

# *Mycobacterium tuberculosis* controls host innate immune activation through cyclopropane modification of a glycolipid effector molecule

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***Mycobacterium tuberculosis* (Mtb) infection remains a global health crisis. Recent genetic evidence implicates specific cell envelope lipids in Mtb pathogenesis, but it is unclear whether these cell envelope compounds affect pathogenesis through a structural role in the cell wall or as pathogenesis effectors that interact directly with host cells. Here we show that cyclopropane modification of the Mtb cell envelope glycolipid trehalose dimycolate (TDM) is critical for Mtb growth during the first week of infection in mice. In addition, TDM modification by the cyclopropane synthase *pcaA* was both necessary and sufficient for proinflammatory activation of macrophages during early infection. Purified TDM isolated from a cyclopropane-deficient *pcaA* mutant was hypoinflammatory for macrophages and induced less severe granulomatous inflammation in mice, demonstrating that the fine structure of this glycolipid was critical to its proinflammatory activity. These results established the fine structure of lipids contained in the Mtb cell envelope as direct effectors of pathogenesis and identified temporal control of host immune activation through cyclopropane modification of TDM as a critical pathogenic strategy of Mtb.**

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Abbreviations used: Mtb, *Mycobacterium tuberculosis*; TDM, trehalose dimycolate.

*Mycobacterium tuberculosis* (Mtb) infection remains a major global health emergency, which has not been controlled by present therapeutic modalities. More effective antimicrobials or vaccines to combat Mtb infection will only be possible through greater understanding of the molecular strategies used by Mtb to facilitate long-term persistence in vivo. An abundance of recent studies have established the *M. tuberculosis* cell envelope as a critical determinant of Mtb–host interactions (1–3). Specific mutations in Mtb that lead to alterations of cell envelope lipids and glycolipids have revealed that some of these may lead to marked reductions in virulence. This has been observed for mutations that lead to a deficiency or failure to secrete phthiocerol dimycocerosate (4, 5), changes in mycolic acid carbon chain length (6) or oxygenation (7), and lack of mycolate modification by cyclopropyl rings (8). However, the defects in growth and pathogenesis observed in mutant

strains lacking these diverse cell envelope products are distinct, suggesting that each compound of the complex Mtb cell envelope has a specialized role in pathogenesis. For example, deficiency of oxygenated mycolic acids or phthiocerol dimycocerosate confers replication defect in mice (4, 5, 7), whereas deficiency of  $\alpha$  mycolate cyclopropanation confers a persistence defect (8). A central unresolved question is whether individual cell envelope compounds mediate pathogenesis indirectly through structural effects on properties of the cell envelope (9) or alternatively act directly as effector molecules that modify host immune responses or interfere with antimicrobial activity (10–15).

The *pcaA* gene (Rv0470c) is one recently defined genetic determinant of Mtb virulence and persistence that encodes an *S*-adenosyl methionine-dependent methyltransferase that catalyzes proximal cyclopropanation of  $\alpha$  mycolate, the major mycolic acid subclass of the Mtb cell envelope (8). Mycolic acids are  $\alpha$  alkyl,  $\beta$  hydroxy-branched fatty acids that are found in mycobacteria and related taxa and can exceed 80 carbons in length. These lipids serve

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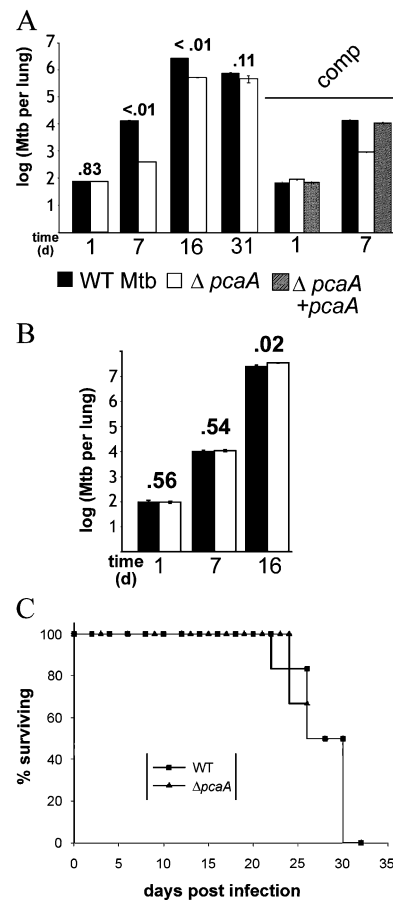
a major structural role in the cell wall of the bacterium, and have also been identified as targets for the adaptive immune response via presentation to T cells by the human CD1b protein (16). In the murine model of infection, *M. tuberculosis* lacking *pcaA* ( $\Delta$ *pcaA*) fails to persist, is attenuated for virulence, and invokes less severe immunopathology than wild-type Mtb. These results suggested that the site specific cyclopropane modification of mycolic acids is an important determinant of Mtb-host interactions. As the cyclopropyl modification of mycolic acids is absent in nonpathogenic mycobacteria, the phenotypes of the  $\Delta$ *pcaA* mutant suggest that this lipid modification system evolved to mediate important pathogenic functions such as interaction with host innate immune receptors. To investigate this hypothesis we focused on trehalose dimycolate (TDM), an inflammatory glycolipid that contains mycolic acids. Here, we show that the cyclopropyl modification of mycolates on TDM modified innate immune recognition of Mtb and had a major effect on the role of these lipids as direct effectors of virulence and pathogenesis.

## RESULTS

### Modulation of the early innate response by *pcaA* during *M. tuberculosis* infection in vivo

Whereas our prior results specifically implicated cyclopropane modification of mycolic acids as a contributor to Mtb-induced immunopathology, the mechanism by which *pcaA* affected pathogenesis was not identified (8). To explore the role of innate host immune recognition in the  $\Delta$ *pcaA* phenotype, we examined in greater detail the behavior of the Mtb  $\Delta$ *pcaA* mutant during the early stages of infection in the lungs. C57BL/6 mice were infected by aerosol inoculation with  $\sim$ 100 of either wild-type Mtb or the  $\Delta$ *pcaA* mutant, and bacterial titers were determined at weekly intervals. Both sets of mice received identical inocula (Fig. 1 A, day 1 time point). Although our previous studies did not demonstrate any growth defect in vivo at 3 wk of infection, a more detailed examination at earlier time points revealed a dramatic initial delay in the growth of  $\Delta$ *pcaA* mutant bacilli (Fig. 1 A). After 1 wk of infection, titers of the  $\Delta$ *pcaA* mutant bacteria were 50-fold lower than wild-type titers, whereas at 2 and 4 wk after infection, wild-type and mutant titers equalized. The early growth defect of the mutant was reversed in the complemented strain (Fig. 1 A, right, comp), demonstrating that the transient early growth defect was due to loss of *pcaA* function. These results indicated that the  $\Delta$ *pcaA* mutant was transiently defective for early lung growth, but not intrinsically defective for replication in vivo, defining *pcaA* as a temporally restricted determinant of bacterial growth after airborne lung infection. In addition, they suggested a possible interaction between the *pcaA*-dependent structural features of mycolic acids and the innate immune mechanisms activated in the earliest stages of infection in a naive host.

**Dependence on TNF of the attenuation of the  $\Delta$ *pcaA* mutant**  
TNF is an important regulator of immune responses and is critically important for host defense against Mtb infection in



**Figure 1. Effect of *pcaA* on early colonization of the lung and its TNF dependence.** (A) Wild-type C57BL/6 mice were infected by aerosol with wild-type Mtb (black bars) or the  $\Delta$ *pcaA* mutant (open bars), and bacterial titers were determined at the indicated time points by serial dilution and plating of lung homogenates. Each bar is the mean of bacterial loads from four mice per group and error bars are SEM. When error bars are not visible they are within the plotted bar. P values (Student's *t* test) for the comparison of WT vs  $\Delta$ *pcaA* mutant Mtb are indicated above each bar set. The 1- and 7-d time points at the far right are a repeat experiment including a complemented *pcaA* mutant (comp, hatched bar). (B) TNF<sup>-/-</sup> mice were infected with either wild-type or  $\Delta$ *pcaA* mutant Mtb as in A, and CFUs of lung tissue were determined at the indicated times. Each bar is the mean of four mice per group. (C) Survival kinetics for TNF<sup>-/-</sup> mice infected with wild-type or  $\Delta$ *pcaA* Mtb. 10 mice each were infected with either wild-type (black line, square symbol) or  $\Delta$ *pcaA* Mtb (gray line, triangle symbol) via the aerosol route and the time to death was recorded.

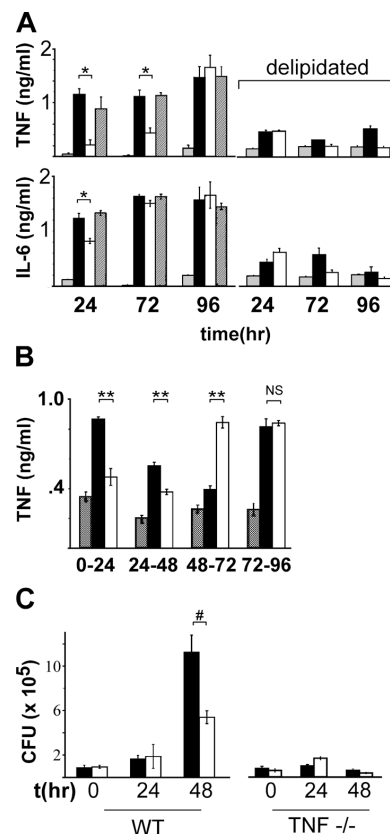
mice and humans (17, 18). In addition to contributing to immune-mediated control of infection, TNF is an important determinant of Mtb-induced granuloma structure, and is likely to be important in preventing reactivation of latent infection (19, 20). Paradoxically, TNF can also facilitate early growth of Mtb in macrophages (21, 22), suggesting that TNF has pleiotropic effects on Mtb that may depend on the stage of infection or cell type examined. Given the effects of the *pcaA* mutation on initial growth of Mtb in vivo, we tested whether the early growth defect of the  $\Delta$ *pcaA* mutant depended on TNF. Infections in TNF-deficient mice revealed that the early growth defect of the  $\Delta$ *pcaA* mutant was

only evident in the presence of TNF, as wild-type and mutant bacterial titers in infected lung were the same at all time points (Fig. 1 B). To test whether the attenuated host mortality of the  $\Delta pcaA$  mutant infection was also TNF dependent, we infected TNF-deficient mice with the wild-type or  $\Delta pcaA$  mutant strains of Mtb and recorded host morbidity. Wild-type mice displayed the predicted accelerated mortality to Mtb infection that has been reported previously (17). In contrast to the attenuation of virulence reported previously with the  $\Delta pcaA$  mutant in wild-type mice (8),  $\Delta pcaA$  mutant-infected TNF-deficient mice succumbed to the infection with the same kinetics as mice infected with wild-type Mtb (Fig. 1 C). Thus, the phenotypic difference between wild-type and  $\Delta pcaA$  mutant Mtb disappeared in mice lacking TNF, suggesting that the mechanism by which cyclopropane modification of mycolic acids contributed to virulence was linked to the induction of TNF during the early phase of infection.

#### Reduction of macrophage cytokine responses to $\Delta pcaA$ mutant Mtb

Because our results suggested that the *pcaA*-dependent modification of the cell envelope altered innate immune recognition of Mtb in a temporally restricted period during the first week of murine infection, we infected murine bone marrow-derived macrophages in vitro with wild-type and the  $\Delta pcaA$  mutant bacteria and measured both cytokine production and bacterial survival during the first few days of infection. Wild-type Mtb induced high levels of interleukin-6 and TNF beginning at 24 h after infection (Fig. 2 A, black bars). In contrast, the  $\Delta pcaA$  mutant induced 5.3-fold lower levels of TNF and 1.5-fold lower levels of IL-6 in culture supernatant at 24 h (Fig. 2 A, open bars). Remarkably, the hypostimulatory activity of the  $\Delta pcaA$  mutant was temporally restricted such that mutant and wild-type strains induced identical levels of TNF by 96 h after infection. Genetic complementation of the  $\Delta pcaA$  mutant with wild-type *pcaA* reversed the mutant phenotype (Fig. 2, striped bars). To more precisely characterize the temporal restriction of the mutant phenotype, we measured TNF production in culture supernatants of infected macrophages by removing supernatants every 24 h such that only the newly synthesized TNF was measured. These experiments confirmed that the  $\Delta pcaA$  mutant was hypoinflammatory during the first 48 h of infection but not thereafter (Fig. 2 B). These results indicated that the  $\Delta pcaA$  mutant was differentially recognized by the innate immune system at the earliest times after infection, but that *pcaA* was dispensable for innate immune recognition later in infection.

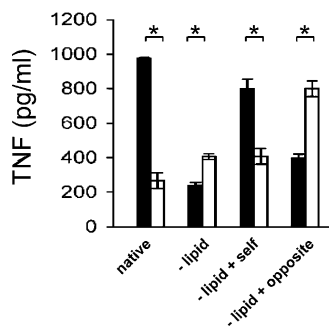
To directly ask whether mycolic acids deficient in cyclopropanation were hypoinflammatory during infection, we delipidated the outer surface of live bacteria using extraction with petroleum ether and infected macrophages with these delipidated organisms. Delipidation of Mtb by this method does not affect bacterial viability and removes a subfraction of cell wall lipids that is >90% TDM when examined by



**Figure 2. *pcaA*-dependent modification of extractable lipids mediates temporally restricted macrophage activation.** (A) Requirement of *pcaA* for early proinflammatory activation of macrophages by Mtb infection. Murine bone marrow-derived macrophages were left uninfected (stippled bar), or infected with wild-type Mtb (black bars),  $\Delta pcaA$  mutant Mtb (open bars), or the complemented  $\Delta pcaA$  mutant (striped bars). At the indicated time points after infection, TNF (top), and IL-6 (bottom) levels were determined by ELISA of culture supernatants. The same experiments were performed with delipidated bacteria, pictured on the right side of A. \*,  $P \leq 0.01$ . (B) Temporal sequence of *pcaA*-dependent macrophage activation. Murine bone marrow-derived macrophages were infected as described in A and supernatants were removed and replaced with fresh media every 24 h. Supernatants from the indicated time periods were assayed for TNF by ELISA. Stippled bar is uninfected, black bar is wild-type Mtb, and white bar is  $\Delta pcaA$  mutant infected. \*\*,  $P < 0.001$ . (C) Effect of *pcaA* on intracellular replication of Mtb in macrophages. Bone marrow-derived macrophages from wild-type C57BL/6 mice (WT) or TNF-deficient mice (TNF<sup>-/-</sup>) were infected with wild-type Mtb (black bars) or the  $\Delta pcaA$  mutant (open bars) and intracellular bacterial titers were determined at the indicated time points. #,  $P = 0.02$ .

thin layer chromatography (unpublished data; 23, 24). Delipidation of the live bacilli markedly reduced TNF production by macrophages infected with wild-type bacteria, but had no effect on the relatively weak TNF production stimulated by  $\Delta pcaA$  mutant bacteria during early infection. (Fig. 2 A), demonstrating that the difference in innate immune recognition between wild-type Mtb and the  $\Delta pcaA$  mutant was mediated by an extractable lipid.

To directly test whether the innate immune recognition of  $\Delta pcaA$  mutant bacteria resulted in more effective bacterial killing, we measured bacterial growth within macrophages.



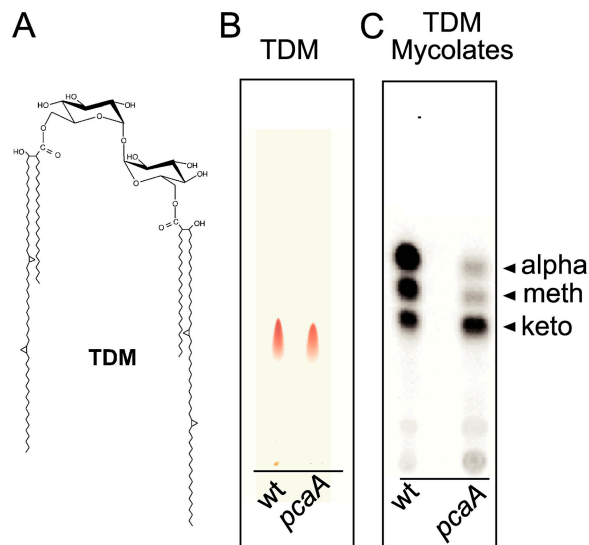
**Figure 3. Extractable lipids are sufficient to transfer the hypoinflammatory phenotype of the *pcaA* mutant.** Murine bone marrow–derived macrophages were infected with wild-type Mtb (black bar) or  $\Delta pcaA$  mutant Mtb (open bars) as in Fig. 2 A. Bacteria were either untreated (native), delipidated with petroleum ether (–lipid), delipidated and reconstituted with lipids from the same strain (–lipid + self), or delipidated and reconstituted with lipids from the opposite strain (–lipid + opposite). TNF levels after 24 h of infection are shown. \*,  $P < 0.001$ .

Wild-type bacteria replicated rapidly in murine macrophages (Fig. 2 C). Intracellular replication of wild-type bacteria was markedly reduced by delipidation (unpublished data) or in macrophages derived from TNF-deficient mice (Fig. 2 C), consistent with previous reports documenting that importance of extractable lipids and host TNF in Mtb intracellular replication (22, 24). In contrast,  $\Delta pcaA$  mutant bacteria were defective for intracellular growth compared with wild-type bacteria, but delipidation did not further reduce intracellular replication of mutant bacteria (unpublished data). These results demonstrated that *pcaA*-dependent modification of the extractable lipids of the Mtb cell envelope mediated proinflammatory innate immune recognition and facilitated early growth within macrophages.

To test whether *pcaA*-modified or wild-type–extractable lipids were sufficient to determine the innate immune recognition of Mtb during infection, we performed a lipid transfer experiment. Wild-type or  $\Delta pcaA$  mutant bacteria were delipidated and the resulting lipid extract was transferred either back onto the parent strain or onto the opposite strain. Remarkably, the hypoinflammatory phenotype of the  $\Delta pcaA$  mutant was transferred to delipidated wild-type bacteria reconstituted by  $\Delta pcaA$  lipids, demonstrating that  $\Delta pcaA$  mutant–extractable mycolates directly mediated the early hypoinflammatory phenotype (Fig. 3, –lipid + opposite, black bar). Conversely, delipidated  $\Delta pcaA$  mutant bacteria were recognized as wild type when reconstituted with wild-type lipids (Fig. 3, –lipid + opposite, white bar). These data were consistent with the conclusion that the altered inflammatory activity of  $\Delta pcaA$ -extractable lipids directly mediated the altered innate immune recognition of this strain by host macrophages that was apparent within the first 24 h of infection.

#### Reduced macrophage stimulation by purified $\Delta pcaA$ TDM

Trehalose dimycolate from Mtb has long been suspected to be a virulence determinant, in part due to its ability to produce granulomatous pathology similar to the pathology of

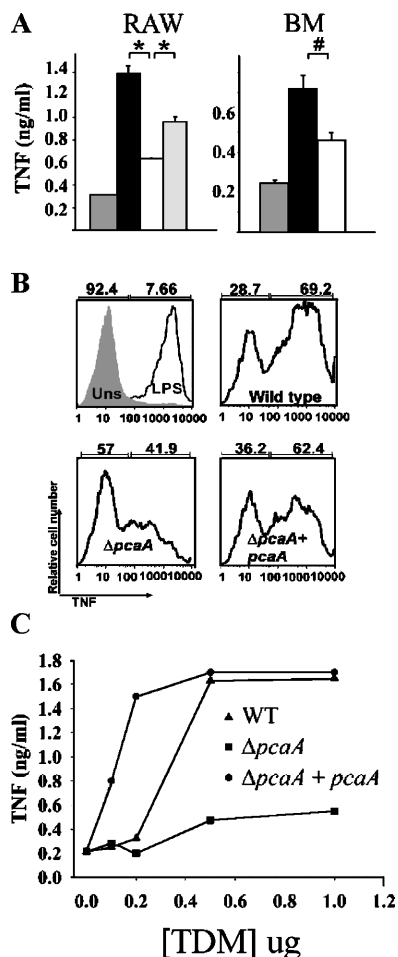


**Figure 4. Purification and characterization of wild-type and  $\Delta pcaA$  mutant TDM.** (A) Chemical structure of TDM. (B) Thin layer chromatography of purified TDM from wild-type and  $\Delta pcaA$  mutant *M. tuberculosis* developed with chloroform/methanol/water (90:10:1, vol/vol/vol). (C) Mycolate subclass analysis of TDM from wild-type and *pcaA* mutant strains.  $^{14}C$ -labeled TDM-derived mycolates were analyzed by high performance thin layer chromatography developed by running three times with hexane/ethyl acetate (95:5, vol/vol). Mycolate subclasses ( $\alpha$ , methoxy, and ketomycolates) are labeled with arrowheads.

Mtb infection. TDM contains a hydrophilic trehalose head group and two mycolic acids esterified at the six positions of each glucose (structure shown in Fig. 4 A). In the absence of *pcaA*, TDM lacks a single cyclopropyl ring in its  $\alpha$  mycolates and has an overabundance of ketomycolates (Fig. 4 C). Because the innate immune recognition of wild-type and mutant Mtb can be transferred by petroleum ether–extractable lipids, which are composed predominantly of TDM (Fig. 3), we hypothesized that the phenotype of the  $\Delta pcaA$  mutant could be completely or partially attributable to changes in the inflammatory activity of TDM. To test this directly, we purified TDM to homogeneity from wild-type Mtb and the  $\Delta pcaA$  mutant (Fig. 4 B) and tested the potency of these purified glycolipids in stimulating cultured macrophages.

Initial studies demonstrated that TDMs added directly to the medium of macrophage cultures did not induce any detectable responses. However, when purified TDM was coated onto the surface of tissue culture plates, it became highly stimulatory to macrophages. Wild-type TDM induced high levels of TNF, as measured both by ELISA of secreted TNF in cell culture supernatants (Fig. 5 A, black bars), and by flow cytometry with intracellular cytokine staining for TNF (Fig. 5 B). In contrast,  $\Delta pcaA$  TDM induced significantly lower levels of TNF from both bone marrow–derived macrophages and the macrophage cell line RAW264.7 (Fig. 5 A, open bars, and Fig. 5 B, lower left). A dose response curve revealed that the hypostimulatory activity of  $\Delta pcaA$  mutant TDM was present across a wide dose range and was reversed by restoration of the *pcaA* gene





**Figure 5. Effects of *pcaA* modification of TDM on innate immune recognition by macrophages.** (A) RAW 264.7 murine macrophage cell line or murine bone marrow–derived macrophages were stimulated with vehicle (stippled bar), or a monolayer of wild-type Mtb TDM (black bar), *pcaA* mutant TDM (open bar), or complemented mutant TDM (striped bar). TNF (ng/ml) in culture supernatants was measured 48 h after stimulation. \*,  $P \leq 0.001$ . #,  $P = 0.03$ . (B) RAW 264.7 cells were stimulated with indicated TDMs for 3 h, incubated for 3 h with Brefeldin A, and stained with monoclonal antibody to TNF. In the upper left plot, the shaded histogram is vehicle stimulated cells, whereas the open histogram is LPS-stimulated cells. Numbers above each plot are the percentage of TNF-positive and -negative cells using the gates established on unstimulated cells in the upper left panel.  $P < 0.01$  for the comparison of WT versus  $\Delta$ *pcaA* TDM by *t* test. (C) Dose response curve for TNF induction by wild-type TDM (triangle),  $\Delta$ *pcaA* mutant TDM (square), or complemented TDM (circle) from RAW cells measured after 24 h of stimulation.

through direct genetic complementation (Fig. 5 C). This difference was not due to generalized failure to stimulate the cells, as levels of IL-10 from TDM-stimulated macrophages were the same for both wild-type and  $\Delta$ *pcaA* TDMs (unpublished data). These results demonstrated that the cyclopropane content of TDM was an important determinant of the inflammatory activity of this glycolipid in macrophages, and identified the *pcaA*-dependent cyclopropanation of the mycolates of TDM as a proinflammatory lipid modification and a target for recognition by innate immunity.

### Proinflammatory effect of *pcaA*-modified TDM in vivo

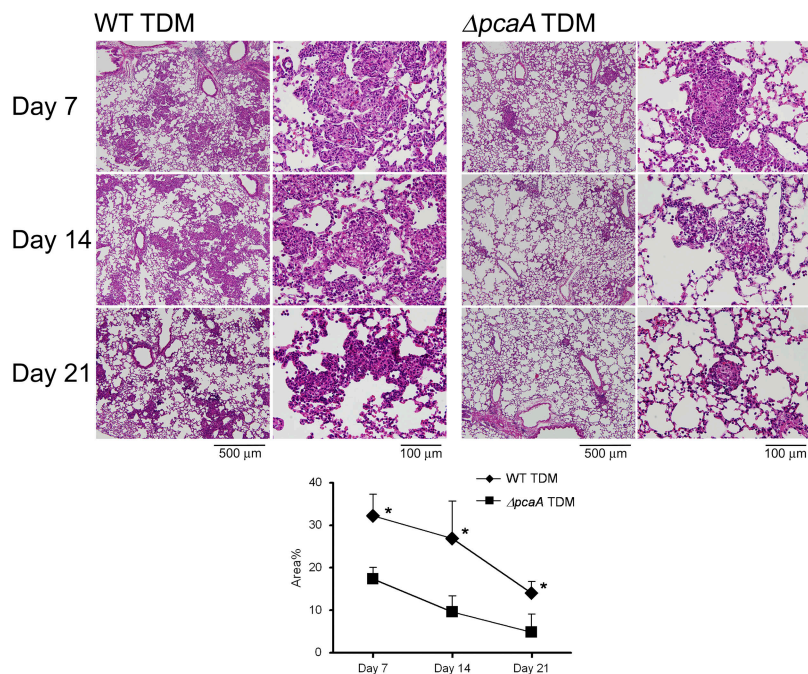
To test whether *pcaA* modification of TDM regulates in vivo inflammatory responses, we tested the potency of these purified glycolipids for inducing granuloma formation in mice. Consistent with the known properties of TDM (25, 26), wild-type Mtb TDM invoked granulomatous pathology in the lungs and liver when injected intravenously in mice as a water–oil–water emulsion (Fig. 6, left). This lung pathology peaked at day 7 and was characterized by mixed inflammatory infiltrates that obliterated the normal air spaces. Strikingly,  $\Delta$ *pcaA* mutant TDM was at least twofold less potent than wild-type TDM at inducing pulmonary granuloma (Fig. 6). These data demonstrated that the fine chemical structure of TDM could dramatically alter its inflammatory potency in vivo, and that *pcaA*-dependent modification of TDM was directly proinflammatory. These results also supported the hypothesis that the hypoinflammatory pathologic phenotype of the *pcaA* mutant strain in mice and macrophages was directly attributable to the altered inflammatory properties of cyclopropane-deficient  $\Delta$ *pcaA* mutant TDM.

### DISCUSSION

The results presented here establish a causal relationship between the fine chemical structure of mycolic acids and innate immune recognition of *M. tuberculosis* at the earliest period after aerosol infection. Whereas previous studies on the role of *pcaA* in virulence emphasized the importance of this gene in the persistence of Mtb and the pathology of the later stages of infection, the current study focused on the effects of deletion of *pcaA* on the earliest events after infection. This has led to the identification of the *pcaA*-dependent modification of mycolic acids, and in particular of the mycolic acids incorporated into TDM, as a critical proinflammatory lipid modification that regulated host-innate immune recognition during the first week of the murine infection in vivo and the first 24 h of macrophage infection in vitro.

Trehalose dimycolate, also named “cord factor,” has been an intensely studied cell envelope compound of *M. tuberculosis* for over 50 yr. TDM was the first virulence determinant proposed for *M. tuberculosis* when it was identified in a petroleum ether extract of *M. tuberculosis* and found to inhibit the migration of neutrophils (27–32). The biologic activity designated cord factor was later identified as TDM and was thought to be responsible both for the cording morphology and mycobacterial virulence. This postulated important role for TDM became less plausible when TDM was isolated from all mycobacteria that produce mycolic acids, most of which are nonpathogenic and do not form serpentine cords (33). However, interest in TDM has remained intense due to its powerful adjuvant properties, chemical properties when interacting with membranes (23, 34, 35), and ability to induce granulomatous inflammation in experimental animals that mimics whole Mtb infection (25, 26, 36).

By analyzing the activities of purified TDMs in vitro and in vivo, the results from the current study also strongly supported the view that the cyclopropane modification of TDM



**Figure 6. Reduced pulmonary granuloma formation by  $\Delta$ pcaA TDM in mice.** C57BL/6 mice were injected intravenously with a water–oil–water emulsion of TDM purified from wild-type (two left panels) or  $\Delta$ pcaA mutant (two right panels) Mtb at a dose of 300  $\mu$ g per mouse. The histology

in the Mtb cell envelope acts directly as an effector of pathogenesis, rather than by inducing indirect effects due to structural modifications of the cell envelope. As such, this study provides proof of principle that the chemical diversity of the Mtb cell envelope has evolved to interact specifically with host cells and not solely as a structural scaffold, as has been noted with other cell envelope mutants with impaired virulence (11). Cyclopropane modification of membrane lipids has been defined in *E. coli* and other bacteria and affects resistance to cold shock and acid (37, 38). However, the immunomodulatory function for cyclopropane modification of bacterial lipids identified in the current study is a novel function for this chemical entity. *M. tuberculosis* expresses a large family of mycolic acid methyl transferases/cyclopropane synthases that modify mycolic acids (39–41), two of which are known to be important for pathogenesis (7, 8). However, the pathogenetic mechanism of cyclopropanation for bacterial virulence or pathogenesis has been unclear. Mycolates are recognized by T cells when presented on CD1, but evidence to date indicates that this recognition is independent of cyclopropane modification (42). Instead, our findings demonstrated that cyclopropane modification of mycolic acids acted directly to promote the virulent behavior of mycobacteria by modulating innate immune activation of macrophages and potentially other cell types during infection. The particular macrophage receptor molecules responsible for these responses to TDM have not yet been identified, and this important point will require further study.

Our results strongly point to TNF as a key mediator of the effects of the normally cyclopropanated TDM molecules

of the lungs was examined at 7, 14, and 21 d after injection. The area of granulomatous inflammation was quantitated as described in Materials and methods and plotted against time. Diamonds represent wild-type TDM whereas squares represent  $\Delta$ pcaA mutant TDM. \*,  $P < 0.05$ .

of wild-type Mtb on the host immune response. Thus, the reduction in growth rate of the  $\Delta$ pcaA mutant seen in the first week of infection was reversed in TNF-deficient mice, and the difference observed previously in survival of mice infected with wild-type versus  $\Delta$ pcaA bacilli was also absent in TNF-deficient mice. Because the  $\Delta$ pcaA mutant elicited a markedly reduced TNF response compared with wild-type Mtb, these findings were consistent with the recent proposal that one effect of TNF may actually be to facilitate the growth of the bacilli early in the course of infection (22). Thus, the reduced stimulation of TNF production by  $\Delta$ pcaA TDM leads to less abundance of TNF during initial infection, and reduced bacterial growth in the first week. The critical importance of TNF in antimycobacterial defense is well established in mice (17) and humans (18). However, the apparent protective effect of TNF is partially due to the defective immune regulation that results from its absence, leading to massive  $T_H1$  type immune activation, tissue necrosis, and death (19, 20). A direct role for TNF in antimycobacterial activity of macrophages has been controversial, and recent data suggest that TNF facilitates growth of virulent, but not attenuated strains of Mtb (21, 22) in cultured human macrophages, suggesting that induction of TNF may be an important virulence strategy of Mtb. Previous studies of Mtb infection in TNF-deficient mice have shown that bacterial burdens are unaffected during the first 2 wk of infection, suggesting either that TNF has no role in early growth of Mtb, or that TNF has equal and opposing effects on bacterial growth during early infection in vivo. In this latter model, loss of a growth promoting effect of TNF in

macrophages would be counterbalanced *in vivo* by loss of a growth restricting effect of TNF produced by other cell types (43). This model is consistent with the data presented here in which the growth of the *pcaA* mutant is restricted in wild-type mice but recovers to wild-type Mtb growth levels in TNF-deficient mice. The data presented here indicate that Mtb has evolved cyclopropane lipid modification to manipulate the host TNF axis. In the case of the  $\Delta$ *pcaA* mutant, defective growth in the first week after aerosol infection and altered innate immune recognition during this period attenuated the later pathology of the infection. As shown in our previous study (8), this dramatically alters the course of chronic Mtb infection, and thus emphasizes the powerful interrelationship between innate and adaptive immunity in this infection.

Our results expand earlier studies that examined the role of cell envelope lipids in immunopathogenesis of Mtb infection. A clinical strain of Mtb that was hypervirulent for mice induced lower levels of TNF in mouse lung at 28 d (44) and was hypoinflammatory in cultured macrophages *in vitro* over the course of a 96-h infection (45). Recent work indicates that these phenotypes are due to production of phenolic glycolipid by this clinical strain (46). Thus, the accumulated data indicate the prolonged suppression of host TNF by PGL promotes bacterial virulence, whereas temporally restricted suppression of host TNF during the first weeks of infection through loss of the *pcaA* modification of TDM is advantageous to the host. These data are consistent with a model in which structurally distinct lipid components of the cell envelope promote or inhibit host inflammatory responses at distinct time periods during the course of infection for the ultimate purpose of achieving microbial symbiosis.

Our past and present results provide new insight into the relationship between TDM and mycobacterial pathogenesis. Our previous work demonstrated that inactivation of the cyclopropane synthase *pcaA* abolished cording and attenuated Mtb in mice (8). In light of prior work with cord factor these results suggested that the cyclopropane modification of TDM was necessary for the cording morphology and explains the lack of cording of saprophytic mycobacteria that contain TDM because these mycobacteria lack cyclopropane modification of mycolic acids. Our present results indicate that *pcaA* modification of TDM with cyclopropyl groups is a proinflammatory modification both in the context of purified glycolipid and whole bacilli. Strikingly, this pathogenic function of this lipid modification is temporally restricted to early infection. This demonstrates not only that cell envelope glycolipids of Mtb are direct effectors of pathogenesis, but that each cell envelope effector may have distinct functions at restricted time points during infection. Although cyclopropane synthases of Mtb are clearly not essential for *in vitro* growth and viability, the findings of the current study suggest that pharmacologic inhibition of members of this enzyme family could reverse pathogen-induced immunomodulation, thereby enhancing host immunity and control or eradication of infection.

## MATERIALS AND METHODS

**Media, strains, and culture conditions.** All *M. tuberculosis* strains were grown in Middlebrook 7H9 liquid media (Becton Dickinson) supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC) (Becton Dickinson), 0.5% glycerol (Fisher Scientific) and 0.05% Tween-80 (Sigma-Aldrich). Where appropriate, hygromycin (Roche) was added at a final concentration of 50  $\mu$ g/ml. The wild-type *M. tuberculosis* strain used in this study was *M. tuberculosis* Erdman, which has been passaged in mice and minimally passaged *in vitro*. The *M. tuberculosis*  $\Delta$ *pcaA* mutant and the  $\Delta$ *pcaA* mutant complemented with a single copy of *pcaA* under its native promoter have been described previously (8). Solid media for the growth of Mtb was Middlebrook 7H10 (Becton Dickinson) with 10% OADC and 0.5% glycerol, and cultures were incubated at 37°C with 5% CO<sub>2</sub>.

RAW 264.7 cells and L929 cells were obtained from American Type Culture Collection and were cultured in DMEM and RPMI-1640, respectively, supplemented with 10% FBS, L-glutamine, Pen-Strep, 50  $\mu$ g/ml Gentamicin, Hepes, and 2-mercaptoethanol (Gibco-BRL). All culture media and cells were tested for LPS by the Limulus amoebocyte assay: QCL-1000 (Cambrex Biosciences) and were below the limit of detection of the assay (0.1 EU/ml). All cell lines and tissue culture reagents were tested routinely for mycoplasma contamination using a PCR-based assay, as described previously (47).

**Purification of TDM from Mtb.** TDM was purified from *M. tuberculosis* grown in liquid media. Cells were harvested by centrifugation and autoclaved to kill viable bacteria. Autoclaved pellets were weighed and sonicated in chloroform/methanol (4:1, vol/vol) for 15 min on ice. Water was added (1/20 total volume) and the organic phase was collected. The aqueous phase was sequentially reextracted with chloroform/methanol (3:1 and 2:1, vol/vol) and the organic phases combined and evaporated completely. The dried pellet was extracted with acetone and the insoluble phase containing TDM was collected by centrifugation. The TDM fraction was precipitated from chloroform by dropwise addition of methanol at 4°C to a final ratio of 1:2 chloroform/methanol (vol/vol). This precipitate was dissolved in tetrahydrofuran and reprecipitated by dropwise addition of methanol at 4°C to a final ratio of 1:2 tetrahydrofuran/methanol (vol/vol). The precipitated TDM fraction was then dissolved in chloroform/acetone (8:2, vol/vol), loaded onto a column of silica gel, and eluted with chloroform/methanol (9:1, vol/vol). The final product was weighed and the purity and quantity were examined by TLC using 10  $\times$  10 cm HPTLC plates (Alltech Associates, Inc.) developing with chloroform/methanol/water (90:10:1, vol/vol/vol). Products were visualized by spraying with 20% sulfuric acid in ethanol and charring for 15 min at 110°C.

For analysis of mycolates derived from TDM, logarithmically replicating bacilli were incubated with 50  $\mu$ Ci 2-[<sup>14</sup>C] acetic acid (PerkinElmer) for 18 h. Labeled bacilli were harvested by centrifugation and extracted with 2:1 chloroform-methanol (vol/vol) for 12 h. The chloroform-methanol extract was dried under nitrogen and mycolic acids were prepared as described previously (39). Mycolates were analyzed by high performance thin layer chromatography using three developments of hexanes/ethyl acetate (95:5) and plates were visualized by autoradiography using a Bio Max Trans-Screen LE (Eastman Kodak Co.).

**Response of macrophages to TDM.** Purified TDM was used to stimulate either RAW 264.7 cells or bone marrow-derived macrophages by a modification of the protocol described previously (48). In brief, TDM was suspended at a concentration of 1 mg/ml in isopropanol and sonicated in a bath sonicator (model 3510; Branson Ultrasonic Corporation) for 5 min. This suspension was then incubated at 60°C for 10 min and sonication repeated. The resulting solution was layered onto 24-well tissue culture plates at the indicated concentrations and incubated at 37°C in order to ensure complete evaporation of the solvent. Control wells were layered with solvent without TDM and incubated at 37°C. To this layer of TDM, either RAW 264.7 cells or bone marrow-derived macrophages were added at a concentration of 10<sup>6</sup> cells in 100  $\mu$ l of medium and incubated at 37°C. At



various time intervals after stimulation, supernatants were collected for analysis of cytokine production by using the commercial ELISA duo-set kit (BD Biosciences) according to the manufacturer's recommendations. Intracellular cytokine staining was performed with the Cytotfix/Cytoperm system (BD Biosciences) according to the manufacturer's instructions. Antibodies used were FITC-labeled anti-CD11b (Mac-1) antibody and APC-labeled anti-TNF- $\alpha$  antibody (BD Biosciences) and CD11b<sup>+</sup> cells were analyzed for expression of TNF in an LSR flow cytometer (BD Biosciences).

**Isolation of bone marrow macrophages.** Marrow cells were isolated from both hind limbs of mice and cultured at a concentration of  $2-5 \times 10^6$  cells/ml in RPMI-1640 containing 20% FBS and 30% L929 cell supernatant at 37°C in Petri-dishes (OPTILUX™; BD Discovery Labware; BD Biosciences). After 2 d, the plates were washed with sterile HBSS in order to remove the nonadherent cells. The adherent population was further incubated in macrophage growth media (RPMI 1640 + 20% FBS + 30% L929 cell supernatant) for 3–4 d after which the cells were harvested.

**Preparation of inoculum and infection of macrophages.** Before infection with mycobacteria, bone marrow-derived macrophages were seeded at a concentration of  $2 \times 10^5$  cells/well in a 24-well tissue culture dish in RPMI 1640 medium containing 10% FBS without antibiotics and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 16–18 h. The mycobacterial strains for infection of macrophages were cultured in Middlebrook 7H9 broth at 37°C to mid-log phase of growth ( $A_{600}$  of 0.5–0.8). The cells were harvested by centrifugation at 3,000 g and washed twice with PBS containing 0.05% Tween-80 in order to remove excess media components. The cells were resuspended in PBS-Tween-80, sonicated for 5 s to disperse clumps, and adjusted to a concentration of  $10^7$ /ml based on the  $A_{600}$ . Macrophages were infected at a multiplicity of infection of 5 for 6 h at 37°C. The cells were washed twice with sterile HBSS in order to remove extracellular bacteria. The levels of secreted cytokines in the culture supernatants were estimated by ELISA. For estimation of the bacterial load, macrophages were lysed by addition of a solution of PBS containing 0.05% SDS and serial dilutions of the lysates were plated onto Middlebrook 7H10 and incubated at 37°C for 3 wk.

**Infection of mice by mycobacteria.** 6–8-wk-old C57BL/6 mice obtained from Jackson ImmunoResearch Laboratories and maintained on standard feed and specific pathogen-free conditions were infected aerogenically with mycobacterial strains in a Middlebrook inhalation exposure system (Glas-Col). All animal procedures were approved by the Memorial Sloan Kettering Institutional Animal Care and Use Committee. The mycobacterial inoculums for murine infection were prepared as described earlier for infection of macrophages. Mtb cells were suspended at a concentration of  $4 \times 10^8$  CFU in 10 ml of sterile distilled water. Mice were infected with a volume of suspension and exposure time calibrated to deliver  $\sim 100$  CFU per animal. The extent of infection was estimated by plating the lung homogenates of animals killed at 24 h after aerosol infection. At various time intervals after infection the lungs and spleen were homogenized in PBS + 0.05% Tween-80 and serial dilutions were plated onto Middlebrook 7H10 agar. After incubation at 37°C in a 5% CO<sub>2</sub> atmosphere, mycobacterial colonies were counted and the bacterial burdens in the organs were calculated.

**Delipidation and lipid reconstitution of mycobacteria.** Delipidation of bacilli was performed by using petroleum ether using a modification of published methods (24). After two 5-min extractions with petroleum ether, delipidated cells were centrifuged and suspended in PBS/0.05% Tween-80 for infection of macrophage cultures. Reconstitution of lipids was done by incubating delipidated cells with the petroleum ether extracts of mycobacterial cultures of identical cell number for 30 min at 25°C, following which cells were harvested by centrifugation and suspended in PBS/0.05% Tween-80. Macrophage cultures were infected with the native, delipidated, and reconstituted bacilli at a multiplicity of infection of 5 as described earlier.

#### Induction of pulmonary granulomatous inflammation in mice.

Granulomatous inflammation was induced by systemic injection of mice with a suspension of purified TDM. To prepare 1 ml of suspension, 1.5 mg of purified TDM was dried completely and then redissolved in 32  $\mu$ l of Incomplete Freund's Adjuvant (Difco Laboratories), to which 32  $\mu$ l of PBS was added. Normal saline with 0.2% Tween-80 was then added to a total volume of 1 ml, and the suspension was extensively mixed using a rotary homogenizer to form a water-oil-water emulsion. C57BL/6 mice were injected intravenously through the tail vein with 200  $\mu$ l of water-oil-water emulsion containing 300  $\mu$ g of TDM. Mice were killed at day 7, 14, and 21 after injection. The lungs were removed and fixed with 10% formalin. The sections were paraffin embedded and stained with hematoxylin-eosin. The areas of granulomatous inflammation were calculated by digital image processing using the Scion Image program (Scion Corp.). The level of granulomatous inflammation was quantitated by determining the area within each section that showed a pixel density greater than a threshold value that was two standard deviations above the average for the entire section. This area was divided by the total lung area in the section and multiplied by 100 to obtain a percentage value for the area of diseased lung.

The authors thank Feng Gao and Paola Bongiorno for outstanding technical assistance.

This work was supported by National Institutes of Health grants AI53417 (M.S. Glickman), AI 45889 (S.A. Porcelli), and AI48933 (S.A. Porcelli). M.S. Glickman is the recipient of the Ellison Medical Foundation New Scholars Award in Global Infectious Diseases, and a grant from the Speakers' fund for Biomedical Research awarded by the City of New York. N. Fujiwara was supported by grants from the Ministry of Health, Labour and Welfare in Japan (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants) and the Mitsubishi Pharma Research Foundation.

The authors have no conflicting financial interests.

Submitted: 18 August 2004

Accepted: 16 November 2004

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