Mycobacterium tuberculosis Hip1 Dampens Macrophage Proinflammatory Responses by Limiting Toll-Like Receptor 2 Activation

Ranjna Madan-Lala,1 Katia Vitorello Peixoto,1† Fabio Re,2 and Jyothi Rengarajan1,3*

Emory Vaccine Center, Emory University, Atlanta, Georgia; Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, Tennessee; and Division of Infectious Diseases, Emory University, Atlanta, Georgia

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Mycobacterium tuberculosis is a highly successful human pathogen that evades host innate immunity by interfering with macrophage functions. In addition to avoiding macrophage microbicidal activities, M. tuberculosis triggers secretion of proinflammatory cytokines and chemokines in macrophages. The levels of proinflammatory cytokines induced by clinical M. tuberculosis isolates are thought to play an important role in determining tuberculosis disease progression and severity, but the mechanisms by which M. tuberculosis modulates the magnitude of inflammatory responses remain unclear. Here we show that M. tuberculosis restricts robust macrophage activation and dampens proinflammatory responses through the cell envelope-associated serine hydrolase Hip1 (hydrolase important for pathogenesis 1). By transcriptionally profiling macrophages infected with either wild-type or hip1 mutant bacteria, we found that the hip1 mutant induced earlier and significantly higher levels of several proinflammatory cytokines and chemokines. We show that increased activation of Toll-like receptor 2 (TLR2)- and MyD88-dependent signaling pathways mediates the enhanced cytokine secretion induced by the hip1 mutant. Thus, Hip1 restricts the onset and magnitude of proinflammatory cytokines by limiting TLR2-dependent activation. We also show that Hip1 dampens TLR2-independent activation of the inflammasome and limits secretion of interleukin-18 (IL-18). Dampening of TLR2 signaling does not require viable M. tuberculosis or phagocytosis but does require Hip1 catalytic activity.

We propose that M. tuberculosis restricts proinflammatory responses by masking cell surface interactions between TLR2 agonists on M. tuberculosis and TLR2 on macrophages. This strategy may allow M. tuberculosis to evade early detection by host immunity, delay the onset of adaptive immune responses, and accelerate disease progression.

* Corresponding author. Mailing address: Emory Vaccine Center, Emory University, 954 Gatewood Road, Room #1052, Atlanta, GA 30329. Phone: (404) 727-8714, Fax: (404) 727-8199. E-mail: jrengar@emory.edu.
† Present address: Institutional Animal Care and Use Committee, Emory University, Atlanta, GA.
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masome, which consists of the NLRP3 scaffold, the ASC adaptors, and caspase-1 (23, 25, 34, 37, 39).

Although several PAMPs purified from M. tuberculosis can stimulate proinflammatory responses through TLR signaling, several studies suggest that M. tuberculosis also inhibits macrophage activation and cytokine induction via cell envelope-associated and secreted mycobacterial factors (3, 15, 18, 19, 38, 41, 42, 45, 47, 61). Modulation of proinflammatory responses is highly relevant to M. tuberculosis pathogenesis, as clinical isolates of M. tuberculosis have been reported to vary in the ability to induce proinflammatory responses in macrophages, with more virulent strains of M. tuberculosis inducing lower levels of proinflammatory cytokines than their less virulent counterparts (28, 30, 40, 44, 47, 59). These studies suggest that limiting proinflammatory responses may lead to rapid progression to disease and provide a selective advantage to M. tuberculosis (44). However, the host pathways that lead to lowered or enhanced inflammatory responses are not clear. In this study, we show that M. tuberculosis dampens TLR2-dependent proinflammatory responses and that the serine hydrolase Hip1 restricts the onset and magnitude of proinflammatory responses induced by M. tuberculosis by limiting TLR2 activation in macrophages.

Rv2224c, which we have named Hip1 (hydrolase important for pathogenesis 1), is a cell envelope-associated serine hydrolase that we and others have previously shown to be critical for the virulence of M. tuberculosis and for its survival in macrophages (29, 49, 50, 65). The hip1 mutant grows poorly in macrophages and is more susceptible to acidic environments and many cell envelope-directed stresses, i.e., lysozyme, SDS, and lipopolysaccharide (49, 50, 64, 65). Hip1 and its M. smegmatis ortholog have been implicated in maintaining M. tuberculosis cell envelope integrity (13, 50). Additionally, mice infected with the hip1 mutant survive significantly longer than C57BL/6 or RAG2−/− mice infected with wild-type (wt) M. tuberculosis strains and have severely reduced granulomas in their lungs, despite only modest decreases in lung bacterial burdens (29, 50, 65). The delayed progression to disease and the mild immunopathology in hip1 mutant-infected mice in the face of large numbers of mutant bacteria suggested that Hip1 may also modulate innate immune responses.

To investigate whether Hip1 modulates innate immune responses, we transcriptionally profiled macrophages infected with either a wt or hip1 mutant strain of M. tuberculosis and found that hip1 mutant-infected macrophages rapidly induced dramatically higher levels of proinflammatory cytokines and chemokines. Defining the host signaling pathways modulated by Hip1 therefore provides a unique opportunity to understand how M. tuberculosis dampens proinflammatory responses. Here we show that the enhanced cytokine production induced by the absence of Hip1 is dependent on TLR2 and M Dubois/88 signaling and does not require live bacteria. Our studies suggest that M. tuberculosis dampens inflammatory responses by limiting cell surface interactions between TLR2 agonists on M. tuberculosis and TLR2 on macrophages. This strategy may allow M. tuberculosis to evade early detection by host immunity and may promote disease progression.
model was applied to the raw data in order to generate processed data. Ratios of gene expression in hip1 mutant-infected macrophages to that in wt-infected macrophages were generated. Genes with a fold change difference in expression of ≥2-fold or more, with a P value of ≤0.01, were included in the study. Data were normalized using the Z score (number of standard deviations from the mean), and a heat map was generated using Spotfire Inc. The expression level of each gene is represented by the number of standard deviations above (red) or below (green) the average value for that gene across all samples.

**Statistical analysis.** The statistical significance of data was analyzed using Student’s unpaired t test (GraphPad Prism 5.0). Data are shown as means ± standard deviations (SD) for one representative experiment of three independent experiments.

## RESULTS

Proinflammatory responses in *M. tuberculosis*-infected macrophages are dampened by the serine hydrolase Hip1. Hip1 is a cell envelope-localized serine hydrolase that is required for *M. tuberculosis* virulence and disease progression in vivo and promotes pathogen survival in macrophages (29, 49, 50, 65). *hip1* mutant strains of *M. tuberculosis* are more susceptible to antimicrobial agents and cell envelope-directed stresses (50, 64, 65), suggesting that the Hip1 hydrolase modifies the *M. tuberculosis* cell envelope. Since the *M. tuberculosis* cell envelope harbors several PAMPs that are recognized by PRRs to elicit innate immune responses in macrophages and dendritic cells, we investigated the hypothesis that Hip1 modulates macrophage responses. We profiled the gene expression of macrophages infected with either a wild-type or *hip1* mutant strain of *M. tuberculosis* at 24 h postinfection, when intracellular bacterial counts are indistinguishable between the two strains (50). We infected BMM from C57BL/6 mice with the wt or *hip1* mutant strain at an MOI of 10 and compared their transcriptional profiles 24 and 48 h after infection by using Affymetrix microarrays. The intracellular bacterial counts in infected macrophages at 4 h postinfection were comparable (data not shown). Statistical analyses of the data revealed that the most significant difference between gene expression levels in wt- and *hip1* mutant-infected macrophages was a dramatic increase in mRNAs corresponding to several proinflammatory cytokines and chemokines in macrophages infected with the *hip1* mutant (Fig. 1A). We confirmed these data by assaying the protein levels of several cytokines and chemokines produced by infected macrophages (Fig. 1B). At 24 h postinfection, cell-free supernatants were harvested from wt- or *hip1* mutant-infected macrophages and analyzed by multiplex ELISA. Levels of several proinflammatory cytokines (IL-1α, IL-1β, IL-6, and TNF-α) and chemokines (LIX, granulocyte colony-stimulating factor [G-CSF], monocyte chemoattractant protein 1 [MCP-1], macrophage inflammatory protein 1α [MIP-1α], MIP-1β, and RANTES) were enhanced 2.5- to 14-fold following infection with the *hip1* mutant compared to the wt (Fig. 1B). The vigorous induction of proinflammatory responses in macrophages by the *hip1* mutant suggests that virulent *M. tuberculosis* dampens proinflammatory cytokine and chemokine production.

*M. tuberculosis* limits the magnitude of macrophage proinflammatory cytokines through Hip1. To further investigate the basis for the increased magnitude of proinflammatory responses induced by the *hip1* mutant, we infected macrophages with bacteria at different MOIs. We observed increased production of representative proinflammatory cytokines (IL-1β, IL-6, and TNF-α) with escalating MOIs of both wt and *hip1* mutant strains (Fig. 2), with an MOI of 10 showing the most significant differences between wt- and *hip1* mutant-induced responses. To confirm that disruption of *hip1* leads to the hyperinflammatory phenotype of the mutant, we ectopically expressed intact Hip1 protein in the *hip1* mutant strain. The high levels of key proinflammatory cytokines (IL-1β, IL-6, and TNF-α) induced by the *hip1* mutant were restored to wild-type levels upon infection with the complemented strain (Fig. 2). Taken together, these results indicate that Hip1 limits the magnitude of macrophage proinflammatory responses.

**Hip1 delays induction of proinflammatory cytokines.** To understand how Hip1 limits robust macrophage responses, we studied the kinetics of IL-1β, IL-6, and TNF-α induction in
macrophages infected with the M. tuberculosis wt or hip1 mutant strain at 5, 8, 24, and 72 h postinfection. We detected significantly enhanced cytokine levels in hip1 mutant-infected macrophages as early as 5 h postinfection (Fig. 3), and this trend continued at 8, 24, and 72 h postinfection, with substantial increases in IL-1β, IL-6, and TNF-α levels. The increased induction of these cytokines by the hip1 mutant preceded any detectable cytotoxicity, as measured by lactate dehydrogenase (LDH) release, and was therefore not due to increased cell death (data not shown). Thus, disruption of hip1 leads to faster and higher levels of proinflammatory responses, indicating that wt M. tuberculosis delays the induction of innate immune responses. The rapid onset and increased magnitude of cytokine responses secreted by hip1 mutant-infected macrophages could result from increased signaling through pathways that are known to be downstream of M. tuberculosis infection or could be due to the involvement of additional pathways that wt M. tuberculosis does not normally engage. To delineate the signaling pathways utilized by the hip1 mutant strain, we first analyzed the production of IL-1β, IL-6, and TNF-α in macrophages derived from mice deficient in MyD88, a cytosolic protein which functions as an adaptor protein for signal transduction through several members of the TLR family. We infected resting BMM from MyD88−/− mice with the wt or hip1 mutant strain and assayed for TNF-α, IL-1β, and IL-6 in the supernatants. We found that the production of these cytokines was severely reduced in MyD88−/− macrophages infected with either wt or hip1 mutant bacteria (Fig. 4A), indicating that the enhanced levels of proinflammatory cytokines induced in the absence of Hip1 are generated through MyD88-dependent pathways. To determine which upstream receptors are responsible for this phenotype, we infected macrophages derived from mice deficient in TLR2,
TLR4, or TLR9 with the wt or hip1 mutant strain of *M. tuberculosis*. These TLRs utilize MyD88 as an adaptor and sense PAMPs derived from *M. tuberculosis*. In macrophages derived from TLR2−/− mice, production of TNF-α, IL-1β, and IL-6 was severely reduced after infection with wt *M. tuberculosis* as well as with the *hip1* mutant (Fig. 4B), indicating that TLR2 signaling mediates the enhanced proinflammatory cytokine responses. In contrast, TLR4 and TLR9 do not contribute to this phenotype, as induction of all three cytokines by either the wt or *hip1* mutant strain was not affected in macrophages derived from TLR4−/− or TLR9−/− mice (Fig. 4C). In addition, signaling through IL-1R1, which also utilizes MyD88 as an adaptor, is not required, as the high levels of IL-1β, IL-6, and TNF-α induced by the *hip1* mutant in C57BL/6 macrophages were maintained in IL-1R1−/− macrophages (Fig. 4C). The amounts of these cytokines were either at or below the limit of detection in all uninfected macrophages (data not shown). These results show that increased signaling through TLR2 and MyD88 leads to high levels of proinflammatory cytokines by *hip1* mutant-infected macrophages and suggest that Hip1 functions by limiting the magnitude of TLR2-dependent innate immune responses. Dampening innate immune responses by

FIG. 4. Enhanced induction of proinflammatory cytokines by the *hip1* mutant requires TLR2 and MyD88 signaling. BMM from C57BL/6 and MyD88−/− mice (A), TLR2−/− mice (B), and TLR4−/−, TLR9−/−, and IL-1R1−/− mice (C) were infected with the wt or *hip1* mutant strain at an MOI of 10. Supernatants were collected at 24 h postinfection and assayed for IL-1β, IL-6, and TNF-α by ELISA. Values are presented as means plus SD, and the data are representative of 3 independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
restricting TLR2 activation thus appears to be an important strategy employed by *M. tuberculosis* to evade optimal detection by host innate immunity.

**Hip1 modulates activation of the NLRP3 inflammasome.** Secretion of IL-1β depends on recognition of PAMPs by both TLRs and cytosolic NLRs and on activation of the inflammasome complex. In response to TLR ligation by microbial ligands, IL-1β is synthesized as inactive pro-IL-1β in the macrophage cytosol; conversion to its biologically active form and its secretion require proteolytic cleavage of pro-IL-1β into mature IL-1β by caspase-1, which in turn is activated by the inflammasome (7, 31). To determine whether the potent IL-1β production in the hip1 mutant was dependent on components of the inflammasome, we infected macrophages from mice deficient in ASC, caspase-1, or NLRP3, each of which has been shown to be required for IL-1β secretion in *M. tuberculosis*-infected macrophages (25, 34, 37). IL-1β secretion induced by wt or hip1 mutant *M. tuberculosis* was severely reduced in the absence of each of these proteins (Fig. 5A), while TNF-α production was unperturbed. The amounts of these cytokines were either at or below the limit of detection in all uninfected macrophages (data not shown). Taken together with the data presented in Fig. 4B, these results show that both TLR2 and the NLRP3 inflammasome complex are required for enhanced secretion of IL-1β.

We also observed 3-fold higher levels of IL-18 in supernatants of hip1 mutant-infected macrophages than in those of wt-infected macrophages (Fig. 5B). Like IL-1β secretion, IL-18 secretion is dependent on activation of the inflammasome complex. However, unlike IL-1β, which is transcriptionally regulated through sensing of PAMPs by TLRs, IL-18 production in response to many bacterial pathogens does not depend on TLR signaling. Pro-IL-18 is expressed constitutively and requires processing by caspase-1 for its maturation and extracellular secretion. To determine whether induction of IL-18 by *M. tuberculosis* is dependent on TLR2, we assayed the IL-18 levels in TLR2−/− macrophages. The absence of TLR2 did not compromise IL-18 production in either wt- or hip1 mutant-infected macrophages (Fig. 5B). Similar results were observed in MyD88−/− macrophages (data not shown). These data indicate that TLR2 signaling does not regulate IL-18 production in either wt or hip1 mutant strains of *M. tuberculosis*. Enhanced IL-18 secretion induced by hip1 mutant-infected macrophages was entirely dependent on components of the inflammasome complex, i.e., ASC, caspase-1, and NLRP3 proteins (Fig. 5C). Since assaying IL-18 gives a direct readout of caspase-1 and inflammasome activation, these results show that in addition to dampening TLR2-dependent signaling, Hip1 modulates TLR2-independent pathways, i.e., it decreases the magnitude of inflammasome activation.

**Heat-killed or gamma-irradiated *M. tuberculosis* retains the capacity to dampen TLR2-dependent cytokines and requires Hip1 catalytic activity.** The rapid induction of TLR2-dependent proinflammatory cytokines by the hip1 mutant suggests that PAMPs present in its cell envelope mediate this response. We reasoned that stronger innate immune responses induced by the hip1 mutant might be a result of alterations in surface-localized and/or extracellular TLR2 ligands. To address whether the viability of *M. tuberculosis* is necessary for the hyperinflammatory phenotype of the hip1 mutant, we tested the ability of live or dead bacteria of the wt and hip1 mutant strains to induce IL-6. Live *M. tuberculosis* is required for inhibition of phagosome maturation, but other immune evasion strategies, for example, TLR2-dependent inhibition of major histocompatibility complex (MHC) class II expression, can be mediated by dead mycobacteria (15, 61). To determine whether live, replicating *M. tuberculosis* is required for augmenting cytokine responses, we infected macrophages with the inactivated (heat-killed or gamma-irradiated) wt or hip1 mutant strain and measured IL-6 levels. We showed that the inactivated (by either heat killing or gamma irradiation) hip1 mutant induced 6- to 16-fold more IL-6 than inactivated wt *M. tuberculosis* (Fig. 6A). Therefore, bacterial viability is not necessary for eliciting enhanced levels of IL-6 in macrophages. Wild-type levels of IL-6 were restored upon infection with the hip1-complemented strain. However, expression of a catalytically inactive form of Hip1 (S228A mutant) failed to restore
components of the TLR2 signaling pathway, or by limiting activation of TLR2 by promoting the masking of TLR2 ligands in the *M. tuberculosis* cell envelope. To distinguish between these two possibilities, we combined wt and hip1 mutant strains in mixed infections and compared these to single infections with either strain. If Hip1 exerted an inhibitory effect on TLR2 signaling, we would expect the phenotype of wt *M. tuberculosis* to dominate in mixed infections. However, if TLR2 ligands were already revealed on the cell surface of the hip1 mutant, we would expect the mutant to retain its ability to elicit enhanced cytokine responses, even in the presence of the wt. Macrophages were exposed to the heat-killed wt and hip1 mutant strains, either singly or in combination (1:1 ratio), at an MOI of 5 or 10. Supernatants were assayed for IL-6 production at 24 h postexposure. The cytokine response elicited by mixed cultures was additive, showing that the wt strains did not suppress the ability of the hip1 mutant to elicit higher levels of IL-6 (Fig. 7A). Rather, heightened cytokine responses were likely driven by increased engagement of TLR2 ligands by the hip1 mutant. These data suggest that Hip1 dampens TLR2 signaling in macrophages by limiting recognition of *M. tuberculosis* TLR2 ligands.

We next examined the basis for limited TLR2 recognition. Increased activation of TLR2 signaling by the hip1 mutant could occur because of an altered repertoire of PAMPs that are recognized by TLR2 on macrophages. In wt *M. tuberculosis*, these TLR2 ligands may be masked by the cell envelope architecture. Unmasking of these TLR2 ligands in the absence of Hip1 could lead to cell surface interactions between *M. tuberculosis* and macrophages upon contact. Alternatively, phagocytosis of the bacteria and damage from antimicrobial agents may be required for release of TLR2 ligands and subsequent sensing by TLR2 on macrophage phagosomes. To distinguish between these possibilities, we either left macrophages untreated prior to infection or pretreated macrophages with cytochalasin D, which inhibits phagocytosis and blocks uptake of *M. tuberculosis* by macrophages (8). Exposure of cytochalasin D-treated macrophages to the inactivated wt or hip1 mutant strain for 6 h did not diminish the enhanced IL-6 production, indicating that phagocytosis is not necessary for induction of these responses (Fig. 7B). Similarly, cytochalasin D did not prevent the induction of IL-6 by live *M. tuberculosis* in C57BL/6 mice (Fig. 7C). These results show that cell surface interactions are sufficient for enhanced induction of IL-6 by the hip1 mutant. In addition, IL-6 production was severely reduced in TLR2-deficient macrophages (Fig. 7D), indicating that interaction of *M. tuberculosis* cell surface-localized TLR agonists with TLR2 on the macrophage cell surface is sufficient for initiating proinflammatory responses. These data suggest that Hip1 dampens TLR2 activation by limiting recognition of TLR2 ligands on the *M. tuberculosis* cell surface.

**DISCUSSION**

Our studies describe a novel strategy utilized by *M. tuberculosis* to dampen proinflammatory responses and show a critical role for the serine hydrolase Hip1 in mediating this effect. We showed that disruption of *hip1* leads to earlier and significantly higher levels of key proinflammatory cytokines and chemokines (Fig. 1 and 3). While these findings differ from what has

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**FIG. 6.** Bacterial viability is not required for Hip1-mediated restriction of TLR2-dependent cytokines. (A) C57BL/6 BMM were infected at an MOI of 10 with the heat-killed (HK) or irradiated (Irr) wt, hip1 mutant, or complemented hip1 (comp) strain or with the hip1 mutant complemented with catalytically inactive Hip1 (*hip1 S228A*). Supernatants collected at 24 h postinfection were assayed for IL-6 by ELISA. (B) C57BL/6 and TLR2 mutant S228A) strain at an MOI of 10. Supernatants collected at 24 h postinfection were assayed for IL-6 by ELISA. Values are presented as means ± SD, and the data are representative of 3 independent experiments. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.
been reported previously (50), the enhanced induction of cytokines in response to infection reported here was characterized extensively in multiple experiments and reflects the true phenotype of the hip1 mutant. The modest reduction in cytokine levels induced by the hip1 mutant in the earlier study was likely due to the lower MOIs used and to differences related to preparation of the inocula. Using macrophages derived from a panel of knockout mice, we showed that the enhanced production of the inocula. Using macrophages derived from a panel of knockout mice, we showed that the enhanced production of proinflammatory cytokines by hip1 mutant-infected macrophages requires MyD88- and TLR2-dependent signaling. Increased transcription of cytokines such as IL-1β, TNF-α, and IL-6, downstream of TLR2 recognition, leads to increased protein levels in macrophage supernatants (Fig. 1 and 4). Reduced levels of these cytokines observed in TLR2−/− and MyD88−/− macrophages (Fig. 4 and 6) suggest that TLR2/MyD88-independent pathways also contribute to proinflammatory responses in M. tuberculosis-infected macrophages, but these responses are independent of TLR4, TLR9, or IL-1R1 signaling (Fig. 4). Overall, we concluded that M. tuberculosis normally limits the magnitude of TLR2 activation and thereby prevents robust activation of macrophage proinflammatory responses. Interestingly, increased cytokine production in response to infection with the hip1 mutant does not appear to be a generalized phenomenon, since the chemokine IP-10 is stimulated to the same extent by wt and hip1 mutant strains (R. Madan-Lala and J. Rengarajan, unpublished data). Several groups, including ours, have shown that in human and murine cells, IP-10 induction in response to TLR agonists or M. tuberculosis infection occurs through the MyD88-independent IRF3 pathway and that TLR2-mediated signaling is unable to induce this chemokine (22, 46, 56, 66).

FIG. 7.ヒップ1変異株誘導による炎症応答はWTを凌駕する。ヒップ1マウスの脾生細胞を熱処理（HK）の細胞のWTまたはヒップ1マウスの脾生細胞をMOIの10または5に混合して感染させ、MOIの10または5でヒップ1変異株を感染させ、24時間後に行う培養液のIL-6レベルをELISA法で測定。WT、ヒップ1変異株、および混合感染群のそれぞれのデータを示す。

Our studies suggest that restricting TLR2 activation contributes to M. tuberculosis pathogenicity, while augmentation of early TLR2 activation, as observed with the hip1 mutant, is associated with reduced virulence (50). Indeed, several studies have suggested that virulent strains of M. tuberculosis are associated with decreased inflammatory responses (28, 40, 44, 47, 59, 67). Clinical isolates corresponding to evolutionarily modern lineages of M. tuberculosis have lowered proinflammatory responses compared to more ancient lineages (44). Thus, it is possible that restricting the magnitude of inflammatory responses may provide a selective advantage to the pathogen. The attenuated virulence exhibited by the hip1 mutant in vivo suggests that increased production of TLR2-dependent proinflammatory cytokines may be beneficial to the host. It is tempting to speculate that dampening the induction of innate cytokines by limiting TLR2 activation enables M. tuberculosis to influence adaptive immunity and accelerate the progression of TB disease. It will be interesting to test whether hip1 expression is altered in different M. tuberculosis lineages, thereby contributing to the various abilities of these strains to induce inflammatory responses.

Many immune evasion mechanisms require replication of live M. tuberculosis organisms within macrophages, but dead mycobacteria are sufficient for other mechanisms. For example, inhibition of MHC class II expression, which is also dependent on TLR2 signaling, does not require M. tuberculosis viability (15, 61). Interestingly, we showed that M. tuberculosis replication is not necessary for the enhanced TLR2-dependent proinflammatory phenotype, as both heat-killed and gamma-irradiated hip1 mutant strains retained the ability to induce higher levels of IL-6 (Fig. 6). This induction requires Hip1 hydrolase activity, as a catalytically inactive Hip1 protein failed to restore wt levels of IL-6 in the mutant (Fig. 6A). To elucidate the mechanism for dampened proinflammatory responses, we next examined whether Hip1 functions by suppressing components of the TLR2 signal transduction pathway. For example, the secreted protein ESAT-6 has been shown to inhibit TLR2 signaling and NF-κB activation by preventing interaction between MyD88 and IRAK (42). We carried out mixed infection experiments using 1:1 ratios of wt and hip1 mutant strains and demonstrated that Hip1 does not suppress the TLR2 signal transduction pathway, since the wt strain was
unable to prevent the hip1 mutant from inducing high levels of IL-6 (Fig. 7A). Rather, these data are consistent with increased recognition of TLR2 ligands by the mutant strain, leading to enhanced production of TLR2-dependent cytokines. Cell surface interactions between TLR2 ligands on the hip1 mutant and TLR2 on macrophages are sufficient to induce rapid and robust cytokine responses, since we showed that phagocytosis is not required for cytokine induction (Fig. 7B and C). These data also imply that damage to the hip1 mutant cell envelope in macrophage phagosomes and subsequent release of PAMPs are not required for engaging the TLR2-MyD88 pathway. Instead, we propose that in wt M. tuberculosis, Hip1 hydrolyase activity masks TLR agonists in the cell envelope that could potentially be recognized by TLR2. This limits TLR2 activation and dampens proinflammatory responses.

Although M. tuberculosis possesses several potential TLR2 ligands, our data suggest that there may be limited recognition of TLR2 agonists in the context of infection with the whole organism. Indeed, despite the ability of several purified lipoproteins (e.g., LpqH, LprG, and LprA) and lipids to act as potent TLR2 agonists in vitro, TLR2-deficient mice are not especially susceptible to acute M. tuberculosis infection (9, 18, 19, 43, 48, 58). However, TLR2/TLR9 doubly deficient mice are more susceptible to infection, and MyD88-deficient mice succumb rapidly to infection (2, 17, 21, 55). These studies suggest redundancy in TLR usage in vivo but may also reflect limited TLR2 engagement during the innate phase of infection. Based on our data, we propose that many M. tuberculosis TLR2 agonists in the cell envelope may not interact with TLR2 on macrophages. They may be inaccessible to macrophages due to Hip1-dependent modifications of the cell envelope that mask these PAMPs, or processing of TLR2 ligands by Hip1 hydrolyase activity may prevent recognition of TLR2 agonists by macrophages. In the absence of Hip1, altered accessibility or changes in the diversity of TLR2 ligands could lead to increased recognition by TLR2 and to enhanced proinflammatory responses.

While TLR agonists on the cell surface of M. tuberculosis are likely to be sufficient for inducing IL-6, the secretion of inflammasome-dependent IL-1β and IL-18 requires sensing of cytosolic PAMPs by NLRs in the macrophage cytoplasm (31). While M. tuberculosis has been shown to induce inflammasome activation (23, 25, 34, 37), stronger activation of the NLRP3 inflammasome by the hip1 mutant raises the interesting question of how NLRP3 senses M. tuberculosis. While the ligands for NLRP3 in M. tuberculosis have not been identified, a wide array of molecules, such as muramyl dipeptide, bacterial RNA, uric acid crystals, ATP, alum, and asbestos, have been implicated in activating the NLRP3 inflammasome, although the molecular basis for activation is not understood (31, 62). It is likely that NLR ligands are able to access the macrophage cytosol. Possible mechanisms for the cytosolic localization and recognition of M. tuberculosis NLR ligands include the rupture of phagosomes due to the particulate nature of M. tuberculosis antigens and the release of PAMPs into the cytosol, as well as entry of M. tuberculosis components into the macrophage cytoplasm (27, 57, 62). This may be mediated in part by ESAT-6, which is implicated in mediating membrane damage and has been reported to be required for activation of caspase-1 and the NLRP3 inflammasome (37). However, ESAT-6 secretion is comparable in both wt and mutant strains (Madan-Lala and Rengarajan, unpublished data). We speculate that the higher susceptibility to cell envelope-directed stresses (50, 65) of the hip1 mutant may facilitate release of mycobacterial cell wall components that can activate NLRP3 into the macrophage cytosol, resulting in increased IL-1β and IL-18 secretion.

Interestingly, disruption of a putative zinc metalloprotease, Zmp1, also results in stronger caspase-1 activation and more IL-1β secretion than those in wt M. tuberculosis, suggesting that similar mechanisms may be involved (32). Overall, these data show that in addition to limiting TLR2 engagement, restricting the magnitude of inflammasome activation is an important mechanism employed by M. tuberculosis for dampening proinflammatory responses and avoiding immune detection.

Elucidating the molecular and biochemical basis for the altered repertoires of PAMPs in wt and hip1 mutant strains should shed light on the nature of the TLR agonists involved. We know from electron microscopy that the cell envelope of the hip1 mutant is not noticeably altered compared to that of the wt (Madan-Lala and Rengarajan, unpublished data). Short-term culture filtrates of the wt and the hip1 mutant show differences in protein composition but induce comparable levels of IL-1β, TNF-α, and IL-6 (data not shown). These data suggest that the differential induction of cytokine levels between these strains is not due simply to altered repertoires of secreted factors or to gross changes in morphology. Rather, alterations in the accessibility or type of cell envelope-associated ligands likely influence how M. tuberculosis modulates macrophage activation. Further studies aimed at defining the protein and lipid profiles of wt and hip1 mutant cell envelopes will provide important insights.

In summary, we show that M. tuberculosis Hip1 modulates the onset and magnitude of proinflammatory responses in macrophages. Limiting recognition of TLR2 ligands on the M. tuberculosis cell surface prevents robust TLR2 activation and leads to dampened proinflammatory responses. Inducing weak innate immune responses is likely to be advantageous to the pathogen during infection.

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