

Intragastric administration of *Mycobacterium vaccae* inhibits severe pulmonary allergic inflammation in a mouse model

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Summary

Background Coexistence with harmless microorganisms such as lactobacilli, saprophytic mycobacteria and some helminths, throughout evolution, may have shaped the host immune system. Exposure to such organisms may have therapeutic benefits by triggering immunoregulatory mechanisms that control inappropriate immune responses to self, gut contents or allergens.

Objective We determined whether treatment with *Mycobacterium vaccae* by gavage influences the host immune response both locally and systemically. We also investigated whether delivery by this route prevents severe symptoms of disease in a murine model of pulmonary allergic inflammation.

Results A single intragastric administration of *M. vaccae* induced a transient increase in the production of IL-10 and IFN- γ by mesenteric lymph nodes cells and splenocytes. In addition, in a mouse model of pulmonary allergic inflammation, a single treatment with *M. vaccae* by gavage not only diminished the total cellular infiltrate and the eosinophilic component induced by subsequent intratracheal allergen challenge, but also biased local and systemic cytokine production towards IL-10. Delivery of *M. vaccae* by gavage was as effective as subcutaneous treatment.

Conclusion This is the first report to suggest that heat-killed mycobacteria can down-regulate symptoms of allergic inflammation by the intragastric route. These data suggest an alternative route of treatment with *M. vaccae* for patients with allergic conditions.

Keywords gavage, hygiene hypothesis, immunoregulation, *Mycobacterium vaccae*, oral delivery, pulmonary inflammation

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Introduction

We previously suggested that common, harmless microorganisms present throughout human evolutionary history, drive immunoregulatory mechanisms that prevent inappropriate immune responses [1, 2]. These microorganisms have been called 'Old Friends' and include lactobacilli, routinely found in fermented food, saprophytic mycobacteria, abundantly present in mud and untreated water, and some helminths, which are commonly harboured without severe consequences [3]. In their presence the host may develop immunoregulation as it learns to distinguish between harmless organisms, to which an immune response is not necessary, and virulent pathogens, which warrant a response [2–4]. In their absence the host's failure to develop immunoregulatory mechanisms may lead to dysregulated immune responses to allergens, self-antigens or gut content. Indeed, the simultaneous increases in diseases of immunodysregulation such as allergies, autoimmune diseases and inflammatory bowel diseases in developed countries have been linked to decreased exposure to these organisms [1, 2]. The common denominator for these

conditions might be decreased activity of regulatory T cells (Tregs) [2, 4, 5]. Patients with mutations in the transcription factor Foxp3, who have defective Tregs, develop a severe inflammatory disorder, which combines autoimmunity, allergy and gastrointestinal inflammation [6, 7].

In the last few years, experimental evidence has accumulated to show that pathologies linked to immunodysregulation can be ameliorated by exposure to Old Friends. For example, experimental helminth infections prevent inflammation during colitis and reduce the severity of autoimmune diseases such as diabetes [8, 9]. Moreover, IL-10-producing Tregs can be induced *in vitro* by schistosomal lyso-phosphatidylserine, which stimulates dendritic cells (DCs) through toll-like receptor 2 (TLR2) [4, 10, 11]. Such experimental findings support clinical trials designed to determine whether exposure to the helminth *Trichuris suis* can provide therapeutic alternatives for patients with inflammatory bowel diseases [12, 13]. Treatment with lactobacilli might also be therapeutic. Clinical trials suggest a significant decrease in the incidence of atopic eczema in the first years of life following administration of lactobacilli during pregnancy, and to the neonates [14, 15]. Further evidence of the potential role of Old Friends in immunoregulation has been obtained from experimental work using *Mycobacterium vaccae*, a non-pathogenic saprophytic mycobacterium. In a murine model

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of allergic pulmonary inflammation, treatment with *M. vaccae* by the subcutaneous (s.c.) route helps to both prevent and treat allergic inflammation by significantly reducing airway eosinophilia [16–18]. *M. vaccae* promotes the development of IL-10-producing allergen-specific CD45RB^{low} Tregs, which suppress pulmonary inflammation [17]. In addition it induces a population of CD11c⁺ cells characterized by an increased expression of the regulatory cytokines IL-10 and transforming growth factor- β (TGF- β) [18]. In the light of these findings, the therapeutic potential of *M. vaccae* is being explored in a number of clinical trials. Preliminary results in atopic asthma using intradermal injections of *M. vaccae* have been encouraging [19].

Because during evolution the most likely route of exposure to *M. vaccae* was oral, we hypothesized that *M. vaccae* administered by the intragastric route would have significant therapeutic effects in a murine model of allergic pulmonary inflammation. Indeed, the intestinal epithelium is easily penetrated by mycobacteria whose size and hydrophobicity are ideal for uptake by M cells on Peyer's patches, leading to an interaction with the mucosal immune system [20, 21]. In this paper we report that a single administration of heat-killed *M. vaccae* by gavage provides strong antigenic stimulation for mesenteric lymph nodes (MLN) and influences the systemic immune response of naïve mice. In addition, administration by this route prevents the development of severe pulmonary inflammation in allergic mice. This beneficial effect is not associated with the development of a T-helper type 1 environment but rather with the preferential induction of the immunoregulatory cytokine IL-10.

Methods

Mice

Female BALB/c mice, 6–8 weeks old (Harlan, Abingdon, UK), were housed under standard conditions with food and water available *ad libitum*. Experimental protocols were ethically approved and complied with the Home Office 1986 Animals Scientific Act.

Mycobacterium vaccae

M. vaccae (NCTC 11659) was supplied by SR Pharma (London, UK), as a preparation containing 10 mg/mL of heat-killed *M. vaccae* suspended in physiological saline. The material (SRP 299) was manufactured under Good Manufacturing Practice guidelines and is currently being tested in phase II clinical trials.

In vitro stimulation of naïve mesenteric lymph nodes cells

MLN were obtained from naïve mice and passed through a 70 μ m cell sieve to obtain a single cell suspension. Cells were washed and resuspended in culture medium consisting of RPMI (Invitrogen, Paisley, UK), 10% fetal calf serum (Invitrogen), glutamine (Invitrogen), 50 U/mL penicillin and 50 μ g/mL streptomycin (Invitrogen). Cells (5×10^6 /mL) were stimulated in a 96-wells plate with PBS (Invitrogen), LPS (5 μ g/mL, Sigma, Dorset, UK) and *M. vaccae* (100, 200 and 400 μ g/mL). Cells were cultured at 37 °C in 5% CO₂ and

supernatants were collected 72 h later for cytokine analysis. Levels of IL-10, IL-12 and IFN- γ were measured using commercially available ELISA kits (lower detection limit 7.8 pg/mL, R&D Systems, Abingdon, UK).

In vivo stimulation of naïve mice

M. vaccae (0.1 mg/100 μ L of sterile water) was administered by gavage on day 0. On days 1, 4 and 7 after treatment mice were sacrificed by exposure to CO₂. MLN and spleens were aseptically removed and individually processed to yield single cell suspensions. Erythrocytes were removed from splenocytes by hypotonic lysis. Cells were washed and resuspended in culture medium. MLN cells (5×10^6 /mL) and splenocytes (10×10^6 /mL) were stimulated *in vitro* with PBS or *M. vaccae* (300 μ g/mL). Supernatants were collected 72 h later for cytokine analysis. Levels of IL-10, IL-12 and IFN- γ were measured using commercially available ELISA kits, as above.

Effects of intragastric Mycobacterium vaccae in a murine model of allergic pulmonary inflammation

Prevention protocol Mice were treated by gavage on day –21 with either *M. vaccae* (0.1 mg/100 μ L of sterile water) or with sterile water alone. On days 0 and 12, mice were rendered allergic by sensitization with 10 μ g of chicken ovalbumin (OVA) (Grade V, Sigma) in 100 μ L of alum gel (AMS, Abingdon, UK) by intraperitoneal (i.p.) injection. On days 19 and 21, mice were challenged intratracheally (i.t.) with 50 μ L of 10 μ g/mL OVA in sterile saline under light halothane anesthesia.

Treatment protocol Mice were rendered allergic by sensitization on days 0 and 12 with OVA in alum. On day 21 mice were treated with *M. vaccae* or sterile water. Two further injections of OVA in alum were administered on days 42 and 54. On days 61 and 63, mice were challenged i.t. as described above.

Where indicated mice were treated with a s.c. injection of *M. vaccae* (0.1 mg) as a positive control. In separate experiments the effects of different doses of *M. vaccae* (1.0, 0.1 and 0.01 mg) were also investigated. Mice were sacrificed 24 h after the second antigen challenge by i.p. injection of sodium pentobarbitone (240 mg/kg). The trachea was cannulated and the bronchoalveolar lavage (BAL) fluid collected by washing three times with 0.3 mL of RPMI supplemented with 50 U/mL penicillin and 50 μ g/mL streptomycin. The number of cells recovered was determined using a Neubauer chamber. Differential cell counts for each BAL sample were obtained from slide cytospins (Cytospin 3, Shandon Scientific, Cheshire, UK) stained with Wright–Giemsa (Sigma). Differential counts on 200 cells were performed using standard cytological criteria. The remaining BAL fluid was centrifuged and the supernatant stored at –20 °C for cytokine analysis. Spleens from each treatment groups were instead pooled and single cell suspensions prepared as described above. Cells (10×10^6 /mL) were cultured *in vitro* in a 96-wells plate and stimulated with PBS and OVA (50 μ g/mL). Supernatants were collected 72 h later for cytokine analysis. Levels of IL-5, IL-10 and IFN- γ were measured using commercially available ELISA kits.

Statistical analysis

Results are represented as mean $\pm$ SEM unless stated otherwise. Data were subjected to a normality test and one-way ANOVA or Student's *t* test were performed as appropriate. A *p* value of ≤ 0.05 was considered significant.

Results

In vitro stimulation of naive mesenteric lymph nodes cells

To determine whether *M. vaccae* induces mucosal immune activation, we investigated the response of naive MLN cells to *in vitro* stimulation. MLN were chosen because they drain the small intestine and are routinely exposed to a variety of antigens. Stimulation of MLN cells with increasing doses of *M. vaccae* resulted in dose-related increased levels of IL-10 production (Fig. 1), but not of IL-12 and IFN- γ , which remained below detection. In contrast, IL-12 was detected following activation by bacterial LPS (137 ± 9 pg/mL, data representative of three separate experiments). We conclude that *in vitro* stimulation with *M. vaccae* preferentially induces IL-10 from cells involved with mucosal immunity.

In vivo stimulation of mesenteric lymph nodes cells and splenocytes

In view of the susceptibility of MLN cells to stimulation with *M. vaccae* *in vitro*, we investigated whether delivery of *M. vaccae* by gavage would influence the local and systemic immune environment *in vivo*. We measured cytokine levels in the supernatants of cultures of MLN cells and splenocytes from mice sacrificed on days 1, 4 and 7 following treatment. Cytokine levels were compared with those of naive mice (day 0). MLN cells, restimulated *in vitro* with *M. vaccae*, released increased levels of IL-10 (Table 1). In contrast, levels of IL-12 (data not shown) and of IFN- γ remained below detection. Cytokine release by systemic

Table 1. Cytokine release by MLN cells and splenocytes from mice that received *Mycobacterium vaccae* by gavage.

	IL-10 (pg/mL)	IFN- γ (pg/mL)
<i>MLN cells</i>		
Day 0	12 $\pm$ 0	b.d.
Day 1	43 $\pm$ 22	b.d.
Day 4	20 $\pm$ 9.6	b.d.
Day 7	24 $\pm$ 6	b.d.
<i>Splenocytes</i>		
Day 0	474 $\pm$ 63	b.d.
Day 1	492 $\pm$ 102	b.d.
Day 4	1300 $\pm$ 180*	223 $\pm$ 114*
Day 7	533 $\pm$ 256	b.d.

Cells were restimulated *in vitro* with *M. vaccae*. These results are representative of two experiments.

*Denotes *p* < 0.05 compared with day 0.

MLN, mesenteric lymph nodes; b.d., below detection.

lymphoid organs such as the spleen was also affected by oral delivery (Table 1). Compared with levels in the supernatants of splenocytes from control mice, on day 4 after oral treatment, both IL-10 and IFN- γ increased upon restimulation with *M. vaccae*. In contrast, IL-12 levels remained low and failed to show any significant changes (data not shown). These data suggest that intragastric administration of *M. vaccae* transiently alters the cytokine environment *in vivo*.

Therapeutic effects of oral delivery of Mycobacterium vaccae on pulmonary allergic inflammation

Allergic pulmonary inflammation is associated with a large cellular infiltrate into the lungs. Compared with sensitized mice challenged with saline i.t., challenge with OVA induces a dramatic influx of cells ($0.87 \pm 0.2 \times 10^5$ /mL vs. $3.42 \pm 0.5 \times 10^5$ /mL, respectively, data representative of at least five separate experiments).

We measured the cellular infiltrate into the lungs of sensitized mice treated with either *M. vaccae* or sterile saline by gavage and challenged with OVA (Fig. 2a). Compared with animals treated with sterile water, mice receiving *M. vaccae* showed a significant reduction in the total cellular infiltrate following i.t. challenge (Fig. 2b). This decrease was similar to that observed following s.c. treatment with *M. vaccae* (Fig. 2b), suggesting that the two routes of administration are both effective.

We determined whether the beneficial effect of an oral preparation of *M. vaccae* was dose-dependent. Whilst treatment with 0.01 mg of *M. vaccae* showed no effect, treatment with 0.1 and 1.0 mg significantly decreased the total cellular infiltrate, albeit to the same extent (Fig. 2c). We conclude that the mucosal immune system is very sensitive to the delivery of *M. vaccae*.

Mice that had received 0.1 and 1.0 mg of *M. vaccae* showed a significant reduction in the number of eosinophils recovered in the BAL fluid (Fig. 2d). No increase in other inflammatory cell populations was observed. In fact, both macrophages (*P* < 0.05), and to a lesser extent neutrophils, were reduced (Fig. 2d). These results suggest that treatment with *M. vaccae*

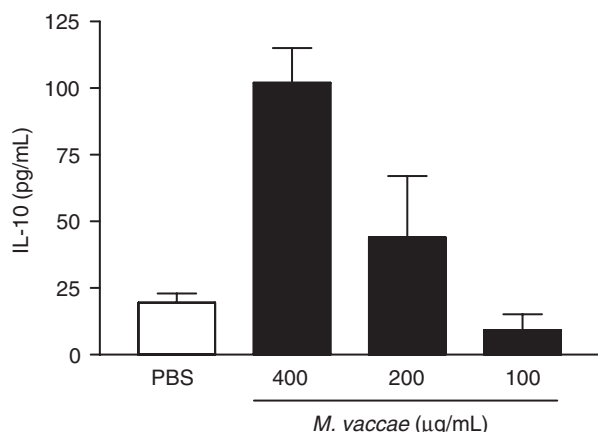


Fig. 1. *In vitro* IL-10 release by mesenteric lymph nodes (MLN) cells from naive BALB/c mice. Compared with stimulation with PBS (white bar), MLN cells released increased levels of IL-10 following *in vitro* stimulation with *Mycobacterium vaccae* (black bars). Data are expressed as mean $\pm$ SD of pooled MLN cells from 3–4 mice. These results are representative of two separate experiments.

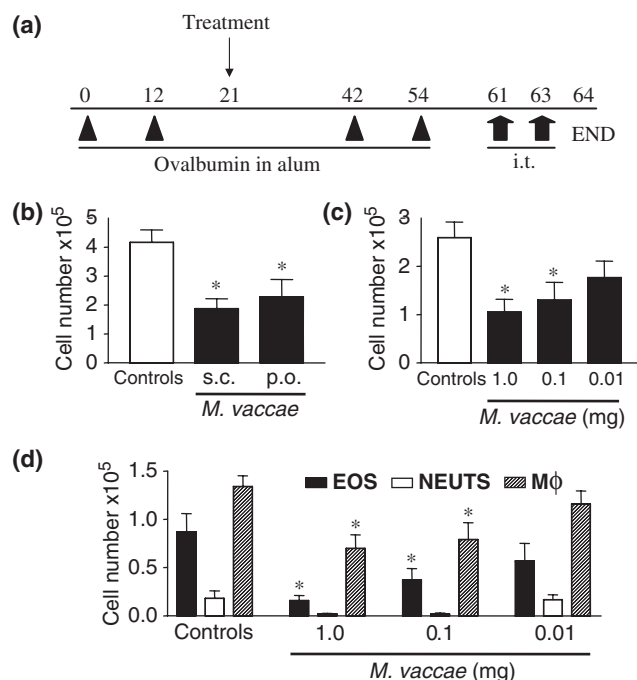


Fig. 2. Treatment with oral *Mycobacterium vaccae* reduces the severity of pulmonary allergic inflammation. (a) A treatment protocol, as described in the relevant Methods section, was used to determine the extent of pulmonary inflammation in sensitized mice treated with sterile water or *M. vaccae* and challenged with ovalbumin. (b) Compared with treatment with water (controls, white bar), treatment by gavage or s.c. injection with 0.1 mg of *M. vaccae* (black bars) significantly reduced the total number of cells recovered in the bronchoalveolar lavage (BAL) fluid. (c) Compared with control mice (white bar), treatment with 1.0, 0.1 but not 0.01 mg of *M. vaccae* by gavage significantly reduced the total number of cells recovered in the BAL fluid. (d) Compared with control mice, treatment with 1.0 and 0.1 mg, but not 0.01 mg of *M. vaccae* by gavage significantly reduced the number of eosinophils (black bars) and macrophages (shaded bars) recovered in the BAL fluid. Data are expressed as mean $\pm$ SEM of 6–8 mice per group. These results are representative of three separate experiments. Similar results were obtained using the prevention protocol. The asterisk denotes $P < 0.05$ when compared with treatment with water.

by gavage significantly reduces the severity of cellular inflammation following allergen challenge in sensitized mice.

To determine whether *M. vaccae* delivered by this route alters the cytokine profile both at the site of inflammation and systemically, IL-5, IL-10 and IFN- γ levels were measured in the BAL fluid and in the supernatant of splenocytes from sensitized mice. IL-10 was significantly elevated in the BAL fluid of sensitized animals treated with *M. vaccae* (40 ± 4.2 pg/mL) compared with levels in the BAL fluid of control mice (22 ± 4.8 pg/mL, data representative of three separate experiments). As previously reported by Zuany-Amorim et al. [16, 17], no differences in IL-5 levels were found in the BAL fluid following treatment with *M. vaccae* (106 ± 18.5 pg/mL) compared with levels in the BAL fluid of control mice (125 ± 18 pg/mL, data representative of three separate experiments). IFN- γ levels in BAL fluids were below detection.

It is intriguing to note that whereas in the BAL fluid, treatment with *M. vaccae* induced a significant elevation of IL-10 but no significant alteration in IL-5, in the supernatant of splenocytes the opposite was observed. Splenocytes from sensitized mice treated with *M. vaccae* released significantly

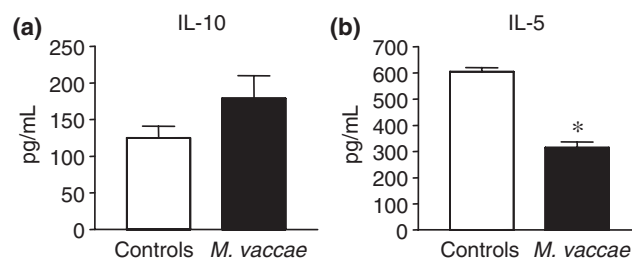


Fig. 3. Treatment with *Mycobacterium vaccae* alters the cytokine profile of splenocytes of sensitized mice. In a treatment protocol, levels of (a) IL-10 and (b) IL-5 were measured in the supernatant of splenocytes from sensitized mice treated with sterile water (white bar) and *M. vaccae* (black bar) and restimulated *in vitro* with ovalbumin. Data are expressed as mean $\pm$ SD of pooled spleens from 6–8 mice. The asterisk denotes $P < 0.05$ when compared with treatment with water. These results are representative of three separate experiments.

less IL-5 compared with splenocytes from control animals (Fig. 3). In contrast, the elevation in IL-10 levels following treatment with *M. vaccae* was consistently observed but failed to reach significance (Fig. 3). We detected no significant differences in IFN- γ levels regardless of whether mice were treated with sterile water (59 ± 5 pg/mL) or *M. vaccae* (36 ± 10 pg/mL, data representative of three separate experiments).

Discussion

In this study we evaluated the effects on the host immune system of treatment with *M. vaccae* by gavage. We report that a single administration of *M. vaccae* altered local and systemic immune responses, as measured by a transient increase in IL-10 and IFN- γ by MLN cells and splenocytes. In addition, in a mouse model of pulmonary allergic inflammation, *M. vaccae* was equally effective when delivered by gavage or by s.c. injection in reducing the severity of allergic inflammation. The decrease in type-2 responses such as pulmonary eosinophilia following treatment was not due to increased type-1 inflammation because both neutrophils and macrophages in the BAL fluid were also reduced. The cytokine environment showed a bias towards IL-10, an immunoregulatory cytokine previously shown to mediate the effects of subcutaneously delivered *M. vaccae* [17]. The s.c. treatment with *M. vaccae* has been shown to reduce symptoms of allergic pulmonary inflammation through the induction of immunoregulatory mechanisms such as the development of IL-10 secreting CD4⁺CD45RB^{low} Tregs [17]. This is the first report that administration of heat-killed mycobacteria by gavage also down-regulates symptoms of allergic inflammation and induces IL-10 release, suggesting an alternative route of treatment with *M. vaccae* for patients with allergic diseases.

The efficacy of oral administration of mycobacteria has been documented in a number of experimental models. Oral vaccination with *M. bovis* bacille Calmette–Guerin (BCG) has been shown to protect against virulent *M. tuberculosis* [20, 22]. Despite these findings, this route of vaccination was abandoned because of the side effects caused by the colonization of the oro-pharyngeal epithelium. In some cases,

oral vaccination with BCG caused cervical adenitis, otitis and, rarely, pharyngeal abscesses [20]. These concerns do not, however, affect oral treatment with *M. vaccae*, which is a heat-killed preparation. In our study we used intragastric treatment to ensure the delivery of known amounts of *M. vaccae*. Experimental evidence using BCG suggests that in the mouse, oral and intragastric administration are comparable and give similar levels of protection against *M. tuberculosis* [22]. However, treatment by gavage bypasses the oro-pharyngeal mucosal system, and it remains to be determined whether the effect reported here could be increased further by stimulation of oral mucosal immunity. Indeed, this might be the case in human patients where the oro-pharyngeal mucosal system is important. The efficacy of oral delivery is facilitated by the rapid uptake of mycobacteria into Peyer's patches due to their size, charge and hydrophobicity [20–22]. We anticipate that exposure of the gut mucosa to *M. vaccae* may further facilitate the induction of Tregs, as this site has long been considered an ideal environment for the development and maintenance of Tregs [23, 24]. This is an important consideration as some of the beneficial effects of treatment with *M. vaccae* are mediated by the induction of Tregs.

There is strong evidence that exposure to certain organisms plays a role in the induction of Tregs, supporting the hypothesis that specific microorganisms such as the Old Friends contribute to immunoregulatory processes [10, 11, 17, 25]. The underlying mechanisms of action remain to be determined, but DCs are likely to be involved [18, 26–28]. Pathogen-associated molecular patterns (PAMPs) on *M. vaccae*, some probiotic lactobacillus strains and certain bacteria of the gut flora, for example, may interact with TLRs or other pattern recognition receptors (PRRs) on the host's DCs. This interaction may influence DC maturation and induce preferential production of specific cytokines [2, 3, 27, 28]. The resulting DC phenotype will determine whether immunity or regulation develops by specifically polarizing the T cell response. *M. vaccae* administered s.c. has been shown to influence cytokine production by DCs infiltrating the lung. Pulmonary CD11c⁺ cells from OVA-sensitized mice treated with *M. vaccae* were characterized by increased expression of the regulatory cytokines IL-10 and TGF- β [18].

Oral intake of saprophytic mycobacteria, once a constant feature of our evolutionary history, has decreased in developed countries. This may be one factor explaining the rise in diseases of immunodysregulation reported in the last few decades. We report that administration of *M. vaccae* by gavage was as effective as s.c. injection in significantly reducing the severity of pulmonary allergic inflammation in a mouse model. These observations raise exciting clinical possibilities. In particular, the ease of administration and the lack of scarring suggest that in patients, the oral route may be a preferred alternative to intradermal injection of *M. vaccae*.

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