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Research Article   **Microbiology**

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Trans-cyclopropanation of mycolic acids on trehalose dimycolate suppresses *Mycobacterium tuberculosis*–induced inflammation and virulence

Vivek Rao,[1] Feng Gao,[1] Bing Chen,[2] William R. Jacobs Jr.,[2] and Michael S. Glickman[1,3]

[1]Immunology Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York, USA.
[2]Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York, New York, USA.
[3]Division of Infectious Diseases, Memorial Sloan-Kettering Cancer Center, New York, New York, USA.

Recent studies have shown that fine structural modifications of *Mycobacterium tuberculosis* cell envelope lipids mediate host cell immune activation during infection. One such alteration in lipid structure is *cis*-cyclopropane modification of the mycolic acids on trehalose dimycolate (TDM) mediated by *proximal cyclopropane synthase of* α *mycolates* (*pcaA*), a proinflammatory lipid modification during early infection. Here we examine the pathogenetic role and immunomodulatory function of mycolic acid cyclopropane stereochemistry by characterizing an *M. tuberculosis cyclopropane–mycolic acid synthase 2* (*cmaA2*) null mutant (Δ*cmaA2*) that lacks *trans*-cyclopropanation of mycolic acids. Although titers of WT and Δ*cmaA2* organisms were identical during mouse infection, Δ*cmaA2* bacteria were hypervirulent while inducing larger granulomas than WT *M. tuberculosis*. The hypervirulence of the Δ*cmaA2* strain depended on host TNF-α and IFN-γ. Loss of *trans*-cyclopropanation enhanced *M. tuberculosis*-induced macrophage inflammatory responses, a phenotype that was transferable with petroleum ether extractable lipids. Finally, purified TDM lacking *trans*-cyclopropane rings was 5-fold more potent in stimulating macrophages. These results establish *cmaA2*-dependent *trans*-cyclopropanation of TDM as a suppressor of *M. tuberculosis*-induced inflammation and virulence. In addition, cyclopropane stereochemistries on mycolic acids interact directly with host cells to both positively and negatively influence host innate immune activation.

Introduction

The ongoing global health crisis of tuberculosis infection has stimulated intense investigation into the molecular basis of *Mycobacterium tuberculosis* pathogenicity (1–5). A substantial subset of genes identified as important for *M. tuberculosis* pathogenesis is involved in biosynthesis of the complex mycobacterial cell envelope. The mycobacterial cell envelope is a complex array of lipids and glycolipids that forms a hydrophobic barrier but also contains substantial adjuvant activity (6–8). Several cell wall determinants implicated in *M. tuberculosis* pathogenesis include phthiocerol dimycocerosate, phenolic glycolipid (PGL), oxygenated mycolates, mycolic acid length, and mycolic acid cyclopropanation (9–15). While some of these lipids appear to mediate pathogenesis through their contribution to a hydrophobic barrier (16), recent evidence strongly supports a model in which specific *M. tuberculosis* cell envelope lipids directly activate or repress host innate immune responses.

One such lipid is PGL, a glycolipid produced universally by *Mycobacterium leprae* but sporadically by *M. tuberculosis* strains (17). Recently, a globally disseminated clinical strain was shown to be hypervirulent for mice. This strain synthesized PGL due to the absence of a frameshift mutation in the *pks1–15* genes present in many TB strains (15). Purified PGL directly inhibited TNF-α

secretion from macrophages, suggesting that PGL in the cell envelope of these strains promoted hypervirulence through suppression of immune activation. Similarly, our recent study with a mutant strain of *M. tuberculosis* deficient in *cis*-cyclopropanation of the α mycolic acid demonstrated that this lipid modification was required for full activation of innate immune responses during early infection (18). This immunostimulatory effect of cyclopropanation was due to altered inflammatory activity of trehalose dimycolate (TDM), a long-suspected glycolipid virulence determinant of *M. tuberculosis* and related pathogenic mycobacteria (19–26). These results implicated *cis*-cyclopropanation of TDM as a proinflammatory lipid modification. Taken together, these 2 examples validated the hypothesis that the *M. tuberculosis* cell envelope is in part a complicated array of lipid effector molecules that interact with host cells (27).

In addition to *proximal cyclopropane synthase of* α *mycolates* (*pcaA*)-mediated *cis*-cyclopropanation of mycolic acids, *M. tuberculosis* expresses a family of cyclopropane synthases that modify mycolic acids at specific locations or with specific stereochemistries of cyclopropane rings (28–32). Genetic deletion of *cyclopropane–mycolic acid synthase 2* (*cmaA2*) abolishes *trans*-cyclopropanation of both methoxy- and ketomycolates while α mycolates are unaffected (30). While *cis*-cyclopropanation of membrane fatty acids is widely distributed in bacteria and plants (33–36), *trans*-cyclopropane rings are exceedingly rare. Here we examine the biological function of *trans*-cyclopropanation in *M. tuberculosis* and show that the *trans*-cyclopropanation of mycolic acids on TDM limits *M. tuberculosis* virulence and suppresses host inflammatory responses during infection.

Nonstandard abbreviations used: *cmaA2*, cyclopropane–mycolic acid synthase 2; *pcaA*, proximal cyclopropane synthase of α mycolates; PGL, phenolic glycolipid; TDM, trehalose dimycolate.

Conflict of interest: The authors have declared that no conflict of interest exists.
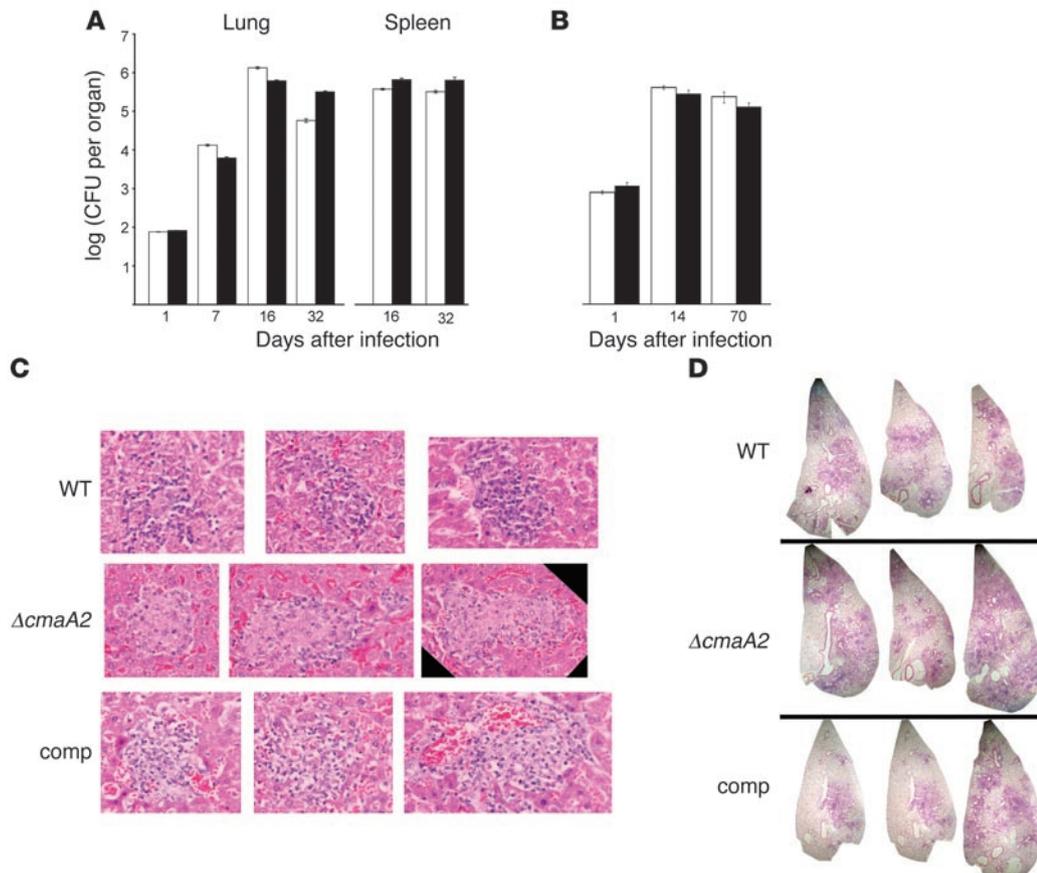
Figure 1
Loss of *cmaA2* does not affect bacterial number but alters granuloma composition. (**A**) WT C57BL/6J mice were infected by aerosol with WT *M. tuberculosis* (white bars) or the Δ*cmaA2* mutant (black bars), and bacterial titers in lungs and spleen were determined at the indicated time points by serial dilution and plating of tissue homogenates. Each bar shows the mean value of bacterial loads from 3 mice per group with error bars indicating SEM. (**B**) Bacterial loads in lungs of animals infected intravenously with WT *M. tuberculosis* (white bars) or the Δ*cmaA2* mutant (black bars). (**C**) Altered granuloma structure in tissues upon infection with Δ*cmaA2.* Representative liver granulomas (3 per infecting strain) from mice infected with WT, Δ*cmaA2*, and complemented (comp) strain. Magnification, ×400. See Results for statistical analysis of granuloma size and cellular composition. (**D**) H&E-stained lungs from C57BL/6J mice infected with the indicated strains and sacrificed 6 months after infection. See Results for quantitation of granulomatous inflammatory lesions.

Results

cmaA2 suppresses M. tuberculosis virulence. To understand the pathogenetic role of mycolic acid *trans*-cyclopropanation in *M. tuberculosis,* we characterized an *M. tuberculosis cmaA2* null mutant that lacks *trans*-cyclopropanation in all oxygenated mycolic acids but synthesizes intact ratios of all mycolic acid subclasses (30). We infected C57BL/6J and BALB/C mice with WT *M. tuberculosis*, a *cmaA2* null mutant, and a genetically complemented *cmaA2* mutant carrying a single copy of WT *cmaA2* expressed from its native promoter. Both BALB/C and C57BL/6J mice received either 100 CFUs by aerosol or 10^5 or 10^6 CFUs by tail vein injection. Regardless of infection route or mouse strain, loss of *cmaA2* did not affect bacterial loads in lungs, liver, and spleen during the intial 70 days of infection (Figure 1, A and B, and data not shown). These results demonstrate that loss of *cmaA2* function in *M. tuberculosis* is not important for bacterial colonization, replication, or persistence in vivo during the first 70 days of infection.

Many recent studies demonstrated that *M. tuberculosis* virulence and immunopathology can occur independently of bacterial titer

(15, 37–41). To examine whether deletion of *cmaA2* caused alterations in the granulomatous histopathology characteristic of *M. tuberculosis* infection, we performed quantitative microscopy on liver granulomas from infected animals at 2.5 weeks after infection. Δ*cmaA2* granulomas were larger than WT granulomas (WT, 12.55; Δ*cmaA2*, 17.19; complemented, 16.35 AU; *P* = 0.004 for WT versus mutant by 2-tailed Student's *t* test) and were lymphopenic both as a fraction of total nucleated cell number per granuloma (WT, 41%; Δ*cmaA2*, 16%; complemented, 30% lymphocytes) and when expressed as lymphocytes per lesion area (WT, 3.98; Δ*cmaA2*, 1.31; complemented, 2.58 AU). These histopathologic differences were also observed in the lungs of infected mice, which displayed more severe inflammatory involvement both early (data not shown) and late in infection (Figure 1D). At 6 months after infection, lungs from Δ*cmaA2* mutant infected animals displayed more severe granulomatous inflammation than WT lungs (Figure 1D): 38% of WT lung area was replaced by inflammatory lesions while Δ*cmaA2*-infected lungs displayed 52% involvement and complemented mutant infected lungs displayed 24% involvement. These results indicate that inactivation of *cmaA2* results in a pathologically more aggressive infection both early and late in infection.

To ask whether these differences in granuloma composition reflected changes in strain virulence, we examined host morbidity in infected mice after intravenous and aerosol infection. Δ*cmaA2 M. tuberculosis*–infected mice succumbed to infection earlier than WT infected mice in both intravenous infection of BALB/C (Figure 2A) and C57BL/6J (Figure 2B) mice and in aerosol infection of C57BL/6J mice (Figure 2C). The mean survival time of C57BL/6J mice infected with 10^5 CFUs of WT or Δ*cmaA2* strains was 320 and 227 days, respectively (*P* < 0.0001) (Figure 2B). BALB/C mice infected with 10^6 of WT, Δ*cmaA2*, or the complemented mutant had mean survival times of 113, 90, and 116 days, respectively (*P* < 0.0001 for WT versus mutant; *P* = 0.003 for complemented
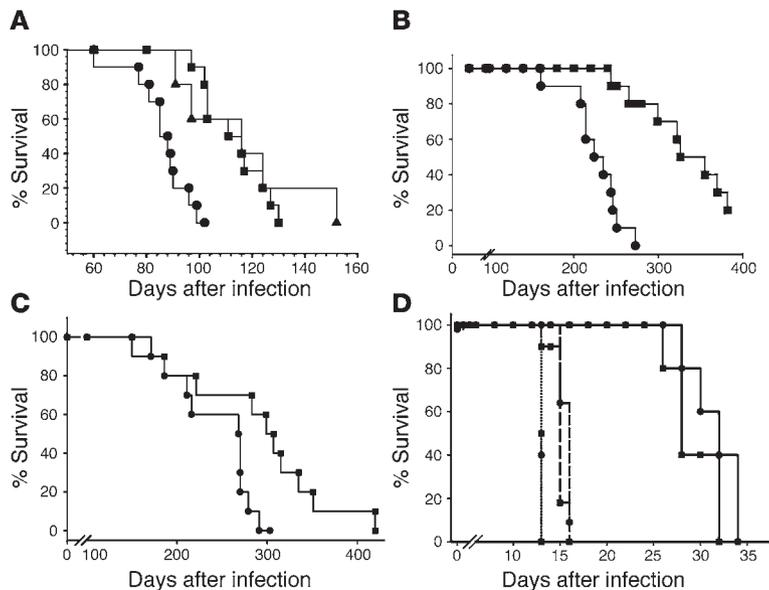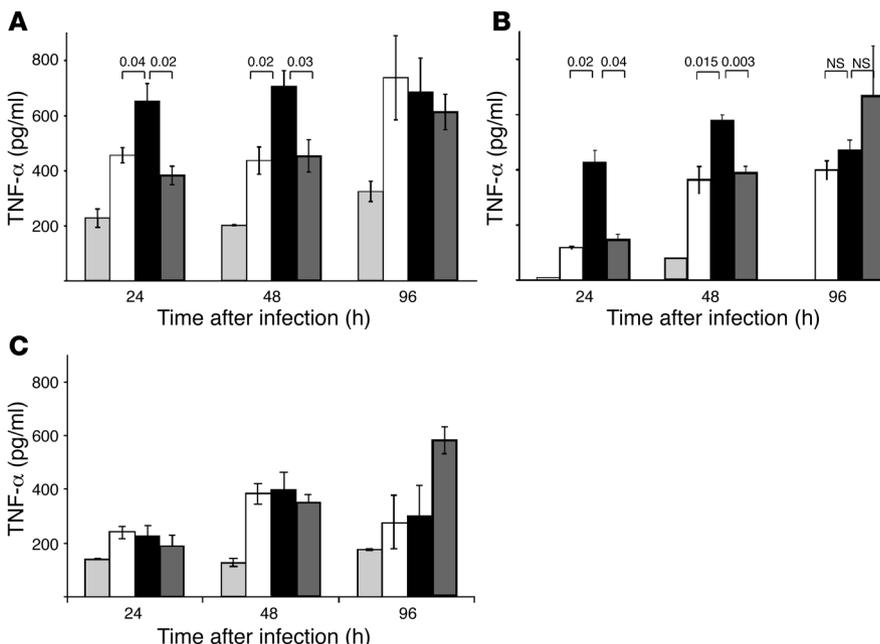
Figure 2
cmaA2 suppresses *M. tuberculosis* virulence in mice. (**A**) Survival kinetics of WT BALB/C mice infected via tail vein injection with 10^6 CFUs of either WT *M. tuberculosis* (squares), Δ*cmaA2 M. tuberculosis* (circles), or the complemented Δ*cmaA2* strain (triangles). (**B** and **C**) Survival kinetics of WT C57BL/6J mice infected with *M. tuberculosis* (squares) or Δ*cmaA2 M. tuberculosis* (circles) by tail vein injection (10^5 CFUs, **B**) or aerosol (100 CFUs, **C**). (**D**) Survival of immunodeficient mice infected with WT (squares) and Δ*cmaA2* (circles) strains. TNF-α–deficient mice (solid lines), IFN-γ–deficient mice (dotted lines), and SCID mice (dashed lines) were infected as described in Methods, and survival was recorded.

versus mutant; *P* = 0.37 for WT versus complemented; Figure 2A). Mean survival times of C57BL/6J mice infected with 100 CFUs of WT *M. tuberculosis* was 314 days and 247 days for Δ*cmaA2* mutant infected mice (*P* = 0.0002) (median survival: WT 318 days; *cmaA2*, 263 days). Taken together, these results demonstrate that the *cmaA2* gene and *trans*-cyclopropanation of cell envelope mycolic acids restrain *M. tuberculosis* virulence and alter granuloma composition.

*Requirement of host immunity for the hypervirulence of the Δ*cmaA2* strain.* Earlier work from several research groups implicated cell-mediated immunity and cytokines such as TNF-α and IFN-γ as important mediators of host defense against *M. tuberculosis* infection in mice and humans (42–46). Our previous results with the *M. tuberculosis* Δ*pcaA* mutant demonstrated that early induction of TNF-α in host macrophages is affected by the *cis*-cyclopropane content of mycolic acids on TDM. To test the role of TNF-α in the

hypervirulent phenotype of the *cmaA2* mutant, we infected TNF-α$^{-/-}$ mice by aerosol and recorded host morbidity. TNF-α–deficient mice succumbed to infection with either strain in approximately 36 days, as reported previously for *M. tuberculosis* aerosol infection (47). In contrast to increased susceptibility of WT mice to Δ*cmaA2* infection, there were no differences in mortality of TNF-α–deficient mice upon infection with either strain (Figure 2D). Similar kinetics of host susceptibility were also observed in both IFN-γ–deficient and SCID mice (Figure 2D) when either strain was administered intravenously (48). Thus, increased virulence of Δ*cmaA2* mutant in WT mice depends on TNF-α, IFN-γ, and intact lymphocyte populations. These results imply that the hypervirulence of the *cmaA2* mutant strain is a manifestation of excessive immune activation, which produces larger and altered granulomas and, ultimately, premature death.

Figure 3
Δ*cmaA2 M. tuberculosis* is hyperinflammatory during macrophage infection. RAW 264.7 macrophages (**A**), BALB/C murine bone marrow–derived macrophages (**B**), and C57BL/6J bone marrow–derived macrophages (**C**) were left uninfected (light gray bars) or infected with WT *M. tuberculosis* (white bars), Δ*cmaA2* mutant *M. tuberculosis* (black bars), or the complemented Δ*cmaA2* mutant (dark gray bars). At the indicated time points after infection, TNF-α levels in culture supernatants were determined by ELISA. Values (pg/ml) indicate mean concentration of TNF-α in triplicate infections, and levels of statistical significance by 2-tailed Student's *t* test are indicated above the bars.
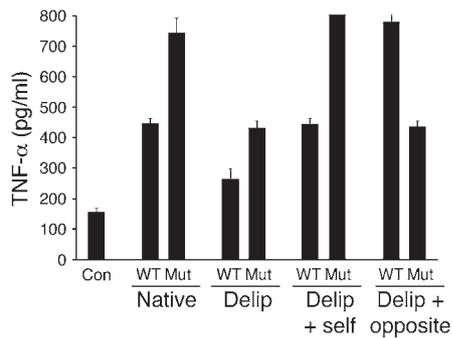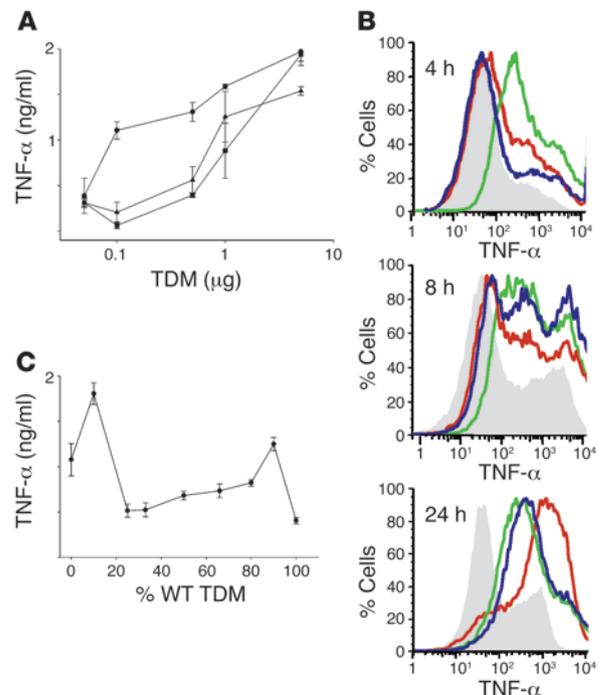
Figure 4

cmaA2-dependent extractable lipid modification suppresses *M. tuberculosis*–induced macrophage activation. Bone marrow–derived macrophages from BALB/C mice were left uninfected (con), or infected with WT *M. tuberculosis* or Δ*cmaA2* mutant *M. tuberculosis* (native), delipidated bacteria (delip), delipidated bacteria reconstituted with self lipids (delip + self), or delipidated and reconstituted with lipids from the opposite strain (delip + opposite). TNF-α levels were determined 24 hours after infection. Values are mean concentrations of quadruplicate wells.

*Early increase in macrophage cytokine responses to Δ*cmaA2* M. tuberculosis*. Multiple prior studies documented complicated relationships between cytokine induction and granuloma structure. To test whether the phenotype of the Δ*cmaA2* strain is associated with altered proinflammatory cytokine release from macrophages, we infected RAW 264.7 macrophages and murine bone marrow–derived macrophages and measured TNF-α release. As early as 24 hours after infection, *cmaA2*-deficient bacilli stimulated significantly higher levels of TNF-α from both RAW and BALB/C bone marrow–derived macrophages (Figure 3, A and B) but not from C57BL6J bone marrow–derived macrophages (Figure 3C). The hyperinflammatory phenotype of the Δ*cmaA2* strain was reversed by genetic complementation by a functional *cmaA2* gene (Figure 3, A and B), demonstrating that the phenotype of the mutant strain was due to loss of *cmaA2* function. Thus, inactivation of *cmaA2* in *M. tuberculosis* causes hyperinflammation during infection of macrophages, which is temporally restricted to early infection.

Cyclopropane-deficient extractable lipids directly mediate the hyperinflammatory phenotype of cmaA2 mutant bacteria. Based on our prior results with the *pcaA* mutant, we hypothesized that the *trans*-cyclopropane modification of extractable lipids in the cell envelope negatively influenced macrophage activation. To analyze the contribution of extractable lipids to the *cmaA2* mutant phenotype, we performed a lipid transfer experiment. Petroleum ether delipidation does not kill *M. tuberculosis* and removes a limit set of extractable lipids that is more than 90% TDM when examined by TLC (18). Native Δ*cmaA2* bacilli induced approximately 2-fold higher levels of TNF-α than WT bacteria (Figure 4; *P* < 0.001). Delipidation reduced the inflammatory capacity of Δ*cmaA2* bacilli to WT levels (Figure 4). Reconstitution of the delipidated bacteria with their corresponding lipid extracts restored the native phenotypes (Figure 4). However, when delipidated WT bacteria were reconstituted with Δ*cmaA2* lipids, the hyperinflammatory phenotype of the Δ*cmaA2* strain was transferred (Figure 4), demonstrating that the hyperinflammatory phenotype was directly attributable to the extractable lipid fraction of *M. tuberculosis*. Conversely, Δ*cmaA2* mutant bacteria acquired the WT inflammatory phenotype when reconstituted with WT lipids.

Trans-cyclopropane–deficient TDM is hyperinflammatory. TDM is an inflammatory glycolipid of the *M. tuberculosis* cell envelope that contains 2 cyclopropanated mycolic acids. Our prior work demonstrated that TDM from the *pcaA* mutant strain of *M. tuberculosis* is hypoinflammatory for macrophages and mice. We hypothesized that the hyperinflammatory phenotype of the Δ*cmaA2* mutant could be due to altered inflammatory potency of Δ*cmaA2*-derived TDM. To test this idea, we purified TDM from WT *M. tuberculosis*, Δ*cmaA2*, and the complemented strains. All 3 preparations were chemically pure when examined by thin layer chromatography and comigrated with commercially available TDM from *M. tuberculosis*, indicating their identity and homogeneity (data not shown).

We stimulated macrophages in vitro with monolayers of different concentrations of purified TDM from the 3 strains and measured TNF-α secretion. Dose-response curves derived from these experiments indicated that Δ*cmaA2* mutant–derived TDM was 4- to 6-fold more potent than either WT TDM or complemented mutant TDM, as assessed by the quantity of glycolipid to achieve half-maximal TNF-α release or the fold increase in TNF-α release at a fixed dose of TDM (Figure 5A). We did not observe any difference between WT and ΔcmaA2 TDM in TNF-α induction from C57BL/6J-derived bone marrow macrophages (data not shown). These results implicate the *trans*-cyclopro-

Figure 5

Trans-cyclopropane–deficient TDM is hyperinflammatory for macrophages. (**A**) Dose-response curve for TNF-α induction by WT TDM (squares), Δ*cmaA2* mutant TDM (circles), or Δ*cmaA2* mutant complemented TDM (triangles) from RAW cells measured after 24 hours of stimulation. (**B**) RAW 264.7 cells were stimulated with vehicle (gray areas), WT TDM (red lines), Δ*cmaA2* mutant TDM (green lines), or complemented TDM (blue lines) for 3 hours, and single cell levels of TNF-α were determined by intracellular cytokine staining for TNF-α. (**C**) Macrophages were stimulated with a total TDM dose of 1 μg in varying ratios of WT/Δ*cmaA2* TDM as indicated, and TNF-α in culture supernatants was measured by ELISA 6 hours after stimulation.

pane ring on the mycolates of TDM as a negative regulator of the inflammatory potency of this glycolipid.

To more completely characterize the temporal induction of TNF-α by these TDMs, we quantitated single-cell TNF-α production by macrophages using intracellular cytokine staining and flow cytometric analysis. Interestingly, Δ*cmaA2* TDM induced very high levels of TNF-α at as early as 4 hours of stimulation in comparison with the low levels of induction by the WT TDM (Figure 5B). Enhanced TNF-α induction by the mutant TDM was also maintained after 8 hours of stimulation. However, by 24 hours of stimulation, the level of TNF-α induction was significantly higher for WT TDM in comparison with the Δ*cmaA2* TDM. These quantitative and kinetic differences in TNF-α induction from macrophage cultures were overcome by complementation of the Δ*cmaA2* mutant with a functional *cmaA2* gene (Figure 5B).

The hyperinflammatory properties of Δ*cmaA2* TDM could be due either to loss of an inhibitory effect of the *trans*-cyclopropane ring in WT TDM or an enhanced stimulatory property of Δ*cmaA2* mutant TDM. If the *trans*-cyclopropane ring on TDM is directly inhibitory, this property should suppress the hyperinflammatory effect of *trans*-cyclopropane–deficient TDM. Alternatively, if *trans*-cyclopropane–deficient TDM is a stronger proinflammatory agonist, then combination of WT and mutant TDMs should be additive or indifferent but not antagonistic. To distinguish these possibilities, we stimulated macrophages with WT, Δ*cmaA2*, or varying ratios of both TDMs, holding the total dose of TDM constant, and analyzed TNF-α secretion. At WT/Δ*cmaA2* ratios of 0.2:0.8, 0.4:0.6, and 0.5:0.5, the hyperinflammatory properties of the cmaA2 TDM were inhibited by the WT TDM (Figure 5C). These results suggest that the *trans*-cyclopropane ring in WT TDM is an inhibitor of macrophage activation.

Discussion

In this study, we examined the pathogenetic role of *trans*-cyclopropanation of mycolic acids in the *M. tuberculosis* cell envelope. We have shown that *trans*-cyclopropanation is a negative regulator of *M. tuberculosis* virulence through suppression of the inflammatory activity of TDM. These results define a novel role for the rare chemical entity of *trans*-cyclopropane rings and expand the diversity of pathogenic functions mediated directly by cell envelope lipids of *M. tuberculosis*.

cmaA2 suppresses M. tuberculosis virulence. The traditional definition of virulence genes is a genetic determinant that enhances pathogenesis. However, there is growing recognition that pathogens that cause persistent infections employ complex strategies of pathogenesis, including functions that limit pathogen virulence or host pathology. Several genetic loci in *M. tuberculosis* are known to restrict virulence and, when mutated, result in hypervirulence in SCID mice (49, 50). In immunocompetent mice, inactivation of the *mceI* operon in *M. tuberculosis* resulted in a hypervirulent strain that grew to higher bacterial loads and caused accelerated host mortality (51). The mechanism by which these genetic loci restrict *M. tuberculosis* virulence is not clear.

Recent work demonstrated that some clinical strains of *M. tuberculosis* are hypervirulent for mice and produce PGL through preservation of an intact *pks1/15* gene (15). This hypervirulence was associated with suppression of macrophage inflammatory responses by PGL, suggesting that this glycolipid directly suppresses innate immune activation to promote bacterial virulence. The present study defines *cmaA2* as a negative regulator of *M. tuberculosis* viru-

lence and provides insight into the mechanism of *cmaA2*-dependent attenuation. *Trans*-cyclopropanation of mycolic acids on TDM restricted *M. tuberculosis*–induced macrophage inflammation during early infection, thereby altering granuloma structure and ultimately restricting virulence. The results presented here, taken together with prior results with PGL and the Δ*pcaA* mutant of *M. tuberculosis*, define 3 lipids that dynamically regulate macrophage activation during early infection and highlight the complex relationship among the mycobacterial cell envelope, innate immune activation, and *M. tuberculosis* virulence. While the Δ*cmaA2* mutant and the HN878 strain share hypervirulence in the mouse, they differ in their inflammatory phenotypes for macrophages, 1 being hyperinflammatory (Δ*cmaA2*) and 1 being hypoinflammatory (HN878). These results are consistent with abundant prior literature demonstrating that appropriate timing and levels of TNF-α are critical in preventing uncontrolled infection (TNF-α deficiency) or excessive tissue destruction (TNF-α excess) and that *M. tuberculosis* actively represses host macrophage activation (52–55).

Although the hypervirulent phenotype of the Δ*cmaA2* strain was present in both C57BL/6J and BALB/C mice, the hyperstimulatory phenotype for macrophages was only observed in BALB/C-derived cells. This may indicate that the in vivo source of TNF-α that mediates hypervirulence is a cell type other than macrophages. There is evidence that T cell– and macrophage-derived TNF-α play distinct roles in the containment of *M. tuberculosis* infection and that T cell–derived TNF-α modifies the granulomatous pathology of *M. tuberculosis* infection (56).

Cyclopropanation of bacterial lipids. Our recent work defines an immunomodulatory function for cyclopropanation of bacterial membrane lipids in the context of microbial pathogenesis. The most extensively studied cyclopropane modification system in bacteria is the CFA synthase (CFAS) of *Escherichia coli*, which *cis*-cyclopropanates membrane fatty acids during entry into stationary phase. *E. coli* deficient for CFAS is sensitive to acid (57), but no role in bacterial pathogenesis has been recognized. An extensive system of S-adenosyl methionine–dependent cyclopropane synthases exists in *M. tuberculosis*, and each enzyme is able to modify mycolic acids with a specific stereochemistry of cyclopropyl group or at a specific location. Although these cyclopropane modifications may indeed play a structural role in the integrity of the cell envelope, evidence presented here and our prior work implicate 2 stereochemistries of cyclopropane rings in pathogen-induced immunomodulation. Such a role is supported by recent studies examining the *M. tuberculosis* transcriptional profile while growing in human lung (58). This study found in vivo induction and repression of mycolic acid cyclopropane synthases, including *pcaA*, *cmaA2*, *mmaA2*, and *mmaA1*, supporting the idea that *M. tuberculosis* modifies its cell envelope in response to the host. Further studies of a complete set of mycolic acid cyclopropane synthase mutants in *M. tuberculosis* will further define the functions of this unique lipid modification.

Innate immune recognition of mycobacterial cell wall products and TDM. TDM has been long recognized as one of the immunomodulatory molecules of mycobacterial cell walls. In mice, TDM induces responses that mimic certain aspects of *M. tuberculosis* infection: development of granulomatous lesions, induction of proinflammatory cytokines, decrease in the presence of serum cortisol, and enhanced procoagulant activity (19, 59, 60). TDM has also been implicated in enhancing survival of mycobacteria by its ability to inhibit phagosome-lysosome fusion and trafficking events during

infection (23). Although several studies have demonstrated these properties of mycobacterial TDM, the molecular mechanisms underlying these responses are not yet completely understood. Prior work has identified the role of lipid antigen presenting molecule CD1d and the C5a receptor in establishment and organization of TDM-induced granuloma formation in mice (61, 62). Mice deficient in either of these molecules developed edematous dysregulated granulomatous responses and also elaborated higher levels of proinflammatory cytokines in response to TDM administration. While *M. tuberculosis* or its cell wall components have been shown to activate macrophages via TLRs, principally TLR2 and TLR4, a recent study demonstrates that TDM-induced activation of macrophages is independent of both of these TLRs (63). However, an active role of MyD88, an adaptor for TLR- and IL-1 receptor–mediated signaling, was reported in TDM-induced cell recruitment and cytokine production, suggesting the role of a receptor other than TLR2 or TLR4 in recognition of TDM. Our present and prior works clearly establish a role for TDM and cyclopropane modification of mycolic acids of TDM in *M. tuberculosis*–mediated immunomodulation and pathogenesis. Identification of a cellular receptor that recognizes TDM as a whole and can distinguish cyclopropane modifications of mycolic acids on TDM is a critical area of investigation for understanding the immunopathogenesis of *M. tuberculosis* infection and its control by cell envelope lipids.

Methods

Media, strains, and culture conditions. All *M. tuberculosis* strains were grown in Middlebrook 7H9 liquid media supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase), 0.5% glycerol, and 0.05% Tween-80 as well as 50 μg/ml of hygromycin and 20 μg/ml of kanamycin when required. For growth in solid media, Middlebrook 7H10 agar supplemented with 10% OADC and 0.5% glycerol was used, and plates were incubated at 37°C with 5% CO_2. An animal-passaged (minimally cultured in vitro) strain of *M. tuberculosis* Erdman was used as the WT strain for this study. The *M. tuberculosis* Δ*cmaA2* mutant and the complemented strain (Δ*cmaA2* with a single copy of *cmaA2* under its native promoter) have been previously described (30).

RAW 264.7 cells and L929 cells were obtained from ATCC and were cultured in DMEM and RPMI-1640, respectively, supplemented with 10% FBS, L-glutamine, Pen-Strep, 50 μg/ml gentamicin HEPES, and 2-mercaptoethanol (Invitrogen). All culture media and cells were tested for LPS by the limulus amebocyte assay QCL-1000 (Cambrex) and were below the limit of detection of the assay. All cell lines and tissue culture reagents were routinely tested for mycoplasma contamination using a PCR-based assay, as described previously (64).

Purification of TDM from M. tuberculosis. TDM was purified from *M. tuberculosis* grown in liquid media. Cells were harvested by centrifugation and autoclaved. Autoclaved pellets were weighed and sonicated in chloroform/methanol (4:1, vol/vol) for 15 minutes on ice. Water was added (1/20 total volume), and the organic phase was collected. The aqueous phase was sequentially extracted with chloroform/methanol (3:1 and 2:1, vol/vol) and the organic phases combined and evaporated completely. The dried pellet was dissolved in chloroform/methanol (3:1), and insoluble material was removed by centrifugation. The supernatant was evaporated to dryness and redissolved with acetone. The acetone 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 × 10 cm HPTLC plates (Alltech Associates Inc.) developed 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 minutes at 110°C.

Response of macrophages to TDM. Purified TDM was used to stimulate either RAW 264.7 cells or bone marrow–derived macrophages as described (18). Briefly, TDM was suspended at a concentration of 1 mg/ml in isopropanol, sonicated for 5 minutes in a bath sonicator (Model 3510, Branson Ultrasonic Corporation), incubated at 60°C for 10 minutes, and again sonicated for 5 minutes. 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 1×10^6 cells in 100 μl of medium and incubated at 37°C. At various time intervals, as indicated, after stimulation, supernatants were collected for analysis of cytokine production by using the commercial ELISA DuoSet kit (BD Biosciences) according to the manufacturer's recommendations. Intracellular cytokine staining was performed with the Cytofix/Cytoperm system (BD Biosciences) according to the manufacturer's instructions. Antibodies used were FITC-labeled anti-CD11b (Mac-1) and APC-labeled anti–TNF-α antibody (BD Biosciences), and CD11b⁺ cells were analyzed for expression of TNF-α in a BD LSR flow cytometer (BD Biosciences). Each analysis was performed on 25,000 CD11b⁺ cells.

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 per ml in RPMI-1640 containing 20% FBS and 30% L929 cell supernatant at 37°C in Petri dishes (OPTILUX; BD Biosciences — Discovery Labware). After 2 days, 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 days, following which the cells were harvested.

Preparation of inoculum and infection of macrophages. Prior to infection with mycobacteria, bone marrow–derived macrophages were seeded at a concentration of 2×10^5 cells per 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_2 for 16–18 hours. 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 seconds to disperse clumps, and adjusted to a concentration of 1×10^7/ml based on the A_{600}, using OD1 = 5×10^8 CFUs/ml. Macrophages were infected at an MOI of 5 for 6 hours 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 measured by ELISA.

Infection of mice by mycobacteria. Six- to eight-week old C57BL/6J or BALB/C mice obtained from Jackson Laboratory and maintained on standard feed and specific pathogen–free conditions were infected either aerogenically or intravenously with mycobacterial strains. TNF-α–deficient and IFN-γ–deficient mice were on the C57BL/6J background. SCID mice were on the BALB/C background. For aerosol infections, a Middlebrook inhalation exposure system (Glas-Col) was used according to procedures approved by the Memorial Sloan-Kettering Institutional Animal Care and Use Committee. Animal care and use for these experiments was approved by the Institutional Animal Care and Use Committee of the Research Animal Resource Center of Memorial Sloan Kettering Cancer Center. The mycobacterial

inocula for murine infection were prepared as described earlier for infection of macrophages. *M. tuberculosis* cells were suspended at a concentration of 4×10^8 CFUs in 10 ml of sterile distilled water. Mice were infected with a volume of suspension and exposure time calibrated to deliver approximately 100 CFUs per animal. For intravenous infections, mice were injected via tail vein with 200 µl of mycobacterial suspension containing either 10^5 or 10^6 mycobacteria. The extent of infection was estimated by plating the lung homogenates of animals sacrificed at 24 hours after aerosol infection, and inocula were equal for all bacterial strains. At various time intervals after infection, the lungs and spleen were homogenized in PBS and 0.05% Tween-80, and serial dilutions were plated onto Middlebrook 7H10 agar; colonies were enumerated after incubation at 37°C in a 5% CO_2 atmosphere. For histopathological analysis, liver lobes were removed and fixed with 10% formalin. The sections were paraffin embedded and stained with H&E. The areas of granulomatous inflammation were calculated by digital image processing using OpenLab software (version 4.0.3; Improvision). For statistical analysis of granuloma size, 14–21 independent liver granulomas 2 weeks after intravenous infection were analyzed for total area, total nucleated cell number, and number of lymphocytes, using visual identification of size and nuclear morphology. Mean areas of the granulomatous inflammation in the different groups were compared by 2-tailed Student's *t* test.

Delipidation and lipid reconstitution of mycobacteria. Delipidation of bacilli was performed by using petroleum ether, using a modification of published methods (22) and as described previously (18). Following two 5-minute 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 minutes 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 an MOI of 5 as described earlier.

Statistics. For statistical significance between 2 groups, the individual values of both groups were analyzed by using the 2-tailed Student's *t* test. A *P* value of less than 0.05 was considered significant.

Address correspondence to: Michael S. Glickman, Memorial Sloan-Kettering Cancer Center, Box 9, 1275 York Avenue, New York, New York 10021, USA. Phone: (212) 639-3191; Fax: (646) 422-2124; E-mail: glickmam@mskcc.org.

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