Pre-treatment with *Mycobacterium avium*-derived lipids reduces the macrophage response to interferon-γ in BCG-vaccinated mice

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*Mycobacterium bovis* Bacille Calmette–Guérin (BCG) is the current vaccine used against *Mycobacterium tuberculosis* (MTB) infection. However, exposure to environmental pathogens, such as *Mycobacterium avium*, interferes with the immune response induced by BCG vaccination. How *M. avium* affects the efficiency of BCG is unclear. In this study, BCG-vaccinated mice pre-treated with *M. avium*-derived lipids (MALs) showed a higher mycobacterial load and increased infiltration of inflammatory cells compared to control mice treated with *Escherichia coli*-derived lipids (ELs). Unexpectedly, there were no changes in cell proliferation or IFN-γ levels in spleen cells stimulated with protein purified derivatives (PPD) or heat-inactivated BCG in MALs-treated mice. However, pre-treatment with MALs decreased the bactericidal effect as well as the production of TNF-α and nitric oxide (NO) in murine macrophages from BCG-vaccinated mice stimulated with IFN-γ. These results suggest that MAL pre-treatment dampens the immune response against MTB and that this dampening is associated with a decreased response to IFN-γ stimulation in murine macrophages. T-lymphocyte responses, however, were unaffected.

INTRODUCTION

Although vaccination with *Mycobacterium bovis* Bacille Calmette–Guérin (BCG) is prevalent in over 160 countries worldwide (WHO, 2008), *Mycobacterium tuberculosis* (MTB) infection is still widespread in many countries, especially China, where there is a high morbidity rate (WHO, 2009). This high rate could be explained by the fact that environmental mycobacteria, such as *Mycobacterium avium*, is an opportunistic pathogen (de Lisle et al., 2005; Buddle et al., 2002), can interfere with BCG vaccination-induced immunity against MTB. The exposure to environmental mycobacteria is prevalent in countries with a low BCG vaccination efficacy (Fine, 1989). Further studies demonstrated that exposure to environmental mycobacteria prior to BCG vaccination could mask the effects of BCG (Howard et al., 2002; Martins et al., 2011). In mice that were not vaccinated with BCG, high levels of *M. avium* affected the host immune response by upregulating CD95 expression in T cells, which suppressed T-cell–macrophage interactions, promoted IL-10 production and reduced the secretion of IL-12, IL-8 and CCL5 (Zhong et al., 2003; Roger & Bermudez, 2001; Wagner et al., 2002). Furthermore, *M. avium*-derived lipids (MALs), which are important components of *M. avium*, were found to be associated with a delay in phagosome maturation and inhibition of Th1-type responses (Horgen et al., 2000; Sweet et al., 2010; Mendoza-Coronel et al., 2011). The current World Health Organization (WHO) guidelines recommend that BCG is administered within the first year of life or at birth (WHO, 2008), suggesting that the exposure to environmental mycobacteria happens after BCG vaccination. Therefore, we studied the immunological effects of *M. avium* exposure after BCG vaccination. In particular, we studied the effects on T-cell–mediated immunity.

We previously confirmed that exposure to low doses of MALs induces a hyporesponsiveness to MTB components in peritoneal macrophages from unvaccinated mice. These mice showed a significant decrease in the clearance of intracellular BCG, which was used as a substitute for MTB in simulating mycobacterial infection in clinical and experimental animal studies (Gao et al., 2009; Olleros et al., 2012). Stimulation with purified protein derivatives (PPD) or heat-inactivated BCG in vitro resulted in the decreased production of TNF-α, IL-6, IL-12 and nitric oxide (NO) (Yang et al., 2012). In this study, we focused on...

**Abbreviations:** BCG, Bacille Calmette–Guérin; DTH, delayed-type hypersensitivity; ELs, *E. coli*-derived lipids; iNOS, inducible nitric oxide synthase; i.p., intraperitoneally; MALs, *M. avium*-derived lipids; MTB, *Mycobacterium tuberculosis*; NO, nitric oxide; PPD, protein purified derivatives; qRT-PCR, quantitative real-time PCR; SC, solvent control.

Two supplementary figures are available with the online version of this paper.
the impact of exposure to low-dose MALs on acquired immunity in BCG-vaccinated mice. We determined that the pre-treatment of vaccinated mice with low-dose MALs attenuated the protective immune response induced by BCG. This attenuation was associated with a decreased response to IFN-γ in murine macrophages. In contrast, this attenuation did not affect lymphocyte responses to MTB PPD or heat-inactivated BCG in mice vaccinated with BCG.

**METHODS**

**Reagents.** Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco. MTB PPD were purchased from States Serum Institute. Middlebrook 7H9 broth, Middlebrook 7H11 broth, oleic acid-albumin-dextrose-catalase (OADC) and albumin-dextrose-catalase (ADC) were purchased from Sigma. Cell counting kit-8 (CCK8) was purchased from Dojindo (Japan). Griess reagent was purchased from Beyotime institute of biotechnology (China). Mouse cytokine ELISA kits were purchased from either E Bioscience or Biolegend. Newborn calf serum (NCS) was obtained from Sijijing Biological Engineering Materials (Hangzhou, China). Pyrogen Plus Limulus Amoebocyte Lysate kit was obtained from Xiamen Pyrogen Limited Company.

**Animals.** Specific-pathogen-free female BALB/c mice were purchased from the Experimental Animal Center of Southern Medical University. Mice were maintained at 25 °C with 50% humidity and given commercial feed and sterile water ad libitum. All animal experiments were approved by the Ethics Committee for Experimental Animals at Southern Medical University and were performed according to the national guidelines for animal welfare.

**Bacterial growth and the preparation of bacterial components.** *M. bovis* BCG was obtained from the Chengdu Institute of Biological Products in China. *M. avium* and *Escherichia coli* (ATCC 25922) were purchased from the National Center for Medical Culture Collections, China. *E. coli* were grown in nutrient agar medium, and mycobacterial stocks were grown in Middlebrook 7H9 broth with OADC enrichment at 37 °C until the mid-exponential phase; aliquots were then frozen at −80 °C. Frozen *M. avium* and *M. bovis* BCG stocks were thawed and briefly sonicated prior to use. Bacterial numbers were confirmed by plate counting on Middlebrook 7H11 agar plates.

**Preparation of MALs and ELs.** *M. avium- and E. coli-derived* lipids (MALs and ELs, respectively) were extracted using the Folch procedure as previously described (Yang et al., 2012; Van Rhijn et al., 2009; Folch et al., 1957). Briefly, *M. avium* and *E. coli* cultures were harvested by centrifugation, lyophilized and stored at −20 °C. Lyophilized *M. avium* or *E. coli* were homogenized in a solution consisting of chloroform and methanol (2:1). Homogenization was then followed by agitation in an orbital shaker at room temperature for 2 h. The samples underwent centrifugation, after which the liquid phase was collected and washed with a 0.9% NaCl solution. The mixture was vortexed for 30 s and then centrifuged at 2000 r.p.m. to separate the different phases. The lower chloroform phase, which contained the lipids, was evaporated with nitrogen. The extracts were then weighed and resuspended in DMSO. Each lipid fraction contained ≤0.02 endotoxin units per 100 µg lipid, as determined using the Pyrogen Plus Limulus Amoebocyte Lysate kit. The samples (10 µg ml⁻¹) contained no proteins, and cytotoxicity was assayed via flow cytometry as described previously (Yang et al., 2012).

**M. bovis BCG vaccination and the evaluation of delayed-type hypersensitivity (DTH) responses.** Female 3- to 4-week-old BALB/c mice were vaccinated by subcutaneous injection in the scuff of the neck with 50 µl *M. bovis* BCG (1 × 10⁶ c.f.u.) diluted in sterile PBS. A DTH response to PPD in *M. bovis* BCG-vaccinated mice was evaluated to test the effectiveness of the vaccination by the injection of 10 µg PPD into the right hind footpad at 5 weeks post-vaccination with BCG. Footpad swelling was measured at 24 and 48 h post-injection using a spring-loaded micrometer according to the following formula: thickness of the PPD-injected right footpad (mm)–thickness of the PBS-injected left footpad (mm) as a control.

**MALs pre-treatment.** The vaccinated mice were injected intraperitoneally (i.p.) with MALs (0.5 mg in 0.1 ml saline) once daily for 5 consecutive days. Control mice received normal saline (NS, 0.1 ml) or ELs (0.5 mg in 0.1 ml saline). All mice were treated at the same time each morning.

**M. bovis BCG challenge and c.f.u. determination.** Vaccinated mice were infected intravenously with 5 × 10⁶ c.f.u. of *M. bovis* BCG 24 h after the last MALs pre-treatment. The level of bacterial burden was determined at 4 weeks post-challenge in the lung and liver by plating serial dilutions of tissue homogenates in triplicate onto Middlebrook 7H11 agar plates. Plates were incubated for 3 weeks at 37 °C, and colonies were counted and calculated. *M. bovis* BCG-vaccinated mice were treated *in vivo* with MALs or control reagents i.p. once daily for five consecutive days. Peritoneal macrophages were collected from these mice and co-incubated with *M. bovis* BCG for 2 h. Extracellular bacteria were removed, and the macrophages were cultured with IFN-γ (10 ng ml⁻¹) for 72 h and then lysed with 0.1% Triton X-100. The cell lysates were plated on Middlebrook 7H11 agar to culture for 3 weeks at 37 °C. Colonies were then counted and calculated.

**Histopathology.** The vaccinated mice were infected intravenously with 5 × 10⁶ c.f.u. of *M. bovis* BCG at 24 h post MALs pre-treatment. Lung tissues were isolated for histological evaluation 5 weeks after *M. bovis* BCG challenge. The tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin.

**Measurement of cytokines.** Murine splenocytes were isolated and cultured in 96-well microplates (2 × 10⁵ cells per well) with PPD (10 µg ml⁻¹) or heat-inactivated *M. bovis* BCG (5 × 10⁶ c.f.u. ml⁻¹) for 72 h. The levels of IFN-γ and IL-12 in the culture supernatants were measured by ELISA following the manufacturer’s guidelines. The peritoneal macrophages were collected as previously described (Zhang et al., 2008; Fortier et al., 1982) and incubated in microplates. Cells (2 × 10⁵ cells per well) were treated with or without MALs for 48 h, washed and then stimulated with IFN-γ (10 ng ml⁻¹) for 24 h. Culture supernatants were harvested, and murine TNF-α was measured by ELISA.

**Quantitative real-time PCR.** Total cellular RNA was extracted with the RNeasy kit (Takara) and then treated with DNase (Qiagen). Quantitative PCR was performed using the Rotor-Gene 3000 system (Corbett Research) according to the manufacturer’s guidelines. Briefly, mRNA was isolated and reverse transcribed using oligo (dT) as the primers. Quantitative real-time PCR (qRT-PCR) was run on the Rotor-Gene 3000. cDNA (1 µl) was incubated at 50 °C for 2 min and 94 °C for 10 min. These incubations were followed by 40 cycles of the following protocol: 94 °C for 30 s, 57 °C for 30 s and 72 °C for 60 s. Fluorescent signals generated during the log-linear phase were used to calculate the relative amount of template DNA. The primers used in this study are listed in Table 1. Data were analysed using the 2⁻ΔΔCT method; mRNA expression is shown as the fold difference compared to untreated control cells.

**Nitrite assay for the evaluation of nitric oxide.** Total nitrite levels in the media were measured using Griess reagent (1% sulfanilamide...
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Table 1. Primers used in this study

<table>
<thead>
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<th>Gene</th>
<th>Primer (5’→3’)</th>
<th>GenBank ID</th>
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<tr>
<td>GAPDH</td>
<td>F: TGTGTCCGCTGGATCTGA, R: TTGCTGTTGAAGTCGCCAGGA</td>
<td>126012538</td>
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<tr>
<td>TNF-α</td>
<td>F: GCCAGGAGAGAAAGAAGA, R: AAGAGGCAGAGAATGGCACCC</td>
<td>133892368</td>
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<tr>
<td>iNOS</td>
<td>F: GGACATTTAACAAGGAAGGA, R: AGTGTCAATGCAAATCTCCTC</td>
<td>146134510</td>
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<tr>
<td>LRG-47</td>
<td>F: AGATTCCAGACAGAGGACCT, R: TGTTGTGATGTCTTCTGAAA</td>
<td>6680350</td>
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and 0.1 % N-(1-naphthyl)ethylenediamine in 2 % phosphoric acid); assays were performed according to the manufacturer’s directions.

Statistical analysis. Data are presented as the mean ± SD. Comparisons between groups were performed using ANOVA; P<0.05 was considered statistically significant.

RESULTS

MALs pre-treatment reduced the protection mediated by M. bovis BCG vaccination

To evaluate the immune response as a result of M. bovis BCG vaccination, DTH and IFN-γ production that were induced by PPD were tested in M. bovis BCG-vaccinated mice. As shown in Fig. S1 (available in JMM Online), M. bovis BCG-vaccinated mice exhibited a footpad response and produced higher levels of IFN-γ compared to the unvaccinated mice, indicating that the M. bovis BCG vaccination was successful. To mimic an asymptomatic response to low-dose M. avium exposure, we pre-treated the mice with 0.5 mg MALs per mouse, which could not induce a detectable inflammatory response in vivo (Fig. S2). The concentration of ELs that induced a similar inflammatory response to MALs was used as a lipid control. The bacterial burdens in the lung and liver as well as the pathological pulmonary injury were examined to evaluate the effects of pre-treatment with MALs in M. bovis BCG-vaccinated mice. MALs pre-treatment significantly reduced bacterial clearance in the lung and liver in M. bovis BCG-vaccinated mice compared to the solvent control (SC) (Fig. 1a), demonstrating that MALs pre-treatment reduced the protection against MTB in M. bovis BCG-vaccinated mice. Moreover, while BCG vaccination protected mice from BCG challenge, pre-treating the mice with MALs remarkably reduced the protection mediated by BCG vaccination in response to BCG challenge (Fig. 1b). The lung tissues from MALs pre-treated mice showed a widespread and severe interstitial pneumonia with intense inflammation. However, mice pre-treated with SC and ELs showed relatively less lymphocyte infiltration and slighter interstitial pneumonia. These results indicate that MALs reduced the protection mediated by M. bovis BCG vaccination.

MALs pre-treatment in vivo did not alter MTB-specific Th1-type lymphocyte responses in M. bovis BCG-vaccinated mice

MTB-specific lymphocyte responses, especially the Th1-type response, play a vital role in mycobacterial clearance in M. bovis BCG-vaccinated mice. Therefore, we determined the effects of MALs pre-treatment on Th1-type lymphocyte responses to mycobacterial components. Splenocytes from vaccinated mice that were pre-treated with MALs in vivo were incubated in vitro with PPD or heat-inactivated M. bovis BCG, and Th1-type cytokines and lymphocyte proliferation were measured. We found that MALs pre-treatment did not affect IFN-γ and IL-12 production and that lymphocyte proliferation did not change in MALs pre-treated mice compared to SC- or ELs-treated controls (Fig. 2). These results indicate that pre-treatment with MALs does not alter MTB-specific Th1-type lymphocyte responses and that the reduced protection against M. bovis BCG in vaccinated mice pre-treated with MALs is not associated with MTB-specific Th1-type lymphocyte responses.

MALs pre-treatment in vivo decreased M. bovis BCG clearance by IFN-γ-activated macrophages

Because the reduction in protection resulting from MALs pre-treatment of BCG-vaccinated mice is unrelated to MTB-specific lymphocyte responses, we determined whether the macrophage response to IFN-γ secreted by T lymphocytes was altered by MALs treatment. The results in Fig. 3 show that mice pre-treated with MALs exhibited no differences at 0 h from controls. However, at 72 h, mice pre-treated with MALs exhibited a higher bacterial burden than controls, indicating that rather than altering phagocytosis, MALs pre-treatment affected the macrophage response to IFN-γ. This reduced response to IFN-γ resulted in an inability to digest and kill the intracellular bacteria.

MALs pre-treatment in vitro decreased TNF-α and NO production in IFN-γ-activated macrophages

Because TNF-α and NO are important effector molecules that are required to control mycobacterial infections (Ehlers et al., 1999; Flynn et al., 1995; Bekker et al., 2000), we examined the effect of MALs pre-treatment on the production of TNF-α and NO from IFN-γ-stimulated macrophages in vaccinated mice. The results indicate that MALs pre-treatment decreased the production of TNF-α and inducible nitric oxide synthase (iNOS) protein as well as the transcription of TNF-α and iNOS mRNA in IFN-γ-activated macrophages (Fig. 4). These results confirm that MALs pre-treatment reduced the response to IFN-γ in activated macrophages from M. bovis BCG-vaccinated mice.
MALs pre-treatment did not alter LRG-47 mRNA expression in IFN-γ-activated macrophages in vitro

LRG-47 is an important molecule modulated by IFN-γ that controls mycobacterial growth. LRG-47 knockout mice exhibit an increased bacterial load and a higher mortality in experimental infections with MTB (MacMicking et al., 2003). Therefore, we examined LRG-47 mRNA expression in MALs-pre-treated macrophages that were stimulated with IFN-γ in vitro. As shown in Fig. 5, LRG-47 mRNA expression did not change in MALs-pre-treated macrophages at any time point compared to the SC or ELs control. These results suggest that the reduced response to IFN-γ in MALs-pre-treated macrophages is not associated with LRG-47.

DISCUSSION

Many studies have demonstrated that chronic environmental mucosal infection can induce lesions in lung and intestinal tissues as well as acute M. avium infection. In contrast, the pre-treatment with low-dose lipids of M. avium in our work is unable to induce an inflammatory response and also unable to change the Th1 response, but is able to induce hyporesponsibility of macrophages. Our aim was to simulate the situation in which most of the people with lower responses to BCG are not infected with M. avium. To explain why the prevalence of MTB infection is high amongst individuals vaccinated with M. bovis BCG, we studied the impact of MALs pre-treatment on M. bovis BCG-vaccinated mice. This study is different from the work studying mycobacterial infection prior to M. bovis BCG vaccination (Buddle et al., 2002; Demangel et al., 2005). In this study, we found that MALs pre-treatment reduced mycobacterial clearance and resulted in more serious infiltration of inflammatory cells in the lungs of M. bovis BCG-vaccinated mice. These results demonstrate that
MTB-specific immunity resulting from M. bovis BCG vaccination is weakened by the exposure to low-dose M. avium, suggesting that M. avium may interfere with the efficiency of M. bovis BCG vaccination in humans.

It is known that the Th1-type response is critical for the clearance of mycobacteria in vivo. In M. bovis BCG-vaccinated mice and humans, T cells activated by MTB or MTB-specific antigens can produce several cytokines, especially IFN-γ, which can activate macrophages to clear mycobacterial infections by secreting a variety of bioactive substances, such as TNF-α and NO, or inducing LRG-47 expression to promote phagosome maturation. It has been reported that MALs can inhibit Th1-type responses in humans (Horgen et al., 2000) and mice (Mendoza-Coronel et al., 2011; Flaherty et al., 2006). Therefore, we

**Fig. 2.** Effects of MALs on MTB-specific lymphocyte responses in M. bovis BCG-vaccinated mice. M. bovis BCG-vaccinated mice were treated with MALs or ELs i.p. once daily for five consecutive days. Murine splenocytes were isolated and then cultured with 10 μg ml⁻¹ PPD (a, c, e) or 5×10⁶ ml⁻¹ heat-inactivated M. bovis BCG (b, d and f). The levels of IFN-γ (a and b) and IL-12 (c, d) in the culture supernatants were measured by sandwich ELISA. MTB-specific lymphocyte proliferation was determined using the CCK-8 kit (e, f). One representative experiment of three (n=7) is shown. **, P<0.01 compared to the SC cultures.
hypothesized that the reduced clearance of *M. bovis* BCG in MALs-pre-treated vaccinated mice may be due to impaired T-cell function. However, MALs pre-treatment did not alter the production of Th1-specific cytokines, such as IFN-γ, or MTB-specific lymphocyte proliferation induced by PPD or heat-inactivated *M. bovis* BCG. These results suggest that low-dose MALs pre-treatment does not affect MTB-specific lymphocyte responses. The discrepancies between reports studying the effects of MALs treatment on T cells may be attributed to different experiment designs. For example, in the work performed by Horgen et al. (2000), Th1-type responses were induced by PHA/PMA after pre-treatment with *M. avium* lipids. This induction occurred via the mitogen receptor, which is not antigen specific. In our studies, however, we used MTB or MTB components as antigenic stimulators on primed T cells pre-treated with low doses of MALs. Mendoza-Coronel et al. (2011) used bone marrow-derived dendritic cells that were exposed to *M. avium* in a ratio of 1:1 and exposed these cells to T cells. However, Flaherty et al. (2006) have demonstrated that exposure of BCG-vaccinated mice to *M. avium* could decrease the clearance of MTB but increase the activated T-cell number in the lung.

Fig. 3. MALs pre-treatment decreased *M. bovis* BCG clearance mediated by IFN-γ-stimulated macrophages. *M. bovis* BCG-vaccinated mice were treated in vivo with MALs and control reagents i.p. once daily for five consecutive days. The peritoneal macrophages were incubated with *M. bovis* BCG for 2 h. Extracellular bacteria were removed, and the macrophages were stimulated with 10 ng ml⁻¹ IFN-γ and lysed at 0 and 72 h. The results are presented as the mean±SD from three independent experiments (n=5). **, P<0.01. Samples are compared to the SC.

Fig. 4. MALs pre-treatment reduced TNF-α and NO production in vitro from IFN-γ-stimulated macrophages. Peritoneal macrophages from *M. bovis* BCG-vaccinated mice were pre-treated with MALs (1 μg ml⁻¹), ELs (1 μg ml⁻¹) or SC for 48 h, and then stimulated with 10 ng ml⁻¹ IFN-γ for 24 h. The levels of TNF-α and NO in culture supernatants were measured (a and b). The same peritoneal macrophages were stimulated with 10 ng ml⁻¹ IFN-γ for 3 h and qRT-PCR was performed to measure TNF-α and iNOS mRNA expression (c and d). Mean TNF-α and iNOS mRNA expression ±SD (in relative units) from three independent experiments (n=7) is shown. NS, not significant; **, P<0.01 compared to cells treated with the SC.
lymph nodes, implying that *M. avium*-induced interference with MTB-specific immunity provided by *M. bovis* BCG vaccination is not associated with the activation of T cells in the lung.

Based on the results that antigen-specific T-cell proliferation and Th1 cytokine production were not changed under the impaired specific immune response induced by MALs, we looked at the macrophage response to MALs pre-treatment. Macrophages are important cellular reservoirs of mycobacteria in the host and are critical for the clearance of pathogenic mycobacteria both in innate and acquired immune responses. Moreover, IFN-γ is indispensable for the activation of macrophages in acquired immunity against MTB in our experimental model. Our results suggest that the MALs-induced reduction in both mycobacterial clearance and production of proinflammatory factors in BCG-vaccinated mice may be attributed to reduced macrophage responses to IFN-γ.

It has been reported that acute infection with *M. avium* could alter macrophage responses by increasing the production of IL-10 (Silva et al., 2001), suppressing the production of IL-12 and CCL-5, and reducing HLA-DR expression on antigen presenting cells (Wagner et al., 2002; Sangari et al., 1999; Tsuyuguchi et al., 1990). Moreover, *M. avium* infection inhibited IFN-γ-induced MHC-II class gene expression on RAW 264.7 cells, which may lead to a reduced response to IFN-γ (Lafuse et al., 2006). Taken together with our previous work on the effect of MALs on innate and acquired immunity against MTB, we hypothesize that pre-treatment with low-dose MALs reduces the activity of macrophages from both BCG-vaccinated and unvaccinated mice.

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