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Therapeutic treatment with heat-killed *Mycobacterium vaccae* (SRL172) in a mild and severe mouse model for allergic asthma

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Abstract

The hypothesis that a lack of early childhood bacterial infections would favor the development of allergic disease suggests that bacteria can be used as a potential treatment for allergic asthma. Therefore, in this study, we investigated the therapeutic potential of heat-killed *Mycobacterium vaccae* in two mouse models of allergic asthma. For this purpose, mice were sensitized i.p. with ovalbumin/alum (severe model) or ovalbumin alone (mild model) and challenged on days 77, 80 and 83 by inhalation of either ovalbumin or saline aerosols. Treatment of mice with *M. vaccae* (s.c. 10^7 or 10^8 colony-forming units) on days 56 and 63, however, did not reduce airway hyperresponsiveness and eosinophilia, IgE and interleukin-5 production 24 h after ovalbumin challenge in either mouse model. We therefore conclude that treatment of sensitized mice with *M. vaccae* before allergen exposure is not able to reduce the allergic and asthma-like response in a mild and a severe model of allergic asthma.

Keywords: *Mycobacterium vaccae*; Allergy; Asthma; Hygiene hypothesis

1. Introduction

The prevalence of allergic asthma and other atopic disorders has increased dramatically to almost epidemic proportions in the last decades, especially in industrialized countries (Hartert and Peebles, 2000). In contrast to this increase, few novel drug therapies for allergic diseases have been developed and proven effective in clinical trials. Existing treatment consists of inhaled bronchodilators and anti-inflammatory drugs, which are effective in most asthmatic patients, but have serious side effects, particularly in children and in patients with severe asthma who require high-dose treatment. Moreover, these therapies require long-term daily administration and do not affect the underlying (immune) responses of allergic asthma. Without a doubt, there is a need for better treatment targeted at the underlying mechanisms of asthma and with a long-term, antigen-specific protective effect.

The "hygiene hypothesis" states that the relative lack of infections early in life could inhibit the development of allergic disease in genetically predisposed individuals (Rook and Stanford, 1998; Strachan, 1989). In line with this hypothesis, certain vaccinations which induce a protective infection could prevent or even treat the development of allergic disease. Support for this idea comes, among others, from rodent models in which bacteria and bacterial products lower the asthma-like response. For instance, bacillus Calmette–Guérin (BCG) immunization before or during sensitization reduced the Th2 responses, eosinophilia and airway hyperresponsiveness after allergen challenge in rodents (Erb et al., 1998; Herz et al., 1998; Hylkema et al., 2002). Furthermore, bacterial products, such as heat-killed BCG (Major et al., 2002), *Mycobacterium vaccae* (Zuany-Amorim et al., 2002a), *Listeria monocytogenes* (Hansen et al., 2000), bacterial unmethylated CpG oligodeoxynucleotide motifs (Broide et al., 1998) and purified protein derivative (PPD) from *Mycobacterium tuberculosis* (Hattori et al., 2002) suppressed the allergic and asthma-like responses in mice as well. Zuany-Amorim et al. (2002b)

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demonstrated that vaccination with heat-killed *M. vaccae* in mice induced allergen-specific CD4+ cells, in particular, T regulatory $CD4^{+}CD25^{+}CD45RB^{lo}$ cells, which are able to diminish the allergic inflammation.

However, in most of these studies, bacteria or bacterial products were given either entirely before or during allergen sensitization (prophylactic), while only few studies looked at the therapeutic potential of these agents. Therefore, in this study, we investigated whether heat-killed *M. vaccae*, which has been demonstrated to be effective to prevent allergic asthma, is able to lower the asthma-like response in a mouse model therapeutically. We hypothesized that the induction of T regulatory cells by *M. vaccae* (Zuany-Amorim et al., 2002b) may be able to lower the allergic response after allergen sensitization as well. To reflect the spectrum of human disease, we used two mouse models for allergic asthma, one reflecting more severe asthma, and one reflecting mild asthma.

2. Materials and methods

2.1. Animals

Specified pathogen-free male BALB/cByJIco mice were obtained from Charles River (Maastricht, The Netherlands) and maintained under SPF conditions in macrolon cages. The mice were provided with food and water ad libitum and used when 5–6 weeks of age. All experiments were conducted in accordance with guidelines of the Animal Care Committee of the Utrecht University.

2.2. Reagents

Vials of heat-killed *M. vaccae* (SRL172, 10 mg or 10^{10} CFU (colony-forming units)/ml) were kindly provided by SR Pharma (London, UK). Diff-Quick staining solutions were purchased from Dade (Düdingen, Switzerland). Ovalbumin (grade V), acetyl-β-methylcholine chloride (methacholine), bovine serum albumin and *o*-phenylenediamine-dichloride substrate (OPD) were purchased from Sigma (St. Louis, MO, USA). Aluminum hydroxide (AlumImject) was obtained from Pierce (Rockford, IL, USA). Digoxigenin, anti-digoxigenin-Fab fragments coupled to horseradish peroxidase and protease inhibitor were purchased from Roche Diagnostics (Basel, Switzerland), and anti-mouse IgE, biotinylated anti-mouse IgG1 and biotinylated anti-mouse IgG2a was obtained from Pharmingen (San Diego, CA, USA). Peroxidase-conjugated streptavidin (Poly-horseradish peroxidase) was purchased from the CLB (Amsterdam, The Netherlands). Enzyme-linked immunosorbent assay (ELISA) buffer contained 0.5% bovine serum albumin, 2 nM EDTA, 136.9 nM NaCl, 50 nM Tris and 0.05% Tween-20.

2.3. Measurement of delayed-type hypersensitivity

For measurement of delayed-type hypersensitivity to heat-killed *M. vaccae* in the ear, mice were injected subcutaneously in the neck with 10^{8} CFU (100 μg) heat-killed *M. vaccae* in 100-μl saline or saline alone on days 0 and 7. On day 21, the mice were anesthetized by an intramuscular injection of a mixture of xylazine and ketamine, and ear thickness of both ears was measured using a spring-loaded caliper (No. 293-561, Mitutoyo, Veenendaal, The Netherlands). All mice were challenged with an intradermal injection of 15-μg heat-killed *M. vaccae* in 20-μl saline in the left ear and saline alone in the right ear. Ear thickness of both ears was measured after anesthesia as described above, 24, 48 and 96 h after ear challenge.

2.4. Sensitization, treatment and challenge

Two mouse models of allergic asthma were used in this study (Table 1). The ‘‘severe’’ model for asthma involved sensitization by two i.p. injections of 10 μg ovalbumin adsorbed onto 2.25 mg aluminum hydroxide in 100 μl saline on days 0 and 14. The ‘‘mild’’ asthma model involved seven i.p. injections of ovalbumin (10 μg/0.5 μl saline) on alternate days between days 0 and 14. *M. vaccae*-treated mice received a subcutaneous injection of 10^{7} or 10^{8} CFU (0.01 or 0.1 mg, respectively) heat-killed *M. vaccae* in 100 μl saline on days 56 and 63. The *M. vaccae* concentrations used are analogous with those used in earlier experiments with this agent (Hernandez-Pando et al., 1997; Hernandez-Pando and Rook, 1994; Janssen et al., 2001; Wang and Rook, 1998). In both protocols, mice were challenged on days 77, 80 and 83 by inhalation of either ovalbumin or saline aerosols in a Plexiglas exposure chamber for 20 min. The aerosols were generated by nebulizing an ovalbumin solution (10 mg/ml) in saline or saline alone using a Pari LC Star nebulizer (Pari Respiratory Equipment, Richmond, VA, USA).

2.5. Determination of airway responsiveness

Airway responsiveness was determined 24 h after the final challenge, in conscious, unrestrained mice, by recording respiratory pressure curves in response to inhaled

Table 1
Experimental design as described in Materials and methods

Protocol	Sensitization	Treatment with *M. vaccae*	Challenge aerosol
“Severe asthma”	days 0 and 14: ovalbumin/alum	days 56 and 63: 10^{7} or 10^{8} CFU	days 77, 80 and 83
“Mild asthma”	between days 0 and 14: 7 × ovalbumin	days 56 and 63: 10^{7} or 10^{8} CFU	days 77, 80 and 83

nebulized methacholine using whole body plethysmography (Buxco, Sharon, CT, USA). Briefly, the mice were placed in a whole-body chamber and pressure differences between this chamber and a reference chamber were recorded. Airway responses were expressed as enhanced pause (Penh), an index of airway obstruction as described previously (Hamelmann et al., 1997). After assessment of baseline Penh values for 3 min, the mice were subsequently exposed to aerosols of saline and increasing concentrations of methacholine (3.13, 12.5, 25 and 50 mg/ml saline) for 3 min. Aerosols were generated by a Pari LC Star nebulizer, and each aerosol was followed by 3 min of recording to assess the average Penh value.

2.6. Bronchoalveolar lavage

After measurement of cholinergic airway responses, the animals were killed and bronchoalveolar lavage was performed. For this purpose, lungs of mice were lavaged once with 1 ml phosphate-buffered saline (PBS) at 37 °C containing 5% bovine serum albumin and protease inhibitor and four times with 1 ml saline at 37 °C. Lung lavage cells of each mouse were collected after centrifugation, pooled and resuspended in 150 μl saline. The supernatant of the first lavage was separated and frozen at −70 °C until further analysis. For differential cell counts, cytospin preparations were made and stained with Diff-Quick. Cells were differentiated into monocytes, eosinophils, lymphocytes and neutrophils by standard morphology.

2.7. Determination of serum levels of ovalbumin-specific immunoglobulins

Blood was withdrawn by heart puncture 24 h after the last allergen challenge to prepare serum for determination of antibody levels in serum by ELISA using microtiter plates from Nunc (Roskilde, Denmark), ELISA buffer for blocking and sample dilution and PBS containing 0.05% Tween-20 for washing between incubations. To determine ovalbumin-specific immunoglobulin E (IgE) levels, wells were coated overnight at 4 °C with 1 μg/ml of anti-mouse IgE in PBS, followed by blocking for 1 h and incubation of the wells with diluted serum samples and duplicate dilution series of an ovalbumin-specific IgE reference serum, prepared as described previously (Hessel et al., 1995) for 2 h. Thereafter, wells were incubated for 1 h with 1 μg/ml of digoxigenin-conjugated ovalbumin followed by incubation with anti-digoxigenin-Fab fragments coupled to horseradish peroxidase, according to the manufacturer's instructions.

To assess ovalbumin-specific IgG1 or IgG2a levels, wells were coated with 10 μg/ml ovalbumin in PBS. After blocking, diluted serum samples or duplicate dilution series of a reference standard serum (obtained from ovalbumin-sensitized and -challenged mice) were added. Hereafter, wells were incubated with 1 μg/ml of biotinylated anti-mouse IgG_1, or 1 μg/ml of biotinylated anti-mouse IgG_{2a} for 2 h, followed by 1:10,000 diluted Poly-horseradish peroxidase for 1 h.

For color development, 0.4 mg/ml of OPD and 4 mM H_2O_2 in PBS were used and the reaction was stopped by adding 4 M H_2SO_4. The optical density was read at 490 nm using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). Results were analyzed using Microplate Manager PC software (Bio-Rad).

2.8. Determination of interleukin-5 in lung lavage fluid

A commercially available ELISA kit (Pharmingen) was used to assess levels of interleukin-5 in the lung lavage fluid, according to the manufacturer's instructions. The detection limit for this ELISA was 32 pg/ml.

2.9. Statistical analysis

All data are expressed as means ± S.E.M. The airway response curves for methacholine and the delayed-type hypersensitivity responses were analyzed with a general linear model of repeated measurements followed by post hoc comparison between groups. Data were $\log_{10}$-transformed before analysis to equalize variances in all groups. Cell counts were analyzed using the Mann–Whitney *U*-test. All other analyses were performed with Student's *t*-test. A probability value $P < 0.05$ was considered significant.

3. Results

3.1. Delayed-type hypersensitivity responses to heat-killed *M. vaccae*

To investigate whether heat-killed *M. vaccae* was able to elicit T cell-mediated responses, we measured delayed-type

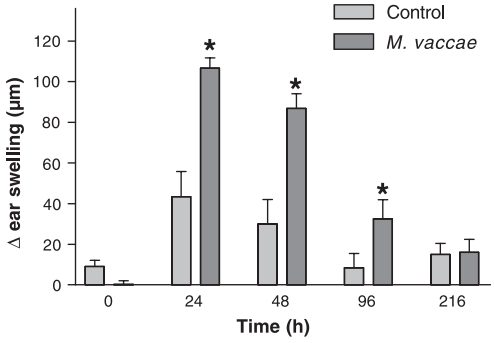

Fig. 1. Ability of *M. vaccae* to induce a delayed-type hypersensitivity reaction. Data are depicted as mean swelling of the left ear compared to that of the control right ear ± S.E.M. in *M. vaccae*-sensitized and nonsensitized mice ($n = 4$), measured 24, 48 and 96 h after challenge. *$P < 0.01$ compared to the nonsensitized group.

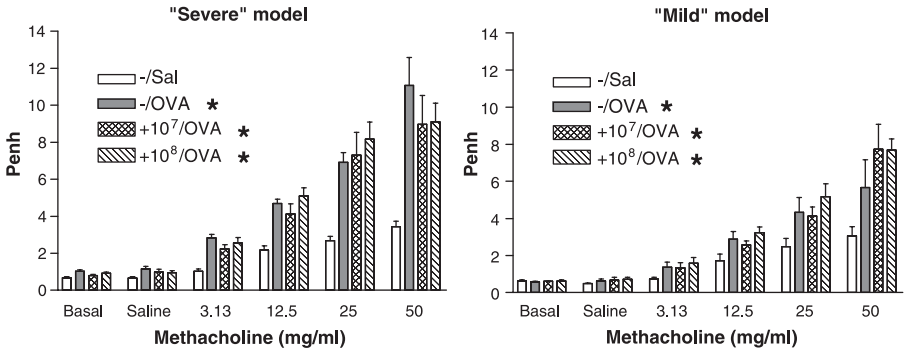

Fig. 2. Airway response to increasing concentrations of nebulized methacholine in ovalbumin-sensitized mice, expressed as Penh values. Measurements were performed 24 h after the final saline (Sal) or ovalbumin challenge of control (− /OVA) mice or mice treated with 10^7 CFU (+ 10^7/OVA) or 10^8 CFU (+ 10^8/OVA) *M. vaccae*. Data are presented as means ± S.E.M., $n = 8$. *$P < 0.05$ compared to the saline-challenged control group.

hypersensitivity responses to this agent. This delayed-type hypersensitivity response was characterized by measuring ear swelling after immunization and ear challenge with *M. vaccae* and defined as the difference in thickness between the left and right ear. Challenge with *M. vaccae* induced a small background swelling in nonsensitized mice (< 45 μm). However, a significantly greater ear swelling was observed in sensitized mice than in nonsensitized mice (Fig. 1) that peaked at 24 h and virtually resolved at 96 h. These results demonstrated that *M. vaccae* is able to elicit a strong and long-term T cell response. No effect of ovalbumin sensitization on the delayed-type hypersensitivity response to *M. vaccae* was observed (data not shown).

3.2. Airway response to methacholine

To study the effect of *M. vaccae* treatment on the development of airway hyperresponsiveness, we measured airway responses to increasing concentrations of methacholine, 24 h after final challenge. The dose–response curves for methacholine in ovalbumin-challenged mice were significantly higher than those of saline-challenged animals (Fig. 2) in either asthma model. This airway hyperresponsiveness was significantly greater in the "severe" asthma model than in the "mild" asthma model. Treatment with heat-killed *M. vaccae*, however, failed to reduce the ovalbumin-induced airway hyperreactivity in either protocol. *M. vaccae* treatment alone did not influence airway reactivity (data not shown).

3.3. Bronchoalveolar lavage cell counts

Furthermore, we studied the effect of allergen exposure and *M. vaccae* treatment on the cellular composition in the airways. Cell counts in the lung lavage fluid obtained 24 h after the final challenge showed that ovalbumin challenges induced a significant increase in the total number of cells in the lavage fluid compared to saline challenge (Fig. 3). This increase in total number of cells was mainly caused by an increase in the number of eosinophils, since no major increase was observed in the number of other cell types (monocytes, lymphocytes or neutrophils) between different groups. The eosinophilic response in the airways was 10 times greater in the "severe" asthma model than in the

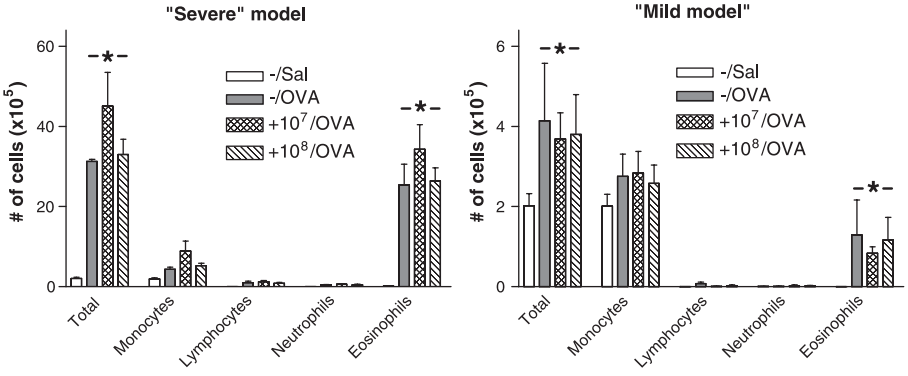

Fig. 3. Number of cells in the lung lavage fluid obtained from ovalbumin-sensitized mice, 24 h after final saline (Sal) or ovalbumin challenge of control (− / OVA) mice or mice treated with 10^7 CFU (+ 10^7/OVA) or 10^8 CFU (+ 10^8/OVA) *M. vaccae.* Data are presented as mean cell number ± S.E.M., $n = 8$. *$P < 0.01$ compared to the saline-challenged control group.

"mild" asthma model. *M. vaccae* treatment did not lower the eosinophil numbers after ovalbumin challenge in either protocol.

3.4. Serum levels of immunoglobulins

Additionally, we analyzed serum prepared from blood collected 24 h after the final challenge to study the possible effects of *M. vaccae* treatment on immunoglobulin levels. Ovalbumin challenge of sensitized mice induced an increase in the levels of ovalbumin-specific IgE compared to those in the saline-challenged animals (Fig. 4A). In addition, ovalbumin challenge markedly increased serum levels of ovalbumin-specific IgG1 and IgG2a (Fig. 4B and C). The increase in IgE and IgG1 was significantly higher in the "severe" asthma model than that in the "mild" asthma model. In contrast, the levels of IgG2a did not differ between the "severe" asthma model and the "mild" asthma model. *M. vaccae* treatment did not affect the levels of ovalbumin-specific IgE, IgG1 or IgG2a after ovalbumin challenge.

3.5. Interleukin-5 in lung lavage fluid

Finally, we measured the levels of interleukin-5 in the lung lavage fluid. The levels of interleukin-5 in the lung lavage fluid of ovalbumin-challenged mice were significantly increased as compared to those in saline-challenged mice (Fig. 5). This increase was greater in the "severe" asthma model than in the "mild" asthma model. *M. vaccae* treatment did not affect the levels of interleukin-5.

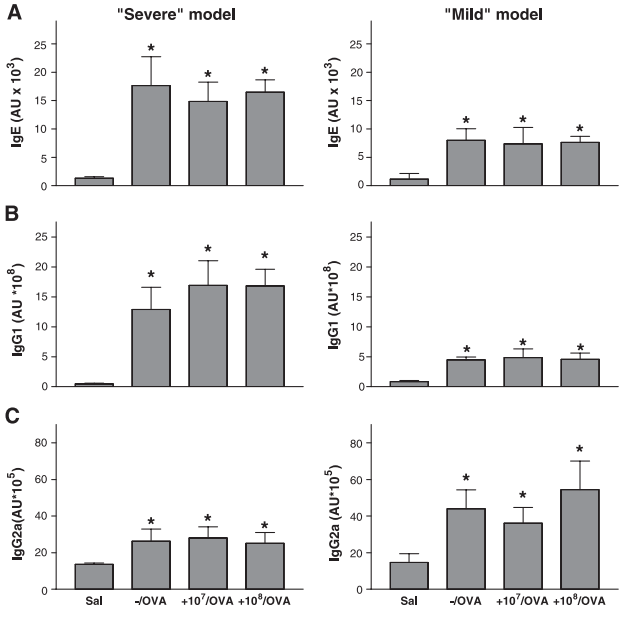

Fig. 4. Levels of ovalbumin-specific IgE (A), ovalbumin-specific IgG1 (B) or IgG2a (C) measured by ELISA in serum of ovalbumin-sensitized mice, 24 h after final saline (Sal) or ovalbumin challenge of control (− /OVA) mice or mice treated with 10^7 CFU (+ 10^7/OVA) or 10^8 CFU (+ 10^8/OVA) *M. vaccae*. Vertical bars indicate arbitrary units (AU) ± S.E.M., $n = 8$. *$P < 0.01$ compared to the saline-challenged control group.

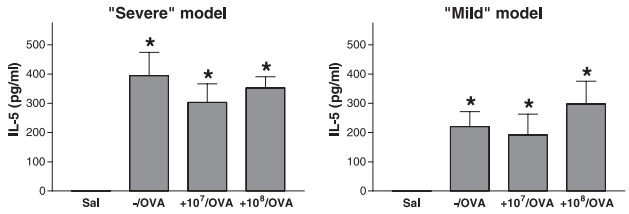

Fig. 5. Levels of interleukin-5 (IL-5) in lung lavage fluid obtained from ovalbumin-sensitized mice, 24 h after final saline (Sal) or ovalbumin challenge of control (− /OVA) mice or mice treated with 10^7 CFU (+ 10^7/OVA) or 10^8 CFU (+ 10^8/OVA) *M. vaccae*. Data are presented as mean levels of interleukin-5 (pg/ml) ± S.E.M., $n = 8$. *$P < 0.01$ compared to the saline-challenged control group.

4. Discussion

This study examined the therapeutic potential of *M. vaccae* to lessen the allergic response in two mouse models of allergic asthma. However, therapeutic treatment with *M. vaccae* starting after sensitization, 3 weeks before ovalbumin challenge, did not lead to an alteration in airway hyperreactivity (as measured indirectly and indicated by Penh values), airway eosinophilia or levels of ovalbumin specific IgE, IgG1 or IgG2a or interleukin-5 after ovalbumin challenge in both a model of mild and one of severe allergic asthma. However, we demonstrated that heat-killed *M. vaccae* was able to elicit a strong delayed-type hypersensitivity response, showing that *M. vaccae* generated strong T cell immunity in our experiments. The delayed-type hypersensitivity results together with those of previous studies demonstrated that administration of both 10^7 and 10^8 CFU heat-killed *M. vaccae* is capable of eliciting a strong Th1 response, while 10^9 CFU elicited mixed Th1 and Th2 responses in spleen cell cultures (Hernandez-Pando and Rook, 1994; Hernandez-Pando et al., 1997; Janssen et al., 2001). For that reason, we may conclude that the timing of administration and dose of *M. vaccae* used in our studies were sufficient to elicit a relatively pure and strong Th1 response, although *M. vaccae*, both viable and heat-killed, is rapidly cleared after immunization (Janssen et al., 2001).

Despite the strong induction of a Th1 response, treatment with *M. vaccae* did not prevent the induction of allergic and asthmatic parameters in our mouse model. These results complement those of earlier experiments (Smit, submitted for publication) in which we demonstrated that *M. vaccae* administration 1 to 3 weeks before allergen sensitization did not lower allergic and asthma-like responses as well. We now investigated the therapeutic effect of *M. vaccae* in two different mouse models for allergic asthma. Since the ovalbumin/alum adjuvant model could push the pathology too far for it to recover, we used a non-adjuvant mild model for allergic asthma in our studies as well. The results showed clearly that the allergic and asthmatic manifestations

measured were far stronger in the severe model than in the mild model. However, treatment with *M. vaccae* did not lessen the allergic and asthma-like response in either protocol. This means that the negative results with *M. vaccae* in our experiments are not a consequence of the model used.

Some explanations for the lack of effect of *M. vaccae* can be given. First, while in most studies, mycobacteria are given before, during or shortly after sensitization (Erb et al., 1998; Herz et al., 1998; Major et al., 2002; Zuany-Amorim et al., 2002b), in the present study, *M. vaccae* was given therapeutically a long period after sensitization. In a murine non-adjuvant ovalbumin model of allergic disease, it was found that after sensitization, Th2 memory was present even after more than 400 days (Mojtabavi et al., 2002). Therefore, it is possible that long after sensitization and allergen exposure, the allergic and asthma-like response is irreversible. Secondly, the route of *M. vaccae* administration might be of importance. Other studies demonstrated that treatment with mycobacteria in allergic asthma models was most effective when mycobacteria were administered locally by the intranasal route. For instance, it was demonstrated that intranasal administration of BCG or *M. vaccae* was superior to administration via intraperitoneal or subcutaneous routes for lowering the asthma-like response in mice (Erb et al., 1998; Hopfenspirger and Agrawal, 2002). On the other hand, in mice, intranasal application of both live and heat-killed BCG is accompanied by serious inflammation in the airways, consisting of macrophages, lymphocytes and neutrophils. Therefore, subcutaneous administration of mycobacteria, as used in our study, has proven to be the safest and thus far only accepted route of administration.

Finally, the time of inoculation may be crucial in treatment of allergic asthma with *M. vaccae*. In this study, *M. vaccae* was administered in the absence of allergen exposure. As suggested by other investigators (Hylkema et al., 2002; Sano et al., 1999), mycobacteria may only be efficient when administered at the time of allergen exposure. Future experiments will investigate whether allergen exposure is necessary for mycobacterial treatment to be effective.

We conclude that heat-killed *M. vaccae*, administered a long time after allergen sensitization, is unable to ameliorate allergic and asthmatic manifestations in a mild and more severe mouse model of allergic asthma. Future research will focus on the most effective moment for *M. vaccae* administration.

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