

Suppression of allergic airway disease using mycobacterial lipoglycans

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Background: Administration of heat-killed mycobacteria can suppress allergic disease in mice and humans. The active components of mycobacteria mediating these effects remain unresolved.

Objective: We sought to identify the active components of mycobacteria mediating suppression of allergic disease and to determine structural features important for function.

Methods: Using a murine model of allergic airway disease, we tested the ability of the lipoglycan fractions of the mycobacterial cell wall to suppress airway eosinophilia.

Lipoglycans isolated from different strains of mycobacteria and chemical modifications were used to explore structure-function relationships. Markers of allergic disease including bronchoalveolar lavage cytokines, spleen and lymph node T-cell cytokine production, and serum specific immunoglobulin (Ig) E/IgG1 were examined.

Results: We identified the mycobacterial cell wall lipoglycans lipoarabinomannan and phosphatidylinositol mannan as components of mycobacteria capable of suppressing airway disease (>70% reduction in airway eosinophilia; $P < .03$). Structure-function analysis identified the acyl chains and mannose groups of the molecules as having a role in mediating this effect. Mechanistic studies provided no evidence for a T-helper cell (Th) 1-mediated suppression of an ongoing Th2 response. An increased capacity of T cells to secrete interleukin 10 in the spleen and lymph node of treated animals was identified, suggesting a potential T-cell-mediated suppression mechanism.

Conclusion: We have identified immunomodulatory component(s) of mycobacteria responsible for the protective effects observed in allergic disease; these findings will lead to the generation of synthetic compounds or agonists devoid of the unwanted characteristics of whole mycobacteria for evaluation in a human clinical setting. (*J Allergy Clin Immunol* 2004;114:302-9.)

Key words: Allergic airway disease, mycobacteria, lipoglycan, suppression, mouse model, T cell, immunoglobulin E, regulatory T cell, interleukin 10

Abbreviations used

AraLAM:	Lipoarabinomannan isolated from avirulent strains that does not contain mannose capping
AAD:	Allergic airway disease
ABTS:	2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
BAL:	Bronchoalveolar lavage
BCG:	Bacillus Calmette-Guérin
HRP:	Horseradish peroxidase
IN:	Intranasal
IP:	Intraperitoneal
cIMDM:	Complete Iscove's modified Dulbecco's medium
LAM:	Lipoarabinomannan
LM:	Lipomannan
ManLAM:	Lipoarabinomannan capped with mannose units
OVA:	Ovalbumin
PIM:	Phosphatidylinositol mannan

Allergic asthma is a chronic respiratory disease characterized by reversible, variable airway obstruction and overreactivity of T-helper (Th) 2 immune mechanisms, which leads to immunoglobulin (Ig) E production and eosinophilic mucosal inflammation.¹ Current clinical approaches to the treatment of asthma have focused on drugs that relieve symptoms of the disease (eg, the targeting of effector mediators). More recently, epidemiologic data has contributed significantly to the search for novel immunoregulatory compounds to treat asthma.² The potential for developing mycobacterial-based vaccines for the treatment or prevention of atopic asthma was first highlighted in a study by Shirikawa et al³ linking natural exposure to *Mycobacterium tuberculosis* with a lower incidence of atopy in Japanese schoolchildren.

Infection with *M bovis* Bacillus Calmette-Guérin (BCG), *M vaccae* (*M vaccae*), or *M tuberculosis* has been shown to suppress Th2-driven allergic airway disease (AAD) in animal models.⁴⁻⁷ Studies using heat-killed mycobacteria instead of live bacteria have shown that the suppressive effects are conserved, suggesting mycobacterial antigen(s) are mediating the effect.⁷⁻⁹ Preliminary clinical studies using heat-killed mycobacterium have been encouraging, suggesting mycobacteria-based therapeutics may show utility in the treatment of atopic dermatitis and asthma in humans.^{10,11}

The immune response to mycobacteria is complex and multifaceted; it lends itself to some side effects if used as

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a therapeutic agent for the treatment of allergy.¹⁰ The ability of mycobacteria to induce this complex immune response has been attributed to the diverse features of the organism. These features include the unmethylated copies of DNA it releases,¹² component proteins (eg, tuberculin-purified protein derivative),¹³ and the constituents of the cell wall.¹⁴ One unique component of mycobacterial cell walls is lipoglycans including lipoarabinomannan (LAM) (Fig 1, A), lipomannan (LM) (Fig 1, B), and phosphatidylinositol mannan (PIM) (Fig 1, C). LAM is a high molecular weight lipoglycan that has been shown to exhibit a wide number of immunogenic effects in vitro¹⁴ and is believed to play a major role in the virulence of mycobacteria.¹⁵ The lower molecular weight lipoglycans LM and PIM also display antigenic properties^{14,16} that may contribute to the immunogenicity of the whole bacterium.

It was hypothesized that lipoglycans may have protective effects against allergy. To this end, the capacity of heat-killed mycobacteria and mycobacterial cell wall components from *M bovis* and *M smegmatis* to suppress Th2-driven AAD in a mouse model was investigated. LAM and PIM have been identified as immunomodulatory components found in mycobacterial cells wall extracts that can suppress AAD. Structure-function analysis identified the acyl chains and mannose groups of the molecules as having a significant role and mechanistic studies using *M bovis*. PIM identified an expansion of interleukin (IL) 10-secreting T cells in the spleen and lymph node, suggesting a role for regulatory T cells in contrast to a Th1-(interferon [IFN] γ)-mediated suppression of an ongoing Th2 response.

METHODS

Mycobacteria cell isolation and fractionation of LAM and PIM

The lipoglycans from mycobacterial species (*M bovis*-AN5, *M smegmatis*-MC²155) were isolated using the Triton X-114 phase separation technique of Severn et al.¹⁷ Lipoglycan extracts were deacylated by treatment with anhydrous hydrazine,¹⁸ and deacylated PIM (dPIM) was further separated by size exclusion chromatography to give dPIM-2. Lipoglycans and modified lipoglycans were identified as described by Severn et al.^{17,18}

Mice

C57BL/6 male mice were bred and housed in a conventional animal facility at the Wellington School of Medicine. All animals used for the experiments were aged between 6 and 10 weeks. Experimental procedures were approved by the animal ethics committee in accordance with University of Otago (Dunedin, New Zealand) guidelines for the care of animals.

Immunization and airway challenge

Mice were injected intraperitoneally (IP) with 2 μ g of ovalbumin (OVA) (Sigma-Aldrich, St Louis, Mo) in 200 μ L of alum adjuvant (Serva, Heidelberg, Germany) on days 0 and 14 ($n = 5$ -8 mice/group). On day 28 mice were anesthetized by an IP injection of a mixture of ketamine and xylazine (Sigma-Aldrich) and intranasally (IN) challenged with 50 μ L of phosphate-buffered saline (PBS) containing 100 μ g OVA.

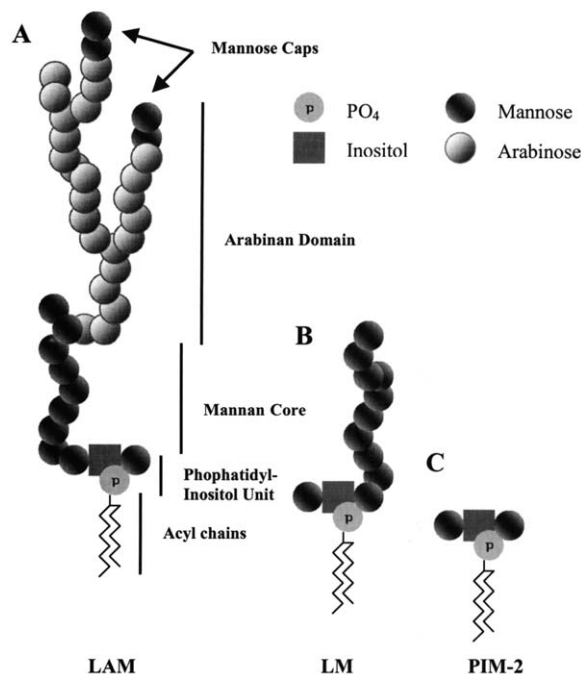


FIG 1. Generic representations of the structure of (A) lipoarabinomannan (LAM), (B) lipomannan (LM), and (C) phosphatidylinositol mannan (PIM).

Treatment with mycobacterial derivatives

One week (day 21) prior to OVA challenge mice were anesthetized and the indicated concentrations of lipoglycans or heat-killed (80°C, 20 minutes) mycobacteria in 50 μ L of PBS was administered IN. Control mice were given 50 μ L of PBS.

Detection of cell types in the bronchoalveolar lavage fluid

Four days (day 32) post-OVA challenge (day 28) the mice were sacrificed and bronchoalveolar lavage (BAL) was performed (3 washes with 1 mL PBS). Total BAL cell numbers were counted; cells were fixed onto slides using a cytospin and stained with the Diff-Quick Staining Kit (Dade Behring, Newark, Ct). The percentages of different cell types were determined microscopically using standard histological criteria.

BAL T-cell activation and intracellular cytokine staining

BAL cells (10^6 cells/well) were cultured for 2 hours (37°C/5% CO₂) on uncoated or 24-well plates coated with anti-CD3 (clone 2C11, 10 μ g/mL) in 1 mL complete Iscove's modified Dulbecco's medium (cIMDM) containing anti-CD28 mAb (1/50 dilution of 37.51 culture supernatant) and IL-2 (50 U/mL). Monensin (Calbiochem-Novabiochem) was added to give a concentration of 2 μ mol/L and the incubation was continued for another 4 hours. Cells were isolated and stained using anti-CD4-fluorescein isothiocyanate (GK1.5), anti-IL-4-phycoerythrin, and anti-IFN- γ -allophycocyanin (BD Pharmingen) as described previously.¹⁹ Data was collected using the Becton Dickinson FACSsort as described by the manufacturer and analyzed using FlowJo (Tree Star Inc, San Carlos, Calif).

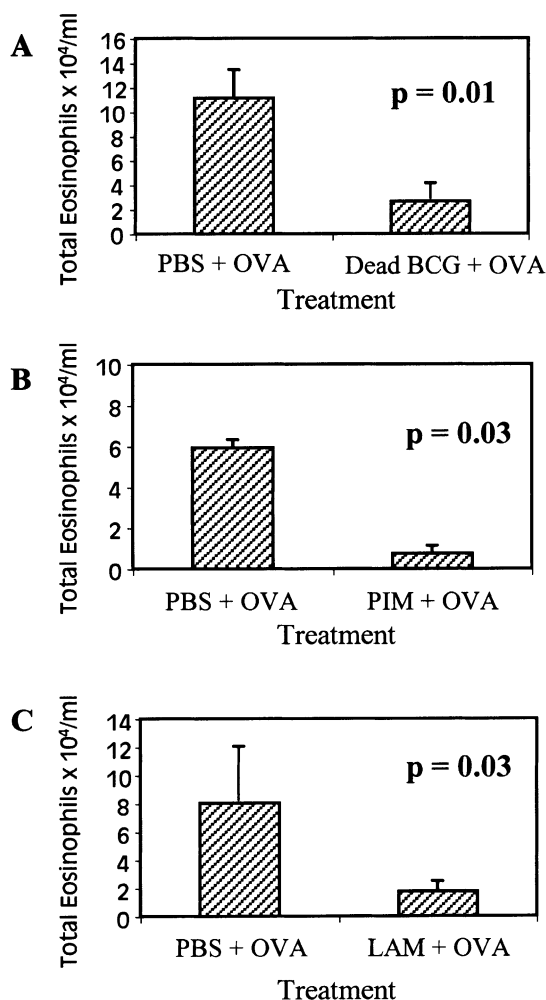


FIG 2. Heat-killed BCG, *M bovis* PIM, and *M bovis* LAM suppress AAD when administered to sensitized mice. The effect of administration of heat-killed BCG (2.5 μ g) (**A**), *M bovis* PIM (0.1 μ g) (**B**), and *M bovis* LAM (0.6 μ g) (**C**) on airway eosinophilia versus PBS control administration. Data are representative of 1 of at least 2 independent experiments $\pm$ SD.

Splenocyte and mediastinal lymph node cell culture

On day 32 of the AAD model, spleens and lymph nodes were removed and pooled into experimental groups. Single-cell suspensions were generated by passing through a 0.2 μ m/L gauze; 10^6 cells/well were cultured for 72 hours (37°C/5% CO₂) on uncoated or 24-well plates coated with anti-CD3 (clone 2C11, 1, 5, 10 μ g/mL) in 0.5 mL cIMDM containing anti-CD28 mAb (1/50 dilution of 37.51 culture supernatant) and IL-2 (50 U/mL). Parallel experiments were also completed stimulating cells with 0, 10, 50, and 100 μ g/m: OVA in cIMDM.

Enzyme-linked immunosorbent assay for the detection of IgE, IgG1, IL-5, IL-10, and IFN γ

On day 32 of the AAD model, blood was withdrawn from the vena cava and serum isolated. Serum OVA specific IgE was captured using anti-IgE mAb (4B-39)—coated enzyme-linked immunosorbent assay (ELISA) plates and detected using OVA-biotin/streptavidin—horse radish peroxidase (HRP) in conjunction with 2,2-azino-bis

(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate (Sigma-Aldrich). Serum OVA specific IgG1 was captured using OVA-coated ELISA plates and detected using goat anti-IgG1-HRP antibody (Serotec, Raleigh, NC) in conjunction with ABTS substrate. The absorbance at 405 nm was used to compare immunoglobulin levels. The cytokines IL-5, IL-10, and IFN γ were detected using commercially available paired antibody kits (BD Pharmingen) in conjunction with tetramethylbenzidine substrate (BD Pharmingen). The absorbance at 450 nm and supplied recombinant standards were used to quantify cytokine levels.

Statistical analysis

Differences between groups were compared using the Mann-Whitney test (Minitab Inc, State College, Pa). $P < .05$ was considered significant.

RESULTS

Heat-killed BCG and *M bovis* LAM and PIM can suppress AAD in sensitized animals

As shown in Figure 2(A), administration of heat-killed BCG (2.5 μ g) resulted in a 77% suppression of total eosinophil cell number ($P = .01$). IN administration of LAM (0.6 μ g) or PIM (0.1 μ g) isolated from *M bovis* resulted in a decrease in airway eosinophilia by 78% ($P = .03$) and 87% ($P = .03$) respectively (Fig 2, B,C; Table I). LM isolated from *M bovis* had no significant effect on airway eosinophilia in this model (Table I, data not shown). These concentrations of lipoglycan used were determined to be optimal for the suppression of airway eosinophilia by titration in the mouse model (data not shown). Total BAL cell numbers including lymphocyte numbers were significantly reduced in lipoglycan-treated mice (data not shown).

M bovis LAM requires mannose capping and intact acyl chains for function

LAM isolated from virulent strains of mycobacteria such as *M bovis* contain mannose caps at the end of the arabinose chains. In avirulent strains such as *M smegmatis* this mannose capping is absent (Fig 1).²⁰ LAM isolated from *M smegmatis* did not suppress airway eosinophilia (Table I). Interestingly, PIM isolated from *M smegmatis* did cause suppression of eosinophilia, although with lower efficacy than PIM isolated from *M bovis* (Table I). Deacylated *M bovis* LAM and PIM did not retain the suppressive properties of the intact molecules (Table I).

Suppression of AAD by *M bovis* PIM does not involve immunoglobulin isotype switch from IgE and IgG1 isotypes

In the sensitized, nonchallenged group (PBS/PBS) there was a lower level of both IgE and IgG1. Following allergen challenge there was a similar increase in serum IgE and IgG1 in both the PBS/OVA (PBS control) and BOVIS PIM (*M bovis* PIM) groups (Fig 3). There was no statistical difference in either IgE or IgG1 levels in the PBS versus BOVIS PIM treatment groups. The increase in IgG1 in the challenged versus nonchallenged groups was statistically significant ($P = .005$). The increase in IgE

TABLE I. Structure-function characterization of lipoglycans

Mycobacteria	Extract	Effective dose (μ g)	% Suppression of eosinophilia*
<i>M bovis</i>	Heat-killed	2.5	77
	LAM	0.6	78
	Deacylated LAM	No effect	—
	PIM	0.1	87
	Deacylated PIM-2	No effect	—
<i>M smegmatis</i>	LM	No effect	—
	Heat-killed	4.8	40
	LAM	No effect	—
	PIM	0.1	51

LAM, Lipoarabinomannan; PIM, phosphatidylinositol mannan.

*Results calculated from at least 2 independent experiments.

levels in the challenged versus nonchallenged groups did not reach statistical significance.

Suppression of AAD by *M bovis* PIM does not involve Th1-mediated suppression of Th2 responses in the lung

Similar percentages of CD4⁺IFN γ ⁺ BAL cells were observed in mice treated with either PBS or *M bovis* PIM. In the PBS control group; 5.04% CD4⁺IFN γ ⁺ cells (Fig 4, A) and in the *M bovis* PIM group 5.39% CD4⁺IFN γ ⁺ cells were present (Fig 4, B). IL-4 production by CD4 cells was not detected (data not shown), in agreement with previous studies using anti-CD3 stimulation of BAL cells in context of this mouse model.⁷ BAL IL-5, IL-10, and IFN γ cytokine levels could not be detected in this model (data not shown), in agreement with others.⁷

Suppression of AAD by *M bovis* PIM is characterized by an increased capacity of T cells to produce IL-10 and a decreased capacity to produce IL-5 and IFN γ in the spleen

IFN γ (Th1-associated), IL-5 (Th2-associated), and IL-10 (regulatory/suppressor function) were measured in cell supernatants from stimulated splenocytes. The greatest level of IFN γ production (136 ng/mL) was observed in the PBS control group (Fig 5, A). The level of IFN γ in the *M bovis* PIM treatment group was lower (peak 54 ng/mL), similar to that observed in the nonchallenged group (peak 47 ng/mL; Fig 5, A). The greatest level of IL-5 production was observed in the PBS control group (peak 233 pg/mL). In the nonchallenged and the *M bovis* PIM treatment group a lower level of IL-5 production was observed (176 and 189 pg/mL, respectively; Fig 5, B). Splenic T cells from the *M bovis* PIM treatment group had the greatest capacity to produce IL-10 (815 pg/mL; Fig 5, C). Lower levels of IL-10 production were observed in both the nonchallenged (377 pg/mL) and PBS control groups (532 pg/mL; Fig 5, C). In a separate series of experiments, splenocytes were stimulated with 10, 50, and 100 μ g/mL OVA for 72 hours; cytokine production was not detected by ELISA (data not shown).

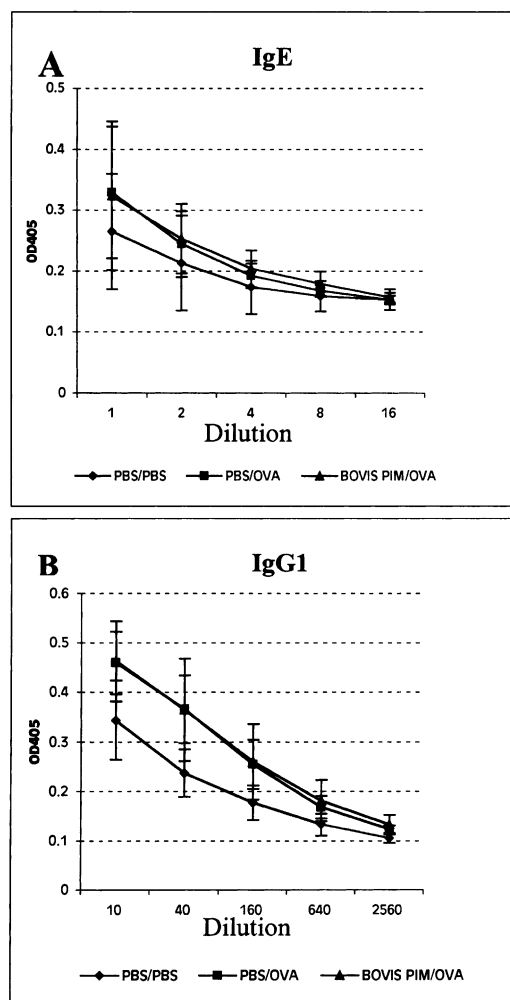


FIG 3. Allergen specific serum IgE and IgG1 levels. **A**, OVA specific serum IgE levels in the PBS/PBS (diamond, sensitized, non-challenged), PBS/OVA (square, PBS control), and the BOVIS PIM/OVA (triangle, *M bovis* PIM [0.1 μ g]) groups. **B**, OVA specific IgG1 levels. Data are representative of 1 of at least 2 independent experiments $\pm$ SD.

Suppression of AAD by *M bovis* PIM is characterized by an increased capacity of T cells to produce IL-5, IL-10, and IFN γ in the mediastinal lymph node

The nonchallenged and *M bovis* PIM treatment groups had a similar capacity to produce IFN γ (503 and 508 ng/mL, respectively), which was higher than the PBS control group (343 ng/mL) (Fig 5, A). IL-5 production levels were highest in the *M bovis* PIM treatment group (4.2 ng/mL) compared to the PBS control group (2.4 ng/mL). The lowest level of IL-5 production was observed in the nonchallenged group (0.5 ng/mL; Fig 5, B). The capacity of T cells from the *M bovis* PIM group to produce IL-10 (13.3 ng/mL) was found to be increased compared to the PBS control group (4.3 ng/mL). A lower level of IL-10 production was observed in the nonchallenged group (0.7

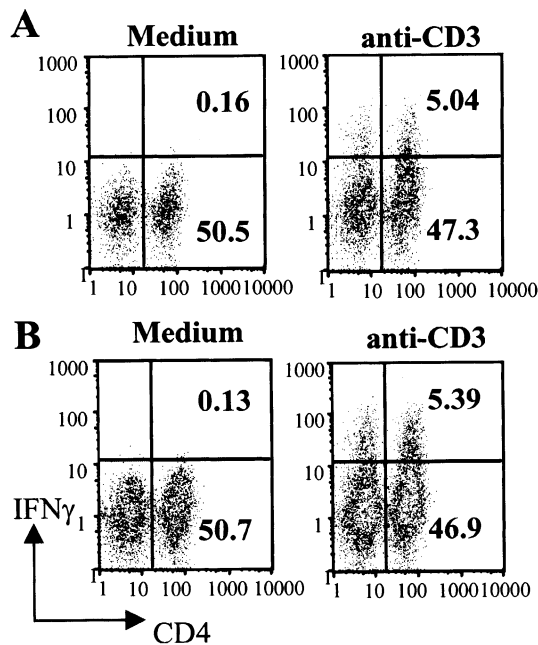


FIG 4. Suppression of AAD by *M bovis* PIM does not involve Th1-mediated suppression of a Th2 response in the lung. **A**, Data for the PBS mouse group. **B**, Data for the *M bovis* PIM group. Data are representative of 1 of at least 2 independent experiments.

ng/mL; Fig 5, C). In a separate series of experiments, when lymph node cells were stimulated with 10, 50, and 100 μ g/mL OVA for 72 hours, cytokine production was not detected by ELISA (data not shown).

DISCUSSION

The use of microbial-derived materials as a potential source of new therapeutics for the treatment of asthma and allergy has lead to intense interest in mycobacteria.² We have previously demonstrated that live *M bovis* BCG infection could suppress Th2-driven allergic airway disease in mice.⁴ Subsequent studies by multiple groups have reproduced these findings and shown that in addition to live organisms, heat-killed *M bovis* BCG or *M vaccae* can mediate the suppression of allergic airway disease in animal models.^{5,6,8,9,21-24} What was lacking from these studies was the knowledge of the active component(s) of mycobacteria mediating these effects.

Using a mouse model of Th2-driven allergic airway disease we have shown that heat-killed BCG, *M bovis* LAM, and *M bovis* PIM can significantly suppress airway eosinophilia. *M bovis* PIM was also shown to suppress airway eosinophilia in an alternative mouse model of AAD based on the KLH antigen (data not shown). Although airway eosinophilia is only one of the many factors associated with allergic asthma, the AAD model is a useful and well-recognized model for investigating the potential for immunomodulation of allergic disease.

Lipoglycans have been shown to have diverse immunomodulatory functions including the activation of

peripheral blood mononuclear cells (PBMCs) leading to cytokine production including TNF α , IL-1 β , IL-6, IL-8, and IL-10.^{14,16} In virulent strains of mycobacteria the arabinan units of LAM are capped with 1 or more mannose units (ManLAM). In contrast, LAM isolated from avirulent strains does not contain mannose capping (AraLAM).²⁰ AraLAM purified from *M smegmatis* did not suppress airway eosinophilia in OVA-sensitized mice, indicating that mannose capping was important for the activity of LAM. The uptake of mycobacteria such as *M tuberculosis* or *M bovis* BCG into cells is associated with recognition of ManLAM by mannose receptors.²⁵ It is therefore reasonable to postulate that the suppression of airway eosinophilia by LAM could involve uptake of the molecule via the mannose receptor. Interestingly, mannosylated LAM has been shown to suppress dendritic cell (DC) function following mannose receptor activation²⁵ and via a DC-specific ICAM-3 grabbing non-integrin-mediated mechanism.^{26,27} These activities may contribute at least in part to the mechanism of suppression of AAD by altering DC function. The finding that PIM isolated from *M smegmatis* caused suppression of airway eosinophilia can be rationalized because the terminal mannose chains are conserved in PIM molecules regardless of the mycobacterial source, further postulating a role for the mannose receptor and highlighting differences in the composition of the extract from different mycobacterial sources.^{28,29} These data suggest a prominent role for the mannose component of the molecules determining activity.

To further define the structural features of the lipoglycans required for the suppression of disease, we examined deacylated *M bovis* LAM and PIM in the context of the AAD model. The removal of acyl chains from *M bovis* LAM and PIM resulted in the complete loss of activity. The significance of the acyl chains of these molecules has previously been recognized; deacylation of *M tuberculosis* LAM results in the complete loss of LAM-induced cytokine production from PBMCs in vitro.¹⁴ Similarly, there was an absolute requirement for *M bovis* PIM to contain at least 1 acyl chain to recruit natural killer cells in a mouse model.²⁸ The requirement for acyl groups on the molecules in order to suppress allergic airway disease may indicate a role in binding to recognition sites on antigen presenting cells (eg, CD1 or toll-like receptors) known to recognize lipoglycan molecules.²⁹⁻³² A potential role for CD1-mediated natural killer T-cell or T-cell activation may be postulated. PIM and LAM from *M leprae* or *M Tuberculosis* activate human CD4⁺CD8⁺ α/β T cells via CD1b, a lipid presentation molecule found on antigen presenting cells.³³

The *M bovis* PIM extract showed the greatest efficacy in the AAD model and was the least complex lipoglycan, which makes PIM a potential lead for future synthetic approaches. *M bovis* PIM was the focus of further study. Serum OVA specific IgE and IgG1 levels were similar in the PBS-treated control and *M bovis* PIM-treated groups, suggesting that suppression of airway eosinophilia did not involve the suppression of these Th2 parameters.

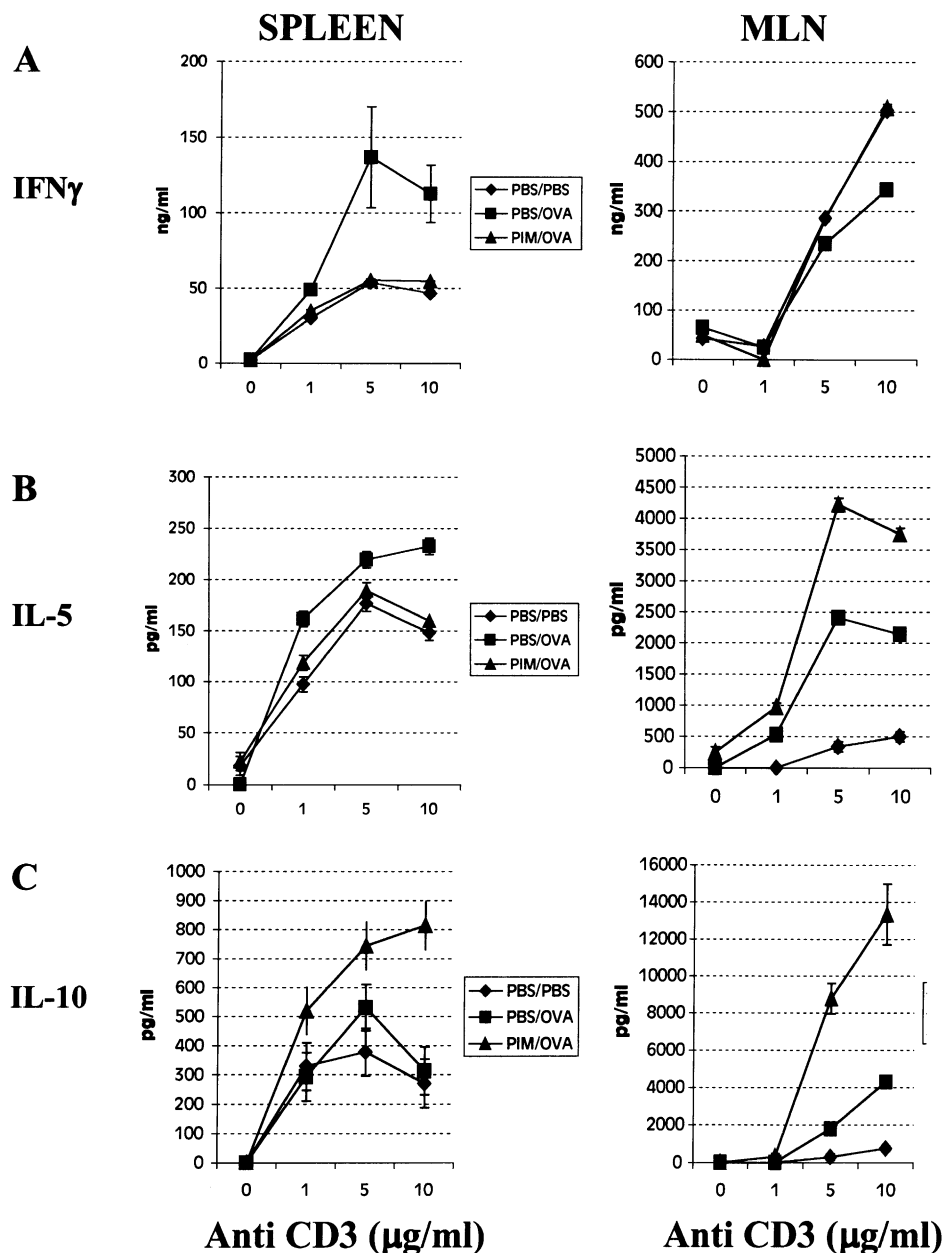


FIG 5. Effect of *M bovis* PIM on IFN γ , IL-5, and IL-10 release in response to in vitro anti-CD3/CD28 activation of splenocytes and mediastinal lymph node cells from OVA immunized mice. Levels of (A) IFN γ and (B) IL-5, and (C) IL-10 production from cultured cells. Data are representative of 1 of at least 2 independent experiments.

These data are in agreement with studies examining the suppression of AAD in mice using live or heat-killed BCG^{4,7,34} but in contrast to others where a reduction in IgE/IgG1 levels in the live BCG treatment group was observed.²¹ These differences may reflect the different protocols utilized by Herz et al including routes of administration and live versus heat-killed mycobacteria.^{2,21}

A long-standing hypothesis for how mycobacteria suppress allergic disease is that they induce a potent

Th1-(IFN γ)—mediated immune response that can suppress the ongoing Th2 response. The BAL cell data show that the percentage CD4⁺IFN γ ⁺ cells were similar in both PBS-treated group and *M bovis* PIM-treated group. These data argue against a Th1-mediated suppression of Th2 responses in the lung. To date this is the only study to directly assess the capacity of BAL CD4⁺ cells to produce IFN γ . Previously, BAL supernatant IFN γ levels have been shown to be both elevated⁷ and not affected²⁴ following BCG or *M vaccae* administration, respectively, in mouse

allergic models. In the current study, BAL cytokines were below the detection limit of the ELISA, in agreement with others utilizing this model.⁷

To further define the mechanism of *M bovis* PIM-mediated suppression of airway eosinophilia, the capacity of splenic and mediastinal lymph node T cells to produce Th1-associated (IFN γ), Th2-associated (IL-5), and regulatory (IL-10) cytokines was determined in vitro. These data show that in the spleen from *M bovis* PIM-treated animals there was an enhanced capacity of T cells to produce the immunoregulatory cytokine IL-10, and this was accompanied by the decrease in the capacity to produce both IFN γ and IL-5. These data suggest an expansion of IL-10-secreting T cells in the *M bovis* PIM treatment group and a potential role for these cells in the mechanism of action. Previous studies examining the spleen of live BCG-treated animals in an allergic model have identified an increased capacity of T cells to produce IFN γ ²¹ or no change in IFN γ production in heat-killed *M vaccae*-treated animals.⁹ These data may reflect the differences between live and heat-killed mycobacteria administration. In agreement with the current study, the capacity of T cells to produce IL-10 was increased following administration of heat-killed *M vaccae* in a mouse allergic model.⁹ Subsequently, the authors identified a splenic CD4⁺/CD45Rb(lo)/IL-10⁺ regulatory T cell responsible for the suppression of airway eosinophilia mediated by *M vaccae* using adoptive transfer studies.⁹ One interpretation of the current study is that the source of the IL-10 is in fact a regulatory T cell of yet undefined phenotype.

IL-5, IL-10, and IFN γ production by in vitro stimulated lymph node cells was elevated in the *M bovis* PIM treatment group. Interestingly, the greatest magnitude of effect was for IL-10 induction, again suggesting a role for T cells capable of producing IL-10 in the suppression of disease. In previous mouse studies examining suppression of AAD by *M vaccae* and BCG, lymph node IL-10 production was not investigated; however, in agreement with the current study, the capacity of T cells to produce IFN γ was increased following live BCG administration versus control in a mouse allergy model.⁵ Interestingly, we observed an increased capacity of lymph node T cells to produce IL-5 in the *M bovis* PIM treatment group versus PBS control. These data are in contrast with other data that showed a decreased capacity to produce IL-5 following live/dead BCG administration⁷ or no effect on IL-5 production following live BCG administration,⁵ suggesting the subtlety of isolated extracts.

The prominent increased capacity of T cells from *M bovis* PIM-treated mice to produce IL-10 is in excellent agreement with other studies that have identified this cytokine as having a central role in *M vaccae*-mediated suppression of AAD in mice.⁹ Previously, administration of IL-10³⁵ or adoptive transfer of CD4 cells engineered to produce IL-10³⁶ have been shown to inhibit airway eosinophilia in mouse AAD models, reinforcing the immunomodulatory role of IL-10 in Th2-mediated disease.

In conclusion, this is the first report identifying the potential of utilizing the mycobacterial lipoglycans LAM and PIM, preferentially from a virulent mycobacterial strain, for the development of a treatment for asthma and other allergic diseases. Structure-function analysis identified the acyl groups and mannose caps as key components of the molecules required for activity. Mechanistic studies did not support a role for Th1-mediated suppression of Th2 responses in the lung, spleen, or mediastinal lymph node and in contrast suggested a potential role for T-cell-derived IL-10. Preliminary human trials of heat-killed *M vaccae* show promise in the treatment of atopic dermatitis¹⁰ and allergic asthma.¹¹ However, only the identification of immunomodulatory component(s) of mycobacteria responsible for these protective effects will lead to the generation of synthetic compounds devoid of the unwanted characteristics of whole mycobacteria.

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