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Mycobacterium tuberculosis Impairs Dendritic Cell Functions through the Serine Hydrolase Hip1

Ranjna Madan-Lala,* Jonathan Kevin Sia,* Rebecca King,* Toidi Adekambi,* Leticia Monin,† Shabaana A. Khader,† Bali Pulendran,*‡ and Jyothi Rengarajan*‡

Mycobacterium tuberculosis is a highly successful human pathogen that primarily resides in host phagocytes, such as macrophages and dendritic cells (DCs), and interferes with their functions. Although multiple strategies used by M. tuberculosis to modulate macrophage responses have been discovered, interactions between M. tuberculosis and DCs are less well understood. DCs are the primary APCs of the immune system and play a central role in linking innate and adaptive immune responses to microbial pathogens. In this study, we show that M. tuberculosis impairs DC cytokine secretion, maturation, and Ag presentation through the cell envelope–associated serine hydrolase, Hip1. Compared to wild-type, a hip1 mutant strain of M. tuberculosis induced enhanced levels of the key Th1-inducing cytokine IL-12, as well as other proinflammatory cytokines (IL-23, IL-6, TNF-α, IL-1β, and IL-18) in DCs via MyD88- and TLR2/9-dependent pathways, indicating that Hip1 restricts optimal DC inflammatory responses. Infection with the hip1 mutant also induced higher levels of MHC class II and costimulatory molecules CD40 and CD86, indicating that M. tuberculosis impairs DC maturation through Hip1. Further, we show that M. tuberculosis promotes suboptimal Ag presentation, as DCs infected with the hip1 mutant showed increased capacity to present Ag to OT-II– and early secreted antigenic target 6–specific transgenic CD4 T cells and enhanced Th1 and Th17 polarization. Overall, these data show that M. tuberculosis impairs DC functions and modulates the nature of Ag-specific T cell responses, with important implications for vaccination strategies.


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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; DC, dendritic cell; ESAT-6, early secreted antigenic target 6; Hip1, hydrolase important for pathogenesis 1; MDC, monocytoid-derived DC; MHC II, MHC class II; MOI, multiplicity of infection; PRR, pattern recognition receptor; TB, tuberculosis; Tg, transgenic.

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the aerogenic mouse model of TB, \textit{M. tuberculosis}–infected DCs have been shown to be important for transporting bacteria from the lungs to the draining mediastinal lymph nodes, where they initiate T cell–mediated immune responses (36–38). Depletion of CD11c+ cells in mice, which includes DCs, caused a delay in CD4 T cell responses and impaired control of \textit{M. tuberculosis} (39). However, \textit{M. tuberculosis} has also been shown to interfere with DC migration and Ag presentation in vivo (36), which likely impact the priming of Th1 responses. Thus, interactions between \textit{M. tuberculosis} and DCs during early stages of infection will directly influence the onset and development of adaptive immunity. Although \textit{M. tuberculosis} employs a number of cell wall–associated and extracellularly secreted bacterial factors to modulate innate immune cells, factors that interfere with DC functions are poorly understood.

In this study, we show that \textit{M. tuberculosis} infection impairs key aspects of DC functions through Hipl (Rv2224c) and thereby impacts adaptive immune responses. Infection of DCs by a \textit{hipl}–deficient mutant induced significantly higher levels of IL-12 and other proinflammatory cytokines compared with wild-type \textit{M. tuberculosis} and enhanced surface expression of MHC II, CD40, and CD86. This enhanced DC maturation induced by the \textit{hipl} mutant was dependent largely on MyDD8 and partially on TLR2/9 pathways. Further, we provide evidence that DCs matured by the \textit{hipl} mutant were more efficient in presenting Ags to CD4 T cells and priming Th1 and Th17 responses. Overall, our data demonstrate that \textit{M. tuberculosis} Hipl impairs DC functions and modulates the nature of Ag-specific T cell responses. Enhancing adaptive immune responses by boosting DC activation and Ag presentation has important implications for developing better vaccines for TB.

Materials and Methods

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Emory University School of Medicine. Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

Bacterial strains and media

\textit{M. tuberculosis} H37Rv (wild-type), \textit{hipl}::tn (\textit{hipl} mutant), and \textit{hipl} mutant–complemented strains were grown at 37°C in Middlebrook 7H9 broth or 7H10 agar supplemented with 10% oleic acid/albumin/dextrose/catalase, 0.5% glycerol, and 0.05% Tween 80 (for broth), with the addition of 25 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO) for the \textit{hipl} mutant and 10 μg/ml streptomycin (Sigma-Aldrich) for the \textit{hipl} mutant–complemented strain. For inactivation of \textit{M. tuberculosis} strains, bacteria were grown in Middlebrook 7H9 until midlog phase, washed twice with PBS, and heat killed by incubating at 80°C for 2 h.

Mice

All mice were housed under specific pathogen-free conditions in filter-top cages within the vivarium at the Yerkes National Primate Center, Walkersville, MD with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM glutamine, 1× 2- ME, 10 mM HEPES, 1 mM sodium pyruvate, 1× nonessential amino acids, and 20 ng/ml murine recombinant GM-CSF (R&D Systems, Minneapolis, MN). Incubations were carried out at 37°C with 5% CO2. Fresh medium with GM-CSF was added on days 3 and 6, and cells were used on day 7 for all experiments. We routinely obtained ~75% CD11c+CD11b+ cell purity by flow cytometry. BMDCs were further purified by using magnetic beads coupled to CD11c+ mAb and passed through an AutoMACS column as per the manufacturer’s instructions, where indicated (Miltenyi Biotech, Auburn, CA). For all experiments, cells were maintained throughout in medium containing GM-CSF. For infection, BMDCs were plated onto 24-well plates (3 × 105 per well). Bacteria were filtered through 5-μM filters, resuspended in complete medium containing 20 ng/ml GM-CSF, and sonicated twice for 5 s each before addition to the adherent monolayers. Each bacterial strain was used for infection (in duplicate or triplicate) at a multiplicity of infection (MOI) of 5 or as indicated. Infection of BMDCs was carried out for 4 h, after which monolayers were washed four times with PBS before replacing with RPMI 1640 medium containing 20 ng/ml GM-CSF. To determine intracellular CFU, one set of DCs was lysed in PBS containing 0.5% Triton X-100 and plated on 7H10 agar plates containing the appropriate antibiotics. Alternatively, BMDCs were infected with heat-killed \textit{M. tuberculosis} at an MOI of 5 or as indicated in RPMI medium containing 20 ng/ml GM-CSF. Cell-free supernatants from DC monolayers were isolated at indicated points and assayed for cytokines by ELISA using Duo Set kits for IL-12p40, IL-12p70, IL-6, TNF-α, and IL-1β (BD Biosciences, San Jose, CA); IL-23 from BioLegend (San Diego, CA); and IL-18 (MBL International, Woburn, MA). Assays were carried out according to the manufacturers’ instructions. Uninfected BMDCs were used as controls for each experiment.

Flow cytometry and Abs

Murine anti-CD11c allopolyocytocin (clone N418) and anti-CD11b FITC (clone M1/70) were obtained from BioLegend; anti-CD40 PE (clone 3/23), anti-CD86 PE (clone GL1), and anti–MHC II PE (clone M5/114.15.2) were purchased from BD Biosciences. Staining for cell-surface markers was done by resuspending ∼1 × 106 cells in 200 μl PBS with 2% FBS containing the Ab mixture. Cells were incubated at 4°C for 30 min and then washed with PBS containing 2% FBS. Data were immediately acquired using a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, San Carlos, CA).

Ag-specific CD4 T cell Ag-presentation assays

CD4 T cells were purified from single-cell suspensions of spleen and lymph nodes from 6–8-wk-old ESAT-6–Tg and OTII-Tg mice originally generated in the laboratory of Dr. S. Akira (2) using magnetic beads coupled to anti-CD11c mAb and passed through an AutoMACS column as per the manufacturer’s instructions. BMDCs were incubated in 24-well plates (3 × 105 per well) with 10 μg/ml ESAT-6, 20 peptide or OVA 323–339 peptides for 6 h, washed with PBS, and incubated with heat-killed wild-type, \textit{hipl} mutant, or medium alone for 24 h. Infected DCs were washed twice with PBS and cocultured with Ag-specific CD4 T cells at a 1:4 ratio for 72 h. Supernatants collected from these cells were analyzed for IFNγ (Mabtech, Cincinnati, OH) and IL-2 (BD Biosciences) by ELISA according to the manufacturers’ instructions.

CD4 T cell polarization assays

CD4 T cells were purified from single-cell suspensions of spleen and lymph nodes from 6–8-wk-old C57BL/6 mice as described above. BMDCs infected with wild-type, \textit{hipl} mutant, or medium alone for 24 h as described above were cocultured with CD4 T cells at a 1:4 ratio for 72 h. Cell-free supernatants collected from these cells were analyzed for IFNγ (Mabtech) and IL-17 (eBioscience, San Diego, CA) by ELISA according to the manufacturers’ instructions.

Aerogenic infection of mice with \textit{M. tuberculosis} strains

\textit{M. tuberculosis} strains, H37Rv, and \textit{hipl} mutant were grown to early log phase (OD600 of ∼0.5–0.6–0.8), washed two times in 1× PBS, and 1-ml aliquots were frozen at −80°C and used for infection after thawing. Single-cell suspensions of these aliquots were used to deliver ∼100 CFU \textit{H37Rv} or the \textit{hipl} mutant into 8–10-wk-old C57BL/6 mice using an aerosol apparatus manufactured by In-Tox Products (Moriarty, NM). Bacterial burden was estimated by plating serial dilutions of the lung homogenates on 7H10 agar plates on day 1.

Tissue harvest and cell preparation

Lungs from infected mice were harvested at 3 wk postinfection and digested with 1 mg/ml collagenase D (Worthington) at 37°C for 30 min. The
upper right lobe of the lung was used for determining CFU. Homogenized single-cell lung suspensions were filtered through a 70-μm cell strainer (BD Biosciences), treated with RBC lysis buffer for 3–5 min, and washed twice with cell culture media. Cells were counted and stimulated with 10 μg/ml ESAT-6,20 peptide for 48 h. Cell-free supernatants were isolated and assayed for IFN-γ and IL-17 by ELISA.

**Human DC infection and Th cell differentiation assays**

PBMCs were isolated from the blood of healthy donors by centrifugation in CPT tubes (BD Biosciences). CD14+ monocytes were purified from PBMCs by positive selection using CD14+ microbeads (Miltenyi Biotech). Cell purity was >80% as assessed by flow cytometry using an FACScalibur (BD Biosciences). To generate immature monocyte-derived DCs (MDCs), CD14+ cells were cultured at 1 × 10^6 cells/ml in RPMI 1640 (Lonza) supplemented with 10% heat-inactivated FBS (HyClone), 1× nonessential amino acids, 20 ng/ml human rGM-CSF (PeproTech, Rocky Hill, NJ), and 40 ng/ml IL-4 (Pepro Tech). Incubations were carried out at 37°C with 5% CO2. Fresh medium with rGM-CSF and IL-4 was added every alternate day. MDCs were harvested on day 6 or 7 for experiments.

For infection, human MDCs were plated onto 24-well plates (3 × 10^5/well) and infected with heat-killed H37Rv or hip1 mutant at an MOI of 10. Cell-free supernatants from DC monolayers were isolated at 24 h postinfection and assayed for cytokines by ELISA using Duo Set kits for IL-12p40 and IL-6 (R&D Systems and BD Biosciences, respectively). Assays were carried out according to the manufacturers’ instructions. For T cell polarization assays, infected DCs were cocultured with autologous PBMCs by positive selection using CD14 + microbeads (Miltenyi Biotech). Cell purity was >95%. PBMCs were isolated from the blood of healthy donors by centrifugation twice with cell culture media. Cells were counted and stimulated with 10^6 cells/ml in RPMI 1640 (Lonza) supplemented with 10% heat-inactivated FBS (HyClone), 1× nonessential amino acids, 20 ng/ml human rGM-CSF (PeproTech, Rocky Hill, NJ), and 40 ng/ml IL-4 (PeproTech). Incubations were carried out at 37°C with 5% CO2. Fresh medium with rGM-CSF and IL-4 was added every alternate day. MDCs were harvested on day 6 or 7 for experiments.

**Statistical analysis**

The statistical significance of data were analyzed using the Student unpaired t test (GraphPad Prism 5.0; GraphPad). Data are shown as mean ± SD of one representative experiment from two to five independent experiments.

**Results**

**M. tuberculosis limits DC production of IL-12 and other proinflammatory cytokines through the serine hydrolase Hip1**

Although *M. tuberculosis* has been shown to infect DCs and impair their functions in vivo (36), the *M. tuberculosis* factors that modulate DC responses during infection are not well understood. Based on the recently identified role for Hip1 in modulating macrophage functions, we investigated whether Hip1 impacts DC functions. We first assessed the ability of wild-type (H37Rv) and *hip1* mutant strains of *M. tuberculosis* to induce IL-12, a cytokine that is critical for inducing the differentiation of naive T cells into the IFN-γ-secreting Th1 phenotype. We infected BMDCs from C57BL/6J mice with the wild-type or *hip1* mutant strains of *M. tuberculosis* at an MOI of 5 and measured the levels of IL-12p40 and p70 subunits at 24 h postinfection in the cell-free supernatants. The intracellular bacterial counts in wild-type and *hip1* mutant–infected BMDCs at 4 h and 8 d postinfection were comparable (data not shown). The *hip1* mutant induced significantly higher levels of IL-12p40 and IL-12p70 in infected DCs compared with wild-type (Fig. 1A), indicating that *M. tuberculosis* limits the production of the key Th1-polarizing cytokine upon infection of DCs. To address whether the viability of *M. tuberculosis* is necessary for the enhanced secretion of IL-12 seen in the absence of Hip1, we infected BMDCs with heat-killed strains of wild-type and the *hip1* mutant. The heat-killed *hip1* mutant induced significantly higher levels of IL-12p40 and IL-12p70 compared with heat-killed wild-type *M. tuberculosis* (Fig. 1B), indicating that bacterial viability is not necessary for eliciting enhanced levels of IL-12 in DCs. In addition, IL-12 induced by the *hip1* mutant was restored to wild-type levels upon infection with the complemented strain (Fig. 1A, 1B), confirming that *M. tuberculosis* limits IL-12 production through Hip1. Although IL-12 is a major Th1-polarizing cytokine secreted by myeloid DCs upon microbial stimulation, DCs also secrete other proinflammatory cytokines, which serve as early triggers of inflammation. In response to infection with the *hip1* mutant, BMDCs secreted high levels of IL-23, IL-6, TNF-α, IL-1β, and IL-18 compared with wild-type, and these levels were restored to wild-type levels by the complemented strain (Fig. 1C, Supplemental Fig. 1). We did not detect significant amounts of IL-10 or IFN-β secretion from infected DCs under these conditions (data not shown). Taken together, these results indicate that *M. tuberculosis* limits the magnitude of IL-12 production, as well as that of additional proinflammatory cytokines in infected DCs, in a Hip1-dependent manner.

**M. tuberculosis impairs DC maturation through Hip1**

Following phagocytosis and Ag capture at the site of infection by immature DCs, interactions between pathogen-associated molecular patterns and PRRs induce maturation of DCs and migration into the local draining lymph nodes, where they prime T cells through cell-surface expression of costimulatory molecules such as MHC II and secretion of cytokines such as IL-12. To determine whether Hip1 influences DC maturation, we infected BMDCs with the wild-type or *hip1* mutant at an MOI of 5 for 24 h and monitored the surface expression of CD40, CD86, and MHC II by flow cytometry. Although wild-type *M. tuberculosis* induced all three markers on CD11c+ BMDCs, the expression levels were much lower than that induced by LPS from *Salmonella*. In contrast, *hip1* mutant induced higher surface expression of CD40, CD86, and MHC II (Fig. 2). This robust maturation of DCs infected with the *hip1* mutant was restored to wild-type levels upon complementation with Hip1 (data not shown). These results indicate that *M. tuberculosis* impairs optimal DC maturation through Hip1.

To investigate whether the impaired maturation of DCs upon *M. tuberculosis* infection is due to direct inhibition of host pathways by Hip1, we asked if *M. tuberculosis* could block DC maturation induced by an exogenous stimulus, such as LPS. We exposed BMDCs to 1 μg/ml LPS or wild-type *M. tuberculosis* at an MOI of 5, either independently or together, and measured the surface expression of CD40 by flow cytometry after 24 h (Fig. 3A). The median fluorescence intensity (MFI) of LPS-induced CD40 on the cell surface was not diminished by the addition of *M. tuberculosis*, demonstrating that wild-type *M. tuberculosis* does not actively inhibit LPS-induced expression of CD40 on BMDCs (Fig. 3A). We next exposed BMDCs to mixed cultures of wild-type and *hip1* mutant strains (1:1) and compared CD40 expression to single infections of either strain. Surface expression of CD40 in the mixed infection setting was comparable to that induced by the *hip1* mutant alone (Fig. 3B), suggesting that the *hip1* mutant phenotype is dominant and that wild-type *M. tuberculosis* does not hinder *hip1* mutant–induced DC maturation. Thus, these data suggest that the presence of Hip1 in wild-type *M. tuberculosis* prevents optimal DC maturation, whereas in the absence of Hip1, interactions between the *hip1* mutant and DCs promote robust DC maturation.

**Inhibition of DC functions by *M. tuberculosis* is dependent on MyD88 and TLR2/9 pathways**

We have previously demonstrated that Hip1-dependent modification of the *M. tuberculosis* cell envelope dampens macrophage proinflammatory responses by limiting interactions between TLR2 agonists on *M. tuberculosis* and TLR2 on macrophages, leading to suboptimal TLR2 activation. Because studies have shown that *M. tuberculosis* engages different TLRs on macrophages and DCs (40), we sought to determine which pathways are engaged by the *hip1* mutant and lead to enhanced cytokine secretion and maturation...
of DCs. We infected BMDCs derived from C57BL/6J mice with the wild-type (wt), hip1 mutant (mut), or hip1 mutant complemented with Hip1 (comp) M. tuberculosis at MOI of 5. At 24 h postinfection, cell-free supernatants were assayed for IL-12p40 and IL-12p70 by ELISA. Purified C57BL/6J BMDCs were infected with heat-killed wild-type (wt), hip1 mutant, or comp strain at MOI of 5. At 24 h postinfection, cell-free supernatants were assayed for IL-12p40 and IL-12p70 (B) and IL-23, IL-6, TNF-α, IL-1β, and IL-18 (C) by ELISA. Data are representative of three independent experiments. Values are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. ud, undetectable.

FIGURE 1. Enhanced inflammatory response in hip1 mutant–infected DCs. (A) Purified BMDCs derived from C57BL/6J mice were exposed to medium alone (ui) or infected with the wild-type (wt), hip1 mutant (mut), or hip1 mutant complemented with Hip1 (comp) M. tuberculosis at MOI of 5. At 24 h postinfection, cell-free supernatants were assayed for IL-12p40 and IL-12p70 by ELISA. Purified C57BL/6J BMDCs were infected with heat-killed wild-type (wt), hip1 mutant, or comp strain at MOI of 5. At 24 h postinfection, cell-free supernatants were assayed for IL-12p40 and IL-12p70 (B) and IL-23, IL-6, TNF-α, IL-1β, and IL-18 (C) by ELISA. Data are representative of three independent experiments. Values are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. ud, undetectable.

FIGURE 2. Enhanced surface expression of CD40, CD86, and MHC II on hip1 mutant–infected DCs. C57BL/6J BMDCs were exposed to medium alone (ui), heat-killed wild-type (wt), or hip1 mutant (mut) at MOI of 5 or 1 μg/ml LPS for 24 h. DCs were labeled with anti–CD11c–allophycocyanin and anti–CD40–PE, anti–CD86–PE, or anti–MHC II–PE. Representative histograms and PE MFI for CD11c+ cells are shown. Isotype control is shown as gray-shaded area. Data are representative of three independent experiments. Values are presented as mean ± SD. **p < 0.01, ***p < 0.001.
the hip1 mutant. We infected BMDCs from mice doubly deficient in TLR2 and TLR9 with wild-type or hip1 mutant and assayed the supernatants for IL-12p40, IL-12p70, and IL-6. As seen in Fig. 4C, IL-12 levels are almost completely abrogated in TLR2 mutant–induced DC maturation. C57BL/6J BMDCs were exposed to 1 μg/ml LPS or heat-killed wild-type M. tuberculosis (wt) at MOI of 5 either independently or together (A) or infected with heat-killed wt, hip1 mutant (mut), or wt + hip1 mutant (1:1) at MOI of 5 (B) for 24 h. DCs were labeled with anti-CD11c–allophycocyanin and anti-CD40–PE. Representative histograms and PE MFI for CD11c+ cells are shown. Isotype control is shown as gray-shaded area. Data are representative of two independent experiments. Values are presented as mean ± SD. *p < 0.05, **p < 0.01.

FIGURE 3. Wild-type M. tuberculosis does not block LPS- or hip1 mutant–induced DC maturation. C57BL/6J BMDCs were exposed to 1 μg/ml LPS or heat-killed wild-type M. tuberculosis (wt) at MOI of 5 either independently or together (A) or infected with heat-killed wt, hip1 mutant (mut), or wt + hip1 mutant (1:1) at MOI of 5 (B) for 24 h. DCs were labeled with anti-CD11c–allophycocyanin and anti-CD40–PE. Representative histograms and PE MFI for CD11c+ cells are shown. Isotype control is shown as gray-shaded area. Data are representative of two independent experiments. Values are presented as mean ± SD. *p < 0.05, **p < 0.01.

M. tuberculosis interferes with DC Ag presentation in a Hip1-dependent manner

DCs are the most effective APCs for activating naïve CD4 T cells. Expression of high levels of costimulatory molecules and MHC II on the cell surface is essential for efficient Ag presentation and T cell activation. We therefore hypothesized that enhanced expression of costimulatory molecules and MHC II in DCs infected with the hip1 mutant would impact Ag presentation to naïve CD4 T cells. To test this hypothesis, we infected BMDCs with wild-type or hip1 mutant at an MOI of 10 for 24 h followed by a coculture with purified CD4 T cells, infected DCs were cocultured with autologous lymphocytes from the respective donors for 3 d, and supernatants were assayed for IFN-γ and IL-17 by ELISA. We found that wild-type–infected DCs elicited significantly lower IFN-γ and IL-2 from ESAT-61–20–specific CD4 T cells as compared with the hip1 mutant (Fig. 5A). The higher IFN-γ and IL-2 production induced by hip1 mutant–infected DCs was also observed using an exogenous Ag. Coculture of hip1 mutant–infected DCs with naïve TCR-Tg CD4 T cells isolated from OT-II mice and OVA23–33 showed enhanced induction of IL-2 and the Th1 cytokine IFN-γ compared with their wild-type counterparts (Fig. 5B). Thus, the absence of Hip1 enhanced the capacity of DCs to present Ag to CD4 T cells and induce Th1 cytokine responses. These data show that suboptimal DC maturation and Ag presentation by M. tuberculosis is dependent on Hip1.

M. tuberculosis Hip1 modulates CD4 T cell differentiation in vitro and in vivo

The increased induction of IFN-γ by DCs matured with the hip1 mutant is likely due to the enhanced IL-12p70 levels, which synergize with costimulatory molecules like CD40 to induce Th1 differentiation. Because the hip1 mutant also induced enhanced production of the cytokines IL-6, IL-1β, and IL-23, which are known to promote differentiation to the Th17 phenotype, we sought to determine whether the interactions between BMDCs and the hip1 mutant–infected DCs induced IL-17–secreting CD4 T cells. We infected BMDCs with wild-type or hip1 mutant at an MOI of 10 for 24 h followed by a coculture with purified CD4 T cells from uninfected C57BL/6J mice. After 72 h, supernatants were assayed for IFN-γ and IL-17 by ELISA. As seen in Fig. 6A, BMDCs infected with the hip1 mutant elicited enhanced IFN-γ and IL-17 levels from CD4 T cells as compared with wild-type M. tuberculosis, indicating that Hip1 controls Th cell differentiation by DCs.

To test whether Hip1 influences Th cell differentiation in vivo, we infected C57BL/6J mice with ∼100 CFU of wild-type or the hip1 mutant by the aerosol route. We harvested lungs at 3 wk postinfection, because at this time point, Ag-specific IFN-γ–producing DC4 T cells have been shown by multiple groups to be present in the lungs of M. tuberculosis–infected mice. Single-cell lung suspensions were stimulated with 10 μg/ml ESAT-61–20 peptide for 48 h, and cell-free supernatants were assayed for IFN-γ and IL-17 by ELISA. As shown in Fig. 6B, lung cells from the hip1 mutant–infected mice show higher levels of IFN-γ and IL-17 in response to ESAT-61–20 peptide stimulation compared with wild-type–infected mice. These data suggest that wild-type M. tuberculosis limit IFN-γ and IL-17 production in lungs early in infection and that Hip1 mediates this effect.

M. tuberculosis interacts with human DCs to impair T cell differentiation

To address whether Hip1 also plays a role in impairing human DC–T cell interactions, we isolated PBMCs from healthy donors and differentiated them in vitro in the presence of GM-CSF and IL-4. These MDCs were infected with the wild-type or hip1 mutant at an MOI of 10 for 24 h. We assayed for representative Th cell–polarizing cytokines IL-12 and IL-6 in supernatants and found that hip1 mutant–infected MDCs from each donor induced significantly higher levels of IL-12p40 and IL-6 compared with wild-type M. tuberculosis (Fig. 7A). To investigate whether MDCs infected by the hip1 mutant also promote IFN-γ and IL-17 production by T cells, infected DCs were cocultured with autologous lymphocytes from the respective donors for 3 d, and supernatants were assayed for IFN-γ and IL-17 by ELISA. As seen in Fig. 7B, the hip1 mutant–infected DCs induced increased production of IFN-γ and IL-17 from human lymphocytes in each donor. Overall, these data extend our observations in mice to human cells and demonstrate that the interaction of M. tuberculosis with DCs impairs their capacity to initiate optimal adaptive immunity.
Discussion

The findings reported in this study reveal new insights into the interactions between DCs and M. tuberculosis and their impact on the initiation of CD4 T cell responses. Although the ability of M. tuberculosis to inhibit macrophage activation and antimicrobial functions has been well studied, the mechanisms by which M. tuberculosis modulates DC functions are poorly defined. By infecting murine and human DCs with a hip1 mutant strain of M. tuberculosis that induced enhanced DC responses, we found that wild-type virulent M. tuberculosis prevents optimal IL-12 production and DC maturation and impairs DC Ag presentation to CD4 T cells. Thus, we show that the M. tuberculosis serine

**FIGURE 4.** M. tuberculosis impairment of DC activation and maturation requires MyD88- and TLR2/9-dependent pathways. Purified BMDCs from C57BL/6J and MyD88−/− (A), TLR2−/− (B), or TLR2/9−/− (C) mice were exposed to medium alone (ui) or infected with heat-killed wild-type (wt) or the hip1 mutant (mut) at MOI of 5 for 24 h, and cell-free supernatants were assayed for IL-12p40, IL-12p70, and IL-6 by ELISA. (D) Infected BMDCs from MyD88−/− and TLR2/9−/− were labeled with anti-CD11c-allophycocyanin and anti-CD40-PE. Representative histograms and PE MFI for CD11c+ cells are shown. Data are representative of three (A, B) or two (C, D) independent experiments. Values are presented as mean ± SD. *p < 0.05, **p < 0.001. ud, undetectable.
hydrolase Hip1 plays a significant role in limiting DC functions that may have important consequences on T cell responses and disease development.

We found that wild-type *M. tuberculosis* prevents robust maturation of infected DCs and limits the secretion of key proinflammatory cytokines such as IL-12. These results support and extend previous reports suggesting that *M. tuberculosis* does not permit optimal DC maturation and thus limits their functions (36, 41, 42). A study using human MDCs showed that *M. tuberculosis* induces minimal upregulation of surface maturation markers as compared with a potent cytokine-maturation mixture, and *M. tuberculosis*-infected DCs were compromised in their ability to induce allogeneic lymphoproliferation (41). *M. tuberculosis* has also been shown to interfere with DC migration and Ag presentation in vivo (36). Our finding that wild-type *M. tuberculosis* prevents robust proinflammatory cytokine and chemokine responses in macrophages through Hip1. Our previous data suggested that Hip1-mediated remodeling of the *M. tuberculosis* cell wall hinders optimal macrophage activation by limiting interactions between TLR2 agonists on *M. tuberculosis* and TLR2 on macrophages and promotes a hypoimmune response that delays detection of *M. tuberculosis* by the host (11). In contrast, hip1-deficient *M. tuberculosis* induced robust MyD88- and TLR2-dependent activation of macrophages and enhanced proinflammatory responses. In this study, we show that the enhanced IL-12 produced by DCs infected with the hip1 mutant is dependent on MyD88 activation. Although the additional requirement for TLR9 is consistent with the increased engagement of TLR9 reported in DCs relative to macrophages (40, 43). Although we do not conclude from these data that Hip1 is directly suppressing MyD88–TLR2/9 pathways, we speculate that Hip1-mediated modification of the *M. tuberculosis* cell envelope prevents optimal MyD88–TLR2/9 activation on DCs during wild-type infection and that the absence of Hip1 enhances MyD88–TLR2/9 activation, resulting in robust DC activation. Although the enhanced surface expression of co-stimulatory markers by the hip1 mutant is dependent on MyD88 pathways, it appears to be largely independent of TLR2/4/9 pathways (Fig. 4 and data not shown), suggesting that yet-unknown MyD88-dependent pathways may be involved. These studies support use of specific TLR agonists as adjuvants to augment DC maturation, cytokine production, and Ag presentation as a strategy

**FIGURE 5.** hip1 mutant augments Ag presentation by BMDCs. Purified C57BL/6J BMDCs in medium alone (ui) or infected with heat-killed wild-type (wt) or hip1 mutant (mut) at MOI of 10 for 24 h were cocultured with ESAT-6–20 peptide and ESAT-6–specific Tg CD4 T cells (A) or OVA233–339 peptide and OT-II–specific Tg CD4 T cells for 3 d (B). Cell-free supernatants were collected and assayed for IFN-γ and IL-2 by ELISA. Data are representative of three independent experiments. Values are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 6.** *M. tuberculosis*–DC interactions modulate CD4 T cell differentiation in vitro and in vivo. (A) Purified C57BL/6J BMDCs in medium alone (ui) or infected with heat-killed wild-type (wt) or the hip1 mutant (mut) at MOI of 10 for 24 h were cocultured with CD4 T cells from C57BL/6J mice for 3 d. Cell-free supernatants were collected and assayed for IFN-γ and IL-17 by ELISA. (B) Single-cell suspensions were prepared from lungs of mice aerogenically infected with live wt or the hip1 mut at 3 wk postinfection, and cells were stimulated with 10 μg/ml ESAT-6–20 peptide for 48 h. Supernatants were collected and assayed for IFN-γ and IL-17 by ELISA. Data are representative of three (A) or two (B) independent experiments. Values are presented as mean ± SD. **p < 0.01, ***p < 0.001. ud, undetectable.
for improving vaccination against TB. Recent data showing that nanoparticles containing TLR4 and TLR7 ligands boost the magnitude and persistence of vaccine-elicted Ab responses, improving vaccine-mediated protection against influenza virus, demonstrate that these approaches are feasible and efficacious in the setting of infectious diseases (44).

To test how the enhanced DC maturation, MHC II expression, and IL-12 production induced by the hip1 mutant affect *M. tuberculosis*–specific CD4 T cell responses, we studied the Ag presentation capacity of DCs and compared the ability of DCs infected with wild-type versus *hip1* mutant *M. tuberculosis* to present the ESAT-6 1–20 peptide to ESAT-6 TCR-Tg CD4 T cells in vitro. We found that the *hip1* mutant promoted increased IFN-γ and IL-2 production upon coculture of DCs and CD4 T cells in the presence of ESAT-6 1–20 peptide, demonstrating that early interactions between DCs and CD4 T cells are likely to influence the kinetics and magnitude of adaptive immune responses. In addition to increased IFN-γ production, we also observed that *hip1* mutant infection led to increased levels of IL-17 in murine and human DC–T cell coculture experiments in vitro as well as in vivo in *M. tuberculosis*–infected mice (Figs. 6, 7). This is consistent with the enhanced production of the cytokines IL-6, IL-1β, and IL-23, which are known to be crucial for driving differentiation of Th17 cells. Although the enhanced IL-17 production in the case of the *hip1* mutant infection is not entirely clear, it has been suggested that early induction of IL-17 promotes recruitment of IFN-γ–producing T cells into the lungs via chemokine signals and improves bacterial killing. IL-17 has also been implicated in protective immunity to TB; intratracheal *M. tuberculosis* infection of mice deficient in IL-17A showed poor control of *M. tuberculosis* infection, and mycobacteria-exposed healthy adults harbored IL-17–producing CD4 T cells in their peripheral blood (54, 55). However, IL-17 production during chronic infection or unchecked Th17 responses may be detrimental by mediating immune pathology (56). Thus, a finely tuned balance between Th1 and Th17 subsets is likely to be required for protective immunity to *M. tuberculosis* infection. Because mice infected with the *hip1* mutant exhibit severely reduced lung pathology relative to wild-type despite high bacterial burdens (21–23), we speculate that robust proinflammatory responses and more efficient Ag presentation during early, acute stages of infection will promote adaptive responses that are less pathologic and may confer protection to the host.

Our studies demonstrating a role for Hip1 in dampening DC responses adds significance to a small but growing body of data showing that *M. tuberculosis*–derived factors modulate DC functions. A few purified *M. tuberculosis* Ags have been implicated in inhibiting DC maturation and functions. The *M. tuberculosis* Ag ESAT-6 inhibited LPS/CD40L-induced maturation of human PBMC-derived DCs and reduced IFN-γ production from T cells (57), and the *M. tuberculosis* cell wall component mannoside-capped lipoarabinomannan inhibited LPS-induced DC maturation by targeting DC-specific intercellular adhesion molecule-3–grabbing nonintegrin (58). However, the role of these factors in the context of whole *M. tuberculosis* remains unclear. In another study, an Ag85A-deficient mutant strain of *M. tuberculosis*, Δ*pba*, induced higher expression of MHC II on murine BMDCs as well as higher levels of IL-12p70; these DCs primed T cells to produce more IFN-γ as compared with wild-type *M. tuberculosis* (42). Further, Δ*pba*-vaccinated mice showed better protection against *M. tuberculosis* challenge compared with those vaccinated with bacillus Calmette-Guérin. Similar studies are ongoing with the *hip1* mutant to assess whether the *hip1* mutant in *M. tuberculosis* or bacillus Calmette-Guérin has potential as a vaccine candidate.

In summary, we have shown that *M. tuberculosis* serine hydrolase Hip1 impairs DC maturation and functions, highlighting its important role in modulating DC–pathogen interactions. Wild-type *M. tuberculosis* induces suboptimal DC maturation and restricts the secretion of IL-12 and other key proinflammatory cytokines. This inhibition of DC maturation and cytokine secretion compromises Ag presentation to CD4 T cells and results in lower IFN-γ and IL-17 compared with the *hip1* mutant. Overall, these findings show that optimal activation of DCs should result in a more efficient T cell responses against *M. tuberculosis* and have important implications for vaccine design.

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References


