CD3⁺ cells transfer the hypersensitive granulomatous response to mycobacterial glycolipid trehalose 6,6'-dimycolate in mice

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The granulomatous response is the characteristic histological feature of *Mycobacterium tuberculosis* infection that is essential for organism containment. Trehalose 6,6'-dimycolate (TDM), a cell-wall glycolipid present on most mycobacterial species, has been implicated in the pathogenesis of *M. tuberculosis* infection. TDM has potent immunoregulatory and inflammatory properties, and can be used to model granulomatous reactions that mimic, in part, pathology caused during active infection. This study examined the hypersensitive granulomatous response, focusing on cellular responses specific to TDM. Lungs from mice immunized with TDM emulsion demonstrated exacerbated histological damage, inflammation, and lymphocytic infiltration upon subsequent challenge with TDM. Splenocytes recovered from these mice demonstrated significant interferon (IFN)- γ production during recall response to TDM, as well as increased production of proinflammatory mediators (tumour necrosis factor- α , interleukin-6 and macrophage inflammatory protein-1 α). The exacerbated response could be adoptively transferred to naïve mice.

Administration of non-adherent lymphocytes or purified CD3⁺ cells from TDM-immunized mice led to increased inflammation, lymphocytic infiltration, and vascular endothelial cell damage upon challenge with TDM. Recipient mice that received immunized CD3⁺ lymphocytes demonstrated significant increases in Th1-type cytokines and proinflammatory mediators in lung tissue following TDM challenge. When CD1d^{-/-} mice were immunized with TDM, they failed to generate a specific IFN- γ response, suggesting a role for this molecule in the generation of hypersensitivity. These experiments provide further evidence for the involvement of TDM-specific CD3⁺ T cells in pathological damage elicited during *M. tuberculosis* infection.

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INTRODUCTION

Historically, the association of delayed-type hypersensitivity (DTH) with resistance to *Mycobacterium tuberculosis* infection has been debated (Reggiardo & Middlebrook, 1974; Rook, 1978; Hussein *et al.*, 1987). However, it is clear that pathological damage during infection is highly dependent upon the host's ability to launch a cell-mediated immune (CMI) response. A major hallmark of this response is the formation of granulomas, nodule-like masses of cells consisting primarily of activated macrophages and T

lymphocytes. Granulomas function to contain the infection and prevent dissemination, yet at the same time contribute to tissue damage within the host (Actor *et al.*, 1999; Algood *et al.*, 2003). Antigen-specific T cells are essential for the maintenance of the granuloma, and secrete cytokines which help to activate macrophages and increase bacterial killing (Dannenberg, 1991; Hahn & Kaufmann, 1981).

Despite initiation of the host immune response, *M. tuberculosis* employs various mechanisms to avoid detection and destruction within the macrophages. The lipid-rich cell wall of *M. tuberculosis* contains several factors that contribute to its survival within the host (Karakousis *et al.*, 2004). Trehalose 6,6'-dimycolate (TDM), a glycolipid located in the mycobacterial cell wall, is an important component of this defence mechanism. The presence of TDM on the surface of mycobacteria has been shown to be

Abbreviations: DTH, delayed-type hypersensitivity; H&E, haematoxylin and eosin; IFN, interferon; IL, interleukin; LWI, lung weight index; MIP-1 α , macrophage inflammatory protein-1 α ; OVA, ovalbumin; TDB, 6,6'-dibehenoyl- α,α' -trehalose; TDM, trehalose 6,6'-dimycolate; TNF, tumour necrosis factor.

crucial for inhibition of phagosome–lysosome fusion events, and survival of the organism following macrophage engulfment (Indrigo *et al.*, 2002, 2003; Silva *et al.*, 1985). Recent studies have shown that *M. tuberculosis* deficient in cyclopropane synthase (*pcaA*), and therefore lacking cyclopropane modification of TDM, exhibits decreased virulence within the host, as well as production of TDM that is less effective at stimulating granuloma formation *in vivo* and activating macrophages *in vitro* (Glickman *et al.*, 2000; Rao *et al.*, 2005). Alterations in the physical properties of TDM on the surface of the organisms lead to distinct changes in pathology upon infection (Rao *et al.*, 2006).

TDM is capable of inducing both non-immune (Behling *et al.*, 1993; Geisel *et al.*, 2005; Perez *et al.*, 2000; Seggev *et al.*, 1982) and immune-type granulomas in susceptible mice (Bekierkunst & Yarkoni, 1973; Guidry *et al.*, 2004; Yamagami *et al.*, 2001). The acute response to TDM is characteristic of non-immune granulomas often observed in early tuberculosis infection, consisting primarily of macrophages with relatively few lymphocytes. Seggev *et al.* (1984) have demonstrated that despite the limited presence of lymphocytes, the acute response to TDM is dependent on the involvement of T cells. *In vivo*, TDM initiates a proinflammatory response, triggering monocytic cells to produce various cytokines and chemokines that contribute to granuloma formation (Bekierkunst, 1968; Perez *et al.*, 2000). On the other hand, an acquired immune response to TDM more closely resembles the pathology seen during post-acute *M. tuberculosis* infection, consisting of T lymphocytes surrounding activated macrophages with a necrotic centre (Guidry *et al.*, 2004). T cells reactive to mycobacterial antigens are essential for regulating responses to *M. tuberculosis* long after acute infection has been controlled (Scanga *et al.*, 2000). The contribution of TDM-specific T cells in this model has not yet been identified.

The focus of this study was to further define the TDM-induced hypersensitive granulomatous response. Identification of the cell phenotype responsible for the formation of hypersensitive granulomas to TDM, and understanding of the generation of that response, may provide further insights into the regulation of immunopathology in *M. tuberculosis* infection.

METHODS

Immunization protocol. Six-to-eight-week-old female BALB/c (CD1d^{+/+}) and BALB/c CD1d knockout (CD1d^{-/-}) mice (Jackson Laboratory), housed in micro-isolator cages under specific pathogen-free conditions, were immunized according to previously published methods (Guidry *et al.*, 2004; Yamagami *et al.*, 2001). Each mouse received, subcutaneously at the base of the tail, 100 µl water–oil (w/o) emulsion containing 100 µg TDM from *M. tuberculosis* (Sigma), emulsified with 100 µg methylated BSA (mBSA; Promega) in 50 µl PBS and 50 µl complete Freund's adjuvant (CFA; Sigma). Mice were then boosted 28 days later with identical preparations. Control groups were either immunized with emulsions lacking the addition of TDM (CFA-immunized), or those containing 100 µg of the irrelevant antigen ovalbumin (OVA; Sigma), formulated in TiterMax adjuvant substituted for CFA (OVA-immunized).

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.

Culture of splenocytes *in vitro*. Single-cell suspensions of naïve and TDM-immunized splenocytes were obtained from at least three mice per group. Cells were plated out at a concentration of 1×10^6 cells per well, and incubated at 37 °C in 5 % CO₂ overnight. TDM- and BSA-coated polystyrene beads (0.78 µm diameter) were prepared as described by Indrigo *et al.* (2003), then added at a 10:1 bead-to-cell ratio. mBSA was not used in the recall response, due to the formulation and complex aggregation of mBSA-coated polystyrene beads. As a control, cells were also incubated with medium alone. Specificity of response was examined using beads coated with 6,6'-dibehenoyl- α,α' -trehalose (trehalose dibehenate; TDB), a synthetic analogue of TDM containing shorter fatty acid chains (Retzinger *et al.*, 1981; Indrigo *et al.*, 2003). Supernatants were collected at 48 h, and analysed in triplicate by ELISA.

Adoptive transfer. Non-adherent and CD3⁺ T cells were purified from immunized mice at 2 weeks post boost, or from negative-control (non-immunized) mice. Animals were sacrificed, and single splenocyte cell suspensions were prepared. Red blood cells were lysed using BioWhittaker ACK lysis buffer (Cambrex). Cells were then plated and incubated at 37 °C for 1 h to remove the adherent cell population. The non-adherent cells were removed and further purified by selecting for CD3⁺ T cells via high-affinity negative selection, using T cell-enrichment columns (R&D Systems) following the manufacturer's suggested protocol. Flow analysis revealed >90 % purity of the CD3⁺ population for all experiments. Naïve 6-week-old female BALB/c mice were used as recipients for adoptive transfer of cells obtained from control or TDM-immunized mice, receiving intraperitoneal injection of 2×10^7 non-adherent cells or 5×10^6 CD3⁺ T cells in 0.5 ml PBS.

Induction of pulmonary granulomas. Two days following adoptive transfer, mice were injected with 100 µl TDM water–oil–water (w/o/w) emulsion, prepared as previously described (Guidry *et al.*, 2004; Yamagami *et al.*, 2001), administered by intravenous tail vein injection. Each injection consisted of 100 µg TDM emulsified in 3.2 µl incomplete Freund's adjuvant (Sigma), prepared by grinding in a homogenizer tube with a Teflon pestle. A w/o emulsion was formed by addition of 3.2 µl PBS, and mixing with the oil phase. Finally, 93.6 µl PBS containing 0.2 % Tween-80 was added to complete the outer phase of the w/o/w emulsion.

Histology, morphometry and immunohistochemistry. Mice (three or four per group) were sacrificed by cervical dislocation at days 3, 4 or 7, and weighed. Lungs were perfused via the right ventricle with 1 ml PBS with 1 mM EDTA. Lungs were visually examined for inflammation, and total lung weights were obtained in order to calculate lung weight index (LWI). The LWI is used as a measure of overall inflammatory intensity by investigators in the field of mycobacteriology (Borders *et al.*, 2005; Collins *et al.*, 1975; Guidry *et al.*, 2004).

$$\text{LWI} = \sqrt{\frac{\text{Lung weight (mg)}}{\text{Mouse weight (g)/10}}} \times 10$$

For histological examination, the left lobe was excised and fixed in 10 % buffered formalin, and embedded in paraffin blocks. Sections (5 µm thick) were prepared and stained with haematoxylin and eosin following standard procedures. The right lung was separated from the left by severing the bronchus distal to the ligature; the right lobe was weighed, minced and transferred to 2 ml Dulbecco's modified Eagle's medium (Sigma) with 10 % heat-inactivated fetal bovine serum (Sigma) and 50 ng penicillin and gentamicin ml⁻¹,

then incubated at 37 °C for 4 h. Cells were removed by centrifugation at 3000 r.p.m. for 10 min, and the resulting supernatant was stored at -20 °C until analysis by ELISA.

ELISA quantification of cytokines and chemokines. Levels of interleukin (IL)-6, IL-12 p40, interferon (IFN)- γ , IL-2, tumour necrosis factor (TNF)- α and macrophage inflammatory protein (MIP)-1 α in cell supernatants were measured by sandwich ELISA using DuoSet kits (R&D Systems) specific for each cytokine or chemokine (Actor *et al.*, 2002). ELISAs were performed according to the manufacturer's directions. A_{570} and A_{450} were read spectrophotometrically on a Spectra Max 250 ELISA plate reader (Molecular Devices). The average of duplicate wells was calculated based on standard curves using recombinant murine standards. The limit of detection was 5–15 pg ml⁻¹ for all assays.

Statistical analysis. All data are presented as the mean $\pm$ SEM. Normally distributed data were analysed by the unpaired two-tail *t* test, where differences between means were compared within groups; differences were considered significant at $P \leq 0.05$.

RESULTS

Induction of recall responses to TDM

Hypersensitive responses to TDM are inducible in BALB/c mice following subcutaneous injections of glycolipid-containing emulsions (Guidry *et al.*, 2004). Whole splenocytes from naïve and TDM-immunized mice were analysed *in vitro* for cytokine and chemokine production in response to TDM- or BSA-coated beads, or medium alone. TDM-immunized cells produced significant amounts of IFN- γ (83.0 ± 1.73 pg ml⁻¹) following incubation with TDM-coated beads, relative to naïve cells that produced little IFN- γ (6.0 ± 1.01 pg ml⁻¹) (Fig. 1). The response was specific to TDM; cells from immunized mice produced substantially less IFN- γ when incubated with BSA-coated beads

(35.7 ± 5.07 pg ml⁻¹). Proinflammatory mediators TNF- α , IL-6 and MIP-1 α were all significantly elevated ($P < 0.01$) in a TDM-specific manner, compared to the levels in control splenocytes. Immunization with TDM elicited antigen-specific responses, with significant levels of cytokines and chemokines secreted upon recall to TDM.

Adoptive transfer of TDM-specific non-adherent cells transfers the hypersensitive response

It has previously been demonstrated that mice exhibit greater granulomatous inflammation and pathological damage upon secondary encounters with TDM antigen (Bekierkunst & Yarkoni, 1973; Guidry *et al.*, 2004; Yamagami *et al.*, 2001). To further investigate the hypersensitive response to TDM, and identify the population of cells responsible for the increased pathological damage, adoptive transfer experiments were performed. Naïve recipients received intraperitoneal injections of 2×10^7 non-adherent cells from TDM-immunized or naïve control mice. Two days later, adoptively transferred mice were challenged with TDM, and lungs were monitored for pathology characteristic of a hypersensitive response. At day 4 post challenge, mice receiving naïve non-adherent cells developed small focal granulomas typical of the acute response to TDM. By day 7, these mice demonstrated an increase in granuloma size, while overall lung architecture remained intact (Fig. 2, upper left). The pathology seen was identical to that in naïve mice receiving TDM for the first time (Fig. 2, lower left). In contrast, mice receiving non-adherent cells from TDM-immunized donors exhibited increased inflammation, cellular infiltration, and tissue destruction at day 4. The pathological damage intensified by 7 days post challenge, with obvious reduction of open

Fig. 1. TDM-specific cytokine and chemokine production from immunized mice. Splenocytes from both naïve (open bars) and TDM-immunized (closed bars) mice were incubated with TDM- or BSA-coated beads, or control medium. TDM-immunized splenocytes demonstrated significant TDM-specific production of IFN- γ , TNF- α , IL-6 and MIP-1 α . Data represent the results from three experiments and are expressed as mean $\pm$ SEM (all $P < 0.01$; *comparison of immunized versus naïve response to TDM-coated beads; **response of immunized groups to TDM- versus BSA-coated beads).

Fig. 2. Adoptive transfer of hypersensitive response. Naïve mice receiving adoptive transfer of non-adherent cells from naïve or TDM-immunized mice were challenged 2 days later with TDM. By day 7, the group receiving the TDM-immunized cells (upper right) showed aggressive pathology characterized by increased lymphocytic infiltrates, similar to that in control mice that were immunized and challenged with TDM (lower right). Mice receiving non-adherent cells from the non-immunized naïve group (upper left) demonstrated typical acute pathology in response to TDM at day 7, comparable to that in mice receiving TDM challenge only (lower left), with restricted development of small mononuclear and focal granulomas. Sections are representative of three mice per group. Haematoxylin and eosin (H&E) stain, $\times 40$. AT, adoptive transfer. Bar, 250 μm .

alveolar spaces, and widespread inflammation being apparent (Fig. 2, upper right). Gross appearance of lungs demonstrated small, focal haemorrhagic petechiae. Higher magnification revealed that adoptive transfer of TDM-immunized cells resulted in lymphocytic cuffing, thickening of vascular walls, and extensive damage to the endothelial cell lining of vascular walls (Fig. 3). Histological manifestation was similar to that of positive-control mice that were immunized and challenged with TDM (Fig. 2, lower right).

The increased inflammatory response in these mice was evident when comparing LWI between the groups. LWI at day 7 was significantly greater ($P < 0.05$) in mice receiving immunized cells (1.543 ± 0.107), compared to that in mice receiving naïve cells (1.160 ± 0.040), and that in naïve control mice (0.880 ± 0.026). These experiments confirm that adoptive transfer of TDM-immunized non-adherent cells was sufficient to transfer the hypersensitive granulomatous response to TDM.

TDM hypersensitive pathology is dependent on the CD3^+ T cell population

The hypersensitive granulomatous response to TDM is characterized by lymphocytic cuffing, previously identified as CD3^+ lymphocytes (Guidry *et al.*, 2004). To determine the relative importance of T cells to this response, purified CD3^+ T cells were adoptively transferred into mice, which were subsequently challenged with TDM. As early as 3 days post challenge, differences in gross pathology were observed between mice receiving TDM-immunized cells and those receiving naïve cells. Gross pathology of lungs revealed that mice receiving TDM-immunized CD3^+ cells developed

focal petechial haemorrhages (Fig. 4, right, arrows), while those receiving naïve CD3^+ cells were normal in outward appearance (Fig. 4, left).

Histological analysis was performed to determine the relative roles of CD3^+ cells, compared to those of non-adherent populations (identified above), in the generation

Fig. 3. Pathological damage caused by adoptive transfer of TDM-immunized cells. Vascular wall and endothelial cell damage was evident in mice receiving TDM-immunized cells. At day 7 post challenge, vascular occlusion and endothelial cell destruction (asterisk) and lymphocyte infiltration (arrows) were readily apparent. Challenge mice that received adoptive transfer of non-immunized naïve cells did not exhibit similar pathological damage. Section is representative of three mice per group. H&E stain, $\times 200$. AT, adoptive transfer. Bar, 50 μm .

Fig. 4. Gross pathology of lungs following adoptive transfer of CD3⁺ cells. Three days post TDM challenge, lungs from mice adoptively transferred with CD3⁺ cells from naïve (left) or TDM-immunized mice (right) were examined. Multiple petechial haemorrhages were observed in mice receiving cells from the immunized group (arrows), as compared to adoptive transfer from the naïve cell group. Results are representative of lungs from four mice per group. AT, adoptive transfer.

of hypersensitive responses to TDM. Transfer of either naïve population (CD3⁺ or non-adherent) did not exacerbate the response (Fig. 5, upper), while transfer of TDM-immunized cells led to development of aggressive and destructive events (Fig. 5, lower). At day 3 post challenge, the pathology following adoptive transfer of both non-adherent and CD3⁺ naïve cells was typical of the acute response to TDM (Fig. 5, upper), with only small monocytic granulomas being apparent. In contrast, histological comparison of lungs from mice given CD3⁺ cells (immunized) with those given non-adherent splenocytes (immunized) revealed similar aggressive histopathology following TDM challenge (Fig. 5, lower). Both groups resulted in development of marked vascular occlusion, lymphocytic cuffing, and cellular infiltration. By 7 days post TDM administration, responses were further exacerbated, leading to even greater occlusion and necrotic events within and surrounding vascular tissue beds (Fig. 6). These experiments confirmed the importance of the CD3⁺ population to TDM-induced pathology indicative of hypersensitive granulomas.

Transfer of TDM-immunized CD3⁺ cells results in increased protein production in lungs of mice

Cytokine and chemokine production correlated with the development of the hypersensitive granulomatous response,

with peak production at 3 days post TDM challenge, as first shown by Yamagami *et al.* (2001). Comparisons in response were made between mice receiving TDM-immunized non-adherent donor cells and those receiving TDM-immunized CD3⁺ donor cells (Fig. 7). Cytokine and chemokine profiles were elevated in both groups. Specifically, Th1-type cytokines IFN- γ , IL-2 and IL-12 were significantly greater in lungs of mice that received purified CD3⁺ cells ($P < 0.05$), compared directly to those receiving only the non-adherent cells. Similarly, significant increases ($P < 0.05$) in inflammatory cytokines TNF- α and IL-6, as well as chemokine MIP-1 α , correlated with adoptive transfer of CD3⁺ cells from TDM-immunized mice. This observation illustrates a critical role for CD3⁺ T cells in the development of the hypersensitive granulomatous response to TDM.

Specificity of the TDM-hypersensitive response

To ensure that the effects observed were due to a TDM-specific response (and not due to transfer of activated cells alone), control mice were immunized and boosted with emulsions containing an unrelated antigen, OVA. At day 3 post challenge, mice receiving CD3⁺ cells adoptively transferred from OVA-immunized mice (Fig. 8, upper left) exhibited only an acute granulomatous response,

Fig. 5. Adoptive transfer of CD3⁺ cells from TDM-immunized mice conferred hypersensitive response at day 3 upon TDM challenge. Comparison of lung histology revealed that adoptive transfer of 5×10^6 CD3⁺ cells from immunized mice (lower right) produced hypersensitive pathology similar to that with adoptive transfer of non-adherent sensitized cells (lower left). Control transfer of non-adherent cells (upper left) or CD3⁺ cells (upper right) from naïve mice did not exacerbate the response. Histological sections from CD3⁺-transferred groups were from lungs described in Fig. 4. Sections are representative of three or four mice per group. H&E stain, $\times 100$. AT, adoptive transfer. Bar, 100 μ m.

Fig. 6. Immunized $CD3^+$ cells were sufficient for transfer of pathology upon subsequent TDM challenge. Transfer of $CD3^+$ cells alone from TDM-immunized mice (right) was sufficient for development of a hypersensitive response to TDM challenge, identical to that with adoptive transfer of the complete non-adherent cell population (left). Increased inflammation, vascular occlusion and endothelial cell damage, and lymphocytic infiltration were evident in both adoptive transfer groups at day 7 post TDM challenge. Lower panels show enlarged images centred on regions of vascular occlusion and damage ($\times 200$). Sections are representative of three or four mice per group. H&E stain, $\times 40$ and $\times 200$. AT, adoptive transfer. Bars: upper panels, 250 μm ; lower panels, 50 μm .

with less overall inflammation than that in the TDM-immunized adoptive transfer groups described above. By day 7, the granulomas increased slightly in size (Fig. 8, lower left), but remained primarily monocytic in nature, with a

noticeable lack of phenotypic lymphocytes observed. For comparison, at 3 days post challenge, mice receiving adoptive transfer of $CD3^+$ cells from mice immunized with the TDM emulsion (Fig. 8, upper right) developed

Fig. 7. Increased cytokine and chemokine production in lungs of mice adoptively transferred with $CD3^+$ TDM-immunized cells. Examination of responses in lungs of mice at 3 days post TDM challenge revealed that mice receiving adoptive transfer of either TDM-immunized non-adherent (Imm NA; closed bars) or purified $CD3^+$ cells (Imm CD3; open bars) produced Th1-type and proinflammatory mediators. The group receiving $CD3^+$ cells demonstrated significantly higher responses than the non-adherent adoptive transfer group. Data are representative of four mice per group, and are expressed as mean $\pm$ SEM ($*P < 0.05$).

Fig. 8. Specificity of the TDM hypersensitive response. Naïve mice receiving adoptive transfer of CD3⁺ cells from mice immunized with a non-tuberculous-related OVA antigen (left) or TDM (right) were examined for response at 3 days (upper panel) and 7 days (lower panel) after challenge with TDM. Adoptive transfer of CD3⁺ cells from the non-related OVA antigen led to a monocytic response alone with limited pathology. Examination of lungs from the group receiving CD3⁺ cells from TDM-immunized mice demonstrated severe responses with evidence of lymphocytic infiltrates, vascular cuffing and endothelial cell damage by 7 days (arrows). Sections are representative of six mice per group. H&E stain, $\times 100$. AT, adoptive transfer. Bar, 100 μm

detectable and increased lung pathology. As seen above, at 7 days post TDM challenge, there was an increase in inflammation and destructive processes within the lung parenchyma of mice receiving cells transferred from TDM-immunized mice (Fig. 8, lower right), with small pockets of lymphocytes identified in the lungs (arrows). The group receiving TDM-immunized cells demonstrated large areas of defined lymphocytic cuffing and intraendothelial and intravascular infiltration in regions of pathology (Fig. 8, lower left). Overall, this indicates that the hypersensitive responses identified in these experiments are specific for TDM, and separate from the residual responses induced by transfer of activated T cells generated in mice to an unrelated antigen.

Contribution of CD1d to generation of recall response to TDM

Previous studies have indicated a difference in innate and hypersensitive responses to TDM in mice lacking CD1d (Actor *et al.*, 2001; Guidry *et al.*, 2004). To examine the role of CD1d in generation of the IFN- γ -inducible hypersensitive response, CD1d^{-/-} mice were immunized and boosted with TDM, and recall responses were assessed (Fig. 9). Splenocytes were analysed *in vitro* for IFN- γ production in response to TDM- or BSA-coated beads, or medium alone. In addition, specificity was addressed through analysis of response to TDB, a synthetic analogue of TDM containing shorter fatty acid chains. As seen in the above experiments, splenocytes from control TDM-immunized mice produced significant amounts of IFN- γ ($360 \pm 116.7 \text{ pg ml}^{-1}$) following incubation with TDM-coated beads, but there was no substantial production of IFN- γ in response to either BSA-coated beads ($73 \pm 47.0 \text{ pg ml}^{-1}$) or to the TDB synthetic analogue ($11 \pm 1.3 \text{ pg ml}^{-1}$). However, splenocytes isolated from the TDM-immunized CD1d^{-/-} mice were significantly deficient in IFN- γ ($86 \pm 7.4 \text{ pg ml}^{-1}$) production

when incubated with TDM-coated beads, similar to the responses to both BSA- ($86 \pm 3.1 \text{ pg ml}^{-1}$) and TDB-coated beads ($95 \pm 3.1 \text{ pg ml}^{-1}$).

DISCUSSION

The requirement for strong DTH and cell-mediated immunity to protect against active tuberculosis remains

Fig. 9. Lack of TDM-specific IFN- γ production from CD1d^{-/-} immunized mice. Splenocytes from both TDM-immunized wild-type (closed bars) and CD1d^{-/-} (open bars) mice were incubated with TDM-, BSA- or TDB-coated beads. Splenocytes from the TDM-immunized BALB/c mice demonstrated specificity of response, while immunized CD1d^{-/-} mice failed to demonstrate significant TDM-specific production of IFN- γ in response to TDM or the shorter-chain analogue TDB. Data are representative of the results from two experiments, measured in duplicate, and are expressed as mean $\pm$ SEM (* $P < 0.05$; comparison of immunized BALB/c splenocyte response to TDM beads against all other groups).

an area of active interest. Recent studies suggest a role for T cells in hypersensitive responses to mycobacterial TDM (Guidry *et al.*, 2004; Yamagami *et al.*, 2001); however, the confirmation of glycolipid antigen-specificity has not yet been firmly established. Our findings confirm that it is possible to generate a CD3⁺ memory response to TDM, and demonstrate the importance of non-adherent cell populations, specifically T cells, in generating hypersensitive granulomas. Following TDM challenge, the lungs of mice receiving adoptive transfer of TDM-immunized CD3⁺ cells demonstrated gross haemorrhages and widespread inflammation, including the development of large destructive granulomas cuffed by numerous lymphocytes. These mice also exhibited increased proinflammatory and Th1-type cytokine and chemokine protein production within the lungs. Earlier, Bekierkunst *et al.* (1973) reported that multiple injections of TDM/oil emulsion are incapable of generating hypersensitive granulomas in mice. However, Yamagami *et al.* (2001) later demonstrated that immunization with emulsion containing CFA and mBSA can generate hypersensitive granulomas following TDM challenge. In the experiments described here, immunization with the addition of purified TDM within the emulsion resulted in a more aggressive hypersensitive pathology post TDM challenge than that of CFA alone (data not shown). Moreover, this study confirms that it is possible to generate TDM antigen specificity through vaccination. TDM-immunized splenocytes exhibited significantly increased cytokine production upon secondary encounter with TDM antigen. This is believed to be the first study to directly link TDM-specific CD3⁺ T cells to pathology associated with the hypersensitive granulomatous response to TDM.

The importance of lipids to the survival and virulence of *M. tuberculosis* has been known for some time. In the early 1950s, Bloch identified a toxic lipid he termed 'cord factor' (later revealed to be TDM), which, when extracted from the surface of virulent mycobacteria, converted them to an avirulent form (Bloch, 1950; Noll *et al.*, 1956). Since then, there has been much controversy over the role of TDM in organism virulence (Hunter *et al.*, 2006). Historically, TDM has been studied for its adjuvant properties, and not necessarily as an immunogen. However, recent findings suggest that the pathophysiological response to TDM manifests itself in multiple ways. TDM involvement in toxicity, inhibition of phagosomal maturation, and primary and hypersensitive granulomatous responses is only now being fully appreciated (Bloch, 1950; Guidry *et al.*, 2004; Hunter *et al.*, 2006; Indrigo *et al.*, 2002; Lima *et al.*, 2001; Seggev *et al.*, 1984; Spargo *et al.*, 1991; Yamagami *et al.*, 2001; Yarkoni & Rapp, 1978). The lipid-rich cell walls of mycobacteria have proven to be an important defence mechanism aiding their survival within the host. Trehalose mycolates (Geisel *et al.*, 2005) and various other lipids, such as the mannose-capped lipoarabinomannan, phthiocerol dimycocerosate, and the 19-kDa lipoprotein, have all been identified as potential contributors to the pathogenesis of mycobacterial infection (Karakousis *et al.*, 2004). In fact,

pharmaceuticals used to successfully treat tuberculosis, such as isoniazid, ethionamide and pyrazinamide, do so by inhibiting mycolic acid or fatty acid synthesis, thereby altering cell-wall virulence (Schroeder *et al.*, 2002). Clearly, lipids are important for survival of the bacilli within the host; however, TDM is the only mycobacterial glycolipid to date demonstrated to induce hypersensitive responses influencing both granuloma formation and activation of cellular responses.

Cytokine and chemokine production during *M. tuberculosis* infection, and initiation and maintenance of the granulomatous response, have been readily established. Arguably, the most important cytokine in this process is the Th1-type cytokine IFN- γ , produced by activated CD3⁺ T cells. The importance of TDM-specific IFN- γ production should not be understated. Mice deficient in IFN- γ production are unable to control or contain infection, and succumb to normally sublethal doses of bacteria (Cooper *et al.*, 1993). TNF- α knockout mice also fail to form proper granulomas, and are unable to control infection (Oswald *et al.*, 1999; Roach *et al.*, 2002; Takimoto *et al.*, 2006). Recently, the importance of TNF- α in maintenance of the TDM-induced granulomatous response has become clearer. Patients with rheumatoid arthritis, receiving treatment with anti-TNF- α , who were latently infected with *M. tuberculosis*, developed clinical disintegration of granulomas, and progressed to active disease (Dimakou *et al.*, 2004; Gomez-Reino *et al.*, 2003). In this current study, TDM-specific production of IFN- γ , as well as proinflammatory mediators TNF- α , IL-6 and MIP-1 α , was demonstrated *in vitro* by TDM-immunized cells. The *in vivo* experiments showed an upregulation of both IL-12 and Th1-type cytokine IFN- γ production in the lung following adoptive transfer of CD3⁺ TDM-immunized T cells. Oswald *et al.* (1977) have demonstrated *in vitro* that TDM is a powerful inducer of IL-12, contributing towards priming of macrophages for antigen presentation. Overall, this would indicate a link for TDM in the generation of acute responses that develop into acquired immune reactivity.

TDB has been used to address the specificity of the response. It has been demonstrated that the biological activities of TDM are dependent on the physical conformation of the molecule (Retzinger *et al.*, 1981), and that the analogue TDB is insufficient to elicit macrophage responses such as those seen towards TDM (Indrigo *et al.*, 2003). This is consistent with recent work showing that mycolic acid structure correlates with survival *in vivo*; *M. tuberculosis* mutants unable to cyclopropanate or oxygenate mycolic acids fail to persist within mice (Dubnau *et al.*, 2000; Glickman *et al.*, 2000). In our studies, immunization with TDM did not elicit an IFN- γ response to TDB, thus showing specificity of response. Although not antigenic, other investigators have shown that the analogue TDB retains partial immunogenic effects similar to those of TDM (Pimm *et al.*, 1979). While BCG vaccine experiments indicate that TDB can be an effective adjuvant when mixed with dimethyl dioctadecyl

ammonium bromide, it has not been proven to work alone, and appears to elicit responses to the incorporated formulated protein antigens (Holten-Andersen *et al.*, 2004).

The precise mechanism for the generation of CD3⁺ cells reactive to TDM in this hypersensitive model remains unresolved. TDM is an atypical glycolipid antigen with large hydrophobic residues, theoretically capable of interacting with the non-classical molecule CD1. Human CD1b, which has been extensively studied for its ability to bind mycobacterial antigens and stimulate T-cell responses, exhibits striking similarities to murine CD1d, in its intracellular trafficking patterns and large hydrophobic antigen-binding grooves (Skold & Behar, 2005). It is not yet clear what role CD1d plays in mycobacterial immunity, especially since CD1d-restricted T cells that specifically recognize mycobacterial antigens have not been identified. While CD1d^{-/-} mice infected with *M. tuberculosis* do not succumb to infection faster than do wild-type mice (Behar *et al.*, 1999), other studies have demonstrated that blocking CD1d presentation results in increased lung c.f.u. and decreased Th1-type cytokine production (Szalay *et al.*, 1999). Models which directly examine lipid-specific responses are able to identify a profound difference in both innate and hypersensitive pathological responses to TDM in CD1d^{-/-} mice, when compared to those in CD1d-sufficient controls (Actor *et al.*, 2001; Guidry *et al.*, 2004). Of interest is the work by Ryll *et al.* (2001), which has demonstrated the ability of TDM to deplete natural killer T cells while upregulating CD1d1 on presenting cells, suggesting a role for CD1 in the presentation of TDM. Higher levels of CD1d1 on presenting cells have the potential to activate invariant natural killer T (iNKT) cells, even in the absence of exogenous antigen (Skold *et al.*, 2005). Transfer of activated, but non-specific, iNKT cells may lead to hypersensitive pathology. Although we cannot rule out secondary activation events, the transfer experiments using the OVA-specific CD3⁺ cells make this interpretation unlikely. Unique to the experiments outlined here was the fact that CD1d^{-/-} mice were not able to generate specific splenocytes capable of producing IFN- γ in response to TDM. These findings suggest a need to pursue delineating mechanisms of TDM presentation within the host, further define CD1d involvement in regulating the hypersensitive response, and clarify exact cellular phenotypes involved in mediating pathology.

In addition to potential contributions through CD1 molecules, mycobacterial TDM may play a significant role in the development of DTH during infection, through alternate molecular means. It has recently been demonstrated that TDM can influence T helper responses through the STAT4 signalling pathway; mice lacking STAT4 protein have significantly reduced DTH, granulomatous and Th1 cytokine responses (Oiso *et al.*, 2005). Other investigators have obtained corollary results in which presensitization (immunization) with mycobacterial glycolipids can limit allergic (Th2) responses, and increase relative ratios of

DTH-related cytokines (Sayers *et al.*, 2004). Alternatively, TDM may work through the toll-like receptor- or IL-1 receptor-mediated pathways, as indicated in the altered responses seen in MyD88^{-/-} mice (Geisel *et al.*, 2005).

Previous studies have focused on the potential role of TDM in initiation and activation of the granulomatous response in relation to *M. tuberculosis* infection (Lima *et al.*, 2001; Rao *et al.*, 2005). The current findings suggest that TDM may play a more complex role, contributing to late-stage reactivation events. TDM is the most abundant lipid on the cell wall of mycobacteria, and is easily extractable from the surface of rapidly growing organisms (Belisle *et al.*, 1997; Brennan, 2003; Retzinger *et al.*, 1981). Any decrease in immune function and subsequent expansion of organisms may result in the release of TDM, at which point hypersensitive (pre-existing) TDM-specific CD3⁺ cell responses may lead to exacerbation of pathology, localized tissue destruction, and development of necrotic granulomas. Furthermore, ensuing destructive events provide an opportunity for micro-organism dissemination, either directly or via transport of infected macrophages through draining lymphatics. Indeed, the role of TDM and TDM-immunized responsive cells in reactivation of latent *M. tuberculosis* infection in mice is of great interest and under current investigation.

In order to design more effective drugs and vaccines for the treatment and prevention of disease, it is important to better understand how *M. tuberculosis* interacts with the host immune system. Mycobacterial cell-wall lipids, such as TDM, may be an important key to unlocking this mystery. The results outlined here provide further insights into the regulation of immunopathology in *M. tuberculosis* infection, and offer evidence for involvement of a TDM-specific, cell-mediated response critical for the development of the pathological damage elicited during infection.

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