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Long-Term Protective and Antigen-Specific Effect of Heat-Killed *Mycobacterium vaccae* in a Murine Model of Allergic Pulmonary Inflammation

Claudia Zuany-Amorim,[1]* Corinne Manlius,* Alexandre Trifilieff,* Laura R. Brunet,[†‡] Graham Rook,[‡] Gareth Bowen,[†] Graham Pay,* and Christoph Walker*

This report examines the effect of heat-killed *Mycobacterium vaccae* in a mouse model of allergic pulmonary inflammation. The s.c. administration of *M. vaccae* 3 wk before the immunization significantly reduced Ag-induced airway hyperreactivity and the increase in the numbers of eosinophils observed in the bronchoalveolar lavage fluid, blood, and bone marrow, even though no detectable changes in either cytokine (IL-4, IL-13, IL-5, and IFN-γ) or total IgE levels were observed. Furthermore, transfer of splenocytes from OVA-immunized and *M. vaccae*-treated mice into recipient, OVA-immunized mice significantly reduced the allergen-induced eosinophilia by an IFN-γ-independent mechanism, clearly indicating that the mechanism by which *M. vaccae* induces its inhibitory effect is not due to a redirection from a predominantly Th2 to a Th1-dominated immune response. The protective effect of *M. vaccae* on the allergen-induced eosinophilia lasted for at least 12 wk after its administration, and the treatment was also effective in presensitized mice. Moreover, the allergen specificity of the inhibitory effect could be demonstrated using a double-immunization protocol, where *M. vaccae* treatment before OVA immunization had no effect on the eosinophilic inflammation induced by later immunization and challenge with cockroach extract Ag. Taken together, these results clearly demonstrate that *M. vaccae* is effective in blocking allergic inflammation by a mechanism independent of IFN-γ, induces long term and Ag-specific protection, and therefore has both prophylactic and therapeutic potential for the treatment of allergic diseases. *The Journal of Immunology,* 2002, 169: 1492–1499.

Allergic asthma is a chronic inflammatory disease characterized by reversible airway obstruction, airway hyperreactivity (AHR),[2] airway infiltration by inflammatory cells, particularly eosinophils and T lymphocytes, and high levels of allergen-specific IgE (1–3). Although current asthma therapy is effective and well tolerated in most patients, there are some limitations (4), and there is a clear medical need for the development of new therapies capable of changing or reprogramming the underlying immune processes to produce a long term, Ag-specific protective immune response. For example, inhaled corticosteroids are very effective in controlling asthma in most cases. However, there are still concerns about side effects, particularly in children and in patients with severe asthma who require high dose treatment. Moreover, although corticosteroids are very effective in suppressing inflammation in asthmatic airways, these drugs do not change the underlying immunological mechanisms, so that when steroids are discontinued, the inflammation and asthma symptoms recur (4, 5). Therefore, corticosteroid and the other currently available treatments for asthma do not have disease-modifying activity.

Epidemiological and clinical studies have provided compelling evidence that suggest a link between the relative lack of infectious diseases and the increase in allergic disorders. There are indeed many studies suggesting that bacterial infections and/or bacterial products can inhibit the development of allergic diseases (6). It has been demonstrated that a positive tuberculin test result suggestive of past infections with *Mycobacterium tuberculosis* was inversely related to the subsequent development of atopy and asthma (7). Moreover, the use of antibiotics during infancy correlated with an increased risk of developing asthma, suggesting that bacterial infections early in life may help to inhibit the development of asthma (8). In animal models of allergic inflammation, bacillus Calmette-Guérin (BCG) inoculation in mice delivered 14 days before allergen immunization reduced the formation of specific IgE in response to allergen and the eosinophilic and AHR responses to allergen with an increase in the production of IFN-γ (9, 10). Bacterial products such as oligonucleotides containing unmethylated CpG motifs have been found to be potent stimuli of IL-12 and IFN-γ production, leading to a strong inhibition of allergic airway inflammation (11). Furthermore, several studies have demonstrated that heat-killed *Listeria monocytogenes*, *Lactobacillus plantarum*, or *Mycobacterium vaccae* could also suppress allergic responses in mice (12–14). In particular, there is evidence that the fast-growing *M. vaccae*, which induces a vigorous cell-mediated immune response and shares several immunodominant epitopes with other mycobacteria, might be a potential candidate for an anti-asthma vaccine. Preliminary clinical studies in adults with grass pollen-induced asthma and rhinitis found that treatment with heat-killed *M. vaccae* was associated with fewer asthmatic symptoms and significantly less use of bronchodilator relief medication

during the pollen season (15). More recently, it has been demonstrated that intradermal administration of a killed *M. vaccae* suspension was associated with an improvement in the severity of the atopic dermatitis in children with moderate to severe disease (16).

To further investigate the potential of mycobacteria to treat, prevent, or even cure atopic diseases, we tested whether heat-killed *M. vaccae,* which in other contexts has undergone extensive investigations in human cancer and tuberculosis patients, was able to alter the Ag-induced inflammation and AHR to inhaled methacholine in a murine model of pulmonary inflammation.

Materials and Methods

Animals

Female BALB/c mice (20–25 g, 5–6 wk old) were obtained from Harlan (Oxon, U.K.), All experimental protocols complied with the Home Office 1986 Animals Scientific Act and were approved by the Novartis Horsham Research Center animal welfare committee.

Ags and M. vaccae *suspension*

OVA was obtained from Sigma-Aldrich (grade V; Sigma-Aldrich, St. Louis, MO). Cockroach extract Ag was obtained from Hollister-Stier Laboratories (Spokane, WA). Sterile vials of *M. vaccae* (0.3 ml of a suspension containing 10 mg heat-killed *M. vaccae*/ml) were provided by SR Pharma (London, U.K.).

Experimental design

Short term immunization protocol. BALB/c mice were immunized i.p. on days 0 and 14 with 10 μg OVA in 0.2 ml alum (Serva, Heidelberg, Germany) as described in Fig. 1. On day 21 animals were exposed for 20 min

to an aerosol of OVA in sterile PBS (50 mg/ml) or sterile PBS alone.

Long term immunization protocol. BALB/c mice were immunized i.p. on days 0, 14, and 42 and s.c. on day 56 with 10 μg OVA in 0.2 ml alum as described in Fig. 1. On day 63 animals were exposed for 20 min to an aerosol of OVA in sterile PBS (50 mg/ml) or sterile PBS alone.

Double immunization protocol. On days 0 and 14 mice received an i.p. injection of OVA in the presence of alum, and on days 42 and 56 of immunization animals were immunized with cockroach extract Ag (CEA; 10 μg in 0.2 ml alum) by i.p. and s.c. routes, respectively. On day 63 mice received an intranasal Ag challenge of 50 μl PBS containing 10 μg CEA. Control animals received 50 μl PBS.

Treatment with M. vaccae

Mice were treated, via the s.c. route, with 0.1 mg *M. vaccae* (SR Pharma) in 200 μl saline. This dose was chosen based on a previous report (14) and from pilot experiments demonstrating an optimal response using these conditions (data not shown). Control animals received an s.c. injection of 200 μl saline alone, and treatment regimens are detailed in Fig. 1. Briefly, in the short term immunization protocol mice were treated with *M. vaccae* 21 days before the first OVA immunization. In the long term immunization protocols *M. vaccae* was administered either before the first or the third immunization to investigate whether the compound could also interfere with an established allergic phenotype. Finally, in the double-immunization protocol animals were treated with *M. vaccae* 21 days before the first OVA immunization or 21 days before the first CEA immunization.

Assessment of inflammation

At the specified time point animals were anesthetized with pentobarbitone sodium (60 mg/kg i.p.). The trachea was cannulated, and bronchoalveolar lavage (BAL) was performed by injecting 0.4 ml PBS into the lung via the trachea. The fluid was withdrawn and stored on ice. This procedure was

Short term immunization protocol

Days	-21	0	14	21
	M.vaccae s.c.	OVA i.p.	OVA i.p.	OVA aerosol challenge

Long term immunization protocol

Days	-21	0	14		42	56	63
	M.vaccae s.c.	OVA i.p.	OVA i.p.		OVA i.p.	OVA s.c.	OVA aerosol challenge

Days		0	14	21	42	56	63
		OVA i.p.	OVA i.p.	M.vaccae s.c.	OVA i.p.	OVA s.c.	OVA aerosol challenge

Double immunization protocol

Days	-21	0	14		42	56	63
	M.vaccae s.c.	OVA i.p.	OVA i.p.		CEA i.p.	CEA s.c.	CEA intranasal challenge

Days		0	14	21	42	56	63
		OVA i.p.	OVA i.p.	M.vaccae s.c.	CEA i.p.	CEA s.c.	CEA intranasal challenge

FIGURE 1. Schematic representation of the protocol of immunizations used in this study.

repeated three times. Total cell count was determined, and cytospin preparation (Shandon Scientific, Cheshire, U.K.) was performed. Cells were stained with DiffQuik (Baxter Dade, Dudingen, Switzerland), and a differential count of 200 cells was performed using standard morphological criteria. The remaining BAL fluid was centrifuged ($400 \times g$ for 10 min), and supernatant was collected and stored at $-80°C$ for cytokine and chemokine measurements. BAL cytokine (IL-5, IL-4, IL-13, and IFN-γ) and chemokine (macrophage inflammatory protein-1α, RANTES, and eotaxin) levels were measured using commercially available kits (R&D Systems, Oxon, U.K.).

Morphometric analysis of eosinophils was accomplished by examining 10 high power fields ($\times 1000$ magnification) in histological sections from each lung. Blood smears were prepared by taking blood from the abdominal aorta. Peripheral eosinophils were identified and counted after staining with DiffQuik stain. Bone marrow cells were flushed from femurs with 1 ml RPMI 1640 medium supplemented with 10% FBS, and eosinophils were enumerated after cytocentrifugation and staining with *O*-phenylenediamine and counterstaining with thiazine.

Measurement of airway reactivity to inhaled methacholine

All mice were immunized i.p. with 10 μg OVA in alum (short term immunization, Fig. 1). Twenty-one days after the beginning of the immunization mice were challenged four times, each 1 day apart, with either 50 mg/ml OVA or PBS for 20 min by aerosol. Twenty-four hours after the last challenge mice were used to assess airway hyperresponsiveness to inhaled methacholine by barometric plethysmography (Buxco Electronics, Troy, NY) using whole body plethysmography as described previously (17). To evaluate airway responsiveness, conscious, spontaneously breathing mice were initially placed in the main chamber of the plethysmograph. Following a short settlement period, baseline airway function over 1 min was recorded and averaged. Mice were then exposed for 1 min to nebulized PBS, and recordings were taken. Following that, mice were exposed to 0.3 M methacholine (Aldrich, Madison, WI) for 1 min, and recordings were taken for a total of 4 min, but were averaged for each minute. The highest of the four 1 min averaged enhanced pause (Penh) values during the 1 min after exposure to methacholine was expressed as a percentage of the baseline Penh value obtained following PBS exposure.

Serum IgE and IgG2a levels

Twenty-four hours after Ag challenge animals were anesthetized with pentobarbitone sodium (60 mg/kg i.p.), a blood sample was taken from the abdominal aorta, serum was prepared, and total IgE and IgG2a Ab titers were determined by ELISAs as previously described (18). Briefly, total serum IgE and IgG2a were measured using sandwich ELISA, 4B3-39 (rat IgG anti-murine IgE mAb; Novartis, Basel, Switzerland) and goat IgG anti-mouse IgG2a were the capture reagent for IgE and IgG2a, respectively. Biotin-conjugated 3-11 (rat IgG anti-mouse IgE mAb) and biotin-conjugated RA33-18-12 (rat IgG anti-mouse IgG2a mAb) were used as detection reagents along with alkaline phosphatase-streptavidin. Affinity-purified monoclonal IgE anti-trinitrophenyl (A3B1; BD Biosciences, Oxford, U.K.) and monoclonal IgG2a anti-PC (Novartis) were used as standards.

Splenocyte activation

To assess the effect of *M. vaccae* on anti-CD3 or OVA-driven IFN-γ release by splenocytes, mice were s.c. treated 3 wk before the immunization with saline or 0.1 mg *M. vaccae*. Briefly, spleens were collected on day 21, and single-cell suspensions were prepared by passing the cells through a cell strainer. RBC were removed by hypotonic lysis. After three washes in RPMI (Life Technologies, Paisley, U.K.), 5×10^5 splenocytes/250 μl RPMI supplemented with 10% FBS (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and 100 U/ml penicillin (Life Technologies) were added to each well in triplicate and then incubated for 72 h at $37°C$ in 5% CO_2 in the presence or the absence of 80 μg/ml OVA. For anti-CD3 activation cells were cultured in 96-multiwell plates coated with 4.5 μg/ml immobilized anti-CD3 mAb 145-2C11 (BD PharMingen, Oxford, U.K.) for 48 h (19). Plates were then centrifuged ($80 \times g$, 5 min, $4°C$), and IL-4 and IFN-γ levels in the supernatants were determined by ELISA (R&D Systems, Oxon, U.K.). The detection limit for both IL-4 and IFN-γ was 2 pg/ml.

Adoptive cell transfer and anti-IFN-γ treatment

Twenty-four hours after the OVA challenge (day 22), spleens from saline- or *M. vaccae*-treated mice were collected, and single-cell suspensions were prepared by passing the cells through a cell strainer. Splenocytes were then isolated by Ficoll-Hypaque density gradient centrifugation. Recipient im-

munized mice received on day 20 an i.v. injection of 50 μl PBS containing 2×10^6 splenocytes or PBS alone. On day 21 mice were challenged with either PBS or OVA, and BAL was obtained 48 h postchallenge.

In another set of experiments immunized mice (day 20) were injected i.p. with 0.5 ml preimmune rabbit serum or an equivalent amount of anti-IFN-γ immune serum (provided by Prof. S. Kunkel, Department of Pathology, University of Michigan Medical School, Ann Arbor, MI) or with saline 2 h before and 48 h after the i.v. injection of 2×10^6 splenocytes from *M. vaccae*-treated and OVA-immunized and -challenged mice. Twenty-four hours after the cell transfer mice were challenged with OVA, and BAL was obtained 48 h postchallenge. This treatment regimen with the anti-IFN-γ immune serum has been previously shown to significantly attenuate systemic levels of IFN-γ in a mouse model of inflammation (20).

Statistical analysis

Results are expressed as the mean $\pm$ SEM of the indicated number of animals. One-way ANOVA was used to determine significance among the groups. If a significant variance was found, an unpaired Student's *t* test was used to assess comparability between means. A value of $p < 0.05$ was considered significant.

Results

M. vaccae *treatment inhibits Ag-induced eosinophil accumulation in BAL fluid*

In a first set of experiments the effect of *M. vaccae* treatment on the eosinophilic inflammation of OVA-immunized and -challenged mice using a short immunization protocol was analyzed. As shown in Fig. 2, single OVA aerosol challenge to immunized mice resulted in a significant increase in the number of total cells, eosinophils, neutrophils, and macrophages in BAL fluid compared with PBS challenged-animals. The increase in eosinophils and macrophages peaked between 24 and 48 h after OVA challenge (Fig. 2, *A* and *C*), whereas the highest number of neutrophils was detected between 4 and 24 h (Fig. 2*B*). To analyze the effect of *M. vaccae*, mice were treated by s.c. injection with 0.1 mg *M. vaccae* 24 h before the immunization, 4 h before allergen challenge, or 21 days before the first immunization. No effect on eosinophilic inflammation was observed in mice treated either 24 h before the immunization (0.71 ± 0.16 and $1.12 \pm 0.37 \times 10^5$ eosinophils/ml for saline-treated and OVA-challenged mice and *M. vaccae*-treated and OVA-challenged mice, respectively) or shortly before the allergen challenge (1.35 ± 0.16 and $1.43 \pm 0.13 \times 10^5$ eosinophils/ml for saline-treated and OVA-challenged mice and *M. vaccae*-treated and OVA-challenged mice, respectively). However, in mice treated 21 days before the first immunization, *M. vaccae* induced a significant reduction of the allergen-induced inflammation, as demonstrated by the significantly reduced numbers of eosinophils, neutrophils, macrophages, and total cells present in the BAL fluid of these animals (Fig. 2). Based on these results the treatment schedule of 21 days before the immunization and the dose of 0.1 mg (see *Materials and Methods*) was selected for all additional experiments.

Treatment with M. vaccae *inhibits the eosinophil accumulation in the peribronchial wall, blood, and bone marrow following Ag challenge*

To further investigate whether the reduction of inflammatory cells in BAL fluid of *M. vaccae*-treated animals was due to the retention of these cells within the bronchial tissue, reduced infiltration from the circulation, or suppressed release from the bone marrow, eosinophil accumulation following allergen challenge in these compartments was analyzed. As shown in Fig. 3, a single aerosol Ag challenge on day 21 to immunized mice induced an increase in the number of eosinophils in bone marrow, blood, and peribronchial wall at 24 h postchallenge compared with PBS-challenged mice.
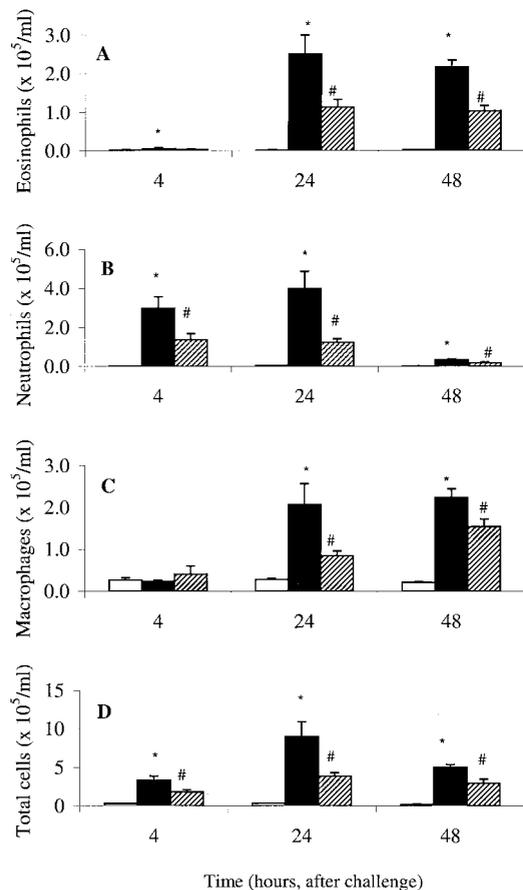
FIGURE 2. Kinetics of inhibition by *M. vaccae* of Ag-induced eosinophil (*A*), neutrophil (*B*), macrophage (*C*), and total cell (*D*) counts in the BAL fluid from immunized mice sacrificed 4, 24, and 48 h after the challenge. Animals were treated s.c. 21 days before the first immunization with saline (■) or with 0.1 mg *M. vaccae* (▨) and challenged with OVA. Control mice were treated with saline and challenged with PBS (□). Data are the mean ± SEM of 8–10 mice for each group. *, $p < 0.05$ compared with saline-treated and PBS-challenged mice. #, $p < 0.05$ compared with *M. vaccae*-treated and OVA-challenged mice.

FIGURE 3. Effect of *M. vaccae* on eosinophil accumulation in the peribronchial wall (*A*), blood (*B*), and bone marrow (*C*). Animals were treated s.c. 21 days before the immunization with saline (■) or with 0.1 mg *M. vaccae* (▨) and were sacrificed 24 h after the OVA challenge. Control mice received an aerosol challenge of PBS and were treated s.c. with saline alone (□). Data are the mean ± SEM of 8–10 mice for each group. *, $p < 0.05$ compared with saline-treated and PBS-challenged mice. #, $p < 0.05$ compared with *M. vaccae*-treated and OVA-challenged mice.

The s.c. administration of 0.1 mg *M. vaccae* 21 days before immunization reduced the numbers of eosinophils in all these compartments (Fig. 3), suggesting that the mechanism of how *M. vaccae* inhibits the inflammatory cell infiltrate found in BAL fluid is not due to a selective blockage of cell movement from these compartments or retention of cells within the bronchial tissue.

Treatment with M. vaccae *does not alter the Ag-induced cytokine and chemokine production in BAL fluid of immunized and challenged mice*

As shown in Fig. 4, the single Ag challenge of OVA to immunized mice induced a characteristic increase not only in the Th2 cell cytokines IL-4, IL-5, and IL-13, but also in chemokines associated with eosinophilic inflammation, such as eotaxin, macrophage inflammatory protein-1α, and RANTES. In contrast, IFN-γ, a cytokine normally associated with a Th1 cell response, was significantly reduced in BAL fluid 24 h following the allergen challenge. However, although pretreatment with *M. vaccae* effectively blocked BAL and lung eosinophilia, no effect on Ag-induced cytokine and chemokine release was observed 24 h after challenge; in particular, no effect on BAL fluid IFN-γ levels was observed, suggesting that the mechanism of action of the *M. vaccae*-medi-
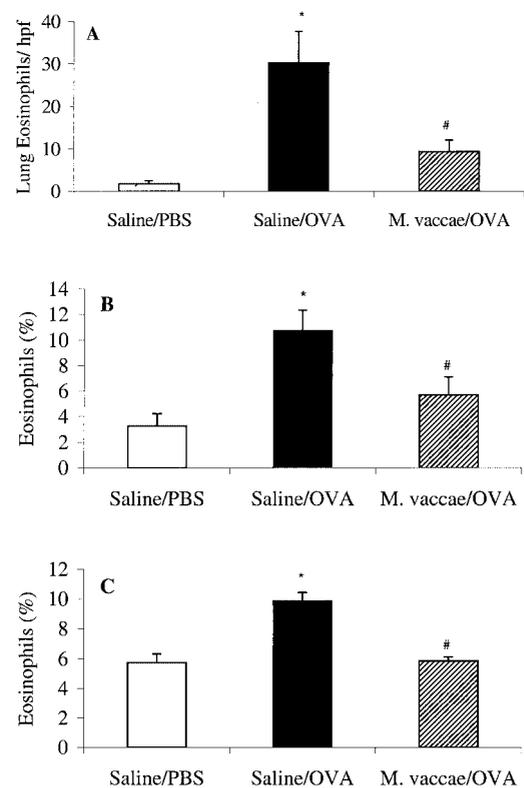
ated anti-inflammatory effect does not involve a strong Th1 cell cytokine production.

Lack of inhibitory effect of M. vaccae *on total IgE and IgG2a levels in serum*

The total IgE level in immunized animals was significantly higher than that in naive mice (753 ± 25 ng/ml), indicating allergic immunization of these animals. When *M. vaccae* at 0.1 mg was administered to mice 21 days before the beginning of immunization, no decrease in the serum levels of IgE was found compared with saline-treated and OVA-immunized and -challenged mice (Fig. 4*C*). Specific OVA-IgE was also unaffected by *M. vaccae* treatment (data not shown). Furthermore, *M. vaccae* was unable to change IgG2a serum levels compared with saline-treated and OVA-immunized and -challenged mice, suggesting again that the main mechanism by which *M. vaccae* induces the suppression of allergic inflammation does not involve the induction of a Th1 cell response, expected to decrease IgE and increase IgG2a levels.

Effect of M. vaccae *on IL-4 and IFN-γ release by splenocytes in response to in vitro anti-CD3 mAb and OVA stimulation*

To confirm that the effect of *M. vaccae* on Ag-induced airway eosinophilia does not involve allergen-specific Th1 cells, splenocytes from saline- or *M. vaccae*-treated and immunized mice (short term immunization, day 21) were stimulated with immobilized anti-CD3 mAb for 48 h. Challenge with anti-CD3 mAb induced a marked IFN-γ release, which was unaffected by *M. vaccae* treatment. In vitro anti-CD3 activation also triggered IL-4 release by
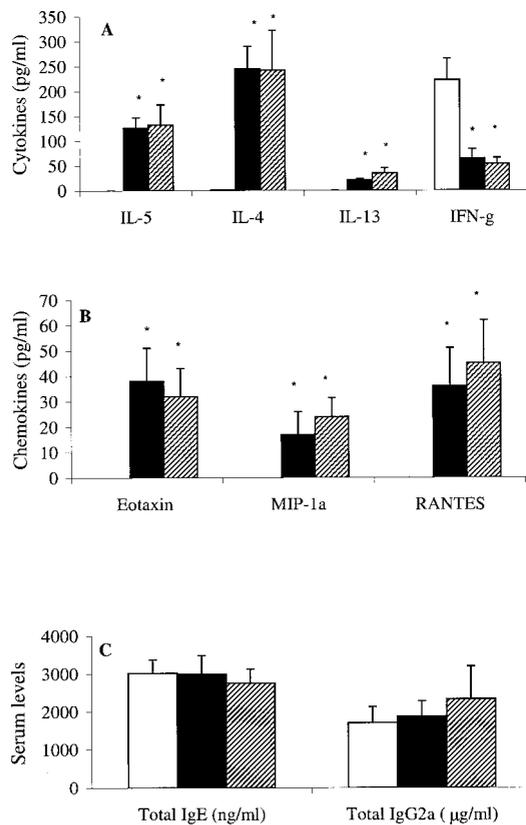
FIGURE 4. Effect of *M. vaccae* on cytokine (*A*) and chemokine (*B*) levels in the BAL fluid and Ig levels (*C*) in the serum of immunized and challenged mice. Animals were treated s.c. 21 days before the immunization with saline (■) or with 0.1 mg *M. vaccae* (▨) and were sacrificed 24 h after the OVA challenge. Control mice received an aerosol challenge of PBS and were treated s.c. with saline alone (□). Data are the mean ± SEM of 5–10 mice in each group. *, $p < 0.05$ compared with saline-treated and PBS-challenged mice.
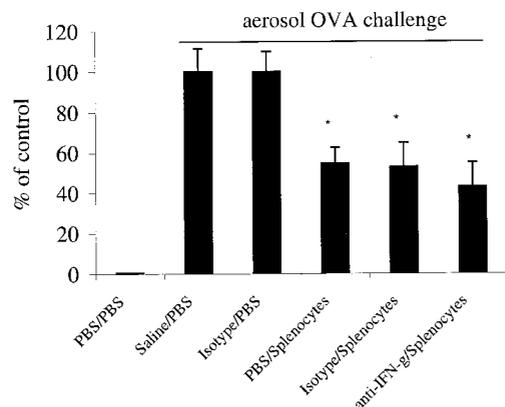

FIGURE 5. Effect of adoptive transfer of splenocytes on OVA-induced eosinophilic inflammation. Recipient OVA-immunized mice (short term protocol, day 20) were treated i.v. 24 h before the allergen challenge with PBS or 2×10^6 splenocytes from *M. vaccae*-treated and OVA-immunized and -challenged donor mice. Two and 48 h after the PBS or splenocyte i.v. injection, immunized recipient mice were injected i.p. with 0.5 ml pre-immune rabbit serum or an equivalent amount of anti-IFN-γ immune serum or with saline. Twenty-four hours after the cell transfer mice were challenged with PBS (PBS/PBS) or OVA, and BAL was obtained 48 h postchallenge. Results are expressed as a percentage of the level in saline-treated and OVA-challenged control mice without cell transfer. Data are the mean ± SEM of 7–10 mice for each group. *, $p < 0.05$ compared with PBS-treated and OVA-challenged mice.

splenocytes, and this response was not modified by previous treatment with *M. vaccae* (Table I).

OVA stimulation of splenocytes from saline- or *M. vaccae*-treated mice resulted in similar IL-4 production. However, unlike anti-CD3 activation, Ag stimulation failed to trigger a significant production of IFN-γ release regardless of the treated group analyzed (Table I).

Effect of adoptive cell transfer on Ag-induced airway eosinophilia

M. vaccae treatment is able to block allergic eosinophilia, without in vitro and in vivo changes in IFN-γ levels. To better understand the mechanism by which *M. vaccae* blocks airway eosinophilia, splenocytes from *M. vaccae*-treated mice were transferred into recipient immunized animals before the Ag challenge. Indeed, the i.v. administration of 2×10^6 splenocytes obtained from *M. vaccae*-treated and OVA-immunized and -challenged mice significantly reduced OVA-induced airway eosinophilia in recipient mice compared with PBS-treated and OVA-challenged mice (45% inhibition; $n = 6$–8; $p < 0.05$; Fig. 5). In contrast, the transfer of splenocytes from saline-treated and OVA-challenged as well as from naive mice to recipient OVA-immunized mice failed to inhibit Ag-induced airway eosinophilia (data not shown). Furthermore, treatment of recipient mice with an Ab against IFN-γ before cell transfer failed to reverse the inhibitory effect of *M. vaccae*-treated splenocytes on airway eosinophilia (Fig. 5).

Effect of M. vaccae on Ag-induced AHR to inhaled methacholine

Next, the effect of *M. vaccae* treatment on the induction of AHR was analyzed using a multiple allergen challenge model (see *Materials and Methods*). OVA immunization followed by multiple challenges induced a marked increase in airway reactivity to inhaled methacholine 24 h after the last OVA challenge compared

Table I. *Effect of* M. vaccae *on IL-4 and IFN-γ release by splenocytes in response to in vitro anti-CD3 mAb and OVA stimulation*[a]

	IL-4 (pg/ml)		IFN-γ (pg/ml)	
	Saline	M. vaccae	Saline	M. vaccae
RPMI	2 ± 1	3 ± 1	22 ± 11	17 ± 6
Anti-CD3	667 ± 63[b]	629 ± 93[b]	1346 ± 10[b]	1333 ± 29[b]
OVA	122 ± 30[b]	154 ± 20[b]	28 ± 6	30 ± 4

[a] Splenocytes from immunized mice treated with either saline or *M. vaccae* were incubated with RPMI alone or were stimulated either with 4.5 μg/ml immobilized anti-CD3 mAb 145-2C11 or 80 μg/ml OVA. The levels of IL-4 and IFN-γ (picograms per milliliter) in the supernatant of cell cultures were determined by ELISA. Data are the mean ± SEM of six mice for each group.
[b] $p < 0.05$ compared with saline or *M. vaccae*-treated and RPMI-stimulated cells.

with control mice (Fig. 6). This OVA-induced AHR to inhaled methacholine was reduced by 72% after treatment with *M. vaccae* 21 days before the first immunization ($n = 8$–10; $p < 0.05$; Fig. 6). In a separate set of experiments the possibility that *M. vaccae* treatment itself might interfere with the bronchial response to methacholine was ruled out, since treatment of OVA-immunized, PBS-challenged mice with *M. vaccae* failed to modify the extent of methacholine-induced bronchoconstriction (increase in Penh of $83 \pm 18.6\%$ and $126 \pm 28.5\%$ for PBS-challenged, *M. vaccae*-untreated and -treated animals, respectively; $n = 5$). AHR to inhaled methacholine was accompanied by airway eosinophilia 24 h after the last OVA challenge. Treatment of mice with *M. vaccae*, given s.c. 21 days before immunization, resulted in 40% inhibition of eosinophil accumulation in BAL fluid at 24 h compared with saline-treated and OVA-challenged mice (saline-treated and OVA-challenged mice, 3.59 ± 0.75 eosinophils $\times 10^5$/ml; *M. vaccae*-treated and OVA-challenged mice, 2.11 ± 0.38 eosinophils $\times 10^5$/ml; $n = 7$–10; $p < 0.05$), demonstrating the protective anti-inflammatory effect of this treatment even after multiple allergen challenges.

Long term protective effect of M. vaccae

The results obtained to date clearly demonstrate that pretreatment with *M. vaccae* results in inhibition of the Ag-induced allergic inflammation and airway hyperresponsiveness in a short term immunization model. However, these experiments do not answer the questions of whether this protective effect is long lasting, or whether *M. vaccae* treatment is also effective in established immunization states. To answer these questions, a long-term immunization protocol was established, which allowed us to analyze the long term and Ag-specific effect of *M. vaccae* treatment. Mice were immunized on days 0, 14, 42, and 56 and were challenged with an aerosol of OVA on day 63 (Fig. 1). As shown in Fig. 7, *A* and *B*, airway eosinophilia induced by a single OVA challenge on day 65 was significantly reduced by a single administration of 0.1 mg *M. vaccae* either 3 wk before the first (day -21) or the third immunization (day 21). When administered before the third immunization (day 21), *M. vaccae* showed a more pronounced inhibitory effect on airway eosinophilia (80% reduction; $n = 8$–10; $p < 0.05$) than when administered before the first immunization (day -21; 42% reduction; $n = 8$–10; $p < 0.05$; Fig. 7*B*), suggesting that *M. vaccae* treatment is able to interfere with an established immunization state and therefore may have therapeutic potential. Similar to the results obtained in the short immunization protocol (Fig. 4), no change in total IgE and IgG2a levels was observed,
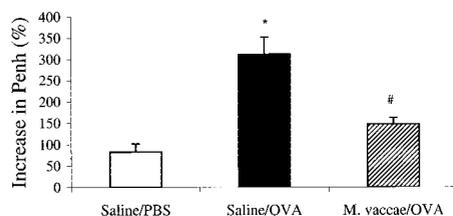

FIGURE 7. Long term protective and specific effect of *M. vaccae* on Ag-induced eosinophilia in the BAL fluid of immunized mice. Animals were s.c. treated with 0.1 mg *M. vaccae* (▨) or saline (■) and were sacrificed at 2 days post-Ag challenge. Control mice received a challenge of PBS and were treated s.c. with saline alone (□). Mice were treated before the first OVA immunization (*A*; day -21) or the third OVA immunization injection (*B*; day 21) with 0.1 mg *M. vaccae* and sacrificed at 2 days post-OVA aerosol challenge (day 65). *C*, Mice were treated with 0.1 mg *M. vaccae* 3 wk before the first immunization with OVA (day 0), and on day 14 they received a booster injection of OVA. On days 42 and 56 animals were immunized with CEA. On day 63 mice received an intranasal Ag challenge of 50 μl PBS containing 10 μg CEA. Control animals received 50 μl PBS. Forty-eight hours after Ag challenge mice were sacrificed for the determination of eosinophil numbers in BAL fluid. Data are the mean $\pm$ SEM of 5–10 mice for each group. *, $p < 0.05$ compared with saline-treated and PBS-challenged mice. #, $p < 0.05$ compared with *M. vaccae*-treated and OVA-challenged mice.
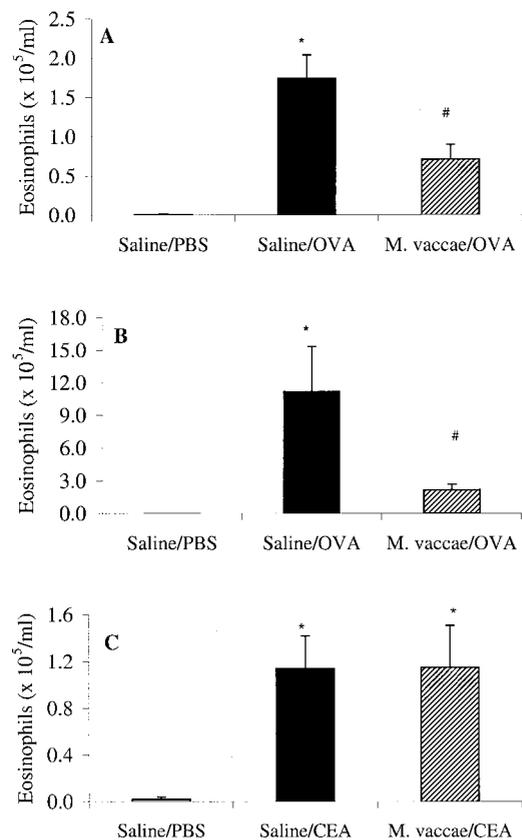

FIGURE 6. Effect of *M. vaccae*, on OVA-induced AHR to inhaled methacholine. OVA-immunized mice were treated with saline and were challenged four times by aerosol with either PBS (□) or OVA (■) or were treated s.c. with 0.1 mg *M. vaccae* 21 days before the immunization and challenged with OVA (▨). Twenty-four hours after the last PBS or OVA challenge animals were processed for AHR. Results are expressed as the percent change in Penh from baseline values in response to 0.3 M inhaled methacholine. Data are the mean $\pm$ SEM of 7–10 mice for each group. *, $p < 0.05$ compared with saline-treated and PBS-challenged mice. #, $p < 0.05$ compared with *M. vaccae*-treated and OVA-challenged mice.

although treatment with *M. vaccae* before the second round of immunization (day 21) slightly, but not significantly, reduced the total serum IgE levels (saline-treated and OVA-challenged mice, $22,700 \pm 3,133$ ng/ml; *M. vaccae*-treated and OVA-challenged mice, $15,363 \pm 2,882$ ng/ml). Moreover, *M. vaccae* treatment failed to inhibit the allergen-induced increase in cytokine or chemokine levels in BAL fluid of OVA-challenged mice (IL-5 levels in saline-treated and OVA-challenged mice, 235 ± 50 pg/ml compared with 263 ± 65 pg/ml in *M. vaccae*-treated and OVA-challenged mice; IL-13, 160 ± 26 vs 210 ± 55 pg/ml; RANTES, 39 ± 7 vs 28 ± 7 pg/ml).

M. vaccae treatment induced Ag-specific inhibition of allergen-induced eosinophilic inflammation

In an attempt to verify the specificity of the *M. vaccae* treatment mice were treated with 0.1 mg *M. vaccae* 3 wk before the first immunization with OVA. On days 42 and 56 of immunization

animals were immunized with CEA and were challenged intranasally with 10 μg CEA on day 63 (Fig. 1). Intranasal CEA challenge to these double-immunized mice resulted in an increase in the number of eosinophils in the BAL fluid at 48 h postchallenge with CEA. In contrast to the results obtained with OVA (Fig. 7*A*), treatment with *M. vaccae* before the OVA immunization had no effect on the eosinophilic inflammation induced by later immunization and challenge with CEA, suggesting that the *M. vaccae*-mediated inhibitory effect is Ag specific (Fig. 7*C*). This is further supported by experiments showing that using this double-immunization, long term protocol, pretreatment with 0.1 mg *M. vaccae* 3 wk before CEA immunization (Fig. 1) inhibited CEA-induced eosinophilic inflammation (saline-treated and CEA-challenged mice, 0.46 $\pm$ 0.1 eosinophils $\times$ 10^5/ml; *M. vaccae*-treated and CEA-challenged mice, 0.25 $\pm$ 0.1 eosinophils $\times$ 10^5/ml; $n =$ 9–10; $p < 0.05$).

Discussion

The present study demonstrates that s.c. treatment with heat-killed *M. vaccae* can prevent the development of allergen-induced bronchial inflammation as well as the induction of airway hyperresponsiveness in a mouse model of allergic pulmonary inflammation. The inhibitory effect was found to be IFN-γ independent, long lasting, and Ag specific and was also effective in previously immunized animals, indicating the therapeutic potential of the treatment.

Pharmacological agents that selectively inhibit Th2 cell activation and cytokine production, antagonize cytokines associated with allergic immune responses, or reprogram the immune response toward a potentially protective Th1 pathway are of considerable interest in the treatment of allergic asthma (21). In this respect it is well documented that many bacterial infections associated with suppression of allergic inflammation may induce profound Th1 responses with high levels of IL-12 and IFN-γ and therefore have the potential to interfere with the mainly Th2-driven allergic response (6, 9–14). In contrast to these studies, our results using s.c. treatment with heat-killed *M. vaccae* had no effect on IFN-γ production and did not modify the BAL fluid levels of IL-4, IL-5, IL-13, or serum IgE. It is therefore very unlikely that a shift from a Th2-dominated immune response to a Th1-dominated immune response is the major mechanism by which *M. vaccae* exerts its inhibitory effect. This conclusion is supported by the findings that 1) no Th1-inducing, Th2-suppressing activity could be demonstrated; 2) the suppressive effect of *M. vaccae* treatment on the allergic pulmonary response was not restricted to eosinophils, but also extended to other inflammatory cell types, such as neutrophils and macrophage; 3) splenocytes from immunized and *M. vaccae* treated-mice when activated in vitro failed to release increased levels of IFN-γ; 4) adoptive transfer of splenocytes from in vivo *M. vaccae*-treated mice into OVA-immunized mice inhibited the allergen-induced eosinophilia, which was not reversible by blocking IFN-γ; and 5) *M. vaccae* treatment had no effect on IgE or IgG2a production. It is noteworthy that the inhibitory effect of *M. vaccae* on the allergic eosinophilia was not observed when the bacterial extract was administered 1 day before the immunization or 4 h before the challenge, suggesting that the timing of exposure to heat-killed *M. vaccae* is crucial for the establishment of an immunomodulatory cascade that needs maturation over a period of several days before being able to induce a suppressive effect. This observation is therefore not compatible with rapid and high level IL-12 or IFN-γ production as seen with other bacterial infections, which subsequently inhibit the Th2-driven inflammatory response (6, 9–14). Moreover, most of the latter studies were performed with live bacteria (e.g., BCG), which may induce a different form

of immune response compared with heat-killed mycobacterial extracts and therefore could explain at least some of the differences observed in our model compared with other reported data (9). On the other hand, very recent studies comparing the immunoregulatory effects of live BCG and *M. vaccae* also demonstrated that although the overall responses were qualitatively similar, *M. vaccae* was much weaker in producing some of the key features associated with a Th1 response, such as IL-12 and IFN-γ production, suggesting differences in the ability to induce a Th1 response in response to different mycobacterial Ags (22).

Another often discussed possibility of how bacterial infection might interfere with Th2-driven inflammation is the exposure to bacterial DNA containing unmethylated CpG motifs. Indeed, oligonucleotides containing unmethylated CpG motifs have been found to be potent stimuli of IL-12 and IFN-γ production, leading to a strong inhibition of allergic airway inflammation (11). Multiple studies demonstrated that such CpG oligonucleotides inhibit allergic inflammatory responses when administered during the immunization period as well as shortly before the challenge, change the cytokine pattern into a predominantly Th1-like profile, and are potent IgE synthesis inhibitors (11, 23, 24). Although the preparation of *M. vaccae* used in our study most likely contains CpG DNA, it is very unlikely that this is a major mechanism of how this bacterial extract induces its inhibitory effect, since no Th1-inducing activity was found, and the treatment was not effective using treatment and Ag sensitization and challenge protocols similar to those described for testing CpG oligonucleotides.

Moreover, and in support of the failure of redirecting the Th2 to a Th1 immune response by *M. vaccae* are the data for serum IgE and IgG2a levels, which were not affected by the treatment. Similar conclusions were reached in a recent study in which intradermal administration of heat-killed *M. vaccae* induced a significant improvement in atopic dermatitis in children with moderate to severe disease (16). In this study the ability of *M. vaccae* to alter Th2 activity was also not convincing, as no effect on serum IgE or blood eosinophil numbers were found. Similar findings were reported by other investigators, demonstrating that a single injection of *M. vaccae* into OVA-preimmunized mice did not cause significant changes in serum IgE levels (25). In contrast, Wang and Rook (14) reported that *M. vaccae* injection to OVA-immunized mice significantly suppressed serum IgE (14). Therefore, it is likely that the discrepancy in effects on IgE levels observed with *Mycobacterium* treatment may be due to different protocols of immunization, which account for a difference in the kinetics of IgE production and turnover.

Besides its effect on the allergic inflammation and AHR in a short term immunization protocol, the present study also demonstrates that *M. vaccae* treatment confers long term and Ag-specific protection and therefore has both prophylactic and therapeutic potential for the treatment of allergic diseases. Of particular importance in this context are the data showing that *M. vaccae* treatment is still able to block eosinophilic inflammation in previously immunized mice (Fig. 7*B*) and that the protection against OVA did not confer protection against CEA-induced eosinophilic inflammation in mice treated 21 days before the first OVA immunization (Fig. 7*C*). One interpretation of these data could be that the treatment with *M. vaccae* induces the generation of specific regulatory cells that might be able to block the allergic response in an Ag-specific way for a long duration of time. The induction of such regulatory cells by *M. vaccae* would also explain why there is a need for a time gap between treatment with the bacteria and immunization with OVA. Supporting this hypothesis, we show here that splenocytes from *M. vaccae*-treated and OVA-immunized

mice are able to prevent airway eosinophilia in recipient immunized mice. This protective effect was not abrogated in recipient mice treated with an anti-IFN-γ Ab, reinforcing the concept that *M. vaccae* attenuates allergic inflammation by an IFN-γ-independent mechanism. The identification of the splenocyte population responsible for this protective effect is currently under investigation.

In conclusion, the ability of s.c. administration of *M. vaccae* to provide long term protection against allergic lung inflammation suggests that it may have therapeutic benefit in asthma and other allergic diseases. Indeed, preliminary clinical studies in adults with grass pollen-induced asthma and rhinitis found that treatment with *M. vaccae* was associated with fewer asthmatics symptoms and significantly less use of bronchodilator relief medication during the pollen season (13). Moreover, it has been demonstrated that intradermal administration of a killed *M. vaccae* suspension was associated with an improvement in the severity of the atopic dermatitis in children with moderate to severe disease (16). These studies clearly support our hypothesis that *M. vaccae* treatment may provide an alternative to the current practice of asthma therapy and also demonstrate that the inhibitory effects of *M. vaccae* noted in murine models of experimental allergic airway inflammation appear to translate well to human allergic diseases. However, the exact mechanism of how *M. vaccae* exerts its effect remains open and is the topic of future studies.

Acknowledgments

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