effect in the modulation of airway histopathological changes in OVA sensitized mice.*

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Key words: Airway histopathology, asthma, Balb/c mice, interleukin-10, interleukin-5, *Mycobacterium vaccae*, OVA sensitization

Over the past few decades, prevalence of allergic diseases has been increasing, especially in populations with a modern lifestyle.[1–5] Many hypotheses have been proposed to explain this increase in prevalence, the most discussed and focused on being the "hygiene hypothesis." In their hallmark study, Shirakawa *et al*. surveyed 867 Japanese children, all immunized with bacillus Calmette-Guerin (BCG), responders to tuberculin had lower levels of total IgE and Th2 cytokines, higher serum levels of IFN-γ, and a lower prevalence of atopy and atopic diseases when compared with tuberculin nonresponders.[6]

Airway inflammation in asthma is accompanied by structural changes including goblet cell metaplasia, smooth muscle cell layer thickening, and subepithelial fibrosis, which are shown not to be reversed by corticosteroids.[7] This allergen-induced airway remodeling can be replicated in murine models of asthma to investigate potential therapeutic targets for this chronic airway inflammation.[8]

In one murine model, it had been shown that immunization with mycobacterial products established a Th1-type immune response that suppressed Th2 cytokines, allergic sensitization, development of increased airway reactivity, and histopathological changes.[9–13] In another murine model of allergen-induced eosinophilia Erb *et al.* had shown that intranasal BCG infection of mice inhibited the accumulation of eosinophils into the airways and IL-5 production by T cells of draining lymph nodes. Furthermore, this effect was strongly reduced in IFN-γ receptor knockout mice and could be reversed partially by intranasal IL-5 application.[9] In another study, BCG immunization of ovalbumin (OVA)-sensitized BALB/c mice markedly hindered the development of IgE/IgG1 antibody responses, IL-4 and IL-10 secretion in splenocytes, eosinophilic infiltration of airways, and IL-4 and IL-5 levels in bronchoalveolar lavage fluid. In parallel, a marked rise in OVA-specific IgG2a antibody and IFN-γ production from splenocytes was detected.[10]

Additionally, Rook and coworkers had indicated that killed *Mycobacterium vaccae* suppressed serum IgE and allergen-specific IL-5 synthesis.[11] In another study,

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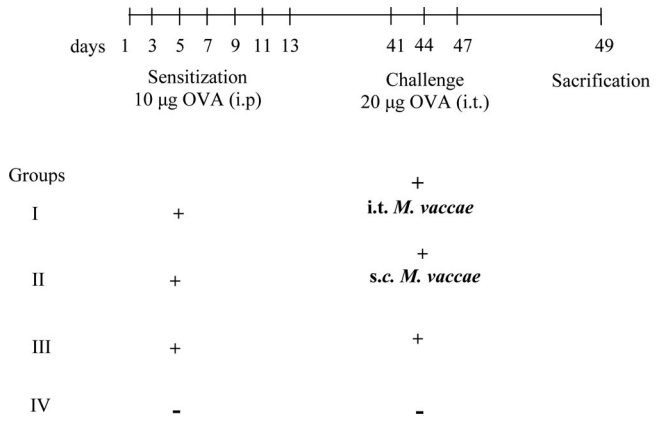

Figure 1. *Study design.*

preimmunization with killed *M. vaccae* in newborn mice ameliorated airway histopathological changes in a murine model of chronic asthma.[13] There is evidence that mechanism of action of a representative of the saprophytic environmental bacteria that shares several immunodominant epitopes with other mycobacteria might be much more complex than is predicted by the Th1–Th2 paradigm. Recently, Zuany-Amorim and colleagues showed that treatment of mice with SRP299, a killed *M. vaccae* suspension up-regulated IL-5 and IL-10 secretion and resulted in induction of allergen-specific $CD4^{+}CD45RB^{Low}$ regulatory T cells, with an inhibitory effect on airway inflammation.[14] In this study, to assess the efficacy of intratracheal or subcutaneous *M. vaccae* administration as a treatment modality in asthma model, we studied parameters of airway histopathology and cytokine levels in splenocyte cultures in BALB/c mice.

MATERIALS AND METHODS

Animals

Female BALB/c mice (5–6 weeks old) were obtained from Turkish Scientific and Technical Research Institute (TUBITAK), and were housed in pathogen-free conditions at the laboratory of Marmara University Research Center in accordance with the guidelines of the animal ethic committee.

Study Groups

Mice were divided into four groups: group I (*M. vaccae* i.t.; $n = 25$), group II (*M. vaccae* subcutaneous; $n = 25$), group III (asthma model group; $n = 20$), and group IV (controls; $n = 11$). Study design is shown in Fig. 1.

Study Design

Mice in groups I–III were sensitized with OVA by 7 (days 1–13) i.p. injections (Fig. 1). Twenty-eight days later, they were challenged with OVA (days 41–47) by intratracheally (i.t.). On the same days of OVA administration, group I mice were immunized with 10^7 CFU of *M. vaccae* in 10 μL of saline additionally. Simultaneously, mice in group II were immunized s.c. with 10^7 colony forming unit (CFU) of *M. vaccae* in 100 μL of saline. Group III mice served as the asthma model group and group IV mice served as controls. On day 49, mice in all groups were killed and lungs and spleens were taken out. Subsequently, lung histopathology and cytokine determinations were performed on days 50 and 51.

Preparation of *M. vaccae* Suspension

M. vaccae (ATCC 29678 strain) was supplied by North Carolina State University. *M. vaccae* was cultured and the vials were autoclaved at 120°C for 25 minutes. Logarithmic dilutions of the cells were made into sterile vials. Aliquots of 1×10^7 CFU/mL in medium for s.c. and i.t. administration were prepared and frozen at −20°C until needed.

Establishment of Asthma Model

Sensitization and Ag Challenge. To establish the asthma model, groups I, II, and III were sensitized with 10 μg of OVA in 100 μL of saline i.p. seven times on each alternate day. Twenty-eight days after the last i.p. injection, they were challenged with 20 μg of OVA three times 2 days apart by i.t. instillation under anesthesia, on days 41, 44, and 47. Mice in group IV served as controls. A number of mice in each group experienced sudden deaths during i.t. administration because of the difficulty of performing OVA in the trachea of mice. Therefore, histopathological and cytokine analyses could be performed on specimens of 13, 10, and 13 mice in groups I, II, and III, respectively.

Immunization with *M. vaccae*

On the same days with intratracheal OVA instillation (days 41, 44, and 47) mice in group I were treated with 10^7 CFU of *M. vaccae* in 10 μL of saline i.t. Mice in group II were treated s.c. with 10^7 CFU of *M. vaccae* in 100 μL of saline on the same days.

Histopathology

Two days after the last i.t. OVA instillation and *M. vaccae* administration (day 49), mice in all groups were killed. Lungs were fixed and inflated by i.t. 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 (3–5 μm) were stained with hematoxylin and eosin (H&E) for the evaluation of epithelial thickness and with periodic-acid Schiff to analyze hyperplasic goblet cells, thickness of smooth muscle, and basement

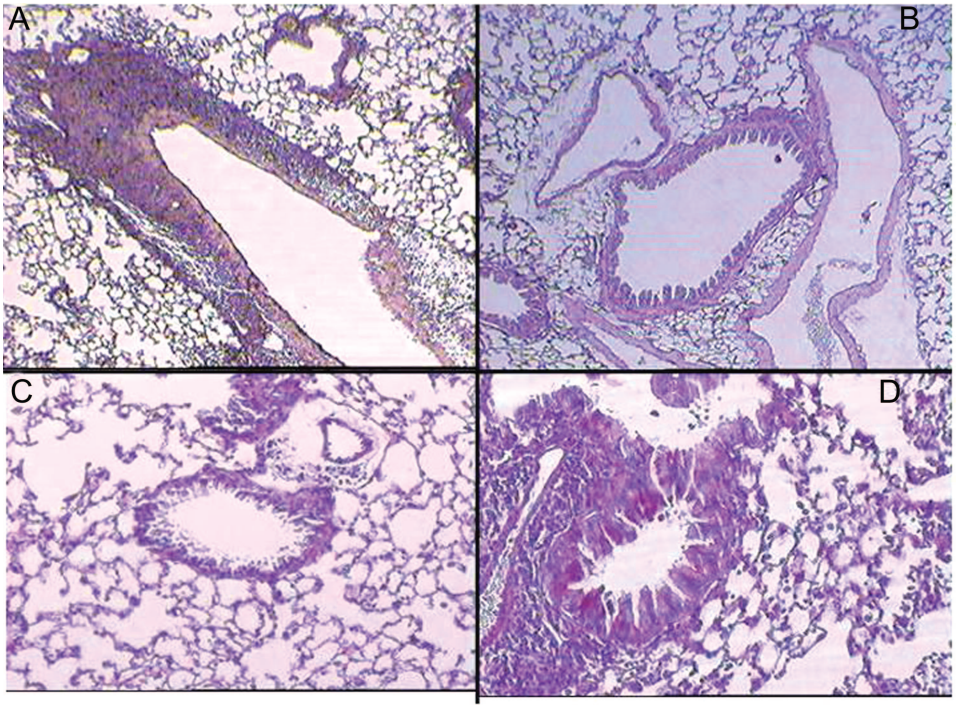

Figure 2. *Lung sections of mice with (a) asthma (b) control (c) i.t.* M. vaccae, *and (d) s.c.* M. vaccae.

membrane. All histological analyses were performed with MS Basic Image Analyser Software (Mikrosistem Computerized Microscope Systems Co., Inc., Istanbul, Turkey), which was adapted on a BH2-RFCA model microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The airways were classified as small (<500 μm), medium (500–1000 μm), and large (>1000 μm) according to their circumferences. Measurements were made on distal airways cut in transverse section and free of branching. Measurement of thickness of epithelial, basement membrane, smooth muscle, number of hyperplasic goblet cells, and severity of inflammation on the airways were recorded. Semiquantitative analysis was achieved by scoring for inflammation severity according to number of inflammatory cell lines surrounding the airway.

Splenocyte Culture and Cytokine Analysis

Spleens were removed and placed in 24-well plates under sterile conditions. Suspensions of splenocytes were obtained by inserting a syringe needle through the capsule to force out unattached cells. Single cell suspensions from the spleens of the different groups of mice were collected 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, potassium bicarbonate, and EDTA for 10–15 minutes to lyse erythrocytes. Then, they were washed and resuspended in RPMI-1640 culture medium. To count viable cells, we used trypan blue and plated in 24-well plates. Cell stimulation was performed by phytohemagglutinin (5 μg/mL), OVA (40 μg/mL), or *M. vaccae* (1×10^9 CFU/mL). Control wells contained no antigen. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Supernatants were collected at 24 hours for IL-10 and IFN-γ and 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 ELISA (Endogen, Inc., Boston, MA). 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.

Statistical Analyses

For statistical analyses, the SPSS (version 11.0, Statistical Package for Social Sciences, SPSS, Inc., Chicago, IL) program was used. Comparisons between groups were made by using the Kruskal-Wallis test that was followed by Bonferroni corrected Mann-Whitney *U* test. For the Kruskal-Wallis test a value of $p < 0.05$ was considered statistically significant.

RESULTS

Airway Histopathology

All of the histopathological characteristics evaluated in small, medium, and large airways were significantly

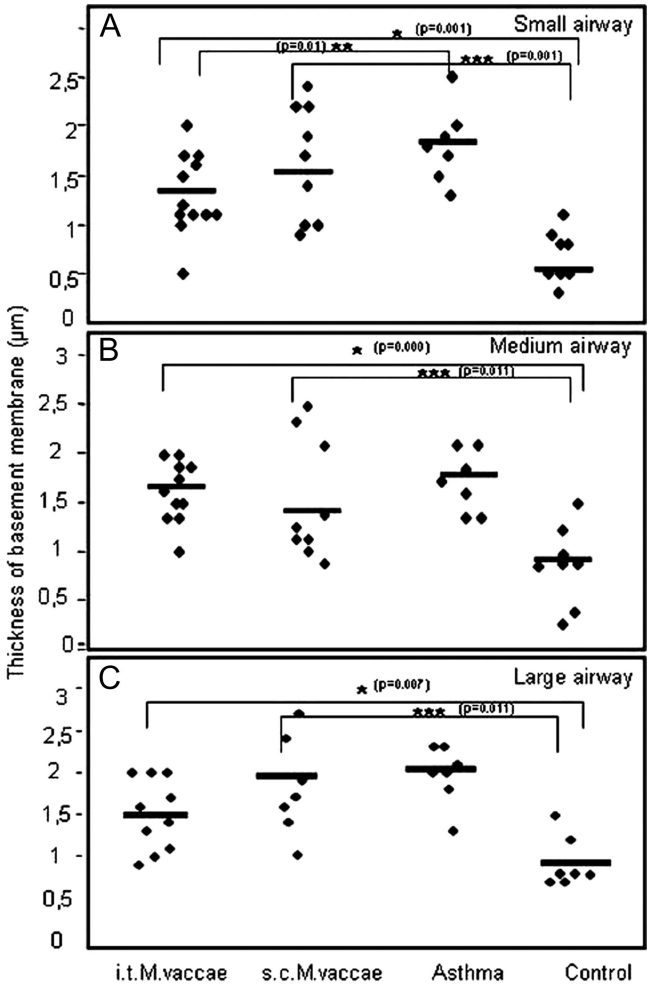

Figure 3. Effect of i.t. or s.c. administration of M. vaccae *on the thickness of basement membrane in lung histopathology in the asthma model. Histopathological evaluation was determined by periodic-acid Schiff and H&E staining, and thickness of basement membrane was measured through use of an image analyzer. *Comparison of group I versus group IV, (a) small airways (p = 0.001), (b) medium airways (p = 0.000), and (c) large airways (p = 0.007); **comparison of group I versus group III, (a) small airways (p = 0.01); ***comparison of group II versus group IV, (a) small airways (p = 0.001), (b) medium airways (p = 0.011), and (c) large airways (p = 0.011).*

more in the asthma model group (group III) when compared with controls (group IV), which indicates that the asthma model was established successfully (Fig. 2).

Intratracheal *M. vaccae* Treatment

Evaluation of airway histopathology parameters showed that thickness of basement membrane (Fig. 3 *a*) and hyperplasic goblet cell numbers in the small airway (Fig. 4 *a*) were significantly more in the asthma model group (group III) when compared with the group treated with i.t. *M. vaccae* (group I). Furthermore, smooth muscle thickness in small and large airways (Fig. 5, *a* and *c*), hyperplasic goblet cell numbers in all sized airways, and epithelial thickness in small and large airways (Fig. 6, *a* and *c*) of this treatment group were not significantly different from controls.

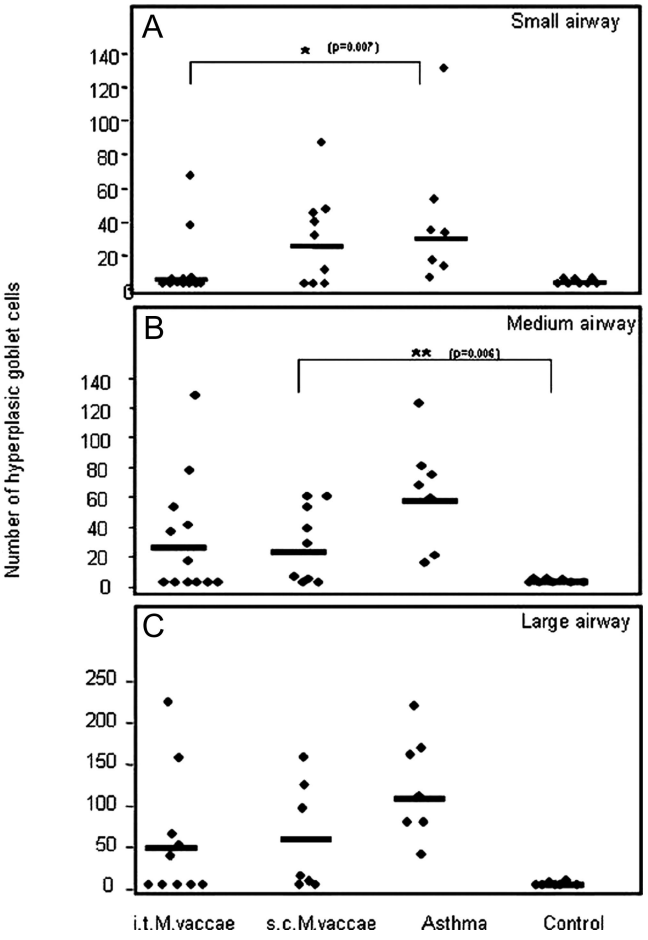

Figure 4. Effect of i.t. or s.c. administration of M. vaccae *on number of hyperplasic goblet cells. *Comparison of group I versus group III, (a) small airways (p = 0.007); **comparison of group II versus group IV, (a) medium airways (p = 0.006).*

Subcutaneous *M. vaccae* Treatment

In the comparison of mice treated with s.c. *M. vaccae* (group II) with the asthma model group (group III), no significant difference was detected. Hyperplasic goblet cells in small and large airways were not significantly different in group II when compared with controls (data not shown).

Comparison of i.t. and s.c. *M. vaccae* Treatment

Comparison of histopathological parameters of two treatment groups (group I and II) revealed no significant differences (data not shown).

Determination of Cytokines

OVA-stimulated IL-5 levels (Fig. 7 *a*) were significantly higher in the group treated with i.t. *M. vaccae*

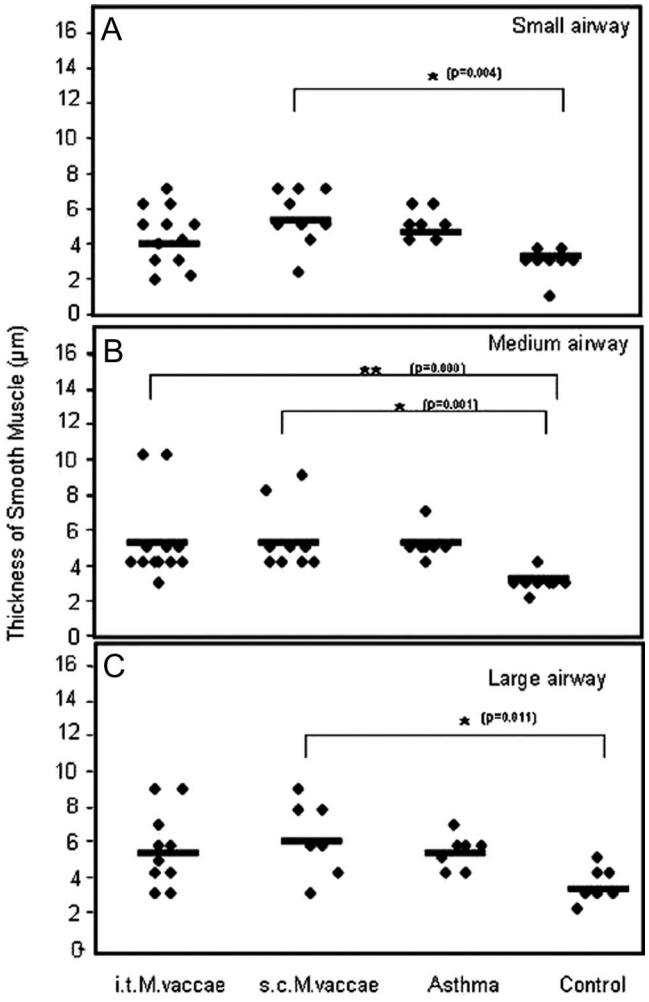

Figure 5. Effect of i.i. or s.c. M. vaccae *treatment on smooth muscle thickness of all sized airways. *Comparison of group II versus IV, (a) small airways ($p < 0.004$), (b) medium airways ($p = 0.001$), and (c) large airways ($p = 0.011$); **comparison of group I versus IV, (a) medium airways ($p = 0.000$).*

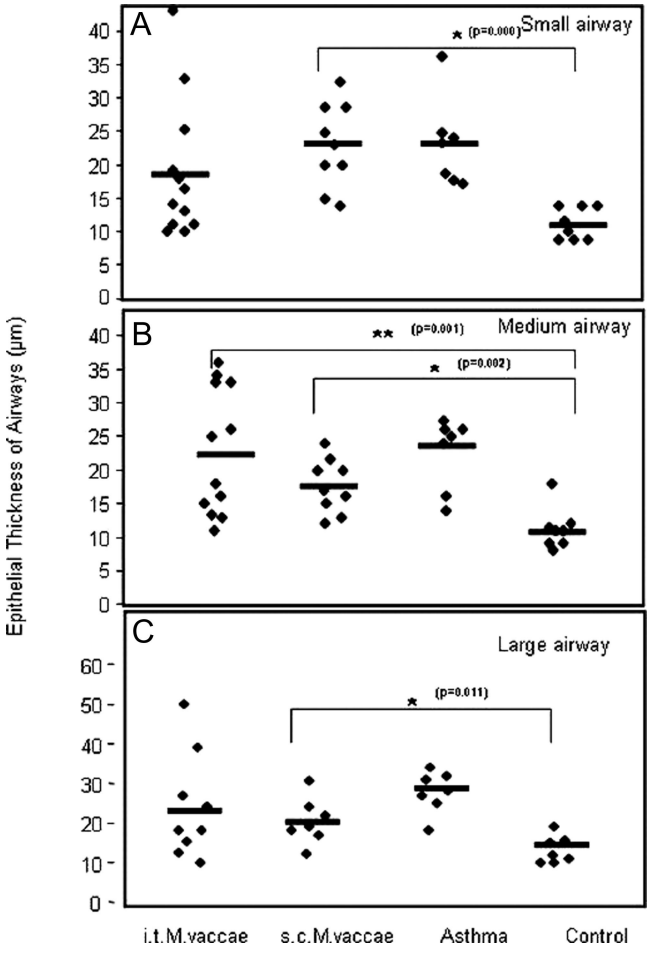

Figure 6. Effect of i.t. or s.c. M. vaccae *treatment on epithelial thickness of all sized airways. *Comparison of group II versus IV, (a) small airways ($p = 0.000$), (b) medium airways ($p = 0.002$), and (c) large airways ($p = 0.011$); **comparison of group I versus IV, (a) medium airways ($p = 0.001$).*

(group I) when compared with the asthma model group. OVA-stimulated IL-5 levels and spontaneous IL-5 levels were not significantly different in group I when compared with controls.

OVA-stimulated IL-5 (Fig. 7 *a*) and spontaneous IL-5 levels (Fig. 7 *b*) were significantly higher in the group treated with s.c. *M. vaccae* than the asthma model group. In addition, there was a significant difference in spontaneous IL-5 between group II and controls (Fig. 6 *b*).

There was no significant difference in comparison of groups with respect to IFN-γ production (data not shown).

DISCUSSION

This study examined the effects of i.t. or s.c. routes of treatment with *M. vaccae* during establishment of a murine model of asthma. Major histopathological changes of asthma[15] exerted by i.t. OVA administration were shown to be modulated by simultaneous *M. vaccae* treatment given *via* s.c. of i.t. route.

According to our results, in the i.t. treated group, thickness of basement membrane layer and number of hyperplasic goblet cells in small airways were significantly inhibited compared with mice in the asthma model group, whereas there was no difference compared with the asthma model group in the s.c. treated mice. Comparison of both the i.t. and s.c. treated groups with controls revealed slightly more severe histopathological changes in the s.c. treated group.

Smit *et al.* previously showed that the s.c route of treatment with *M. vaccae* during allergen challenge was able to suppress airway hyperresponsiveness, airway eosinophilia, and IL-5 production in bronchoalveolar lavage fluid in OVA-sensitized mice.[16] However, the same group also subsequently had shown that treatment with heat-killed *M. vaccae* failed to reduce OVA-induced airway hyperreactivity, airway eosinophilia,

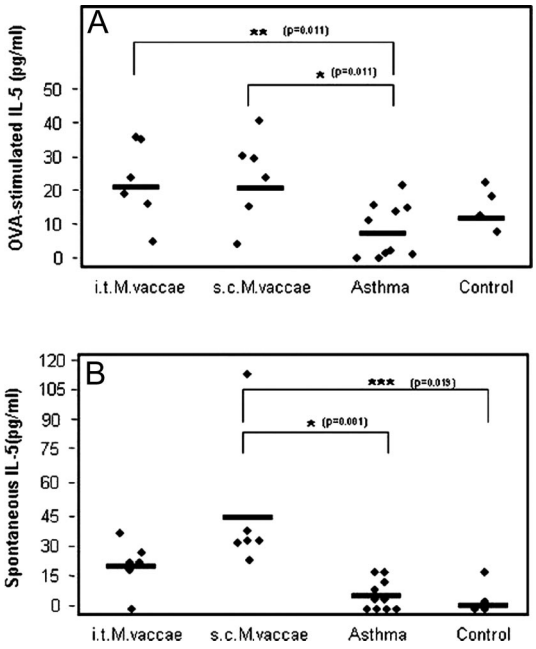

Figure 7. *Effect of i.t. and s.c.* M. vaccae *treatment on cytokine production in splenocyte culture supernatants. (a) OVA-stimulated IL-5, *group II versus group III ($p = 0.011$) and **group I vs group III ($p = 0.011$); (b) spontaneous IL-5, *group II versus group III ($p = 0.001$), and ***group II versus group IV ($p = 0.019$).*

specific antibody, and cytokine levels when administered after sensitization and 3 weeks before OVA challenge.[17] Furthermore, in a previous study, we had indicated that subcutaneous *M. vaccae* administration in newborn mice before OVA sensitization was able to ameliorate the histopathological changes in lungs.[13]

Taken together, the effect of administration of heat-killed *M. vaccae* along with the antigen when given simultaneously may provide a more favorable effect on lung histology. On the other hand, the optimal site of *M. vaccae* administration—systemic or local—warrants additional investigation.

An alternative explanation for the communication between epithelial mesenchymal trophic unit (EMTU) and Th2 inflammation is a new paradigm. Epithelial damage and Th2 act in concert to cause a functional discordance of the EMTU, which leads to myofibroblast activation and a remodeling response that occur in chronic asthma. An inherited or acquired epithelial susceptibility to environmental agents may result in a growth arrest and prolonged epithelial repair, which can enhance cell–cell communication within the EMTU. This event then leads to myofibroblast activation and propagation of remodeling. These processes depend on expression and release of Th2 cytokines, which are targets of specific or nonspecific immunotherapy regimens by inducing T-cell cytokine responses to immune regulation.[7]

Zuany-Amorim reported that *M. vaccae* treatment gives rise to $CD4CD45RB^{Lo}$ T-regulatory cells, displaying a protective effect on allergic pulmonary inflammation through IL-10 and TGF-β production[14]; this finding has not been supported in the current study.

The beneficial effect of *M. vaccae* on human allergic diseases was by Arkwright previously. In this study, 41 children with moderate to severe atopic dermatitis were given either one intradermal injection of killed *M. vaccae* (SRL172) or placebo. A significant reduction in the severity of children's atopic dermatitis was shown but the same effect could not be observed on serum total IgE level or eosinophil count.[18] On the other hand, in the report of Shirtcliffe and coworkers, 43 patients with stable moderately severe asthma sensitized to house-dust mites received two intradermal injections of placebo, heat-killed *M. vaccae*, or delipidated deglycolipidated *M. vaccae* (DDMV), and eosinophil and IgE levels or T cell proliferative cytokine responses were studied, showing no significant effect.[19] In another study of the same group, there was no significant improvement in 37 patients with moderately severe asthma.[20]

Based on evidence obtained from humans and animal models there seems to be a discrepancy regarding the preventive and therapeutic effect of *M. vaccae* on atopic diseases. Factors causing this conflict could be caused by the timing and route of immunization, as well as, genetic background. The time of inoculation may be crucial in the treatment of allergic asthma with *M. vaccae.* Future experiments will investigate whether allergen exposure is necessary for mycobacterial treatment to be effective. Taken together, we showed that i.t. *M. vaccae* administration along with allergen has an ameliorating effect in the modulation of airway histopathological changes in OVA-sensitized mice.

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