

***Mycobacterium vaccae* administration during allergen sensitization or challenge suppresses asthmatic features**

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Summary

Background and objective The hygiene hypothesis suggests that a lack of bacterial infections would favour the development of allergic disease. For this reason, bacteria or their components can be used as potential treatment for allergic asthma. We investigated whether heat-killed *Mycobacterium vaccae* is either able to suppress the induction of allergic asthma or able to suppress already established allergic asthma.

Methods Mice were sensitized with ovalbumin (OVA)/alum on days 0 and 14. Thereafter, mice were challenged on days 35, 39 and 42 by inhalation of either OVA or saline aerosols. *M. vaccae*-treated mice received an injection with 10^6 , 10^7 or 10^8 CFU heat-killed *M. vaccae* on days 0 and 14 or 10^7 CFU on days 35 and 39. On day 43, the airway responsiveness of the mice to increasing concentrations of methacholine was assessed, blood was withdrawn to measure serum parameters, and lung lavage was performed to detect cytokines and inflammatory cell number.

Results Treatment of OVA-sensitized mice with 10^7 CFU *M. vaccae* either during sensitization or challenge suppresses airway hyper-responsiveness, airway eosinophilia and IL-5 production after OVA challenge. The increases in OVA-specific serum IgE and in IL-4 by respiratory challenges with OVA were only diminished after *M. vaccae* treatment (10^7 CFU) during sensitization.

Conclusions Heat-killed *M. vaccae* prevents allergic and asthmatic manifestations in a mouse model and, more importantly, *M. vaccae* treatment during challenge suppresses features of asthma, which opens up possibilities for new therapeutic interventions.

Keywords airway hyper-responsiveness, allergy, asthma, eosinophilia, lung, *Mycobacterium vaccae*, Th1/Th2 cells

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Introduction

Atopic asthma is a disease characterized by (i) airway hyper-responsiveness to a variety of specific and non-specific stimuli, (ii) chronic pulmonary inflammation with eosinophilia and (iii) IgE-mediated immune responses [1]. The underlying mechanisms of the development of atopic asthma have become clearer over the past decade. Nevertheless, the prevalence of asthma has steadily increased by 75% during the last decade in the USA [2]. The specific cause for this rise is not known, but one explanation that has gained much attention is the 'hygiene hypothesis'. It suggests that the increase in allergic diseases is caused by a cleaner environment and fewer childhood infections [3, 4]. Mechanistic explanations for the hygiene hypothesis initially comprised a disbalance in Th1 and Th2. Th1 responses are induced during most bacterial and viral infections, and may be able to down-

regulate the allergic Th2 responses. Accordingly, a reduction in the overall microbial burden will result in weakening Th1 responses and therefore unrestrained Th2 responses, resulting in an increase in allergy [5, 6].

This possible mechanism is contradicted by the observations that Th2-mediated helminth infections are negatively associated with allergy and that the prevalence of Th1-mediated autoimmune diseases is increasing as well [7, 8]. Therefore, it has recently been suggested that both allergic and autoimmune diseases are a result of disordered immunoregulation instead of a misbalance in Th1 or Th2 [9, 10]. Most of the regulatory feedback of the immune system is performed by the specialized regulatory T cells [11]. Infection or colonization with various microbial agents (whether Th1 or Th2 stimulatory) may lead to an up-regulation of the regulatory feedback of the immune system, which results in the suppression of the underlying predisposition to allergy and asthma. In line with this mechanism, it may be possible to use certain bacterial species as protective or therapeutical vaccines against allergic diseases like asthma. Good candidates in this regard may be the group of mycobacterial infections. In animal models, inoculation with bacillus

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Calmette-Guérin (BCG) or *M. vaccae* reduced allergic sensitization, eosinophilic and airway hyper-responsiveness responses to allergen [12–15]. Interestingly, Zuany-Amorim and co-workers clearly 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 mediated the suppression of allergic inflammation. In contrast with the results in rodent models, several retrospective human studies failed to demonstrate a correlation between BCG vaccination during early childhood and the subsequent development of atopy or asthma (reviewed in [16]). It seems that differences exist between prophylactic or therapeutic treatment of allergic disorders with mycobacteria in humans, since some investigators demonstrated a positive therapeutic effect of BCG or *M. vaccae* on established asthma [17, 18] or atopic dermatitis [19].

The purpose of this study was to investigate in a mouse model whether heat-killed *M. vaccae* (SRL172) is not only able to suppress the induction of allergic asthma but can also down-regulate established allergic asthma. In addition, we investigated whether the treatment with *M. vaccae* is dependent on allergen exposure in our model. Here, we show that *M. vaccae* is able to inhibit both development of allergic asthma and established allergic asthma in a mouse model. This beneficial effect, however, is dependent on the timing and dose of *M. vaccae* administration.

Materials and methods

Animals

Specified pathogen-free male BALB/cByJico mice were obtained from Charles River (Maastricht, the Netherlands) and maintained under Specific Pathogen Free conditions in macrolon cages. They were provided with food and water *ad libitum* and used when 5–6 weeks of age. All experiments were conducted in accordance with the Animal Care Committee of the Utrecht University. Each experimental group consisted of eight animals.

Reagents

Vials of heat-killed *M. vaccae* (SRL172, 10 mg or 10¹⁰ CFU/mL) were kindly provided by SR Pharma Ltd. (London, UK). Diff-Quick staining solutions were purchased from Dade A.G. (Düdingen, Switzerland). Ovalbumin (OVA, grade V), acetyl- β -methylcholine chloride (methacholine), bovine serum albumin (BSA) and *o*-phenylenediamine-dichloride substrate (OPD) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Aluminium hydroxide (AlumImject) was obtained from Pierce (Rockford, IL, USA). Digoxigenin (DIG), anti-DIG-Fab fragments coupled to horseradish peroxidase and protease inhibitor were purchased from Roche Diagnostics Corp. (Basel, Switzerland) and anti-mouse IgE, biotinylated anti-mouse IgG₁, biotinylated anti-mouse IgG_{2a} were obtained from Pharmingen (San Diego, CA, USA). Peroxidase-conjugated streptavidin (Poly HRP) was purchased from the CLB (Amsterdam, the Netherlands). ELISA buffer contained 0.5% BSA, 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris and 0.05% Tween-20.

Sensitization, treatment and challenge

All mice were sensitized by two intraperitoneal (i.p.) injections with 10 μ g OVA adsorbed onto 2.25 mg aluminium hydroxide in 100 μ L saline (OVA/alum) on days 0 and 14 (Fig. 1). Hereafter, mice were challenged on days 35, 39, 42 by inhalation of either OVA or saline aerosols in a Plexiglas exposure chamber for 30 min. The aerosols were generated by nebulizing an OVA solution (10 mg/mL) in saline or saline alone using a Pari LC Star nebulizer (Pari Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1 μ m) driven by compressed air at a flow rate of 6 L/min. *M. vaccae* treated mice received a subcutaneous injection in the neck with 10⁶, 10⁷ or 10⁸ CFU heat-killed *M. vaccae* in 100 μ L saline on days 0 and 14, immediately before the i.p. OVA/alum injection (Fig. 1). A separate group of mice received a subcutaneous injection in the neck with 10⁷ CFU heat-killed *M. vaccae* in 100 μ L saline on days 35 and 39, immediately before receiving an OVA aerosol.

Determination of airway responsiveness

Airway responsiveness to inhaled nebulized methacholine was determined 24 h after the final challenge, in conscious, unrestrained mice using whole-body plethysmography (Buxco Corp., Sharon, CT, USA). The airway response was expressed as enhanced pause (P_{enh}), as described previously [20, 21]. Briefly, animals were placed in a whole-body chamber to record differences in pressure between this chamber and a reference chamber for the calculation of P_{enh} values. After assessment of baseline P_{enh} values for 3 min, mice were subsequently subjected 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 P_{enh} value from 10 or 5 valid breaths.

Bronchoalveolar lavage

After measurement of cholinergic airway responses, animals were killed and bronchoalveolar lavage was performed. For this purpose, the lungs of mice were lavaged once with 1 mL PBS at 37 °C containing 5% BSA 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 μ L saline. Total numbers of cells were determined using a Bürker-Türk chamber (Omnilabo, Breda, the Netherlands). For differential cell counts, cytopspin preparations were made and stained with Diff-Quick. Cells

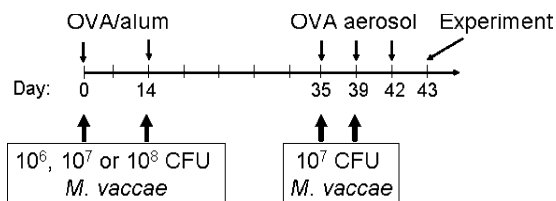


Fig. 1. Schematic representation of experiment protocol. Mice were sensitized and challenged as described in Materials and methods. Mice were treated with *M. vaccae* as indicated in Materials and methods.

were differentiated into monocytes, eosinophils, lymphocytes and neutrophils by standard morphology. At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated. The supernatant of the first lavage was separated and frozen at -70°C until further analysis.

Determination of serum levels of OVA-specific immunoglobulins

Blood was withdrawn by heart puncture 24 h after the last allergen challenge to prepare serum for the determination of antibody levels in serum by ELISA using microtitre plates from Nunc A/S (Roskilde, Denmark), ELISA buffer for blocking and sample dilution, and PBS containing 0.05% Tween-20 for washing between incubations. To determine OVA-specific IgE levels, wells were coated overnight at 4°C with $1\text{ }\mu\text{g/mL}$ 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 OVA-specific IgE reference serum, prepared as described previously [22] for 2 h. Thereafter, wells were incubated for 1 h with $1\text{ }\mu\text{g/mL}$ of DIG-conjugated OVA followed by incubation with anti-DIG-Fab fragments coupled to horseradish peroxidase, according to the manufacturer's instructions.

To assess OVA-specific IgG1 or IgG2a levels, wells were coated with $10\text{ }\mu\text{g/mL}$ OVA in PBS. After blocking, diluted serum samples or duplicate dilution series of a reference standard serum obtained from multiply OVA-boosted mice were added. Thereafter, wells were incubated with $1\text{ }\mu\text{g/mL}$ of biotinylated anti-mouse IgG1 or $1\text{ }\mu\text{g/mL}$ of biotinylated anti-mouse IgG2a for 2 h, followed by 1 : 10,000 diluted Poly-HRP for 1 h.

For colour 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 analysed using Microplate Manager PC software (Bio-Rad).

Determination of IL-4, IL-5 and IL-10 in lung lavage fluid

Commercially available ELISA kits (Pharmingen) were used to assess IL-4, IL-5 and IL-10 levels in the lung lavage fluid, according to the instructions of the manufacturers. The detection limits for IL-4, IL-5 and IL-10 were 8, 32 and 60 pg/mL , respectively.

Statistical analysis

All data are expressed as mean $\pm$ SEM. The airway response curves to methacholine were statistically analysed by a general linear model of repeated measurements followed by *post hoc* comparison between groups. Data were log₁₀ transformed before analysis to equalize variances in all the groups. Cell counts were statistically analysed using the Mann–Whitney *U*-test. All other analyses were performed using Student's *t*-test. A probability value $P < 0.05$ was considered to be significant.

Results

Airway response to methacholine

To investigate the effect of *M. vaccae* treatment on the development of airway hyper-responsiveness, airway responses of OVA-sensitized mice to increasing concentrations of methacholine, 24 h after final OVA or saline challenge, were measured and expressed as Penh (Fig. 2). The dose-response curves to methacholine of OVA-challenged mice were significantly higher than those of the saline-challenged animals, demonstrating OVA-induced hyper-responsiveness. Treatment with 10^7 and 10^8 CFU heat-killed *M. vaccae* during OVA sensitization lowered the airway hyper-responsiveness significantly, in contrast to 10^6 CFU *M. vaccae*. Interestingly, when 10^7 CFU *M. vaccae* was administered during OVA challenge, it was also effective in lowering the airway hyper-responsiveness. *M. vaccae* treatment alone did not influence the airway reactivity (data not shown).

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 final challenge showed that OVA challenge 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 was mainly caused by an increase in the number of eosinophils, although the number of other cell types (monocytes, lymphocytes and neutrophils) was increased to a certain extent as well. Treatment with 10^7 CFU *M. vaccae* during OVA sensitization significantly reduced the number of eosinophils in the lung lavage fluid, while 10^6 or 10^8 CFU *M. vaccae* did not significantly lower the number of eosinophils. In addition, treatment with 10^7 CFU *M. vaccae* during challenge was effective in lowering the eosinophilia in the lung as well.

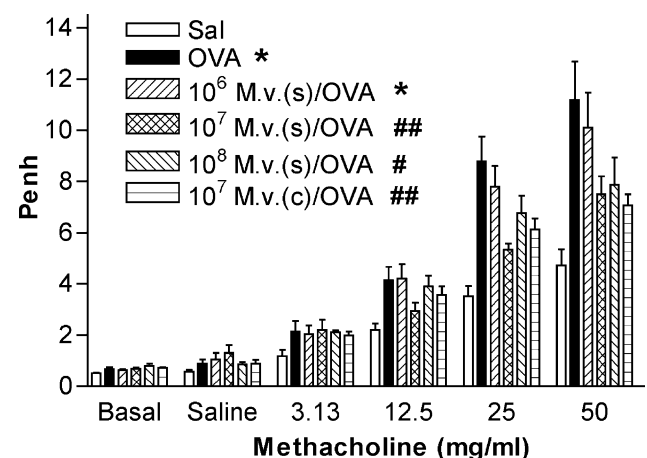


Fig. 2. Airway response to increasing concentrations of nebulized methacholine in OVA-sensitized mice, expressed as Penh values. Measurements were performed 24 h after the final saline (Sal) or OVA challenge. Mice were treated with 10^6 (10^6 M.v.(s)/OVA), 10^7 (10^7 M.v.(s)/OVA) or 10^8 (10^8 M.v.(s)/OVA) CFU *M. vaccae* during OVA sensitization or with 10^7 (10^7 M.v.(c)/OVA) CFU *M. vaccae* during OVA challenge. Data are represented as mean $\pm$ SEM, $n = 8$. * $P < 0.01$ compared to the saline-challenged control group. # $P < 0.05$ or ## $P < 0.01$ compared to the OVA-challenged control group.

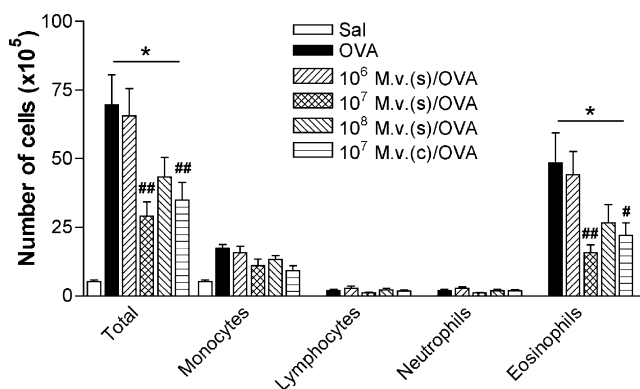


Fig. 3. Number of cells in the lung lavage fluid obtained from OVA-sensitized mice 24 h after final saline (Sal) or OVA challenge. Mice were treated with 10⁶ (10⁶ M.v.(s)/OVA), 10⁷ (10⁷ M.v.(s)/OVA) or 10⁸ (10⁸ M.v.(s)/OVA) CFU *M. vaccae* during OVA sensitization or with 10⁷ (10⁷ M.v.(c)/OVA) CFU *M. vaccae* during OVA challenge. Data are represented as mean cell number $\pm$ SEM, $n = 8$. * $P < 0.01$ compared to the saline-challenged control group. # $P < 0.05$ or ## $P < 0.01$ compared to the OVA-challenged control group.

Serum levels of immunoglobulins

Additionally, we analysed the serum prepared from blood collected 24 h after the final challenge, to study the possible effects of *M. vaccae* treatment on allergen-specific antibody levels. OVA challenge of sensitized mice induced an increase in the levels of OVA-specific IgE (Fig. 4), IgG1 and IgG2a (Fig. 5) compared to saline-challenged mice. Treatment with 10⁷ CFU *M. vaccae* during sensitization significantly reduced the serum levels of OVA-specific IgE and IgG1, in contrast to *M. vaccae* treatment during challenge, which did not affect the levels of these immunoglobulins. *M. vaccae* treatment did not change the serum levels of OVA-specific IgG2a.

Cytokines in lung lavage fluid

Finally, cytokine levels were measured in the lavage fluid to study the cytokine profile in the lung 24 h after challenge. Lung lavage fluid of OVA-sensitized mice contained no detectable levels of IL-4 and IL-5 and low levels of IL-10 after saline challenge (Fig. 6). OVA challenge caused a significant increase in the levels of these cytokines in both the strains as compared to the saline-challenged controls. Interestingly, the increase in IL-4 was significantly lower in mice treated during sensitization with 10⁷ CFU *M. vaccae*. In addition, the level of IL-5 was not only reduced in mice treated during sensitization with 10⁷ CFU *M. vaccae*, but also in mice treated with *M. vaccae* during challenge. No effect of *M. vaccae* treatment was observed on the levels of IL-10 in the lavage fluid. IFN- γ was not detectable in the lavage fluid, irrespective of OVA challenge or *M. vaccae* treatment (data not shown).

Discussion

This study demonstrates that heat-killed *M. vaccae* reduces important manifestations of atopic asthma in a mouse model, in which we used two approaches of *M. vaccae* treatment, during sensitization or during establishment of allergic asthma. We demonstrated that *M. vaccae* treatment during

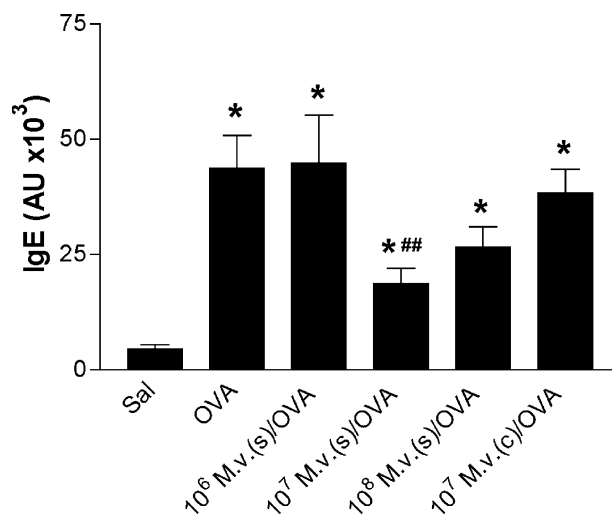


Fig. 4. Levels of OVA-specific IgE measured by ELISA in the serum of OVA-sensitized mice 24 h after final saline (Sal) or OVA challenge. Mice were treated with 10⁶ (10⁶ M.v.(s)/OVA), 10⁷ (10⁷ M.v.(s)/OVA) or 10⁸ (10⁸ M.v.(s)/OVA) CFU *M. vaccae* during OVA sensitization or with 10⁷ (10⁷ M.v.(c)/OVA) CFU *M. vaccae* during OVA challenge. Bars indicate arbitrary units (AU) $\pm$ SEM, $n = 8$. * $P < 0.01$ compared to the saline-challenged control group. ## $P < 0.01$ compared to the OVA-challenged control group.

sensitization suppressed the airway hyper-responsiveness, eosinophilia, serum levels of IgE, and the Th2 cytokines IL-4 and IL-5 in the lung lavage fluid after OVA challenge. This effect was dose-dependent, with 10⁷ CFU being the optimal dose for *M. vaccae* treatment, while 10⁸ CFU appeared less and 10⁶ CFU not effective in lowering the allergic and asthmatic response. Moreover, therapeutic treatment with 10⁷ CFU *M. vaccae* during challenge was effective in lowering the airway hyper-responsiveness and airway eosinophilia after challenge, interestingly accompanied by a lower level of IL-5 in the lavage fluid.

The results presented in this study complete earlier experiments in which 10⁷ or 10⁸ CFU *M. vaccae* administered 1–3 weeks before OVA sensitization or challenge did not decrease parameters of atopic asthma in the same mouse model [23]. These data and the present study suggest that the timing of *M. vaccae* administration is crucial; *M. vaccae* should be present during allergen exposure to be effective. These results seem to be in contrast with those by other investigators who demonstrated that treatment with 10⁸ CFU heat-killed *M. vaccae* 3 weeks before OVA sensitization or OVA challenge was able to lower airway eosinophilia and airway hyper-responsiveness to methacholine [13, 24]. Differences in study design may account for this observed difference, such as the dose and frequency of *M. vaccae* and the time of sensitization and challenge.

Next to the observed effects of *M. vaccae* on the development of airway hyper-responsiveness and eosinophilia, we demonstrated that *M. vaccae* down-regulates allergen-specific IgE responses and the Th2 cytokines IL-4 and IL-5 in the lung as well. Although the first study describing the beneficial effects of heat-killed *M. vaccae* demonstrated that 10⁷ CFU *M. vaccae* down-regulated the production of IgE and IL-5 by splenic cells [12], the following studies using 10⁸ CFU *M. vaccae* failed to do so [13, 24]. It seems that a lower dose of *M. vaccae* (10⁷ CFU) is more effective than a

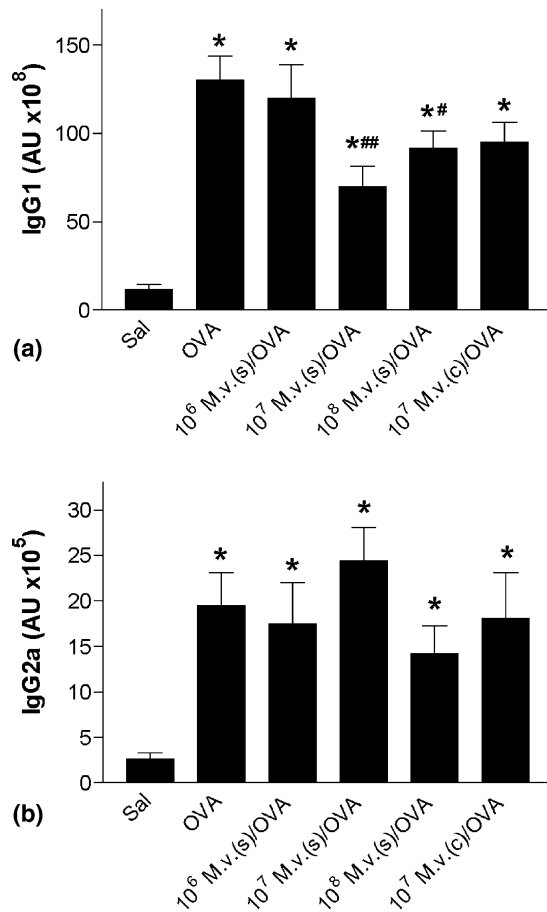


Fig. 5. OVA-specific IgG1 (a) and IgG2a (b) levels as measured by ELISA in serum of OVA-sensitized mice 24 h after final saline (Sal) or OVA challenge. Mice were treated with 10^6 (10^6 M.v.(s)/OVA), 10^7 (10^7 M.v.(s)/OVA) or 10^8 (10^8 M.v.(s)/OVA) CFU *M. vaccae* during OVA sensitization or with 10^7 (10^7 M.v.(c)/OVA) CFU *M. vaccae* during OVA challenge. Vertical bars indicate arbitrary units (AU) $\pm$ SEM, $n = 8$. * $P < 0.01$ compared to the saline-challenged control group. # $P < 0.05$ or ### $P < 0.01$ compared to the OVA-challenged control group.

higher dose (10^8 CFU) in lowering Th2-type responses. Interestingly, a low dose of *M. vaccae* (10^7 CFU) has been described to prime a Th1 response in the absence of a Th2 component, while a higher dose (10^8 and 10^9 CFU) induces a mixed Th1/Th2 response in the spleen of mice [25]. The difference in cytokine secretion related to the *M. vaccae* immunizing dose may explain the observed effects on Th2 responses in our model.

We observed a lowering in the IL-4 and IL-5 response after *M. vaccae* treatment during sensitization and, interestingly, a lowering in IL-5 after treatment during challenge as well. This lowered Th2 response in both treatment regimes may provide a possible mechanism for the observed effects of *M. vaccae* on allergen-induced eosinophilia and airway hyper-responsiveness, both downstream manifestations of asthma. Many subsequent studies in mice and humans have confirmed the pivotal role of allergen-specific Th2 cells in the pathogenesis and maintenance of allergic asthma, their cytokines orchestrating the inflammatory immune response resulting in airway symptoms (reviewed in [26]). Regardless of the down-regulation of Th2 cytokines in the lavage fluid, we did not

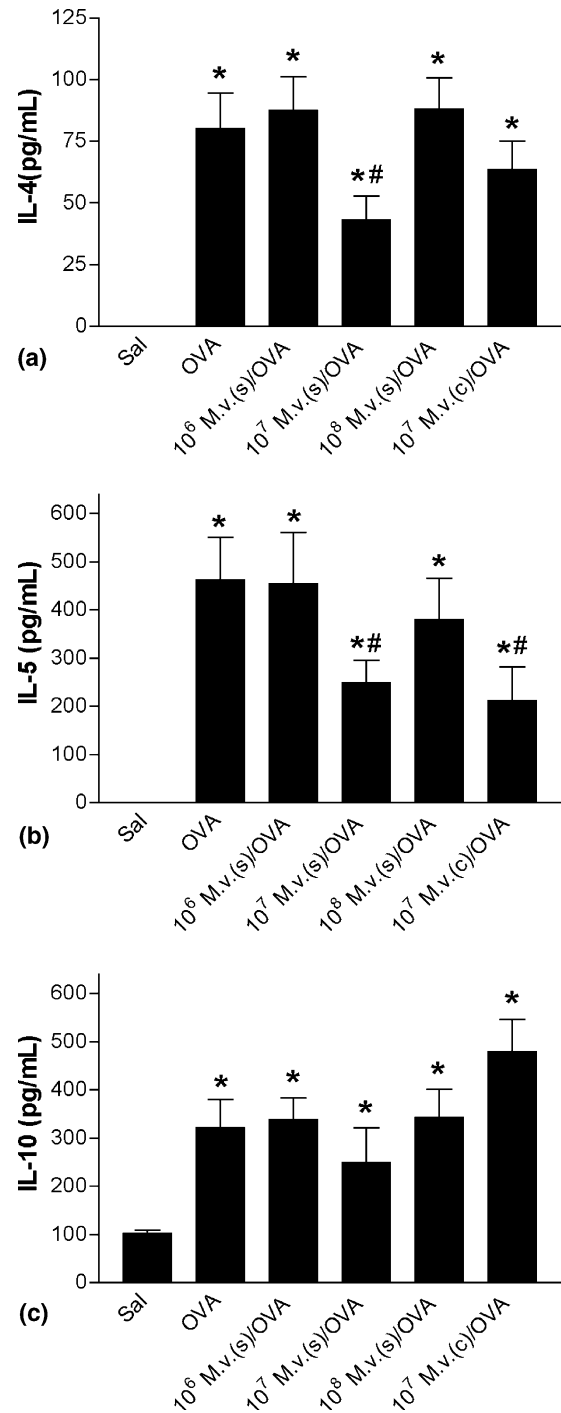


Fig. 6. Levels of IL-4 (a), IL-5 (b) and IL-10 (c) in lung lavage fluid obtained from OVA-sensitized mice 24 h after final saline (Sal) or OVA challenge. Mice were treated with 10^6 (10^6 M.v.(s)/OVA), 10^7 (10^7 M.v.(s)/OVA) or 10^8 (10^8 M.v.(s)/OVA) CFU *M. vaccae* during OVA sensitization or with 10^7 (10^7 M.v.(c)/OVA) CFU *M. vaccae* during OVA challenge. Data are represented as mean pg/mL $\pm$ SEM, $n = 8$. * $P < 0.01$ compared to the saline-challenged control group. # $P < 0.05$ compared to the OVA-challenged control group.

observe an up-regulation of Th1 responses, as characterized by the levels of IFN- γ in the lung lavage fluid or the Th1-related IgG2a antibodies in serum in our experiments. The beneficial effects of mycobacteria, including BCG and *M. vaccae*, in treating allergic disease might be caused by their

capacity to strongly induce Th1-type responses. Since both Th1 and Th2 cells cross-regulate each other's development and activity [27], the up-regulation of Th1 responses by mycobacteria might have dampened the Th2 response. The lack of a Th1 response in our experiments suggests that mechanisms other than simple changes in the balance between Th1 and Th2 are responsible for the observed effects.

Another mechanism for the observed effects of *M. vaccae* comes from recent studies by Zuany-Amorim which showed that *M. vaccae* gives rise to induction of CD4⁺CD45RB^{lo} T regulatory cells, which conferred protection against airway inflammation, probably mediated through IL-10 and TGF- β [24]. Studies in mouse models confirmed a role for IL-10 in suppressing airway inflammation and cytokine production, although paradoxical effects on airway hyper-responsiveness have been found [28, 29]. For this reason, we measured IL-10 in the lung lavage fluid after final challenge. We did not observe a significant change in the levels of IL-10 in the lung lavage fluid after *M. vaccae* treatment, either during sensitization or challenge. Therefore, the role of IL-10 in the observed effects of *M. vaccae* remains unclear in our model, although we cannot exclude a role of IL-10 in direct cell-cell interactions or temporal responses after *M. vaccae* treatment, since we measured cytokines in lavage fluid 24 h after the final challenge.

Interactions of ligands of *M. vaccae* with several receptors on host cells play a crucial role in the immune response to *M. vaccae* and its efficacy in treatment either during sensitization or challenge. Mycobacteria express a whole range of ligands that trigger signalling through pattern recognition receptors such as the mannose receptor, Toll-like receptor (TLR) 2 and/or TLR 4 on host phagocytes and other cells, resulting in the activation of phagocytes and the release of pro- or con-inflammatory mediators. *M. vaccae* express mycobacterial heat-shock proteins [30] and lipoarabinomannan (LAM). While slow-growing mycobacteria such as BCG and *M. tuberculosis* express mannose-capped LAM (manLAM), fast-growing mycobacteria express arabinofuransyl-terminated LAM (araLAM), which potentially induces cytokine release from macrophages [31]. To date which type of LAM *M. vaccae* expresses unknown. This might be of importance since DC-SIGN, an important recently discovered receptor for LAM, binds only manLAM [32]. Induction of TLRs and DC-SIGN by heat-shock protein or LAM influences not only the induction or inhibition of inflammatory responses but also the development of antigen-specific adaptive immunity, including regulatory T cell development [33, 34]. In addition, the relatively rapid effects, within a few days, in the protocol of *M. vaccae* administration during challenge may be explained by a protective effect of either heat-shock proteins [35] or other components of *M. vaccae*.

Several other animal studies showed that mycobacteria are effective in lowering allergic and asthmatic responses [13–15, 36]. The use of a heat-killed vaccine as used in our study is preferable above a viable preparation, in particular when converting to possible human use. Heat-killed *M. vaccae* has undergone extensive toxicological and safety assessment in humans, while BCG may exert side-effects and poses a risk of bacterial disease. In addition, BCG immunization provides variable protection rates against tuberculosis [37], illustrating

the incapacity of this agent to elicit a Th1 cell response in a substantial proportion of the human population. In addition, the subcutaneous route of administration that we used in our experiments is strongly preferable above administration of mycobacteria directly at the site of allergen exposure, which is often used in animal experiments. Intranasal treatment with viable or heat-killed BCG most effectively lowered asthmatic responses, when compared with systemic administration, but this was always accompanied by significant inflammation in the lung [14, 38].

In conclusion, as shown in our study, *M. vaccae* down-regulates allergen-induced Th2-mediated allergic and asthmatic responses, during the development of allergic disease. Importantly, *M. vaccae* is able to lower these responses during the establishment of allergic disease as well. Furthermore, *M. vaccae* must be administered during allergen exposure to be effective in our model. Therefore, the contradictory results in clinical trials using *M. vaccae* [18, 39, 40] may be due to the duration and level of exposure to allergens during the treatment period in these studies. New insights into the mechanism of action of *M. vaccae* need to be gained, and possible genetic factors influencing *M. vaccae* treatment remain to be investigated.

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