Mycobacterial glycolipid trehalose 6,6'-dimycolate-induced hypersensitive granulomas: contribution of CD4⁺ lymphocytes

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The granulomatous response is a characteristic histological feature of *Mycobacterium tuberculosis* infection responsible for organism containment. The development of cell-mediated immunity is essential for protection against disease, as well as being required for maintenance of the sequestering granulomatous response. Trehalose 6,6'-dimycolate (TDM; cord factor), a glycolipid associated with the cell wall of mycobacteria, is implicated as a key immunogenic component in *M. tuberculosis* infection. Models of TDM-induced hypersensitive granulomatous response have similar pathologies to that of active tuberculosis infection. Prior immunization (sensitization) of mice with TDM results in exacerbated histological damage, inflammation and lymphocytic infiltration upon subsequent TDM challenge. Adoptive transfer experiments were performed to ascertain the cell phenotype governing this response; CD4⁺ cells were identified as critical for development of related pathology. Mice receiving CD4⁺ cells from donor TDM-immunized mice demonstrated significantly increased production of Th1-type cytokines IFN- γ and IL-12 within the lung upon subsequent TDM challenge. Control groups receiving naïve CD4⁺ cells, or CD8⁺ or CD19⁺ cells isolated from TDM-immunized donors, did not exhibit an exacerbated response. The identified CD4⁺ cells isolated from TDM-immunized mice produced significant amounts of IFN- γ and IL-2 when exposed to TDM-pulsed macrophages *in vitro*. These experiments provide further evidence for involvement of a cell-mediated response in TDM-induced granuloma formation, which mimics pathological damage elicited during *M. tuberculosis* infection.

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INTRODUCTION

Tuberculosis is not only a disease of the past; it is a disease of the future. More than 2 million people die each year from *Mycobacterium tuberculosis* infection, making it the most deadly of all infectious organisms. With nearly 2 billion people infected worldwide, tuberculosis remains a serious health threat (WHO, 2006), so continued research is needed to learn how this organism evades detection and destruction within the host.

The cell wall of the *M. tuberculosis* bacilli is rich in lipids. They constitute more than 50 % of the dry weight of the organism and have been implicated as potential virulence factors (Kolattukudy *et al.*, 1997). More than a half a century ago, Hubert Bloch identified cord factor as a 'toxic substance' that when extracted from virulent organisms caused them to lose their virulence (Bloch, 1950), and when

added during infection exacerbated disease (Bloch & Noll, 1955). This substance was later identified as the mycobacterial glycolipid trehalose 6,6'-dimycolate or TDM (Noll *et al.*, 1956). TDM has been extensively studied over the years and many unique properties have been attributed to its presence. Removal of TDM from the surface of *M. tuberculosis* results in enhanced trafficking to acidic vesicles and decreased survival of the organism within macrophages (Indrigo *et al.*, 2002, 2003). Relative to surface-associated TDM, organisms genetically altered to produce less TDM, or irregular TDM, exhibit reduced growth and survival both *in vivo* and *in vitro* (Armitige *et al.*, 2000; Copenhaver *et al.*, 2004; Glickman *et al.*, 2000; Rao *et al.*, 2005).

Perhaps TDM's most interesting attribute is its ability to induce cytokine and chemokine production leading to granuloma formation in the lungs of responsive mice (Behling *et al.*, 1993; Bekierkunst, 1968; Guidry *et al.*, 2004; Lima *et al.*, 2001; Perez *et al.*, 2000; Yamagami *et al.*, 2001). In mice, hypersensitive granulomas produced in response to TDM produce pathology which mimics in many ways

Abbreviations: BMM, bone-marrow derived macrophages; RT-qPCR, real-time quantitative RT-PCR; TDM, trehalose 6,6'-dimycolate; TLR, Toll-like receptor.

that seen in *M. tuberculosis* infection in man. Previous studies suggested a role for T cells in this response; however, antigen specificity still remained to be shown (Seggev *et al.*, 1982, 1984; Yamagami *et al.*, 2001). Recently, it was demonstrated that a T cell-mediated response could be generated in cells recovered from mice immunized with TDM. These cells produced inflammatory and Th1-type cytokines in a TDM-specific manner. Adoptive transfer of CD3⁺ TDM-immunized cells was sufficient in transferring the hypersensitive granulomatous response, while transfer of T cells immunized with an alternative antigen did not transfer the hypersensitive pathology, indicating that this response was TDM specific (Guidry *et al.*, 2006).

The studies reported here sought to pinpoint the cell phenotype responsible for the development of the hypersensitive granulomatous response to mycobacterial TDM. In these experiments a potential role was identified for the contribution of TDM to generating CD4⁺ T cells that play a role in granuloma development during infection. The importance of the contribution of T cells to *M. tuberculosis* infection is well established (Caruso *et al.*, 1999; Flynn & Chan, 2001; Grotzke & Lewinsohn, 2005; Muller *et al.*, 1987; Scanga *et al.*, 2000; Serbina & Flynn, 2001; Serbina *et al.*, 2001). These experiments add to those findings by examining the potential function of TDM in generating reactive CD4⁺ T cells during development of *M. tuberculosis*-related pathology.

METHODS

Mice. Female 6–8-week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in microisolator cages under specific pathogen-free conditions. All experiments were conducted under approval of the University of Texas-Houston Health Science Center Animal Welfare Committee (documents AWC-01-034 and AWC-04-065). Three to six mice were used per group.

Immunization. Mice were immunized according to previously published methods (Guidry *et al.*, 2004, 2006; Yamagami *et al.*, 2001). Briefly, mice received 100 µl of a water/oil emulsion containing 100 µg TDM (Sigma) emulsified in complete Freund's adjuvant (Sigma) and 100 µg methylated BSA (Promega) administered by subcutaneous injection at the base of the tail. TDM was >95 % pure, as demonstrated by thin-layer chromatography and gel analysis (Indrigo *et al.*, 2002). Mice were boosted 28 days later with the same preparation, prior to use in the experiments described.

Preparation and adoptive transfer of donor cells. Single-cell suspensions were prepared from splenocytes from naïve or TDM-immunized mice. CD4⁺ and CD8⁺ T cells, as well as CD19⁺ B cells, were isolated via magnetically assisted cell sorting (MACS; Miltenyi Biotec) according to the manufacturer's instructions. The purity of the fractionated cells was consistently >95 % as assessed by flow cytometry (data not shown). Naïve or TDM-immunized cells (2 × 10⁶ per treatment) were resuspended in 0.5 ml phosphate-buffered saline (PBS) and administered by intraperitoneal injection into naïve wild-type BALB/c mice.

Induction of pulmonary granulomas. Two days after adoptive transfer, mice were injected with 100 µl of a water/oil/water emulsion

prepared as described previously (Guidry *et al.*, 2004; Yamagami *et al.*, 2001), administered by intravenous tail vein injection. To prepare 100 µl of sample, 100 µg TDM was added to 3.2 µl incomplete Freund's adjuvant (Sigma) and ground in a homogenizer tube with a Teflon pestle. A water/oil emulsion was formed by addition of 3.2 µl 0.1 PBS to the tube and mixing it with the 'oil' phase. Then 93.6 µl PBS containing 0.2 % Tween 80 was added to complete the outer phase of the water/oil/water emulsion.

Histology and pathology. Mice were sacrificed and lungs were perfused with 1 ml PBS with 1 mM EDTA via the right ventricle. Lungs were grossly monitored for inflammation and pathological damage, evaluating tissue according to lung weight index (LWI), as detailed previously (Guidry *et al.*, 2004, 2006). The right lung was separated from the left by severing the bronchus distal to the ligature; the right lower lobe was snap-frozen in RNazol B (TelTest) and stored at –70 °C for RNA extraction and qRT-PCR. The remaining right upper lobe was weighed, minced, and transferred to 2 ml Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10 % heat-inactivated fetal bovine serum (FBS, Sigma), 50 ng ml^{–1} of penicillin G (Sigma) and gentamicin (Sigma), and 0.01 % HEPES (Sigma) and L-arginine (Sigma), then incubated at 37 °C for 4 h. Cellular debris was removed by centrifugation at 3000 r.p.m. for 10 min. Supernatant was collected and stored at –20 °C for later cytokine evaluation by ELISA. For histological examination, the left lung was excised and fixed in 10 % buffered formalin and embedded in paraffin blocks. Sections (5 µm thick) were prepared and stained with haematoxylin/eosin.

In vitro T cell overlay. Bone-marrow-derived macrophages (BMM) were prepared as described previously (Indrigo *et al.*, 2002; Perez *et al.*, 2000) with minor modification. Briefly, bone marrow cells were isolated from BALB/c femurs and differentiated at 1 × 10⁶ cells ml^{–1} in McCoy's base containing 10 % heat-inactivated FBS, 10 ng ml^{–1} recombinant murine GM-CSF (Cell Sciences), 100 µg penicillin G ml^{–1} and 50 µg gentamicin ml^{–1} (complete medium) at 37 °C in 5 % CO₂ for 6 days. Adherent cells were then washed with 1 × PBS and rested in DMEM supplemented with 10 % heat-inactivated FBS and 0.01 % HEPES and L-arginine overnight prior to use. J774A.1, a murine monocyte/macrophage cell line, was obtained from the ATCC (#TIB-67). Cells were maintained at 37 °C with 5 % CO₂ in DMEM containing 10 % heat-inactivated FBS. Cells (1 × 10⁶ per well) were added to 24-well tissue culture plates in a total volume of 1 ml and rested for 4 h before use. TDM-coated polystyrene beads (Indrigo *et al.*, 2003) were added at a 10:1 bead:macrophage ratio and incubated for 4 h as described by Indrigo *et al.* (2002). Cells were washed prior to incubation with lymphocytes. Purified lymphocyte populations (3 × 10⁶) were resuspended in DMEM containing 100 µg penicillin G ml^{–1} and 50 µg gentamicin ml^{–1}, 0.005 % 2-mercaptoethanol (Gibco), and 0.01 % HEPES and L-arginine, then overlaid onto macrophages (BMM or J774A.1). Cell supernatants were collected 48 h later for cytokine analysis by ELISA.

ELISA quantification of cytokines. Levels of cytokines from lung supernatants were measured by sandwich ELISA using DuoSet kits (R&D Systems) specific for each cytokine (Guidry *et al.*, 2004). ELISAs were performed according to the manufacturer's instructions. Absorbance was read at 570 nm and 450 nm on a Spectra Max 250 ELISA plate reader (Molecular Devices). The mean of duplicate wells was calculated based on a standard curve constructed for each assay, using recombinant murine IFN-γ, IL-12(p40), IL-2 and IL-4. The lower limit of detection was 5 pg ml^{–1} for all assays.

RNA isolation and RT-qPCR. RNA was isolated from lung tissue as described by Guidry *et al.* (2004), with modifications. Briefly, lung tissues were homogenized in RNazol B (Tel-test) using a plastic pestle. Chloroform (0.1 vol.) was added, and the sample was shaken

vigorously, chilled, and centrifuged at 13 000 r.p.m. for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube, and an equal volume of 2-propanol was added. The pelleted precipitate was washed with 75 % ethanol, and then resuspended in water containing 1 mM EDTA. RNA was DNase treated (Invitrogen) to eliminate potential genomic DNA contamination. Real-time quantitative RT-PCR (RT-qPCR) was performed utilizing a 7700 Sequence Detector (TaqMan; Applied Biosystems) (Bustin, 2000; Heid *et al.*, 1996). Specific quantitative assays for β -actin, IFN- γ and IL-12(p40) were developed using Primer Express software version 1.0 for Macintosh (Applied Biosystems) following the recommended guidelines based on GenBank sequences. Primer sequences are listed in Table 1. The limit of sensitivity for all assays was between 190 and 200 molecules, with approximately 95 % assay efficiency (with less than 5 % primer dimers or incomplete amplification after >35 cycles).

cDNA was synthesized in 10 μ l total volume by the addition of 6 μ l per well of RT master mix, consisting of 400 nM assay-specific reverse primer, 500 μ M deoxynucleotides, Stratascript buffer and 10 U Stratascript reverse transcriptase (Stratagene), to a 7700 96-well plate (ISC Bioexpress) followed by 4 μ l of sample (25 ng μ l⁻¹). Samples were measured in triplicate plus a control without reverse transcriptase. Each plate also contained an assay-specific sDNA (synthetic amplicon oligo) standard spanning a 5-log concentration range and a no-template control. Plates were preincubated in a PTC-100 thermocycler (MJR) (30 min at 50 °C, 72 °C for 10 min), followed by addition of 40 μ l PCR master mix (400 nM forward and reverse primers; IDT, Coralville, IA), 100 nM fluorogenic probe (Biosource, Camarillo, CA), 5 mM MgCl₂ and 200 μ M deoxynucleotides, PCR buffer and 1.25 U *Taq* polymerase (Invitrogen) directly to each well. RT master mixes and all RNA samples were pipetted by a Tecan Genesis RSP 100 robotic workstation (Tecan US); PCR master mixes were pipetted by a Biomek 2000 robotic workstation (Beckman). Plates were run in the 7700 Sequence Detector (Applied Biosystems) using the following cycling conditions: 95 °C, 1 min; followed by 40 cycles of 95 °C, 12 s and 60 °C, 30 s. The resulting data were analysed using SDS 1.9.1 software (Applied Biosystems) with ROX (Invitrogen) as the reference dye.

Synthetic DNA oligos used as standards (sDNA) encompassed the entire 5'-3' amplicon for the assay (Biosource). Each oligo standard was diluted in 100 ng μ l⁻¹ yeast tRNA-H₂O (Invitrogen) and spanned a 5-log range in 10-fold decrements starting at 0.8 pg μ l⁻¹. Standards for housekeeping gene assays started 10-fold higher for abundant transcripts. The final data were normalized to β -actin and are presented as (molecules of transcript)/(molecules of

β -actin) $\times$ 100 (% β -actin). Data were also generated using bioluminescent RT-PCR on independently derived samples (Actor *et al.*, 1998; Guidry *et al.*, 2004), with similar values obtained.

Statistical analysis. All data are presented as the mean $\pm$ SEM. Each experiment was repeated at least twice. Normally distributed data were analysed by the unpaired *t*-test when the difference between two means was compared within groups. A difference between group means was considered significant at a level of $P < 0.05$.

RESULTS

CD4⁺ recall response following TDM immunization

Mice were immunized subcutaneously at the base of the tail with TDM water/oil emulsion, and CD4⁺ cells were isolated from splenocytes and analysed for recall responses to TDM (Fig. 1). The CD4⁺ cells and the remaining CD4⁻ population were overlaid onto J774.1 macrophages preincubated with TDM. Supernatants were examined for presence of cytokines to indicate T cell phenotypic responses; IFN- γ and IL-2 (T_H1) and IL-4 (T_H2) were measured. Analysis of cell supernatants revealed a significantly increased production of cytokines IFN- γ and IL-2 from the CD4⁺ population which was absent in the CD4-depleted population ($P < 0.005$). No significant IL-4 production was present in either group.

Purified CD4⁺ cells were further examined for recall responses to TDM presented by bone-marrow-derived macrophages (BMM) isolated from BALB/c mice. Purified CD4⁺ cells were overlaid onto BMM previously incubated with TDM and monitored for cytokine production (Fig. 2). Comparisons were made with TDM-immunized CD8⁺ cells, as well as control CD4⁺ or CD8⁺ cells isolated from non-immunized mice. Overlay of TDM-immunized CD4⁺ T cells resulted in significant IFN- γ and IL-2 production. However, CD8⁺ cells obtained from TDM-immunized mice and overlaid onto presenting macrophages produced no significant IFN- γ or IL-2. Likewise, control CD4⁺ cells

Table 1. Oligonucleotide primers and probes

Target*	Primer/probe†	Sequence (5'-3')
β -actin (NM_007393)	Se	TCTGGCTCCTAGCACCATGA
	AS	CCACCGATCCACACAGAGTACT
	Probe	ATCAAGATCATTGCTCCTCCTGAGCGC
IFN- γ (NM_008337)	Se	AGCAACAGCAAGGCGAAA
	AS	CTGGACCTGTGGGTGTTGA
	Probe	TCAAACCTTGGCAATACTCATGAATGCATCCT
IL-12(p40) (NM_008352)	Se	AAGTGTGAAGCACCAAATTACTC
	AS	TTCAAGTCCATGTTTCTTTGC
	Probe	ACGGTTCACGTGCTCATGGCT

*Accession numbers are in parentheses.

†Se, sense; AS, antisense.

Fig. 1. TDM-elicited cytokine production from immunized CD4⁺ cells. CD4⁺ cells (CD4⁺ Imm; black bars) and the resultant CD4-depleted population of cells (CD4⁻ Imm; white bars) isolated from TDM-immunized mice were examined for cytokine response to TDM presented by J774.1 macrophages. CD4⁺ cells isolated from TDM-immunized mice produced significantly more IFN-γ and IL-2 than the CD4-depleted population, while IL-4 production was slightly above the limit of detection for both groups. Results represent mean ± SEM, with similar data obtained from repeated experiments. **P* < 0.005.

from non-immunized (naïve) mice did not respond to presented TDM.

CD4⁺ T cells transfer hypersensitive pathology to TDM

The cells analysed above were used in adoptive transfer experiments to investigate the contribution of TDM-immunized CD4⁺ cells to development of the hypersensitive response to TDM. CD4⁺ T cells purified from TDM-immunized and naïve mice were injected intraperitoneally into naïve recipients. Mice were later intravenously challenged with TDM, and sacrificed 3 days post-challenge. Non-immunized mice receiving TDM challenge alone (Fig. 3, top left), developed small granulomas characteristic of the acute response to TDM. Mice previously immunized against TDM exhibited a hypersensitive response following challenge (Fig. 3, top right), manifesting as a rapid onset of inflammation with presence of infiltrating lymphocytes around vascular beds. Adoptive transfer of naïve CD4⁺ cells (Fig. 3,

middle left) had no influence on the response, with lung histology strikingly similar to that of the non-immunized control. In contrast, mice receiving TDM-immunized CD4⁺ cells (Fig. 3, middle right) developed large granulomas and widespread inflammation in the lung. Adoptive transfer of CD8⁺ cells (Fig. 3, bottom left) or CD19⁺ B cells (Fig. 3, bottom right) from immunized mice did not lead to changes in histopathology. In summary, only the adoptive transfer of the TDM-immunized CD4⁺ population of cells led to the development of hypersensitive pathology.

The lung weight index (LWI) was calculated as a measure of inflammation and cellular recruitment. Non-immunized mice showed significant increase in LWI at 3 days after TDM challenge compared to naïve lungs (1.12 ± 0.36 vs 0.094 ± 0.02). The LWI values were further increased after TDM challenge in mice that received adoptive CD4⁺ cells from the immunized animals ($LWI\ 1.41 \pm 0.16$). Similarly, the positive control immunized mice exhibited a significant increase in LWI values (1.54 ± 0.09).

Fig. 2. *In vitro* analysis of purified cell populations. CD4⁺ purified from naïve mice (Naïve CD4⁺; white bars) or TDM-immunized mice (TDM imm CD4⁺; black bars) were examined for cytokine production when overlaid onto BMM previously incubated with TDM. The results indicate high IFN-γ and IL-2 production only from the CD4⁺ immunized group, with no significant response either from naïve CD4⁺ cells or from CD8⁺ cells isolated from TDM-immunized mice (TDM imm CD8⁺; striped bars). IL-4 production was not significant for all groups. Results represent mean ± SEM, with similar data obtained from repeated experiments. **P* < 0.001.

Fig. 3. Adoptive transfer of CD4⁺ cells from TDM-immunized mice leads to exacerbated lung response after TDM challenge. Naïve mice challenged with TDM (top left) form small focal granulomas within the lung, whereas previous immunization with TDM (top right) results in aggressive, 'hypersensitive' reactivity. Adoptive transfer (AT) of CD4⁺ cells from TDM-immunized (middle right) transferred the hypersensitive pathology, while mice receiving naïve CD4⁺ cells (middle left) demonstrated reactivity similar to that of the naïve challenge control. Adoptive transfer of TDM-immunized CD8⁺ T cells (bottom left) or TDM-immunized CD19⁺ B cells (bottom right) failed to transfer the hypersensitive pathology. Sections representative of three or four mice per group. Haematoxylin/eosin staining, 40 $\times$.

This hypersensitive granulomatous response was further examined at 7 days post-challenge (Fig. 4). Nearly identical pathologies were observed in both the immunized/challenge control mice (Fig. 4, left) and the adoptive transfer group that received TDM-immunized CD4⁺ T cells (Fig. 4, right). Inflammation was present throughout the lung, with both groups demonstrating profound lymphocytic infiltration and cuffing of vascularized regions, as well as indication of endothelial cell damage.

Increased IFN- γ and IL-12 production in response to TDM challenge via adoptive transfer of immunized CD4⁺ cells

Lungs were analysed for *in vivo* cytokine production at 3 days after TDM challenge. IFN- γ and IL-12 have been identified as factors important in development and maintenance of the granulomatous response during tuberculosis infection and are indicators of a strong Th1

Fig. 4. Heightened immunopathology due to specific transfer of a CD4⁺ TDM-specific population. Mice receiving adoptive transfer of TDM-immunized CD4⁺ cells (right panels) develop an aggressive lung pathology that peaks at 7 days after TDM challenge, with marked lymphocytic vascular cuffing (arrow), intravascular occlusion (star) and endothelial damage, and exacerbated monocytic infiltration. The exhibiting pathology was indistinguishable from TDM-challenged, positive control immunized mice (left panels). Haematoxylin/eosin staining; top panels, 40 $\times$; bottom panels, 100 $\times$.

response. Production of these cytokines was monitored using RT-qPCR (Fig. 5). Adoptive transfer of TDM-immunized CD4⁺ cells led to significant increases in IFN- γ and IL-12(p40) message, as compared to challenge controls. IFN- γ transcript levels were elevated as much as 18-fold above that of the challenge controls. Cytokine message for both IFN- γ and IL-12(p40) remained significantly lower in the lungs of mice receiving naïve CD4⁺ T cells. Lungs were also monitored for cytokine protein production (Fig. 6). As suggested from the mRNA analysis, mice receiving adoptive transfer of TDM-immunized CD4⁺ cells demonstrated significantly more IFN- γ and IL-12 protein in the lungs than both those receiving naïve CD4⁺ cells and the challenge control. These results demonstrate the importance of TDM and related T cell activation in the production of cytokines essential in the hypersensitive granulomatous response.

DISCUSSION

Previous studies shed light on the potential involvement of T cells in the hypersensitive granulomatous response to TDM (Guidry *et al.*, 2004; Yamagami *et al.*, 2001). More recently, the contribution of CD3⁺ T cells to the

generation of this hypersensitive pathology was confirmed (Guidry *et al.*, 2006). The current study identifies the importance of CD4⁺ cells to this response. Adoptive transfer of distinct lymphocyte subpopulations verified that only the CD4⁺ phenotype, and not CD8⁺ or CD19⁺ cells, could transfer the hypersensitive granulomatous pathology with resultant increased production of Th1-type cytokines *in vivo* following TDM challenge.

The granulomatous response is an essential immune component for the containment of *M. tuberculosis* during infection. While the granuloma is critical for protection against disease, it can also act as a double-edged sword. On the one hand, it isolates infectious organisms, preventing dissemination to peripheral organs; yet on the other hand, it results in localized tissue damage which may ultimately lead to disease transmission (Actor *et al.*, 1999; Kobayashi *et al.*, 2001; Russell, 2007). It is therefore essential to learn more about mycobacterial granulomatous pathology and the factors that contribute to its development and maintenance. The most common experimental models used to study *M. tuberculosis* infection are insufficient and fail to reproduce the pathologies observed in man (Hunter *et al.*, 2006a, b). New models that examine the granulomatous response elicited by mycobacterial glycolipid TDM

Fig. 5. Increased IFN- γ and IL-12(p40) mRNA transcript following TDM challenge in lungs of CD4⁺ adoptive transfer mice. RT-qPCR was used to quantify IFN- γ and IL-12 transcript levels in the lungs of mice following TDM challenge. Adoptive transfer of TDM-immunized CD4⁺ cells (black bars) resulted in increased production of IFN- γ and IL-12(p40) transcripts compared to adoptive transfer of naïve CD4⁺ cells (white bars) or that of non-immunized challenge controls (striped bars). Data are normalized to β -actin transcript levels and represented as the mean percentage of β -actin $\pm$ SEM ($n=3$ or 4 mice per group). * $P<0.02$.

may provide novel insights into pathological manifestation; studies using gel matrix technologies are now able to characterize glycolipid-induced leukocyte trafficking (Rhoades *et al.*, 2005). Historically, acute TDM-induced responses to TDM have been well characterized (Behling *et al.*, 1993; Bekierkunst, 1968; Bekierkunst & Yarkoni, 1973; Perez *et al.*, 2000). The lungs of injected mice demonstrate increased production of proinflammatory cytokines, which correlate well with the development of small, focal monocytic granulomas. These initial reports indicated TDM's involvement in the initial phases of granuloma development due to mediators of proinflammatory responses, with at least one strong influence from complement factor C5a (Borders *et al.*, 2005). However, more recent studies have revealed novel characteristics of TDM and its ability to induce hypersensitive (immune-type) granulomas which more closely represent the pathology seen during *M. tuberculosis* infection (Guidry *et al.*, 2004; Hunter *et al.*, 2006a, b; Yamagami *et al.*, 2001). It is likely that the physical nature of TDM plays a role in this response (Rao *et al.*, 2005, 2006). The hypersensitive response to TDM is characterized by development of larger, more complex granulomas, which are often destructive in nature and include a strong lymphocytic component. Moreover, this hypersensitive response is associated with an increased production of Th1-type

Fig. 6. IFN- γ and IL-12 protein production is elevated in lungs of adoptive transfer mice. Significant increases in IFN- γ and IL-12 protein were identified at 3 days post-challenge in the lungs of mice receiving adoptive transfer of TDM-immunized CD4⁺ cells (black bars), compared to those receiving naïve CD4⁺ cells (white bars) or the non-immunized challenge control (striped bars). Data are means $\pm$ SEM ($n=4$ mice per group). * $P<0.05$.

cytokines, as well as proinflammatory cytokines, that are essential to granuloma maintenance. These models suggest a potential role for TDM (and perhaps other mycobacterial glycolipids) in the later stages of disease. The TDM-induced hypersensitive granulomatous response may therefore serve as an alternative method to study lung pathologies relating to disease.

Development and maintenance of the granulomatous response is highly dependent on the host's ability to launch an effective cell-mediated immune response. Individuals with impaired T cell-mediated responses often progress to active disease, leading to transmission or even death. This is clearly illustrated by the increased susceptibility of AIDS patients to active *M. tuberculosis* infection. These patients exhibit severe reduction in responding CD4⁺ cells, characterized as the most important phenotypic population for protection against *M. tuberculosis*. Mice deficient in CD4⁺ cells exhibit severe defects in granuloma formation, resulting in increased bacterial load and death (Caruso *et al.*, 1999). Additionally, depletion of CD4⁺ T cells has been shown to lead to reactivation of latent disease (Scanga *et al.*, 2000). Despite the obvious importance of these cells during infection, their reactivity to the surface glycolipid TDM has not previously been established. Previous immunohistochemical analysis revealed increased presence of CD3⁺ T cells surrounding hypersensitive granulomatous lesions, suggesting recruitment of T cells for this response (Guidry *et al.*, 2004; Yamagami *et al.*, 2001). Adoptive transfer of TDM-immunized CD4⁺ T cells in the experiments reported

here confirmed the importance of this cell phenotype in development of the TDM-induced hypersensitive granulomatous response.

Production of proinflammatory cytokines is important in the cellular recruitment and regulation of the immune response directed against *M. tuberculosis*. Activated macrophages produce IL-12, encouraging the differentiation and activation of T cells into the Th1 phenotype (Seder *et al.*, 1993). IFN- γ , secreted by activated T cells, helps to promote Th1 differentiation as well as activating macrophages to effectively kill organisms. In the hypersensitive response to TDM, both of these cytokines peak at the first histological manifestation of granuloma development. Previous studies show that STAT4-deficient mice fail to produce Th1 cytokines and are incapable of developing hypersensitive granulomas to TDM, suggesting that TDM induces Th1 cytokines in a STAT4-dependent manner (Oiso *et al.*, 2005). Certainly, TDM by itself can invoke a strong cell-mediated immune response (Davidsen *et al.*, 2005); immunization with TDM adjuvant allows generation of IFN- γ producing CD4⁺ T cells to vaccine incorporated antigens. These studies, and others (Guidry *et al.*, 2004, 2006; Yamagami *et al.*, 2001), indicate that this also occurs when TDM is the antigen. It is demonstrated here that adoptive transfer of TDM-immunized CD4⁺ T cells results in an upregulation of both IL-12 and IFN- γ following challenge, suggesting that TDM-induced production of these cytokines may play a role during infection. Moreover, *in vitro* analysis of the recall response to TDM by immunized CD4⁺ cells revealed an elevated production of IFN- γ as well as the T cell differentiating cytokine IL-12 by these cells. Together, these experiments indicate the potential for TDM-specific CD4⁺ cells in the production of cytokines essential to maintenance of the granulomatous response.

The experiments outlined here allow the consideration of T-cell responses generated which are specific for TDM moieties. However, an alternative possibility is that TDM affects antigen-presenting cell activation events, leading to secondary responses that dictate CD4⁺ cell activities. Indeed, TLR-2 and TLR-4 are important to control infection (Reiling *et al.*, 2002) and mycobacterial lipids are known to be ligands for TLRs, triggering production of IL-12 and other factors. As suggested by Roura-Mir *et al.* (2005), mycobacterial cell wall lipids may provide two distinct signals. One signal may lead to the activation of lipid-reactive T cells while the other may act as a lipid adjuvant to activate antigen-presenting cells through TLR-2. With that said, although TLR-2 and TLR-4 play a role with other mycobacterial lipids, the TDM response to active macrophages appears to be independent of those TLRs (Geisel *et al.*, 2005).

Earlier studies revealed that absence of a strong T cell-mediated immunity correlated with the inability to form cohesive granulomas after TDM challenge. Furthermore, mice deficient in CD1d were unable to develop TDM-associated pathologies, whether acute or hypersensitive in

nature (Actor *et al.*, 2001; Guidry *et al.*, 2004). The current study indicated that CD4⁺ T cells may be generated to the mycobacterial lipid antigen. It will be of major interest to further define the exact phenotype of these CD4⁺ cells, and examine the potential for natural killer T cells as mediators of response. Likewise, future experiments will investigate the activation status of the CD4⁺ cell, examining activation and memory markers. At this time it remains unclear if these CD4⁺ cells are CD1-restricted in response. What is shown in these experiments is that CD8⁺ T cells are not the reactive phenotype in this model, with little IFN- γ and IL-2 produced in response to TDM with this population. Additionally, *in vivo* CD8⁺ T cells failed to transfer the hypersensitive pathology.

These observations will require further study to dissect the true importance of the immune response to TDM during active infection, in an environment that contains not only glycolipid antigens but multiple immunogens that are present during infection. Past studies indicate that TDM has properties that influence innate immune responses. It appears that TDM may also play an important role in adaptive responses contributing to maintenance and maturation of the granuloma.

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