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A role for tumour necrosis factor-α, complement C5 and interleukin-6 in the initiation and development of the mycobacterial cord factor trehalose 6,6′-dimycolate induced granulomatous response

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Abstract

Trehalose 6,6′-dimycolate (TDM) is a glycolipid component of the mycobacterial cell wall that causes immune responses in mice similar to *Mycobacterium tuberculosis* (MTB) infection, including granuloma formation with production of proinflammatory cytokines. The precise roles of tumour necrosis factor (TNF)-α, complement C5 and interleukin (IL)-6 in the molecular events that lead to the initiation and maintenance of the granulomatous response to TDM have not been fully elucidated. Macrophage proinflammatory responses from wild-type and complement-deficient mice after infection with MTB were assessed, and compared to responses from organisms in which surface TDM had been removed. Removal of TDM abolished proinflammatory responses, markedly so in the complement-deficient macrophages. Mice deficient in TNF-α, C5a and IL-6, along with wild-type C57BL/6 controls, were intravenously injected with TDM in a water-in-oil emulsion, and analysed for histological response and cytokine production in lungs. Wild-type C57BL/6 mice formed granulomas with increased production of IL-1β, IL-6, TNF-α, macrophage inflammatory protein-1α (MIP-1α), IL-12p40, interferon-γ (IFN-γ), and IL-10 protein and mRNA. TNF-α-deficient mice failed to produce a histological response to TDM, with no increases in cytokine production following TDM administration. While C5a-deficient mice exhibited inflammation, they did not form structured granulomas and initially

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had decreased production of proinflammatory mediators. IL-6-deficient mice initiated granuloma formation, but failed to maintain the granulomas through day 7 and demonstrated decreased early production of proinflammatory mediators in comparison to wild-type mice. These data suggest that TNF-α is critical for initiation of the granulomatous response, C5a is necessary for formation of cohesive granulomas, and IL-6 plays a key role in the granuloma maintenance response to mycobacterial TDM.

INTRODUCTION

Mycobacteria cause a wide range of infectious pathologies that are a significant cause of morbidity and mortality. Disease due to *Mycobacterium tuberculosis* (MTB) is responsible for an estimated two million deaths each year (WHO, 2003). Infection is classically described as a pulmonary pathology in which the formation of granulomas results in containment of organisms. However, disseminated disease may also occur in which any organ system can be infected, including the kidneys, bones, central nervous system and those of the digestive tract (Cagatay *et al.*, 2004). The mechanisms underlying protective granuloma formation which lead to the prevention of disseminated disease have not been fully elucidated. In light of the increasing number of therapeutics being developed to target cytokines in the treatment of chronic immune-mediated disease, it is clinically relevant to understand the role these molecules play in the initiation and maintenance of the granulomatous response.

Tumour necrosis factor-α (TNF-α) is thought to be the major cytokine responsible for the formation and maintenance of mycobacterial antigen-induced granulomas (Chensue *et al.*, 1995). More importantly, TNF-α is of high importance in triggering molecular mechanisms that provide protection against mycobacterial disease (Algood *et al.*, 2005). TNF-α exhibits a wide range of biological functions, including the activation of immune and endothelial cells, induction of apoptosis, and thymocyte proliferation (Hernandez-Caselles & Stutman, 1993). Depletion of TNF using neutralizing antibodies prevented granuloma formation and the ability to contain infection with bacille Calmette–Guérin (BCG) in mice (Kindler *et al.*, 1989). Mice lacking the TNF receptor, as well as wild-type mice treated with a TNF-α neutralizing antibody, were rapidly killed upon infection with MTB (Flynn *et al.*, 1995). Furthermore, TNF-deficient mice failed to form granulomas in response to mycobacterial infection, exhibiting delayed expression of C-C and C-X-C chemokines and delayed recruitment of $CD11b^+$ cells (Roach *et al.*, 2002). TNF has also been shown to be critical to control long-term and persistent infections, with depletion resulting in failure to maintain granuloma pathology (Botha & Ryffel, 2003).

Complement C5 is another critical component in the granulomatous response. Its cleavage product, C5a, is a potent anaphylotoxin that recruits cells to inflammatory sites and induces the production of cytokine subsets (Schulman *et al.*, 1988). Complement C5-deficient A/J mice exhibit increased mortality and a markedly increased inflammatory response in the absence of granuloma formation in a murine model of MTB infection (Actor *et al.*, 2001). The complement-deficient A/J mice also exhibited exacerbation in an induced chronic infection, significantly earlier reactivation of drug-cured disease, and their infected

macrophages showed a reduction in secretion of cytokines and chemokines compared to complement-sufficient mice (Jagannath *et al.*, 2000). An investigation by Borders *et al.* (2005) demonstrated that mice lacking the C5a receptor exhibited an exacerbated inflammatory response with failure to form granulomas. Thus, complement C5 likely plays an important role in early maturation of the granulomatous response, related in part to mediation of chemotaxis of regulating proinflammatory cells to the site of inflammation.

Interleukin (IL)-6 is a proinflammatory cytokine produced by monocytes, fibroblasts, T-cells and B-cells that is hypothesized to play a role in mycobacterial infection through its involvement in adaptive cell activation, acute-phase protein production and immunoglobulin production (Van Snick, 1990). *In vitro* studies suggest a pathological role for IL-6 in mycobacterial infection, but these experiments are in need of further definition. IL-6 production by macrophages infected with MTB suppresses the T-cell response (VanHeyningen *et al.*, 1997) as well as blocking transcriptional activation of interferon-γ (IFN-γ) in nearby, uninfected macrophages (Nagabhushanam *et al.*, 2003). *In vivo* studies yielded mixed results. One investigation found that MTB infection was lethal for mice deficient in IL-6 (Ladel *et al.*, 1997); however, another study using IL-6-deficient mice found that the deficient mice had delayed production of IFN-γ but were still able to contain the infection (Saunders *et al.*, 2000). Clearly IL-6 plays a role in the response, but to date the precise function of IL-6 in mycobacterial infection and granuloma formation is unclear.

Trehalose 6,6′-dimycolate (TDM) is a glycolipid component of the mycobacterial cell wall. In mice, purified TDM causes immunopathology that mimics in part MTB infection, including the formation of granulomas and proinflammatory cytokines (Geisel *et al.*, 2005; Perez *et al.*, 1994). TDM is emerging as important in multiple roles during the development of pathogenesis during MTB infection (Hunter *et al.*, 2006b; Rao *et al.*, 2006). Recent findings implicate this molecule in the development of caseation in the lung post-experimental infection of mice (Hunter *et al.*, 2006a). Taken together, the TDM model system provides a useful tool to dissect the factors required for granulomatous response. Because the exact roles for TNF-α, C5 and IL-6 remain unknown in the initiation and maintenance of granuloma formation to isolated TDM, experiments were performed to analyse responses in mice deficient in these critical immune factors.

METHODS

Animals

Three- to five-week-old, female C57BL/6 mice, A/J mice (C5a deficient), and mice deficient in TNF-α (B6;129S6-Tnftm1Gkl/J), C5a (B10.D2-Hc0H2dH2-T18c/oSnJ), or IL-6 (B6.129S2-Il6tm1Kopf/J) were obtained from Jackson Laboratories. Animal studies were conducted under the approval of the UTHHSC Institutional Review Board, document AWP 04-065. Four to six mice were used per group, per time point indicated.

Bone marrow-derived macrophage (BMM) isolation, challenge with TDM-coated beads, and infection with mycobacteria

BMMs from C57BL/6 and complement C5a-deficient (A/J) mice were established as previously described (Indrigo *et al.*, 2002). Femurs were flushed with PBS and 3×10^7 cells were added to 75 cm^2 tissue culture flasks (Corning). Cells were grown in Eagle's minimal essential medium (EMEM; Sigma) containing 10 % fetal bovine serum (FBS), 2 mM glutamine, 10 ng ml^{-1} recombinant murine granulocyte/macrophage colony stimulating factor (GM-CSF; Chemicon), 100 U penicillin (Sigma) ml^{-1} and 100 µg streptomycin (Sigma) ml^{-1}. Cells were incubated at 37 °C in 5 % CO_2 overnight. Non-adherent cells were collected, incubated for 7 days and given two additional media changes containing GM-CSF. Finally, adherent cells were removed, washed and resuspended in EMEM containing 2 % FBS. Cells were then adjusted to 1×10^6 cells ml^{-1} and added to 24-well tissue culture plates (Corning) in 1 ml total volume.

TDM-coated beads were prepared by previously detailed methods, as were control BSA-coated beads (Indrigo *et al.*, 2003). Beads were washed thoroughly and sonicated before use in experiments, and were added to cells at a ratio of 10 beads per cell. TNF-α and IL-6 protein production was assayed in the supernatants after 72 h using an ELISA (see below).

Mycobacterium tuberculosis (MTB) (Erdman, ATCC 35801) was cultured to exponential phase in Dubos broth (Difco) supplemented with 5 % BSA and 7.5 % glucose. For delipidated MTB, mycobacterial surface lipids were extracted with petroleum ether, as previously described (Indrigo *et al.*, 2002; Silva *et al.*, 1985). After addition of petroleum ether (Sigma), the bacteria were vortexed vigorously for 2 min, followed by 5 min incubation at room temperature. The culture was centrifuged at 500 ***g*** for 10 min. The supernatant was removed and the process was repeated twice more and suspended in PBS. Petroleum ether extraction by these methods does not affect viability of the bacteria after 14 days' growth in Dubos broth and has no impact on the acid-fastness of the organisms (Indrigo *et al.*, 2002; Silva *et al.*, 1985). HPLC and TLC analyses have demonstrated that TDM is the primary extracted component (Indrigo *et al.*, 2002; Silva *et al.*, 1985). Delipidated MTB was reconstituted by addition of a 0.01 % (50 µg ml^{-1}) solution of purified TDM (Sigma; 100 % pure by TLC, >98 % as 6,6′-mycolate esters) in petroleum ether (Indrigo *et al.*, 2002; Silva *et al.*, 1985). Solvent was evaporated, and the bacteria were resuspended in PBS. The amount of surface glycolipids recovered is indistinguishable from untreated bacteria by 7 days post-extraction (Indrigo *et al.*, 2002).

Bacteria were adjusted to 5×10^6 bacteria ml^{-1} in DMEM containing 2 % FBS and sonicated to disperse clumps. Serial dilutions were plated on Middlebrook 7H11 agar (Remel) to confirm infectious dose. Colony-forming units were enumerated after incubation of plates at 37 °C for 21 days. Matured BMMs were infected 24 h after seeding (m.o.i. 5 : 1). Immediately before use, monolayers were washed extensively with PBS. One millilitre of native MTB, delipidated MTB, delipidated MTB reconstituted with purified TDM, or medium alone was added, and the infection was allowed to proceed for 4 h at 37 °C with gentle rotation. Cells were infected in triplicate wells; they were then washed to remove extracellular bacteria, and fresh DMEM containing 2 % FBS was added.

Administration of TDM, processing and histology

A TDM water-in-oil emulsion was prepared as previously described (Perez *et al.*, 2000), with modifications discussed below. C57BL/6 mice, or mice deficient in TNF-α (B6;129S6-Tnftm1Gkl/J), C5a (B10.D2-Hc0H2dH2-T18c/oSnJ), or IL-6 (B6.129S2-Il6tm1Kopf/J), were injected intravenously in the tail vein with 50 µl emulsion, prepared by dissolving 25 µg purified TDM (Sigma) in 9 : 1 (v/v) hexane/ethanol followed by evaporation of the solvent. Then 1 µl Drakeol (Penreco) was added and homogenized. Finally, 48 µl 0.2 % Tween 80 (Mallinckrodt) in PBS (Mediatech) was admixed. Mice were sacrificed at days 0, 4, 7 and 14 post-TDM challenge. Lung tissues were immediately aseptically removed, weighed, and processed for cytokine analysis or fixed for histological studies. Calculation of a lung weight index (LWI) was performed as a measure of inflammatory intensity (Borders *et al.*, 2005; Guidry *et al.*, 2004; Pelletier *et al.*, 1982) using the following equation:

$$\mathrm{LWI} = \sqrt{\{[\text{Lung weight (mg)}]/[\text{Mouse weight (g)}/10]\}}/10$$

Approximately 30 mg lung tissue was homogenized and placed into 2 ml Dulbecco's modified Eagle's medium (Sigma) containing 0.01 % L-arginine (Sigma), 0.01 % HEPES (Sigma), 10 % FBS (Sigma), 100 µg penicillin (Sigma) ml^{-1} and 50 µg gentamicin (Sigma) ml^{-1}. Samples were incubated for 4 h at 37 °C with 5 % CO_2. The resulting supernatants were stored at −20 °C for later analysis by ELISA. For histological analysis, the left lung was fixed in 10 % buffered formalin, sectioned (5 µm thick) and stained with haematoxylin and eosin by standard procedures. The remaining lung section was snap-frozen in RNAzol B (TelTest) and stored at −70 °C for RNA extraction and analysis by real-time RT-PCR. No significant difference in histopathology or lung weight index was seen in the emulsion-alone treated controls when compared to naive controls, as previously detailed (Guidry *et al.*, 2004). Experiments were repeated three times through 7 days, and once through 14 days.

ELISA analysis of lung cytokines and chemokines

Levels of IFN-γ, IL-1β, IL-6, IL-10, IL-12p40, macrophage inflammatory protein-1α (MIP-1α) and TNF-α in cell supernatants were measured by a sandwich ELISA according to the manufacturer's instructions (R&D Systems). Briefly, capture antibody coated Costar 96-well plates (Corning) were washed (0.5 % Tween-20 in PBS) and blocked (1 % BSA, 5 % sucrose, 0.05 % NaN_3 in PBS). Supernatants were incubated for 2 h, followed by detection using biotin-conjugated secondary antibodies, with visualization using streptavidin-horseradish peroxidase (R&D) and TMB Microwell Peroxidase Substrate (KPL). Reactions were halted using 1 M H_2SO_4, and the absorbance was read at 570 nm and 450 nm on an ELISA plate reader (Molecular Devices). The mean of duplicate wells was calculated based on a standard curve generated for each assay using manufacturer supplied recombinant molecules (R&D). The lower range limit for detection sensitivity was 15–32 pg ml^{-1}.

Isolation and purification of mRNA, reverse transcription and quantitative PCR

Lung tissue was homogenized in 1 ml RNAzol, and RNA extracted as previously described (Guidry *et al.*, 2006). cDNA was synthesized from 2 µg RNA in buffer (250 mM Tris/HCl,

pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) (Invitrogen), with final concentrations of 0.1 M DTT (Invitrogen), 2.5 mM deoxynucleotide triphosphates (dNTPs) (Invitrogen), 80 U random hexamer oligo-nucleotides (Roche Diagnostics), and 20 U RNase inhibitor (Promega). SuperScript II reverse transcriptase (200 U; Invitrogen) was added after heating to 70 °C for 5 min; incubation continued at 42 °C for 50 min after which the reaction was terminated by heating to 70 °C for 15 min. The sample was diluted 1 : 8 with distilled water prior to analysis.

Quantification of cDNA was performed using the Taqman assay with fluorogenic probes that were FAM (6-carboxifluorescein) reporter and BHQ-1 (black hole quencher) quencher dyes (Biosearch). The sequences of the primers and probes used are shown in Table 1. The reaction mix contained 200 nM dNTPs (Invitrogen), 1 × ROX Reference Dye (Invitrogen), 400 nM of each primer (Integrated DNA Technologies), 1 × PCR buffer (20 mM Tris/HCl pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween 20, 0.5 % Igepal CA-630) (5 Prime), 1 U/45 µl *Taq* DNA polymerase (5 Prime), and 100 nM probe. Amplification in the ABI Prism 7700 Sequence Detection System (Applied Biosystems) was achieved by heating to 95 °C for 1 min, followed by 40 cycles of a 12 s step at 95 °C and a 1 min step at 60 °C. Data were analysed by the $-2^{\Delta\Delta CT}$ method as previously described (Livak & Schmittgen, 2001) using β-actin message as the calibrator.

Statistics

Data are presented as the mean ± 1 SD. Normally distributed data were analysed by an unpaired *t*-test where the difference between two means was compared within groups. Two-way ANOVA was used for statistical analysis between strains for results of *in vivo* experimentation. Differences between means were considered significant at a level of $P < 0.05$.

RESULTS

Inflammatory response to TDM in normal and C5a-deficient bone marrow-derived macrophages

TDM is recognized as a major inducer of proinflammatory cytokines from monocytes (Perez *et al.*, 2000). The levels of TNF-α and IL-6 were significantly elevated in culture supernatants of both C5a-sufficient (C57BL/6) and C5a-deficient (A/J) BMMs treated with TDM-coated beads (Table 2). Previous investigations demonstrated that removal of TDM from the surface of MTB led to significantly less proinflammatory response (Indrigo *et al.*, 2002). Recent reports identified the importance of complement factors in this response (Actor *et al.*, 2001; Borders *et al.*, 2005); therefore, TNF-α and IL-6 were also measured in C5a-deficient BMMs infected with native *M. tuberculosis* (*Mtb*), or organisms treated to remove surface TDM (delipidated; d*Mtb*). Comparisons were made to responses in the C5a-sufficient derived cells. For both groups, delipidation led to significantly diminished magnitude of response. Of interest, the response from C5a-deficient derived cells was always significantly less than that from matched C5a-sufficient derived cells. The response was nearly completely restored when cells were challenged with organisms reconstituted

with purified TDM (r-d*Mtb*), confirming the relative importance of the TDM glycolipid in the initial proinflammatory response to mycobacteria.

Inflammatory response to TDM in TNF-α-, C5a-and IL-6-deficient mice

LWIs were calculated as a measure of general inflammatory response for wild-type and deficient mice following intravenous administration of TDM (Table 3). The wild-type, C5a-deficient (OSN) and IL-6-deficient mice demonstrated significantly ($P < 0.05$) elevated LWI at 4 and 7 days post-TDM administration in comparison to untreated control mice, or compared to emulsion-alone controls (not shown; Actor *et al.*, 2001). In contrast, the mean LWIs of the TNF-α-deficient mice were 0.99 ± 0.01 on day 4 and 1.01 ± 0.01 on day 7, compared to wild-type mice that had LWIs of 1.31 ± 0.07 and 1.63 ± 0.23 on days 4 and 7, respectively, suggesting the TNF-α-deficient mice failed to initiate a significant inflammatory response to TDM.

TNF-α-, C5a- and IL-6-deficient mice demonstrate altered TDM-induced lung histopathology compared to control challenged mice

Wild-type C57BL/6 mice formed cohesive and transient granulomas after a single intravenous injection of TDM (Fig. 1). Focal, small, histiocytic clusters were evident at day 4 post-TDM administration. The clusters became more complex at day 7, increasing in both size and number. The granulomas were located within the lung parenchyma, with no obvious occlusion of blood vessels or lymphatics. Responses were primarily monocytic, with larger phenotypic responders containing activated intracellular vesicles. By day 14, there was complete resolution of the granulomatous response.

In contrast to the wild-type mice, the TNF-α-deficient mice largely failed to demonstrate a histological response to TDM. Inflammation was not apparent at 4 days after TDM administration. Examination of tissue at day 7 revealed slight accumulation of cellular infiltrates without the formation of granulomas; infiltrating cells were monocytic, with few or no visible intracellular inclusions. There was no obvious occlusion of vasculature, nor was there evidence of oedema or pneumonitis.

C5a-deficient mice also failed to form granulomas in response to isolated TDM. However, the pathology was different from that of the TNF-α-deficient mice. The complement-deficient mice showed a marked non-focal inflammatory response at day 4. By 7 days, they completely lacked cohesive or structured cellular aggregates and had histological evidence of considerable lymphocytic and monocytic infiltration. The lung parenchyma was distorted and demonstrated some vessel occlusion with small pockets of pulmonary oedema. This reaction appeared to be transient, although resolution of the response remained incomplete at day 14.

The IL-6-deficient mice initially formed granulomas in response to TDM with focal monocytic clusters apparent at day 4. However, they failed to maintain this response. By day 7 post-challenge, acute inflammation was increased with marked monocytic infiltration. Lymphocytic cuffing around occluded vesicles was evident, as was oedema and alveolar cell-wall thickening. Of interest, the response was transient, and largely resolved by day 14.

Altered proinflammatory cytokine and chemokine protein and mRNA profiles in deficient mice after TDM challenge

Cytokine and chemokine protein patterns were examined in the lungs of mice challenged with TDM (Figs 2 and 3). Challenged wild-type mice had significantly elevated levels ($P < 0.05$) of IL-1β, IL-6, TNF-α, MIP-1α, IL-12p40 and IL-10 on both days 4 and 7 in comparison to non-injected mice. In addition, there was a significant, but transient, rise in IFN-γ on day 4. Cytokine protein returned to near baseline levels by day 14 post-TDM administration.

The differences in protein production in the lung between controls and knockout strains were readily apparent on day 4 post-TDM administration. The TNF-α-deficient mice did not produce an elevation in cytokine or chemokine levels in response to TDM. The levels of IL-1β, IL-6, MIP-1α, IFN-γ, IL-12p40 and IL-10 did not differ from that of non-injected mice, and were thus significantly below levels produced by wild-type mice at those time points (Figs 2 and 3). TNF-α was not detectable in the TNF-α-deficient mice (data not shown).

The complement C5-deficient mice had significantly decreased production of IL-1β, IL-6, TNF-α, MIP-1α and IFN-γ in lungs on day 4, compared to the wild-type challenged mice. However, protein levels were comparable to the wild-type mice on day 7. Specifically, amounts of the proinflammatory cytokines IL-1β, IL-6 and TNF-α on day 4 were 5536.43 ± 4316.35, 950.57 ± 758.49 and 10 602.18 ± 9694.66 pg per lung, respectively, in the C5a-deficient mice compared to 16 113.19 ± 941.15, 2199.75 ± 1319.66 and 22 510.79 ± 2384.84 pg per lung in the wild-type mice. MIP-1α, a chemokine that controls the migration of numerous effector cells, was significantly reduced in the C5a-deficient mice at day 4, with levels of 1867.43 ± 1701.89 pg per lung compared to wild-type levels of 4835.34 ± 480.04 pg per lung. IL-12p40 was also significantly decreased on days 0, 4, 7 in comparison to the complement-sufficient mice.

The IL-6-deficient mice also demonstrated reduced production of IL-1β, TNF-α, IFN-γ and IL-12p40 in lung tissue at day 4 post-TDM challenge. TNF-α was 8330.44 ± 2384.84 pg per lung in the IL-6-deficient mice whereas it was 22 510.79 ± 2384.84 in the wild-type mice at day 4. The amount of IL-12p40 in the IL-6-deficient mice was 1040.28 ± 253.31 pg per lung in comparison to wild-type levels of 1912.53 ± 124.45 pg per lung at day 4. The amount of these cytokines became similar to that of wild-type mice on day 7. IL-10 levels significantly decreased at day 7 in the IL-6-deficient mice, with 148.79 ± 51.30 pg per lung compared to wild-type levels of 278.03 ± 112.82 pg per lung. Levels of MIP-1α did not differ significantly from the wild-type mice on any days tested. Protein production of IL-6 in the IL-6-deficient mice was not detectable (data not shown).

Evaluation of mRNA in the wild-type and knockout mice treated with TDM yielded similar results to the protein levels identified within lung tissue (Table 4). The wild-type C57BL/6 mice had a marked increase (20-fold or greater) in message for the proinflammatory mediators IL-1β, IL-6, TNF-α and MIP-1α message compared to untreated or emulsion-alone controls (not shown, Guidry *et al.*, 2004). A modest increase in expression of IL-12p40 occurred on days 4 and 7. There was an early increase in expression of IFN-γ and

IL-10 that decreased by day 7. The deficient mice had decreased expression of all cytokines tested, except IL-12p40, compared to the wild-type mice. Significant differences in individual message levels for each knockout strain compared to the wild-type control mice ($P < 0.05$) are indicated in Table 4. Expression of TNF-α or IL-6 in the TNF-α-deficient or IL-6-deficient mice, respectively, was not observed (data not shown).

DISCUSSION

Roughly one-third of the world's population is latently infected with MTB (WHO, 2003). The formation of granulomas is a critical host defence mechanism for containment of organisms and is a process that requires on-going concerted regulation of proinflammatory mediators (Russell, 2007). The growing number of therapeutics that target cytokines to treat chronic immune-mediated disease makes it clinically relevant to identify how these molecules are involved in the initiation and maintenance of the granulomatous response. For example, patients treated with infliximab, a TNF-α neutralizing antibody, experienced altered inflammatory patterns that led to reactivation of latent pulmonary tuberculosis, resulting in greater occurrence of disseminated disease (Keane *et al.*, 2001). In addition, the use of cytokines as adjunct therapeutics to augment antibiotic efficacy is being considered for tuberculosis due to the emergence of multidrug-resistant strains and in treating immunodeficient patients (Bermudez & Kaplan, 1995; Murray *et al.*, 1996). Understanding the implications of dysregulation of these mediators is paramount when considering treatment in these individuals.

Numerous investigations implicate TDM as a major immunomodulatory component of the mycobacterial cell wall. Remarkably similar proinflammatory molecules are induced in mice and BMMs challenged with TDM as observed in MTB infection (Actor *et al.*, 2000; Perez *et al.*, 1994, 2000). We demonstrate that removal of MTB surface lipids diminished macrophage TNF-α and IL-6 production, with restoration of this response upon reconstitution with TDM. These data confirm the critical involvement of surface glycolipids in the macrophage response to MTB. The studies outlined here extend these findings to investigate the role of these particular cytokines, as well as complement C5a, in development of the granulomatous response against purified TDM.

TNF-α is a key mediator involved in the initiation of the granulomatous response. TNF-α-deficient mice failed to form granulomas in response to MTB infection, with subsequent development of necrotic lesions devoid of epithelioid cells (Kaneko *et al.*, 1999). The TNF-α-deficient mice in the studies reported here failed to produce a histological response to TDM, with severely limited inflammatory protein or message production. Of interest, these knockout mice also demonstrated decreased levels of the T_H1 cytokines INF-γ and IL-12p40. An investigation by Kindler *et al.* (1989) noted that TNF neutralization inhibited granuloma formation in response to BCG and dissolved established granulomas. Transgenic mice that express high levels of the human soluble TNF receptor 1 also failed to form granulomas in response to BCG and had delayed production of both IFN-γ and IL-12p40 (Guler *et al.*, 2005). The lack of granuloma formation in TNF-α-deficient mice may be due to an inability to produce an inflammatory cascade that includes chemokine production to recruit monocytes and T-cells to the lung. Neutralization of TNF in macrophages infected

with MTB caused a decrease in CCL5, CXCL9 and CXCL10 (Algood *et al.*, 2004). This same study found that $CD11b^+$ cells isolated from mice lacking the 55 kDa TNF receptor infected with MTB had delayed production of inflammatory chemokines. Another investigation found that TNF-α was essential for TDM to prime peritoneal macrophages (Oswald *et al.*, 1999), perhaps indicating an autocrine mechanism towards initiation of responses observed in this study. Furthermore, TNF-α is a critical regulator of the T_H1 immune response essential for the control of mycobacterial infections. Macrophages treated with a TNF-neutralizing antibody and macrophages lacking the TNF receptor 1 failed to produce IL-12 after infection with BCG (Flesch *et al.*, 1995). TNF-α was required for induction of the IL-12-mediated T_H1 response in BALB/c mice (Shibuya *et al.*, 1998). In addition, Ahlers *et al.* (2001) noted that a synergism between IL-12 and TNF-α was necessary to upregulate IFN-γ and the IL-12Rβ2 chain to promote development of T_H1 cells. The overall importance of T-cells in response to TDM-induced granulomas has recently been investigated (Guidry *et al.*, 2006; Yamagami *et al.*, 2001), with T-helper cells of major importance for development of hypersensitive immunopathology (Guidry *et al.*, 2006; Oiso *et al.*, 2005).

The experiments described here indicate that complement C5 is essential for both initiation of the granulomatous response and continued development of cohesive granuloma formation in response to the mycobacterial glycolipid TDM, once initiated. TDM activates the alternative pathway of complement, and complement component C5a has been found to increase transcription and translation of TNF-α, IL-6 and IL-1β (Gross & Andus, 1992; Ramanathan *et al.*, 1980; Schindler *et al.*, 1990). C5 is secreted by numerous cell types, including macrophages, and is cleaved to C5a by extracellular proteases to activate macrophages by autocrine binding to C5aR (Czermak *et al.*, 1999). The hypothesis that macrophages from C5-deficient mice would have defective responses to MTB and TDM was explored. BMMs derived from C5a-deficient mice had diminished levels of TNF-α and IL-6 in response to MTB infection and stimulation with TDM-coated beads compared to BMMs derived from complement-sufficient C57BL/6 mice, implicating complement in the initial induction of proinflammatory mediators that lead to granuloma formation. Deficiency of C5 resulted in the development of a pneumonitis with monocytic infiltration and oedema with delayed resolution of the response after *in vivo* challenge with TDM, suggesting the importance of C5 in the maintenance and further resolution of granulomas. In addition, the complement-deficient mice had delayed production of proinflammatory mediators concurrent with consistently lowered production of IL-12p40. The histology and cytokine response of the C5a-deficient mice challenged with TDM have been identified as mediating chemokine profiles that moderate cellular infiltrates in the lung (Borders *et al.*, 2005), studies that correlate very well with deficient mouse models of MTB infection. A/J mice, which lack the fifth component of complement, failed to form granulomas and rapidly succumbed to MTB infection (Jagannath *et al.*, 2000). An investigation utilizing the same C5a-deficient (OSN) mice as used in this study found enhanced pulmonary growth of MTB as well as lack of productive granulomatous response (Actor *et al.*, 2001). Both studies noted early decreases in cytokine and chemokine protein and mRNA, similar to data presented here for the β chemokine MIP-1α. Complement C5 is essential for a number of additional or synergistic processes that may be involved in the formation and maintenance of granulomas.

C5a induces NFκB, which regulates expression of numerous inflammatory molecules (Hsu *et al.*, 1999). C5a and C5b induce expression of adhesion molecules on macrophages, neutrophils and endothelial cells (Fleming *et al.*, 2003; Foreman *et al.*, 1994). Finally, C5 appears to be essential for IL-12-induced cell-mediated immunity (Karp & Wills-Karp, 2001).

The results presented here also suggest a role for IL-6 in granuloma maintenance, but not in initiation of response. The IL-6-deficient mice formed granulomas upon TDM challenge at day 4, similar to wild-type mice. Of interest, this occurred despite lowered levels of inflammatory cytokines. However, granuloma cohesiveness was not maintained through day 7, even in the presence of cytokine levels comparable to that of the wild-type mice. Studies on the role of IL-6 in the control of mycobacterial diseases have yielded conflicting results, with even less understood regarding the role of IL-6 in protective granuloma formation during MTB infection. Ladel *et al.* (1997) demonstrated that IL-6-deficient mice had much higher bacterial burdens and succumbed to infection, while Saunders *et al.* (2000) found that mice deficient in IL-6 were ultimately able to contain the infection despite higher bacterial load and decreased early production of IFN-γ. The discrepancies between these studies may be the result of the route and number of organisms used for the infectious process. To further complicate interpretation, treatment of *Mycobacterium avium*-infected mice with IL-6 neutralizing antibodies resulted in increased bacterial growth; however, no differences in granuloma number or size were noted (Appelberg *et al.*, 1994). Infection of IL-6-deficient mice with *M. avium* produced fewer necrotic lesions compared to wild-type mice (Florido *et al.*, 2002). Infection of IL-6-deficient mice with *Rhodococcus aurantiacus*, a short-chain TDM-producing organism that induces T_H1 granulomas similar to mycobacterial agents, found considerable inflammatory infiltrates following challenge, with larger granulomas that had central necrosis compared to wild-type mice (Yimin *et al.*, 2003). IL-6 is generally considered a T_H2 cytokine (Rincon *et al.*, 1997; Van Snick, 1990); however, this is not a stand-fast rule in all intracellular infection models (Romani *et al.*, 1996). IL-6 is identified with the development of a T-cell response against *M. avium* (Appelberg *et al.*, 1994). It is clear that maintenance of granulomas requires antigen-specific T-cells (Dannenberg, 1991). Therefore, IL-6 may be a regulator of the T-cell responses critical for the maintenance of granulomas, especially during infection when persistent antigen is present.

The TDM-induced granulomatous response mimics in part many aspects of the mycobacterial immunopathology identified early during aerosol infections of mice. As such, this model system is ideal for investigation of the roles of complement and cytokine components towards early response to mycobacterial antigens. The studies described here indicate that TNF-α is a critical mediator involved in initiation of the granulomatous response to the TDM. Once the granulomatous process has begun, factors such as complement C5 and IL-6 mediate secondary responses. C5 most likely is a critical mediator of responses culminating in a cohesive and structured granuloma immunopathology, while IL-6 regulates the maintenance of granulomas once established. The end result of deficiency of any of these components is an inability to effectively regulate the early granulomatous response, and would therefore also likely be critical in control during mycobacterial infections.

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Abbreviations

BCG	bacille Calmette–Guérin
BMM	bone marrow-derived macrophage
IFN-γ	interferon-γ
IL	interleukin
LWI	lung weight index
MIP-1α	macrophage inflammatory protein-1α
MTB	*Mycobacterium tuberculosis*
TDM	trehalose 6,6′-dimycolate
TNF	tumour necrosis factor

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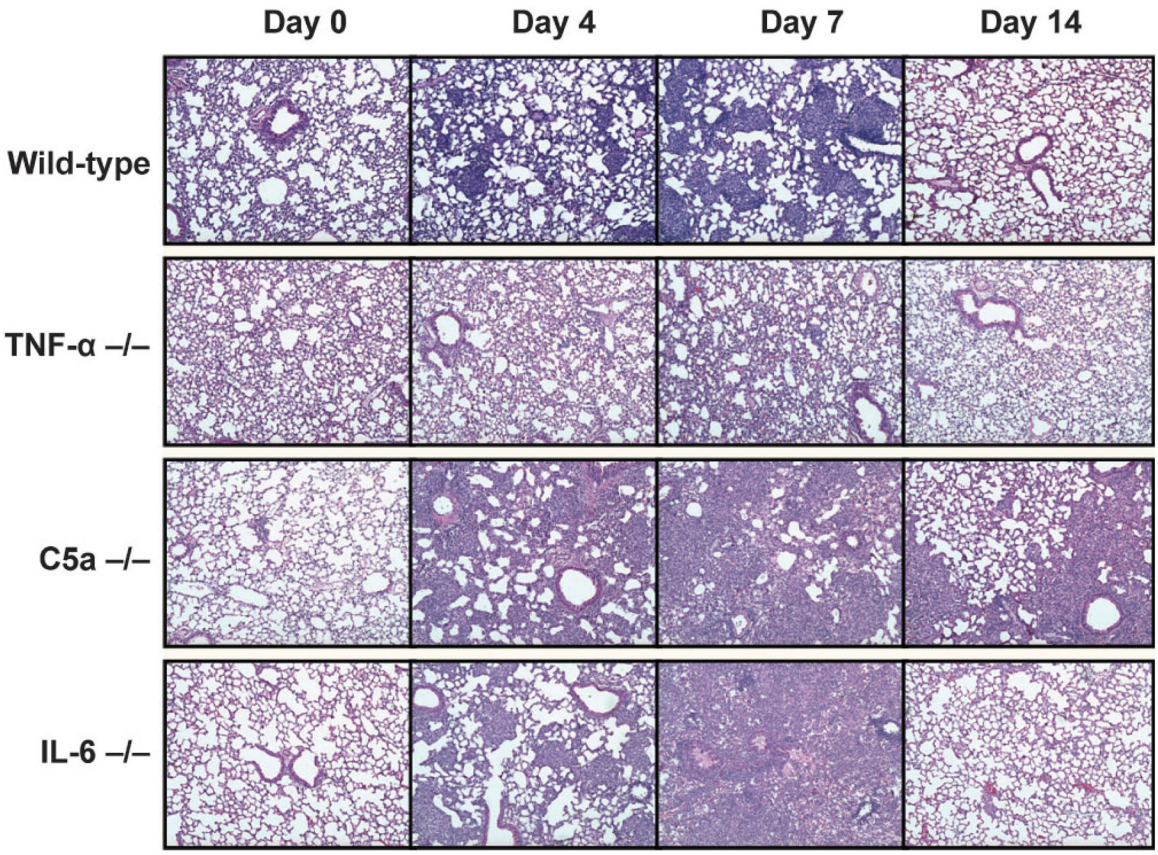

Fig. 1.

Granulomatous response to TDM in mice deficient in TNF-α, C5a, or IL-6. Wild-type mice challenged with mycobacterial TDM demonstrate small focal pulmonary granulomas by 4 days post-TDM administration, which peak at day 7 and resolve by day 14. In comparison, challenge of TNF-α-deficient mice did not elicit significant histopathology, with failure to mount significant inflammation in the lung. Mice with deficiency in C5a demonstrated a non-focal inflammatory response by day 4, and with no cellular aggregation of accompanying lymphocytic and monocytic infiltration or true resolution by 14 days post-challenge. The IL-6-deficient mice initiated granuloma histopathology, but by day 7 exhibited marked monocytic infiltration, lymphocytic cuffing around occluded vesicles, and oedema with accompanied alveolar cell wall thickening. Sections representative of 4–6 mice per group. Haematoxylin and eosin staining; magnification ×40.

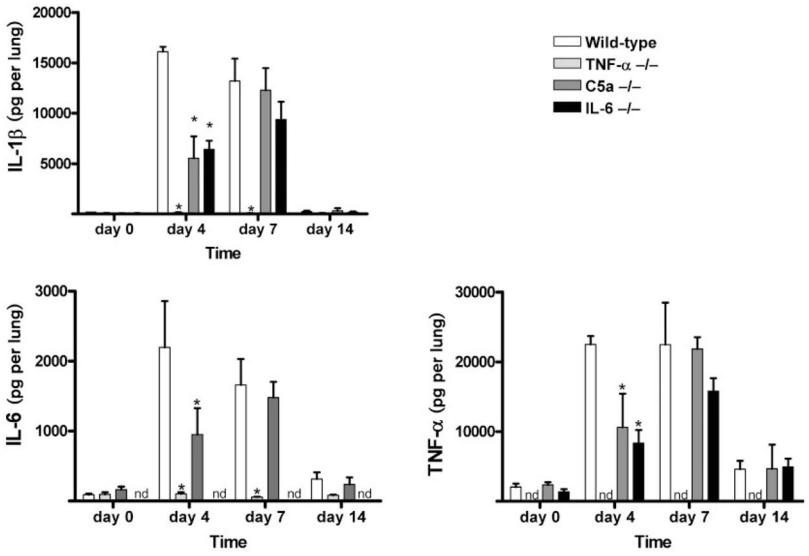

Fig. 2.

TDM-elicited proinflammatory mediator production from lungs of deficient and wild-type mice. Levels of pulmonary proinflammatory mediators IL-1β, IL-6 and TNF-α were quantified post-challenge in the lungs of mice administered TDM. Mean values per lung are shown for mice deficient in TNF-α, C5a or IL-6 prior to challenge (day 0) and at 4, 7 and 14 days post-TDM challenge. Comparisons are made to the wild-type control animals administered TDM. Data are represented as the mean ± SD for duplicate wells per mouse (n = 4–6 mice per group per time point). *$P < 0.05$; nd, none detected.

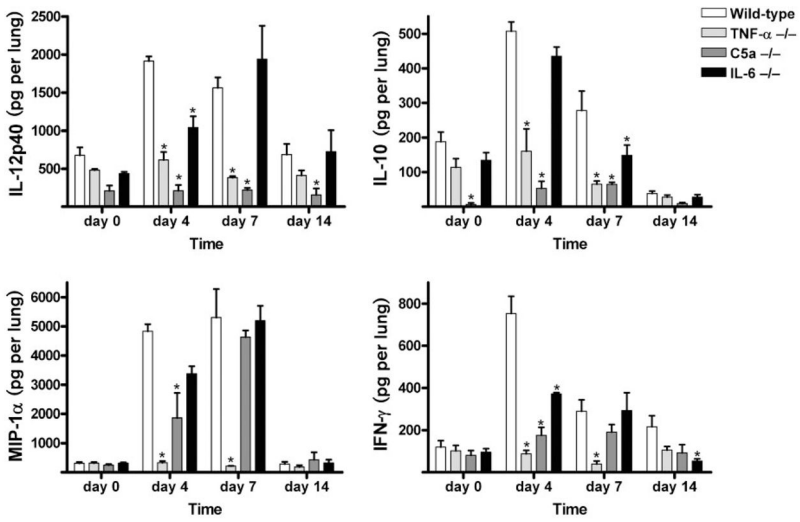

Fig. 3.

TDM-elicited cytokine production from lungs of deficient and wild-type mice. Levels of pulmonary cytokines IL-12p40, IL-10, MIP-1α and IFN-1γ were quantified post-challenge in the lungs of mice administered TDM. Mean values per lung are shown for mice deficient in TNF-α, C5a or IL-6 prior to challenge (day 0) and at 4, 7 and 14 days post-TDM challenge. Comparisons are made to the wild-type control animals administered TDM. Data are represented as the mean ± SD for duplicate wells per mouse (n = 4–6 mice per group per time point). *$P < 0.05$.

Table 1

Oligonucleotide primers and probes

Protein		Primer (5′–3′)	Probe (5′–3′)	Product size (bp)
β-Actin	S*	TCTGGCTCCTAGCACCATGA	ATCAAGATCATTGCTCCTCCTGAGCGC	72
	AS*	CCACCGATCCACACAGAGTACT		
IFN-γ	S	AGCAACAGCAAGGCGAAA	TCAAACTTGGCAATACTCATGAATGCATCCT	72
	AS	CTGGACCTGTGGGTTGTTGA		
IL-1β	S	CTCATTGTGGCTGTGGAGAA	TGGCAGCTACCTGTGTCTTTCCCG	78
	AS	GGTGCTCATGTCCTCATCCT		
IL-6	S	CCCAATTTCCAATGCTCTC	TAGCCACTCCTTCTGTGACTCCAGCT	77
	AS	TGAATTGGATGGTCTTGGTC		
IL-10	S	CAGCCGGGAAGACAATAACTG	CCCACTTCCCAGTCGGCCAG	67
	AS	CCGCAGCTCTAGGAGCATG		
IL-12p40	S	AAGTGTGAAGCACCAAATTACTC	ACGGTTCACGTGCTCATGGCT	71
	AS	TTCAAGTCCATGTTTCTTTGC		
MIP-1α	S	ACTAAGAGAAACCGGCAGAT	TGCGCTGACTCCAAAGAGACC	77
	AS	TTCAGTTCCAGGTCAGTGAT		
TNF-α	S	CCGATGGGTTGTACCTTGTCT	TCTTCAAGGGACAAGGCTGCCCC	76
	AS	TGGGTGAGGAGCACGTAGTC		

*S, sense; AS, antisense.

Table 2

TDM-induced cytokine production in C5a-sufficient and C5a-deficient bone marrow-derived macrophages

Production of TNF-α and IL-6 by BMMs in response to BSA-coated beads, TDM-coated beads, native *Mtb*, delipidated *Mtb* (d*Mtb*), delipidated *Mtb* reconstituted with TDM (r-d*Mtb*), or untreated cells. Values were measured by ELISA and expressed as mean pg protein per 10^6 BMMs (±SD); three replicates per time point.

	BSA beads	TDM beads	*Mtb*	d*Mtb*	r-d*Mtb*	Uninfected
TNF-α						
C5a-sufficient	24 (2)	1294 (173) *	541 (70)	253 (39) †	461 (44)	19 (5)
C5a-deficient	14 (1)	322 (19) *	303 (50)	166 (32) †	339 (50)	10 (1)
IL-6						
C5a-sufficient	7 (1)	245 (4) *	335 (75)	102 (6) †	227 (14)	5 (1)
C5a-deficient	<10	24 (4)	30 (1)	<10	<10	<10

* $P < 0.05$ between treatment groups (TDM-coated vs BSA-coated or untreated cells) as analysed by Student's *t* test.

† $P < 0.05$ between groups compared against d*Mtb* or against TDM-reconstituted organisms; all responses from C5a-deficient (A/J) derived BMMs were significantly lower ($P < 0.05$) than the C5a-sufficient (C57BL/6) BMMs treated in the identical manner. Experiments were repeated two or three times with similar results.

Table 3
Lung weight indices following TDM administration

LWIs were calculated at days 0, 4, 7 and 14 post-TDM administration. Individual values are mean responses (±SD) and compared to wild-type C57BL/6 mice at each indicated time point.

	Day 0	Day 4	Day 7	Day 14
Wild- type	1.00 ± 0.033	1.31 ± 0.073	1.63 ± 0.232	1.06 ± 0.154
TNF-α −/−	0.98 ± 0.026	0.99 ± 0.012 *	1.01 ± 0.010 *	0.96 ± 0.035
C5a −/−	0.93 ± 0.144	1.32± 0.156	1.53 ± 0.157	1.04 ± 0.196
IL-6 −/−	1.02 ± 0.048	1.30 ± 0.051	1.55 ± 0.082	1.19 ± 0.216

* $P < 0.05$.

Table 4

Relative change in mRNA following TDM administration

Proinflammatory mediator mRNAs in lungs of mice deficient in TNF-α, C5a, or IL-6 were evaluated by RT-PCR and compared to wild-type controls, after challenge with TDM. Results shown are normalized to β-actin, and represented as fold-change from non-injected mice. Data are expressed as means ± SD.

	IL-1β	IL-6	TNF-α	MIP-1α	IFN-c	IL-12p40	IL-10
Day 4							
Wild-type	23.89 ± 9.37	32.01 ± 7.71	20.71 ± 3.67	29.57 ± 10.59	23.09 ± 14.60	2.99 ± 0.32	29.17 ± 17.71
TNF-α −/−	3.79 ± 2.24*	5.45 ± 3.41*	–	4.61 ± 1.87*	4.12 ± 1.76*	3.21 ± 1.74	7.10 ± 4.02*
C5a −/−	15.25 ± 1.94*	16.57 ± 6.76	31.34 ± 13.99	19.59 ± 20.04	12.43 ± 2.10*	1.35 ± 0.98	11.45 ± 7.86*
IL-6 −/−	5.02 ± 1.42*	–	5.72 ± 1.50*	5.54 ± 2.00*	7.02 ± 2.48*	2.04 ± 1.02	7.02 ± 3.22*
Day 7							
Wild-type	32.96 ± 10.66	27.87 ± 26.62	43.59 ± 13.60	42.51 ± 16.84	9.63 ± 5.95	2.19 ± 0.72	11.84 ± 3.34
TNF-α −/−	2.54 ± 1.73*	1.08 ± 0.88*	–	2.32 ± 1.77*	2.31 ± 1.36	0.89 ± 0.68	2.49 ± 1.64
C5a −/−	15.94 ± 4.17*	3.28 ± 0.97*	18.44 ± 10.55*	16.36 ± 10.88*	10.57 ± 5.37	1.79 ± 0.45	5.73 ± 1.20
IL-6 −/−	5.04 ± 1.77*	–	5.97 ± 2.21*	7.04 ± 1.89*	2.18 ± 0.87	0.95 ± 0.55	2.07 ± 0.81
Day 14							
Wild-type	0.61 ± 0.20	0.53 ± 0.05	10.29 ± 15.73	0.72 ± 0.56	1.75 ± 1.04	1.09 ± 0.29	0.67 ± 0.30
TNF-α −/−	2.30 ± 1.25	1.50 ± 1.53	–	2.14 ± 1.14	3.42 ± 1.97	1.13 ± 0.69	2.16 ± 1.39
C5a −/−	0.68 ± 0.55	0.44 ± 0.22	0.63 ± 0.60	0.94 ± 0.55	1.37 ± 1.48	1.58 ± 1.19	1.79 ± 2.24
IL-6 −/−	2.01 ± 1.32	–	3.29 ± 2.72	2.65 ± 1.05	2.56 ± 1.71	1.35 ± 0.60	3.41 ± 2.29

*$P < 0.05$.