

Analysis of recombinant mycobacteria as T helper type 1 vaccines in an allergy challenge model

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SUMMARY

The potential for development of mycobacteria as T helper type 1 (Th1) vaccines capable of induction of Th1 responses to recombinant antigens was explored in a model system based on an immunodominant peptide from house dust mite. Different recombinant mycobacterial preparations were compared for their ability to induce a Th1 response to the peptide. It was found that mycobacterial viability was not a prerequisite for Th1 immunogenicity. A dominant interferon- γ (IFN- γ) response to peptide was observed in splenocytes from C57BL/6J mice immunized with live or heat-killed preparations of recombinant *Mycobacterium vaccae* or with live attenuated bacillus Calmette–Guérin (BCG) vaccine expressing the antigen. Interleukin-5 (IL-5), a marker of a Th2 response, was detected only in mice receiving live *M. vaccae*. A similar pattern was observed in BALB/b mice, although the magnitude of the IFN- γ response was much lower. Control and immunized mice were subsequently exposed to allergen using a Th2-inducing challenge protocol. A significant shift from a Th2 to a Th1 response was observed in immunized mice, as judged by cytokine expression by splenocytes and by subclass of circulating antibody. The effect was seen in three inbred mouse strains differing in their innate bias towards Th1 or Th2 responses. It was dependent on the presence of specific antigen in the mycobacterial preparation and, under the immunization conditions tested, was more pronounced with dead *M. vaccae* than with live BCG as carrier vaccine. The results demonstrate the potency of killed mycobacteria as Th1 adjuvants and suggest a potential application for recombinant mycobacteria in antigen-specific immune modulation.

INTRODUCTION

The majority of vaccines in routine clinical use promote induction of antibody-mediated immunity to toxins and to capsular proteins of viral and bacterial pathogens. Effective immunization requires the induction of a humoral response, in which T helper type 2 (Th2) lymphocytes promote maturation of antibody-secreting B cells. Classical strategies for vaccine development have proved less effective for the broad range of diseases in which macrophage activation mediated by a Th1 response is required for protection. These include intracellular microbial infections, such as tuberculosis and leishmaniasis. In addition, allergic disorders which are characterized by

a pathological Th2 response, may benefit from interventions designed to promote Th1 activation.¹ To extend the range of diseases that can be prevented by vaccination, there is a need to identify safe and effective procedures for induction of Th1 responses.

The bacillus Calmette–Guérin (BCG) vaccine – an isolate of *Mycobacterium bovis* attenuated by laboratory passage in the early 1900s – is the most widely used Th1-inducing vaccine.² It has a long record of safe use in man and is a potent activator of a Th1 response as detected by delayed-type hypersensitivity or by interferon- γ (IFN- γ) production by peripheral blood lymphocytes.^{3–6} Although BCG has proved variable in its ability to protect against its primary target disease of pulmonary tuberculosis,^{2,7} it has demonstrated consistent efficacy against childhood forms of tuberculosis and against the related mycobacterial infection of leprosy.⁸ With the recent development of genetic manipulation of mycobacteria, many researchers have investigated the possibility of harnessing the Th1-inducing properties of BCG for delivery of heterologous recombinant antigens.^{9–11} Experimental analysis of these

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recombinant BCG vaccines in animal models confirms this as a promising approach to the generation of novel Th1 vaccines.¹² The strong Th1-inducing properties of mycobacteria were further illustrated in a study by Erb *et al.*, which showed that BCG infection in the lungs of mice could prevent the subsequent induction of an allergic response at this specific site.¹³ A study in Japanese schoolchildren indicated that exposure to *M. tuberculosis* and development of allergy were inversely correlated.¹⁴ However, whether BCG vaccination in humans can also prevent the development of allergy later in life is still unclear.^{15,16} This may suggest that the Th1-inducing properties of mycobacteria *per se* are not enough to modulate allergic responses. To explain this apparent discrepancy we reasoned that simultaneous exposure to mycobacteria and allergen may be necessary to induce an allergen-specific Th1 response strong enough to prevent the induction of allergy.

In order to study this possibility we have taken advantage of recently developed recombinant expression systems to engineer mycobacteria that express an immunodominant T- and B-cell epitope of *Der p* I of house dust mites.^{17,18} To test the contribution of the mycobacterial carrier in this model, the epitope was expressed in BCG and in *Mycobacterium vaccae*, two strains of mycobacteria with different biological properties. *Mycobacterium vaccae* is a rapid-growing soil organism that has been used as a therapeutic vaccine in a series of clinical trials in tuberculosis and other diseases.^{19,20} In common with BCG, *M. vaccae* is safe for human use and is a potent inducer of Th1 responses.²¹ In contrast to BCG, it is delivered as a heat-killed vaccine. First, we wanted to study the Th1-inducing properties of these mycobacteria in mouse strains of distinct genetic background which are known to be either strong Th1 responders or strong Th2 responders. Secondly, we wanted to evaluate whether the presence of an allergen-specific Th1 response could prevent the subsequent induction of a Th2 response in these mouse strains.

MATERIALS AND METHODS

Bacterial cultures and plasmids

Mycobacterium vaccae NCTC 11659 (supplied by Dr John Stanford, Royal Free and University College London Medical School) was grown in Middlebrook 7H9 medium supplemented with 0.5% glucose. The *M. bovis* BCG (strain P3) was grown in Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase (ADC) supplement as recommended by the manufacturer (Difco, West Molesey, UK), or on Middlebrook 7H11 agar plates with oleic acid-albumin-dextrose-catalase (OADC) supplement. When appropriate, hygromycin B (Sigma, Poole, UK) was added at 50 µg/ml.²² Transformation of mycobacteria was carried out by electroporation.²² Plasmid p23.1 encodes the wild-type superoxide dismutase (SOD) of *M. tuberculosis* and carries a *Bam*HI site in the position of the *sodA* gene, corresponding to amino acids 51–53.²³ Plasmid SOD-P1 contains a linker insertion in the *Bam*HI site of p23.1 encoding for peptide 111–139 of *Der p* I of house dust mites.²³ Recombinant mycobacteria expressing chimeric SOD:*Der p* I^{111–139} are denoted *M. vaccae*-pI and BCG-pI. Control strains expressed native SOD protein and are denoted *M. vaccae*-SOD and BCG-SOD. For immunization purposes, *M. vaccae* and BCG were grown on plates and in liquid culture, respectively.

Analysis of expression of chimeric SOD proteins

For analysis of protein expression, 10–20 µg of sonicated extracts of recombinant *M. vaccae* or BCG were analysed by Western blotting.²⁴ Blots were developed with a monoclonal antibody (D2D) against mycobacterial SOD²⁵ and visualized by chemoluminescence (Amersham, UK).

Preparation of antigens

Suspensions of recombinant mycobacteria (10^9 – 10^{11} colony forming units/ml) were frozen at -70° in 15% glycerol. For immunization, the glycerol was washed away, the bacteria were heat-killed (20 min, 80°) where necessary, and the preparations were resuspended at the appropriate concentration in phosphate-buffered saline (PBS). Soluble native *M. vaccae* antigens for *in vitro* stimulation and enzyme-linked immunosorbent assay (ELISA) were prepared by sonicating the bacterial suspensions in PBS and filtering them using a 0.22-µm filter. Peptide p111–139 of *Der p* I (sequence: FGISNYCQIYPP-NANKIREALAQTSAIA²⁶) was made on an ABIMED 422 synthesizer (ABIMED, Germany) using the simultaneous peptide synthesis method. The purity of the peptide was verified by reverse-phase C18 high-performance liquid chromatography (Lichrospher, Merck, Germany) and was shown to be routinely over 75%.

Animals and immunization protocols

Female C57BL/6J and 129Sv mice (6–8-week-old) from Broekman Institute (Someren, the Netherlands) or from Harlan-Olac (Bicester, UK) were used in the study. Female BALB/b mice were purchased from the Animal Resources Centre (Perth, Australia). Groups of four to six mice, were immunized subcutaneously (s.c.), twice with a 3-week interval in the left flank with live or heat-killed recombinant *M. vaccae* (10^8 – 10^9) or once with live recombinant BCG (10^7). Ten days after the second *M. vaccae* immunization, or 4 weeks after BCG immunization, mice were immunized s.c. with 2 µg peptide 111–139 in incomplete Freund's adjuvant (IFA) in the right flank.

Cell culture

Single cell suspensions of splenocytes or lymph node cells were cultured in 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark) at 2×10^6 cells/ml in 200 µl/well RPMI-1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, 20 IU/ml penicillin, 20 µg/ml streptomycin (Gibco, Grand Island, NY) and 50 µM 2-mercaptoethanol (Sigma, St Louis, MO) at 37° in humidified air containing 5% CO₂. Cells were incubated in triplicate wells alone, or with varying concentrations of antigens, for the assessment of proliferation or the level of cytokines in supernatants at 72 hr (previously determined as the optimum time-point). Proliferation was measured by pulsing for the last 16 hr with 0.6 µCi/well [³H]thymidine ([³H]TdR; Amersham, UK), harvesting the contents of each well onto glass-fibre mats and determining the incorporation of [³H]TdR using a β-plate counter (Canberra Packard, Meriden, CT).

Cytokine assays

IFN-γ was measured by ELISA using the rat anti-mouse coating antibody R4-6A2 and biotinylated detector antibody XMGI.2 pair (Pharmingen, San Diego, CA). Interleukin-4

(IL-4) and IL-5 were also measured by ELISA using coating monoclonal antibodies 11B11 or TRFK-5 and biotinylated detector monoclonal antibodies BvD6-24G2 or TRFK-4 (Pharmingen). The binding of biotinylated antibody was detected with alkaline phosphatase-conjugated streptavidin (Amersham) followed by *p*-nitrophenylphosphate (Sigma, UK) at 1 mg/ml in Tris-HCl buffer, pH 9.6 as substrate. Optical density at 405 nm (OD_{405}) of the product was measured using a Biorad ELISA reader. Recombinant murine IFN- γ , IL-4 and IL-5 (Pharmingen) were used to construct a standard curve.

ELISA for specific antibodies

Der p I peptide p111–139 (5 μ g/ml) was coated onto Maxisorp microtitre plates (Nunc) in bicarbonate coating buffer (Sigma, UK) overnight at 4°. After blocking (1 hr, PBS with 1% bovine serum albumin) and washing, serum dilutions were incubated in duplicate for 2 hr at 37°. After washing, the amount of specific immunoglobulin G (IgG) isotypes bound was detected using alkaline phosphatase-conjugated rat anti-mouse IgG1 and IgG2a (Pharmingen) for 1 hr at 37°. The enzyme substrate *p*-nitrophenylphosphate was added and the soluble product was measured as for the cytokine ELISAs. To determine the ratio between IgG1 and IgG2a, antibody titres were determined as $-\log_{10}$ of the highest serum dilution that resulted in an extinction value which was higher than OD 0.1. Extinction values of preimmune serum were generally between OD 0.03 and 0.05. To measure IgE levels, rat anti-mouse IgE antibody (R35-72, Pharmingen) was coated onto Maxisorp microtitre plates in bicarbonate coating buffer overnight at 4°. After blocking (1 hr, PBS with 5% skimmed milk) and washing, serum dilutions were incubated for 2 hr at 37°. The amount of IgE was detected using biotinylated rat anti-mouse IgE (R35-92, Pharmingen) followed by alkaline phosphatase-conjugated streptavidin and *p*-nitrophenylphosphate as described for the cytokine ELISAs. To generate a standard curve purified IgE was used (Pharmingen).

Statistics

Statistical analysis was performed using Student's *t*-tests and a *P*-value <0.05 was considered significant.

RESULTS

Preparation of recombinant mycobacteria

We elected to study immune responses to an immunodominant peptide (amino acid residues 111–139) from the *Der p* I protein, a major allergen from house dust mites. This peptide is capable of inducing strong immune responses in mice carrying the H-2^b haplotype.^{17,18} It includes overlapping epitopes presented by major histocompatibility complex (MHC) class I and class II molecules (residues 111–119, and 113–127, respectively), allowing recognition by CD4⁺ as well as CD8⁺ T cells.^{27,28} In addition the peptide is recognized by B cells of C57BL/6J mice. We took advantage of a recently developed epitope delivery system to express the house dust mite peptide in recombinant mycobacterial vaccines.²³ This involved insertion of the peptide within a permissive loop of the *M. tuberculosis* SOD enzyme, and allowed a high level expression of the recombinant antigen. The recombinant SOD protein is visible as a band with a slightly lower mobility than the endogenous SOD protein

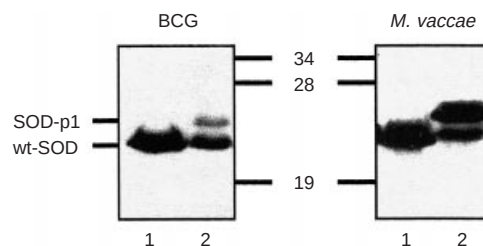


Figure 1. Expression of chimeric SOD proteins. Western blot analysis of sonicated extracts from BCG or *M. vaccae*-expressing wild-type SOD (lane 1) or SOD containing peptide 111–139 of *Der p* I (lane 2). Blots were stained with a monoclonal antibody to SOD. In addition to the recombinant SOD, both BCG and *M. vaccae* also express endogenous SOD which is visible as a 23 000 MW band.

which is also present in BCG and *M. vaccae* (Fig. 1). The level of expression of the chimeric SOD in the recombinant *M. vaccae*-pI construct was approximately five-fold higher than that observed in BCG-pI.

Immunogenicity of recombinant mycobacteria

The Th1-inducing properties of the recombinant mycobacterial vaccines were initially evaluated in C57BL/6J mice by subcutaneous immunization without adjuvant. For *M. vaccae* vaccines, mice were immunized at day 0 and day 21 with doses ranging from 10^7 to 10^9 mycobacteria. We compared the response to *M. vaccae* delivered as viable bacteria, to that of the same preparation killed by heating prior to immunization. Spleens were harvested and sera were collected 10 days after the second immunization. For BCG vaccines, mice were given a single immunization with 10^7 live organisms and spleens were harvested after 4 weeks. The difference in immunization protocol was chosen to reflect the difference in the biological properties of the two vaccines; BCG being able to replicate within the host.

Splenocytes were cultured in the presence of peptide 111–139 of *Der p* I or with a sonicated extract of *M. vaccae*, and IFN- γ production and IL-5 production in supernatants were measured as indicators of Th1 and Th2 responses, respectively. The results of immunization of C57BL/6J mice are shown in Fig. 2. Immunization with increasing doses of heat-killed or live *M. vaccae*-pI (10^8 – 10^9 bacteria) resulted in a progressive increase in the production of IFN- γ in response to the *Der p* I peptide (Fig. 2a). Splenocytes from control mice immunized with *M. vaccae* expressing the wild-type SOD produced no detectable IFN- γ under these conditions (Fig. 2a). Effective vaccine delivery was confirmed by detection of a similar strong IFN- γ response from all of the mice when *M. vaccae* was added as antigen to splenocyte cultures; approximately 20 ng/ml of IFN- γ was detected following immunization with *M. vaccae*-pI or *M. vaccae*-SOD. There was no significant difference between the heat-killed and live *M. vaccae* in terms of IFN- γ response. A difference was observed in the IL-5 response, however (Fig. 2b). Surprisingly, splenocytes from mice immunized with live but not heat-killed *M. vaccae*-pI produced IL-5 in response to the *Der p* I peptide, indicating that the response to live *M. vaccae* is not completely polarized towards Th1. Immunization with 10^7 recombinant BCG-pI also resulted in induction of a peptide-specific IFN- γ response in splenocytes (Fig. 2a),

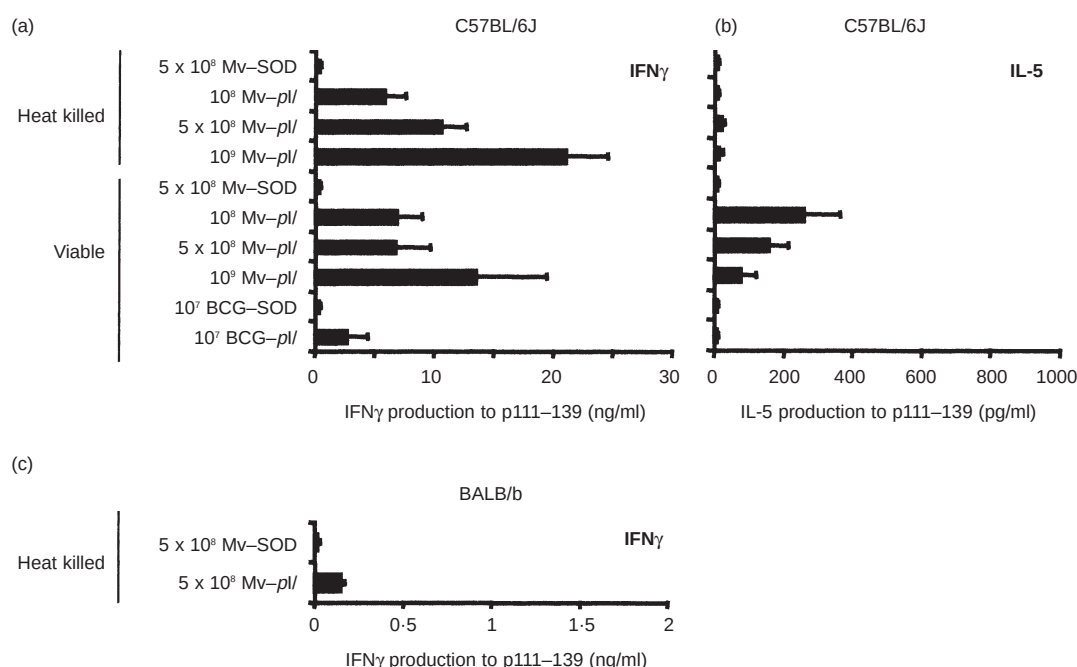


Figure 2. Immunogenicity of recombinant mycobacteria. C57BL/6J mice (a,b) or BALB/b mice (c) were immunized twice with a 3-week interval with viable or heat-killed *M. vaccae* expressing SOD containing peptide 111–139 of *Der p I* (*M. vaccae*-pI) or once with viable BCG-expressing control SOD (BCG-SOD) or SOD-pI (BCG-pI). Spleen cells were isolated 10 days after the second *M. vaccae* immunization or 4 weeks after BCG immunization and stimulated with 2 μ M of peptide 111–139. IFN- γ (a,c) and IL-5 (b) were determined in the supernatants after 72 hr of culture. Data of a representative experiment are expressed as the mean cytokine production of a group ($n=4$) $\pm$ SE.

although the response was lower than that observed in mice immunized with 10^8 – 10^9 *M. vaccae*. Animals immunized with BCG expressing the wild-type SOD control did not produce any IFN- γ in response to the *Der p I* peptide. No IL-5 was detected following vaccination with live BCG.

Antibody responses to the *Der p I* peptide were detectable in mice receiving the highest doses of the *M. vaccae*-pI vaccine. Antibodies were of the IgG2a isotype indicative of a Th1 response; no IgG1 was detectable. IgG2a production was similar in mice immunized with the live or heat-killed vaccine (end-point titres 1.36 ± 0.13 and 1.63 ± 0.13 , respectively). No antibodies were detected in mice receiving recombinant BCG (data not shown).

Comparison of inbred mouse strains

The data in C57BL/6J mice were consistent with our assumption that mycobacteria are potent Th1 inducers. Since C57BL/6J mice are known to be very strong IFN- γ producers, we decided to study the response raised against mycobacterial vaccines in BALB/b mice as an example of an H-2^b strain characterized by a relatively low level of IFN- γ production. Mice were immunized twice over a 21-day interval with 5×10^8 heat-killed *M. vaccae* and, 10 days after the boost, spleen cell responses were evaluated. Both the control group and the group receiving *M. vaccae*-pI produced high levels of IFN- γ to *M. vaccae* sonicate. The response, around 5 ng/ml, was approximately four-fold lower than that observed in C57BL/6J mice. In contrast to results in the C57BL/6J mice, only a low level of peptide-specific IFN- γ production could be detected in

the *M. vaccae*-pI group (Fig. 2c). Small proliferative responses to the peptide could also be detected in this group [stimulation index (SI) of 2.2 ± 0.6 for the *M. vaccae*-pI immunized mice as compared to 1.1 ± 0.14 for *M. vaccae*-SOD immunized mice] indicating that T cells could indeed be primed with the mycobacterial vaccine. No IL-5 production was detectable. These data indicate that although mycobacteria are potent Th1 inducers, the magnitude of this response is highly dependent on the genetic background of the mice.

Modulation of the T-cell response to allergen challenge

We next evaluated the ability of the recombinant mycobacterial vaccines to modulate the immune response to challenge with allergen delivered using a Th2-inducing protocol. The test system entailed a single s.c. injection of 2 μ g of *Der p I*^{111–139} in IFA. Splenocytes were removed 2 weeks later and cultured with peptide *in vitro*. The PBS control (white bars in Fig. 3a,b) compares the effect of the challenge protocol in C57BL/6J and BALB/b mice. In each case, a Th2-dominant profile was observed, characterized by release of relatively large amounts of IL-5 and low amounts of IFN- γ . As expected, there was a significant difference in the response of the strains, with the BALB/b splenocytes producing much less IFN- γ than the corresponding cells from C57BL/6J mice in response to *in vitro* stimulation with the peptide. Furthermore, there was a higher level of IL-5 production by the BALB/b splenocytes, resulting in a marked difference in Th2:Th1 ratio between the strains (0.21 and 7.1 for C57BL/6J and BALB/b mice, respectively).

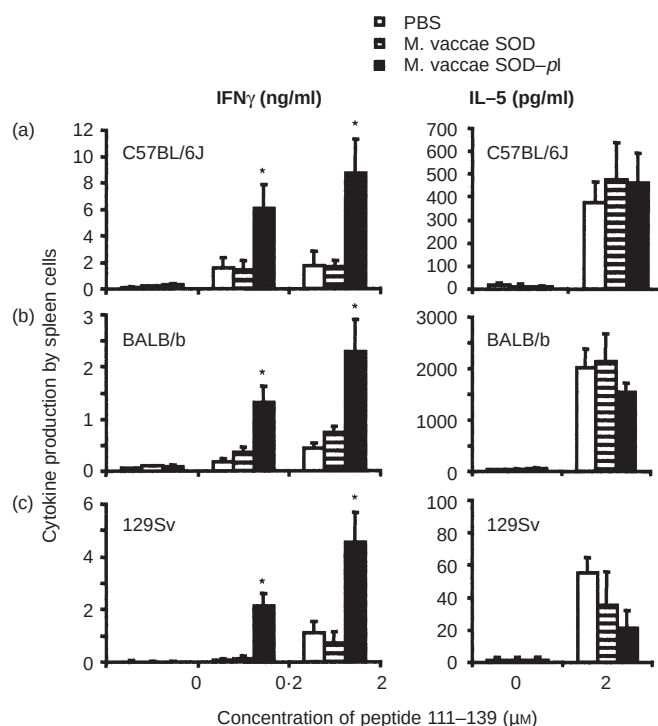


Figure 3. Vaccination with recombinant *M. vaccae*. C57BL/6J mice (a), BALB/b mice (b) or 129Sv mice (c) were vaccinated s.c. twice with a 3-week interval with PBS, 10^8 heat-killed *M. vaccae* expressing the wild-type SOD, or with 10^8 heat-killed *M. vaccae* expressing SOD-pI. Ten days later mice were challenged s.c. with 2 μg of peptide 111–139 in IFA and 2 weeks later spleen cells were harvested and cultured in the presence of increasing amounts of peptide. IFN-γ production (left panel) and IL-5 production (right panel) in supernatants collected at 72 hr were measured. Data are expressed as the mean cytokine production of individual mice ($n=4-6$) of a representative experiment $\pm$ SE. For C57BL/6J mice data are expressed as the mean cytokine production of individual mice ($n=8$) of two independent experiments $\pm$ SE. * $P < 0.05$ as compared to the PBS group and the *M. vaccae*-SOD group.

The same challenge protocol was then used in mice that had been immunized twice with mycobacterial vaccine several weeks earlier. Based on the immunogenicity results described above, we chose to focus on immunization with two doses of 10^8 heat-killed *M. vaccae* vaccine. Control mice were injected with PBS, while test mice received either *M. vaccae* expressing wild-type SOD or the *M. vaccae*-pI vaccine. Splenocyte responses from a representative experiment are shown in Fig. 3. In C57BL/6J mice, prior vaccination with *M. vaccae*-pI resulted in approximately four-fold increase in the IFN-γ response to subsequent *Der p* I challenge, whereas IFN-γ responses of splenocytes from mice receiving the *M. vaccae*-SOD construct were indistinguishable from PBS controls (Fig. 3a). IL-5 production was not affected by the vaccination procedure.

A similar pattern was seen in BALB/b mice. Even though primary responses to *M. vaccae*-pI were significantly lower than in C57BL/6J, the response to subsequent allergen challenge with peptide was characterized by a six-fold increase in IFN-γ production (Fig. 3b). Again, the IL-5 response was unaltered, resulting in a decreased Th2:Th1 ratio. A third strain of H-2^b mice – 129Sv – was also tested. This strain is resistant to *Leishmania major* infection,²⁹ indicative of a Th1 response, although it is susceptible to the induction of lung eosinophilia using a respiratory syncytial virus infection model.³⁰ In our vaccination and allergy challenge model

129Sv mice also responded with enhanced IFN-γ production following allergen challenge in *M. vaccae*-pI vaccinated mice (Fig. 3c) although the response to peptide 111–139 immunization was much less vigorous than that observed in C57BL/6J mice (PBS group Fig. 3c). IL-5 production was also very low in 129Sv mice but, as in the other two strains, there was no significant difference in IL-5 response to peptide in the vaccinated animals. No IL-4 production was detected in C57BL/6J or 129Sv. In BALB/b mice IL-4 production was very low but detectable (between 5 and 20 pg/ml), although again there was no significant difference between vaccinated and control groups.

The effect of vaccination with recombinant BCG was tested using the allergen challenge protocol in C57BL/6J mice. Again there was no effect on IL-5 levels, but a 1.5–2-fold enhancement of the specific IFN-γ response to peptide stimulation was observed in the splenocytes of mice receiving BCG-pI (Fig. 4a,b). This effect was less clear than in the case of the *M. vaccae* vaccine and did not reach statistical significance.

Modulation of the antibody response to allergen challenge

The effect of the mycobacterial vaccines on IgG subclass production was also evaluated using IgG1 as a marker for the Th2 response and IgG2a for Th1. C57BL/6J mice challenged with peptide 111–139 in IFA mounted a predominantly IgG1

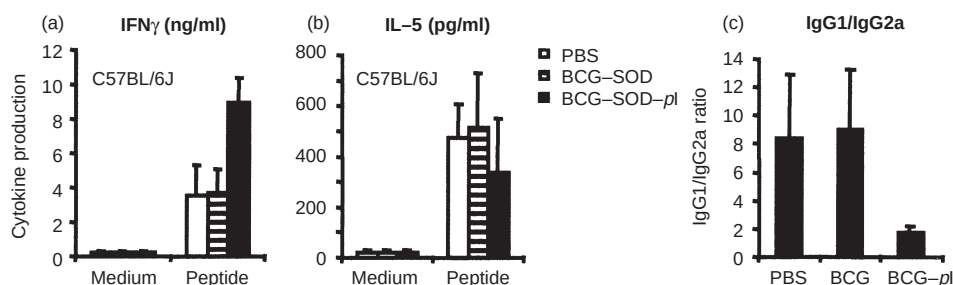


Figure 4. Vaccination with recombinant BCG. Mice were vaccinated s.c. with PBS, 10^7 viable BCG expressing the wild-type SOD, or with 10^7 viable BCG expressing SOD-*pI*. After challenge with 2 μ g of peptide 111–139 in IFA spleen cells were cultured in the presence of increasing amounts of peptide and IFN- γ production in response to 0.2 μ M of peptide 111–139 (a) and IL-5 production in response to 2 μ M peptide 111–139 (b) was measured. Data are expressed as the mean cytokine production of individual mice ($n = 4-5$) $\pm$ SE. Sera of mice were collected and tested for the presence of *Der p* 1 peptide 111–139-specific IgG1 and IgG2a antibodies. The OD measured at different serum dilutions were evaluated and the IgG1 : IgG2a ratio was determined (c). The ratio displayed was determined at a serum dilution of 1 : 30.

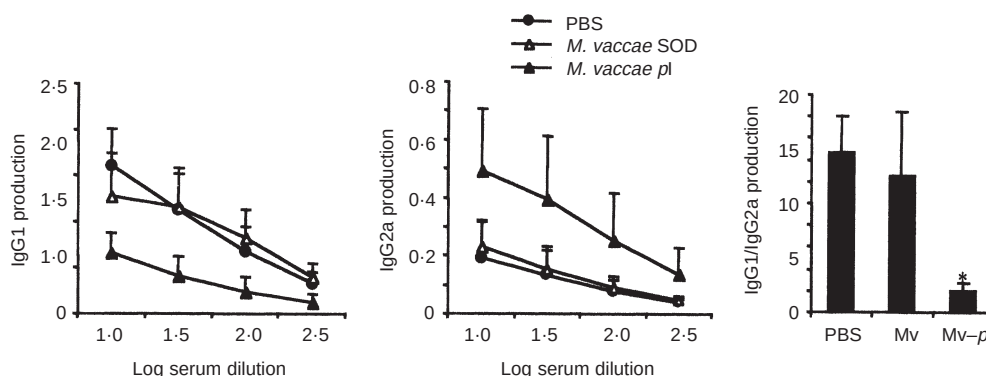


Figure 5. Antibody responses of *M. vaccae*-vaccinated C57BL/6J mice. Sera of mice vaccinated and challenged as described in the legend to Fig. 3 were collected and tested for the presence of *Der p* 1 peptide 111–139-specific IgG1 (a) and IgG2a (b) antibodies. The OD measured at different serum dilutions were evaluated and the IgG1 : IgG2a ratio was determined (c). The ratio displayed was determined by calculating end-point titres as described in the Materials and Methods. * $P < 0.01$ as compared to the PBS group.

response (Fig. 5a), resulting in a high IgG1 : IgG2a ratio (Fig. 5c). The response of mice vaccinated with *M. vaccae* expressing wild-type SOD was indistinguishable from the control group. In contrast, *M. vaccae*-*pI*-vaccinated mice not only produced lower amounts of IgG1, but also higher amounts of IgG2a (Fig. 5a,b), resulting in a much lower IgG1 : IgG2a ratio (Fig. 5c). Thus, the antibody responses are consistent with the cytokine assays in demonstrating polarization towards a Th1 response in the *M. vaccae*-*pI*-vaccinated group. Similar effects on the IgG1 : IgG2a ratio were observed in BCG-*pI*-vaccinated mice (Fig. 4c). In this case the change in ratio was mainly due to a decrease in IgG1 titre, however, the decrease in ratio was just a trend and did not reach statistical significance. BALB/b mice produced only very low amounts of antibodies to the peptide, and the antibody response was unsuitable for assessing Th1/Th2 balance in these mice. Total IgE levels were also determined. Although there was a marked difference in the amount of IgE produced by the three strains of mice the vaccination protocol did not alter IgE production (Table 1).

Table 1. Comparison of total IgE production by *M. vaccae*-vaccinated C57BL/6J, BALB/b and 129Sv mice

Mouse strain	Vaccination	Total serum IgE (ng/ml)
C57BL/6J	PBS	133 + 37
	<i>M. vaccae</i> SOD	153 + 38
	<i>M. vaccae</i> <i>pI</i>	92 + 48
BALB/b	PBS	1145 + 91
	<i>M. vaccae</i> SOD	1552 + 103
	<i>M. vaccae</i> <i>pI</i>	1152 + 194
129Sv	PBS	1511 + 64
	<i>M. vaccae</i> SOD	1786 + 130
	<i>M. vaccae</i> <i>pI</i>	1745 + 180

Mice were vaccinated on day 0 and day 21 with 10^8 heat-killed bacteria or with PBS and were challenged with peptide 111–139 in IFA on day 31. Total serum IgE was measured on day 45.

DISCUSSION

The incidence of allergy and asthma has risen dramatically in recent years in parts of the world in which infectious diseases have declined to historically low levels. Indirect evidence of an inverse correlation between the incidence of infection and the incidence of allergy is provided by the observation that allergic responses are more prominent in individuals who failed to mount a skin test response to mycobacteria.¹⁴ Together with the observed associations of Th2 responses with allergy and Th1 responses with infection, these findings have stimulated investigation of the possible use of microbial vaccines in the control of allergy.^{1,31} Prior BCG vaccination has been reported to reduce allergic responses in an ovalbumin model of airway eosinophilia¹³ and administration of *M. vaccae* resulted in reduced expression of Th2 cytokines in mice with a pre-existing allergic response to ovalbumin.³² Whereas exposure to *M. tuberculosis* has been associated with a lower incidence of allergy,¹⁴ it is still unclear whether BCG vaccination in humans prevents the development of allergy.^{15,16} While previous mouse studies have focused on the interplay between responses to separate Th2 and Th1 stimuli, the model developed in the present study allows us to investigate the potential contribution of antigen-specific responses in Th1/Th2 modulation.

Following immunization with recombinant mycobacteria expressing a house dust mite allergen we observed that splenocytes from C57BL/6J mice produced IFN- γ at high levels whereas those from BALB/b mice produced low levels of IFN- γ . This finding is consistent with previous reports of an innate bias towards Th1 and Th2 responses in these strains which was shown to be related to a difference in maintenance of IL-12 responsiveness between the strains.³³ Recently a locus designated T-cell phenotype modifier 1 (Tpm1) was implicated in this process.³⁴ In terms of priming of an IFN- γ response to the peptide, heat-killed *M. vaccae* were as potent as the live organisms, and as good as, if not better than, recombinant BCG vaccine. A surprising finding was that, in contrast to the response to the heat-killed recombinant, the T-cell response to viable *M. vaccae*-pI was not completely polarized towards Th1 as reflected in detectable levels of IL-5 production to the *Der p* I peptide. It has long been recognized that live mycobacteria are more effective than dead mycobacteria in induction of protection against challenge with *M. tuberculosis*,^{35,36} but the immunological basis of this effect is unclear. The live organisms may present the immune system with a different repertoire of antigens; they may stimulate different types of response as a result of differential trafficking within infected cells or within the whole animal; or enhanced persistence may result in their induction of a more effective memory response. The results of the present study suggest that mycobacterial viability is not a prerequisite for effective Th1 vaccination and that viable mycobacteria can even induce a mixed Th1/Th2 response. Recently it was shown that DNA containing non-methylated CpG motifs polarizes T-cell responses towards Th1.^{37–38} Since our data indicate a clear difference between viable and non-viable mycobacteria in polarizing responses towards Th1 this indicates that our observations cannot solely be ascribed to CpG motifs in the mycobacterial preparations.

When mice immunized with recombinant mycobacteria were subsequently challenged with peptide 111–139 of *Der p* I, we observed a switch in the response to allergen from a Th2 to a

Th1 profile. This response was specific for the recombinant vaccines expressing the allergen peptide and, in contrast to the primary immunization results, the genetic background of the mouse strain did not influence this. Even in BALB/b mice which did not show a strong Th1 response after primary immunization the response to peptide challenge was more polarized towards Th1. As in the studies of primary immunogenicity, killed *M. vaccae* was at least as effective as live BCG in the challenge model. In evaluating the relative merits of the two vaccine carriers, it is important to take into account that in contrast to *M. vaccae*, which dies rapidly after immunization (I. Brown and R. Janssen, unpublished observation), BCG can persist for long periods of time in murine tissues. The maximum time-frame between vaccination and challenge in the BCG experiments was 4 weeks, and we cannot exclude a possible advantage of live BCG vaccination in establishing a longer-lived memory response. In addition the expression level of the house dust mite peptide was lower in BCG than in *M. vaccae*.

Three previous studies have investigated the interplay between immune responses to mycobacteria and allergens in murine models. Erb *et al.* demonstrated localized suppression of Th2 responses to allergen in response to BCG infection in the lung,¹³ and Wang and Rook observed a systemic reduction in IL-5 and IgE production during *M. vaccae* therapy of mice with a pre-existing allergic response.³² The third study by Herz *et al.*³⁹ showed that intravenous BCG vaccination resulted in enhanced IFN- γ production and reduced IL-4 production in response to mitogen stimulation, although in their study no antigen-specific responses were measured. An important difference in the present study is that we have been able to examine the effect of delivering the allergen as a component of the mycobacterial vaccine. Our results differ from previous studies in two important aspects. Firstly, although we observe a similar overall trend from Th2 to Th1 immunity in association with mycobacterial vaccination, the antigen-specific modulation in the recombinant system is reflected predominantly in an enhancement of the Th1 response rather than in a reduction in Th2 cytokines. Secondly, our study deals with vaccination against allergy and not with the treatment of pre-existing allergic conditions. Unlike in our vaccination protocol, treatment of pre-existing allergic responses can result in a non-specific local¹³ or systemic³² suppression of the Th2 component of the response. Taken together these data suggests that mycobacteria can exert multiple immunomodulatory effects. One could for instance hypothesize that in addition to changing the Th1/Th2 balance they may stimulate immunoregulatory mechanisms. Together such effects may ensure the development of a well-balanced, non-pathological immune response to harmless antigens.

While the immediate non-specific reduction of Th2 immunity may be anticipated to provide relief for patients suffering from allergy, priming of allergen-specific Th1 responses could have a particular benefit in establishing a memory response capable of long-term protection against allergen-specific Th2 responses. Allergic patients treated with classical immunotherapy show improvement of clinical symptoms and although the precise mechanisms for this effect are unknown, some studies have indicated a correlation between an increase in IFN- γ production and a decrease in clinical symptoms^{40,41} indicating that induction of allergen-specific

Th1 responses may indeed be beneficial for prevention of allergy. In addition Till *et al.*⁴² have shown that immunotherapy is not accompanied by a reduction in IL-5 production. While the possibility has to be borne in mind that a strong Th1 response to the allergen could in itself have pathological effects associated with chronic inflammation, our data in BALB/b mice indicating that the presence of a strong Th1 response is not a prerequisite for the immunomodulatory effect of mycobacteria, suggests that potentially harmful effects of a Th1 response may be limited. The combined ability of mycobacterial vaccines to stimulate antigen-specific induction of Th1 immunity, in addition to non-specific suppression of Th2 responses, suggest a potential application in both prevention and treatment of allergy.

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