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Deletion of BCG Hip1 protease enhances dendritic cell and CD4 T cell responses

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Abstract

Dendritic cells (DCs) play a key role in the generation of CD4 T cell responses to pathogens. *Mycobacterium tuberculosis* (Mtb) harbors immune evasion mechanisms that impair DC responses and prevent optimal CD4 T cell immunity. The vaccine strain *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) shares many of the immune evasion proteins utilized by Mtb, but the role of these proteins in DC and T cell responses elicited by BCG is poorly understood. We previously reported that the Mtb serine protease, Hip1, promotes sub-optimal DC responses during infection. Here, we tested the hypothesis that BCG Hip1 modulates DC functions and prevents optimal antigen-specific CD4 T cell responses that limit the immunogenicity of BCG. We generated a strain of BCG lacking *hip1* (BCG *hip1*) and show that it has superior capacity to induce DC maturation and cytokine production compared to parental BCG. Furthermore, BCG *hip1*-infected DCs were more effective at driving the production of IFN- γ and IL-17 from antigen-specific CD4 T cells *in vitro*. Mucosal transfer of BCG *hip1*-infected DCs into mouse lungs induced robust CD4 T cell activation *in vivo* and generated antigen-specific polyfunctional CD4 T cell responses in the lungs. Importantly, BCG *hip1*-infected DCs enhanced control of pulmonary bacterial burden following Mtb aerosol challenge compared to transfer of BCG-infected DCs. These results reveal that BCG employs Hip1 to impair DC activation, leading to attenuated lung CD4 T cell responses with limited capacity to control Mtb burden after challenge.

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CONFLICT OF INTEREST DISCLOSURE

The authors have declared no conflicts of interest.

Keywords

Vaccine; immunogenicity; tuberculosis; immune evasion

INTRODUCTION

Critical to the success of *Mycobacterium tuberculosis* (Mtb) as a pathogen is its ability to evade host innate and adaptive immunity. Mtb dampens macrophage functions and impairs the ability of dendritic cells (DCs) to induce optimal antigen-specific CD4 T cell responses. As the major antigen presenting cell (APC) in the immune system, DCs are central to the generation of CD4 T cell responses after infection and vaccination. However, immunomodulatory factors expressed by Mtb promote sub-optimal DC maturation, cytokine production and antigen presentation to CD4 T cells, which adversely affects T cell immunity and impedes control of Mtb infection (1, 2). BCG is an attenuated strain of *Mycobacterium bovis* and the only licensed vaccine for tuberculosis (TB) in humans. While BCG vaccination protects children under the age of five from disseminated forms of TB disease, BCG has limited efficacy against pulmonary TB in children and adults (3, 4). However, the immunological basis for sub-optimal immunity induced by BCG remains unclear. The genome of the BCG parent strain, *M. bovis* shares over 99.95% sequence identity with the Mtb genome (5) and BCG retains many of the genes shown to encode immune evasion proteins in Mtb. We therefore reasoned that retention of immune evasion strategies that are present in virulent mycobacteria by BCG may impede generation of effective innate and adaptive immune responses induced by the vaccine. Thus, we hypothesized that deleting immune evasion genes in BCG that impair DC functions has the potential to improve innate and adaptive immune responses induced by BCG.

We have previously demonstrated that an Mtb cell wall-associated serine protease, Hip1 (Hydrolase important for pathogenesis 1, Rv2224c), is involved in impairing DC functions (6). Since Hip1 from BCG and Mtb are 100% identical, we hypothesized that BCG Hip1 may contribute to sub-optimal DC and CD4 T cell responses and that deletion of *hip1* from BCG would augment innate and adaptive immune responses. In this study, we generated a BCG (Danish) strain lacking *hip1* (BCG *hip1*) to investigate whether deletion of *hip1* in BCG enhances DC functions and improves CD4 T cell responses *in vitro* and *in vivo*. We show that DCs infected with BCG *hip1* produce significantly enhanced levels of pro-inflammatory cytokines and express higher levels of major histocompatibility complex (MHC) class II and costimulatory molecules compared to DCs infected with the parent BCG strain. Additionally, deletion of *hip1* from BCG augmented DC antigen presentation to CD4 T cells *in vitro*. Moreover, mucosal priming of immune responses via intratracheal instillation of BCG *hip1*-infected DCs improved antigen-specific CD4 T cell responses in the lungs and enhanced control of Mtb burden following aerosol challenge, compared to transfer of BCG-infected DCs. Our results demonstrate that BCG subversion of DC functions through Hip1 impedes the generation of robust CD4 T cell responses and provides a rationale for targeting *hip1* to improve BCG immunogenicity.

MATERIALS AND METHODS

Bacterial strains and culture conditions

BCG (Danish), BCG *hip1*, and BCG *hip1* complemented with *hip1* (BCG *hip1* comp) were grown at 37°C in Middlebrook 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.2% glycerol and 0.05% Tween 80 or on Middlebrook 7H10 agar supplemented with 10% OADC, 0.5% glycerol, and 0.2% Tween 80. Media for complemented BCG *hip1* was supplemented with 20µg/ml of streptomycin (Sigma-Aldrich, St. Louis, MO), and media for BCG *hip1* was supplemented with 50µg/ml of hygromycin (Roche Diagnostics, Indianapolis, IN). For growth curves, bacterial strains were inoculated into supplemented 7H9 medium at OD₆₀₀ 0.05, and the OD₆₀₀ measurements were taken daily.

Construction of BCG *hip1* and complemented strains

BCG was transformed via electroporation with 3µg of pEBOP-2 (pYUB657 suicide vector containing a *hip1* allele, a selectable hygromycin resistance marker, and a counter selectable *sacB* marker). Resulting transformants that were resistant to hygromycin were then patched onto 7H10 plates containing 2% sucrose. Colonies that displayed hygromycin resistance and sucrose sensitivity were considered to have undergone a single crossover event resulting in incorporation of pEBOP-2 into the BCG genome. These colonies were then grown to saturation for a week in 5ml of 7H9 broth, and then serial dilutions were plated in duplicate onto 7H10 plates supplemented with 2% sucrose. Colonies arising on these plates were patched onto hygromycin-containing plates. Colonies that were both hygromycin sensitive and sucrose resistant were grown in 7H9 broth, and genomic DNA was extracted using the protocol adapted from Belisle and Sonnenberg (7). Genomic DNA was then subjected to Southern blot analysis. DNA was digested with NcoI, and then probed with a DIG-labeled DNA amplicon corresponding to a 1kb region present in both the genome and pEBOP-02. Deletion of *hip1* was also confirmed via amplification of the deleted region using primers upstream (forward primer 5'-CGGCCACCCGCTACCGCCCTCG-3') and downstream (reverse primer 5'-GCACGGCGAATGTCAGATAGGG-3') of the 1kb regions of homologous recombination, resulting in a 4.5kb amplicon from the BCG *hip1* genome, and a 6kb amplicon from the wild-type BCG genome (supplemental Fig. 1C). These amplicons were then sequenced for further confirmation of gene deletion. BCG *hip1* was complemented with *hip1* expressed from its natural promoter on an integrated plasmid.

Mice

All mice were housed under specific pathogen-free conditions in filter-top cages within the vivarium at the Yerkes National Primate Center, Emory University, and provided with sterile water and food ad libitum. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-II TCR transgenic mice specific for OVA₃₂₃₋₃₃₉ peptide were obtained from Dr. Bali Pulendran (originally generated in the laboratory of Dr. F. Carbone, University of Melbourne), and bred at the Yerkes animal facility.

BMDM and BMDC generation and infection

Bone marrow-derived macrophages (BMDMs) were generated as previously described (8). Bone marrow cells were isolated from C57BL/6J mice and differentiated for 7 days at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Lonza, Walkersville, MD) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT), 2mM L-glutamine, and 10% L-cell conditioned medium (LCM). Adherent cells were collected, and macrophages were plated onto 24-well plates at 3×10⁵ per well and rested overnight. For heat-killed BCG infections, bacteria suspended in DMEM/F-12 medium containing 10% FBS, 2mM L-glutamine, and 5% LCM were added to differentiated BMDMs in 24 well plates at indicated MOIs. Murine bone marrow derived dendritic cells (BMDCs) were generated as previously described (9). Bone marrow cells isolated from C57BL/6J mice were grown and differentiated for 8 days in Roswell Park Memorial Institute (RPMI) medium (Lonza, Walkersville, MD) supplemented with 10% FBS (HyClone, Logan, UT), 2mM L-glutamine (Gibco, Grand Island, NY), 20ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) (R&D systems, Minneapolis, MN), 50 μM beta-mercaptoethanol (Gibco, Grand Island, NY), 1X non-essential amino acids (Gibco, Grand Island, NY), 10mM HEPES buffer (Lonza, Walkersville, MD), 1mM sodium pyruvate (Lonza, Walkersville, MD). Non-adherent cells were harvested after 8 days and purified using CD11c microbeads (Miltenyi, Gladbach, Germany), plated at 3×10⁵ per well, and rested overnight. For heat-killed BCG infections, bacteria were suspended in DC media without rmGM-CSF and added to differentiated BMDCs in 24 well plates at indicated MOIs and incubated at 37°C. For live infections, BCG strains suspended in DC media without rmGM-CSF, were added to BMDCs at indicated MOIs and allowed to incubate at 37°C for 4 hours. Monolayers were then treated with 200μg/ml amikacin for 45 minutes. Cells were then washed thrice with PBS, and DC media without rmGM-CSF was then added to infected cells. To determine the CFU of bacteria within infected cells, one set of BMDCs was first washed with PBS then lysed using PBS containing 0.5% TritonX, and serial dilutions of the lysate were plated onto 7H10 plates. To assess BCG growth within BMDCs, cell lysates were plated on days 1, 3 and 5 after initial infection. CFU were enumerated after 21 days of incubation. Supernatants from infected BMDCs or BMDMs were collected at indicated timepoints and analyzed via ELISA for cytokine levels according to the manufacturers' instructions: BD OptEIA kits for IL-6, IL-1β, IL-12p70 and IL-12p40 (BD Biosciences, San Jose, CA) and IL-23 (Biolegend, San Diego, CA) per manufacturer's instructions.

BMDC-T cell co-cultures

BMDCs were co-cultured with CD4 T cells 24 hours after infection with BCG strains. Briefly, CD4 T cells were isolated from splenocytes collected from OT-II TCR transgenic mice and purified using CD4 magnetic microbeads (Miltenyi, Gladbach, Germany). Purified CD4 T cells were suspended at 1×10⁶/ml in supplemented RPMI and co-cultured with BMDCs to achieve a 1:4 DC:T cell ratio. Supernatants from co-cultures were collected after 72 hours, spun down to remove cells, and frozen. Cytokine levels were analyzed via ELISA according to manufacturers' instructions: IFN-γ (Mabtech, Cincinnati, OH), IL-2 (BD Biosciences, San Jose, CA), and IL-17 (eBioscience, San Diego, CA)

Intratracheal instillation of BMDCs

BMDCs were generated as described, purified using CD11c microbeads (Miltenyi), and stimulated with either BCG or BCG *hip1* at an MOI of 30 or left unstimulated in media for 24 hours. BMDCs were then washed, resuspended in PBS and intratracheally instilled (1×10^6 per mouse in 50 μ l PBS) into isoflurane-anesthetized C57BL/6J hosts.

Assessment of antigen-specific responses

6 days after intratracheal BMDC transfer, lungs were harvested and processed for further analysis. Briefly, organs were minced and placed in harvest medium consisting of HBSS containing 10 mM HEPES, 2% FBS, 0.1% collagenase type IV (Worthington, Lakewood, NJ), and 0.01% DNase I (Worthington, Lakewood, NJ) for 30 minutes at 37°C. Following incubation, organs were processed into single cell suspension utilizing the gentleMACs tissue dissociator (Miltenyi, Gladbach, Germany). Cells were thoroughly washed, counted, and 1×10^6 cells were plated for phenotypic analysis or antigen restimulation. Cells were exposed to media (unstimulated), PMA/ionomycin (80ng/ml and 500ng/ml, respectively), or 10 μ g/ml whole cell lysate (WCL). Cells were then incubated at 37°C for 1.5 hours before addition of BFA (5 μ g/ml) and monensin (1:1500) followed by a further incubation at 37°C for 4.5 hours (for media and PMA/ionomycin stimulations) or overnight (for WCL stimulations). Cells were then spun down, washed, and stained with the following fluorophore conjugated antibodies purchased from BD Biosciences, Biolegend, or eBioscience for flow cytometric analysis: anti-CD8 PerCP (clone 53-6.7, BD), anti-CD44 APC-Cy7 (clone IM7, BD), anti-TCR $\gamma\delta$ BV605 (clone GL3, Biolegend), anti-CD3e V450 (clone 500A2, BD), anti-CD4 Alexa700 (clone RM4-5, BD), anti-TNF- α PE-Cy7 (clone MP6-XT22, BD), anti-IL-2 FITC (clone JES6-5H4, BD), anti-IL-17 PECF594 (clone TC11-18H10, BD), and anti-IFN- γ APC (clone XMG1.2, eBioscience). Live cells were determined by exclusion of amine-reactive dye (Live/Dead Fixable Aqua Dead Cell Stain kit, Life Technologies, Carlsbad, CA). Samples were acquired using an LSRII flow cytometer and analyzed by FlowJo (FlowJo, LLC).

Mtb aerosol challenge and enumeration of bacteria

Groups of five mice were intratracheally instilled with 1×10^6 BMDCs, rested for 14 days, and then challenged with a low dose (~ 100 CFU) of Mtb H37Rv using an Intox aerosol apparatus. Lungs from infected mice were harvested 28 days post-challenge, homogenized, plated on 7H10 agar plates, and incubated for 21 days in 37°C prior to CFU enumeration.

RESULTS

Construction of a BCG *hip1* strain

To determine the role of *hip1* in BCG-induced DC responses, we generated an in-frame, unmarked deletion of *hip1* in the BCG Danish strain. We utilized the suicide vector, pYUB657, which expresses a hygromycin resistance cassette and a counter-selectable marker, to introduce an allelic exchange-based deletion of *hip1* (supplemental Fig. 1A). The resulting BCG *hip1* strain harbored a complete deletion of *hip1* from its genome, which we verified via PCR amplification of the genomic region as well as through Southern blot

analysis (supplemental Fig. 1B and 1C). Next, we sought to determine the effect of deleting *hip1* on BCG growth kinetics. We observed no significant differences between BCG, BCG *hip1*, or a BCG *hip1* strain complemented with *hip1* (BCG *hip1* comp) on growth in 7H9 broth (Fig. 1A). Additionally, these strains grew comparably in bone marrow-derived dendritic cells (BMDCs) over 5 days of culture (Fig. 1B).

BCG *hip1* elicits robust DC cytokine responses compared to BCG

DC cytokine production is a canonical signal driving differentiation of naïve CD4 T cells to specific CD4 T-helper (Th) subsets. We therefore sought to compare cytokine responses from DCs infected with BCG or BCG *hip1*. We infected BMDCs from C57BL/6J mice with BCG, BCG *hip1* or BCG *hip1* comp and measured DC cytokine production by ELISA. DCs infected with BCG *hip1* produced significantly higher levels of IL-6 and IL-12p40 than DCs infected with BCG. Importantly, cytokine levels induced by BCG *hip1* were restored to BCG levels after infection with the complemented strain (Fig. 2A), indicating that BCG limits DC cytokine production through *hip1*. We next infected DCs with BCG or BCG *hip1* at multiplicities of infection (MOI) of 10 and 20 and assessed cytokine levels in the supernatant at 24, 48 and 72 hours after infection by ELISA. DCs infected with BCG *hip1* produced significantly higher levels of IL-6 and IL-12p40 relative to BCG infection at all MOIs and time points tested (Fig. 2B). Notably, incubation of DCs with heat-killed BCG *hip1* also resulted in significantly higher levels of cytokines compared to heat-killed BCG, indicating that enhanced cytokine production by DCs infected with BCG *hip1* was not dependent on viability of the bacteria (Fig. 2C). Further, we found that bone marrow-derived macrophages (BMDMs) infected with BCG *hip1* produced higher levels of IL-6 and IL-1 β compared to BCG-infected BMDMs at all MOIs tested (Fig. 2D). These data demonstrate that deletion of *hip1* in BCG results in significantly augmented pro-inflammatory cytokine production from both infected DCs and macrophages.

BCG *hip1* enhances expression of costimulatory molecules on infected DCs

Following infection, DCs undergo maturation, which is required for optimal antigen presentation and initiation of antigen-specific CD4 T cell responses. DCs present antigens via MHC class II complexes and provide critical costimulatory signals to CD4 T cells through upregulation of molecules such as CD40 and CD86. We determined the expression levels of MHC class II and the costimulatory molecules CD40 and CD86 on DCs infected with BCG or BCG *hip1* by flow cytometry. DCs infected with BCG *hip1* expressed higher levels of MHC class II, CD40, and CD86 when compared to BCG-infected DCs (Fig. 3). These data suggest that deletion of *hip1* in BCG enhances DC maturation and expression of costimulatory molecules.

Enhanced polarization of IFN- γ and IL-17- producing antigen-specific CD4 T cells by DCs infected with BCG *hip1*

IL-12 is known to drive the polarization of IFN- γ -producing Th₁ subsets while IL-6, IL-1 β , TGF- β , and IL-23 drive the polarization and expansion of IL-17-producing Th₁₇ subsets (10). Since DCs infected with BCG *hip1* induced higher levels of pro-inflammatory cytokines (Fig. 2) and displayed an enhanced maturation profile (Fig. 3) compared to BCG infection, we hypothesized that BCG *hip1*-infected DCs would more effectively polarize

antigen-specific CD4 T cells towards Th₁ and Th₁₇ subsets compared to BCG-infected DCs. To test this, we co-cultured DCs infected with BCG or BCG *hip1* with naïve ovalbumin-specific TCR transgenic CD4 T cells (OT-II) for 3 days in the presence of cognate peptide (OVA_{323–339}). Levels of IFN- γ , IL-17, and IL-2 were measured via ELISA (Fig. 4A). DCs infected with BCG *hip1* promoted significantly higher levels of IFN- γ and IL-17 from antigen-specific CD4 T cells after co-culture (Fig. 4A). This was consistent with higher levels of the Th₁-polarizing cytokines IL-12p40 and IL-12p70 induced by BCG *hip1*-infected DCs as well as higher levels of Th₁₇-polarizing cytokines IL-6 and IL-1 β (Fig. 4B). We do not detect significant levels of TGF- β under any of our culture conditions. Further, compared to BCG-infected DCs, BCG *hip1*-infected DCs produced higher levels of IL-23, which is a cytokine known to maintain Th₁₇ lineage commitment (11). These data demonstrate that BCG *hip1*-infected DCs have an enhanced capacity to induce antigen-specific IFN- γ and IL-17 responses compared to BCG-infected DCs.

Intratracheal instillation of DCs infected with BCG *hip1* enhances lung CD4 T cell responses *in vivo* and improves control of Mtb burden following aerosol challenge.

Mucosal administration of DCs has been utilized to assess early antigen-specific T cell responses to mycobacteria in the lungs of mice (12), and antigen-loaded DCs have previously been shown to confer protection against Mtb challenge (9, 13–15). Therefore, we used a tractable DC intratracheal instillation model to assess antigen-specific CD4 T cell responses *in vivo* following transfer of BCG or BCG *hip1*-infected DCs, or control uninfected DCs, into the lungs of mice. After 6 days, we harvested lungs and assessed, by flow cytometry, antigen-specific CD4 T cell responses upon *ex vivo* stimulation of lung cells with Mtb whole cell lysate (WCL). We measured the number of activated CD44⁺ CD4 T cells in the lungs following intratracheal instillation of BCG or BCG *hip1*-infected DCs, which reflects levels of CD4 T cell activation. We found higher numbers of CD44⁺ CD4 T cells in the lungs of mice that received DCs infected with BCG *hip1* compared to mice that received BCG (Fig. 5B), indicating that BCG *hip1*-infected DCs induced better activation of CD4 T cells *in vivo*. Next, we assessed the functionality of antigen-specific CD4 T cells in the lungs by measuring cytokine responses by intracellular cytokine staining (ICS) and flow cytometry following restimulation with WCL. We found higher numbers of antigen-specific CD4 T cells producing IL-2, IFN- γ , TNF- α , and IL-17 in the lungs of mice that received BCG *hip1* DCs compared to BCG DCs (Fig. 5C). Triple cytokine-producing CD4 T cells, conventionally termed polyfunctional CD4 T cells, are thought to be indicative of a more protective adaptive immune response (16). Interestingly, we observed higher frequencies of CD4 T cells producing IFN- γ , IL-2, and TNF- α in animals that received BCG *hip1* DCs compared to BCG DCs (Fig. 5D). To investigate whether mucosal administration of DCs exposed to BCG *hip1* would provide enhanced bacterial control compared to BCG after low dose aerosol Mtb challenge, we intratracheally instilled BCG DCs or BCG *hip1* DCs, rested mice for 2 weeks prior to aerogenic challenge with low-dose Mtb H37Rv, and determined lung Mtb bacterial burden 4 weeks post-challenge. As shown in Fig. 5E, mice that received BCG *hip1*-infected DCs harbored significantly less Mtb CFU post-challenge compared to mice that received BCG-infected DCs (Fig. 5E). These results demonstrate that mucosal-targeted approaches using BCG *hip1* can augment antigen-

specific CD4 T cell responses compared to BCG and lead to enhanced control of Mtb burden.

DISCUSSION

The interplay between mycobacteria and DCs is a critical consideration for rational development of efficacious vaccines for TB. In this study, we demonstrate that deletion of the BCG serine protease, Hip1, promotes robust DC activation and enhances antigen-specific lung CD4 T cell responses. We have shown that BCG *hip1*-infected DCs produce higher levels of cytokines, express elevated levels of costimulatory molecules, and enhance CD4 T cell responses both *in vitro* and *in vivo* compared to DCs infected with BCG. These data provide insight into the sub-optimal immunogenicity of BCG and demonstrate that deletion of the immune evasion gene, *hip1*, in BCG promotes enhanced DC-T cell crosstalk that leads to better control of Mtb burden.

The underlying reasons for the variable efficacy of BCG as a TB vaccine are unclear. Since DCs are the key cells linking innate and adaptive immunity, the interaction between DCs and BCG is a critical factor in generating anti-mycobacterial T cell responses. Our results are consistent with a growing body of literature suggesting that BCG impairs innate and adaptive immune responses. BCG has been shown to adversely impact antigen-specific CD4 T cell activation through the upregulation of PD-L1 and PD-L2 (17), and diminish activation of antigen-specific CD8 T cells by inducing DC death (18). Furthermore, BCG-infected DCs produce significantly lower levels of IL-23, IL-1 β , TNF- α , and IL-12 than Mtb-infected DCs (19), indicating that BCG stimulates weaker innate immune responses than Mtb. Interestingly, a study by Satchidanandam *et al* showed that overexpression of an Mtb glycosylated protein, Rv1860, in BCG impaired DC maturation, attenuated Th₁ and Th₁₇ polarization, and led to subsequent loss of protection against Mtb challenge conferred by BCG vaccination (20), suggesting that Mtb proteins that negatively impact DC responses can attenuate the protective effect of BCG vaccination. Conversely, relatively little is known about BCG genes that retain immunomodulatory properties and thus promote impaired DC responses and sub-optimal T cell immunity. Our data showing that BCG Hip1 contributes to impaired DC cytokine production and maturation demonstrates that BCG retains immunomodulatory factors that negatively impact DC and T cell responses, leading to impaired control of Mtb after challenge.

Several avenues have been explored for improving BCG immunogenicity and efficacy, including introduction of immunodominant proteins from Mtb (20–23), and expression of host proteins (24–30). The majority of studies utilizing recombinant or mutant strains of BCG are primarily focused on enhancing macrophage driven responses and functions, such as phagosomal maturation, apoptosis of infected macrophages, and bacterial escape from phagosomal compartments (31–36). For instance, a recombinant strain of BCG lacking urease production and expressing listeriolysin from *Listeria monocytogenes* (BCG *ureC::hly*) was shown to enhance apoptosis of infected macrophages (33), leading to increased central memory T cell responses (37), enhanced Th₁ and Th₁₇ immunity (38), and cross-presentation to CD8 T cells (34). Interestingly, deletion of anti-apoptotic gene *nuoG* in BCG *ureC::hly* showed enhanced efficacy over BCG *ureC::hly* (39). Notably, we show

evidence that BCG *hip1*-infected macrophages display enhanced cytokine production relative to BCG-infected macrophages (Fig. 2). Since we utilized a mucosal transfer approach that exclusively utilized DCs, it will be important to address the role that macrophages may play at priming antigen-specific CD4 and CD8 T cell responses after vaccination with BCG *hip1*.

Relatively few studies have elaborated on BCG factors that can be targeted to improve DC responses. However, targeting DCs has proven to be a viable approach to improve Mtb-specific CD4 T cell responses after vaccination. H56, a subunit vaccine incorporating Ag85B, ESAT-6, and Rv2660, was shown to provide enhanced protection relative to BCG (40), and utilizes a liposome-based adjuvant (CAF01) that targets DCs (41). Furthermore, improving DC antigen-presentation by induction of autophagy has been shown to improve BCG immunogenicity and improve control of Mtb burden after challenge (42). Our data show that transfer of BCG *hip1*-DCs leads to enhanced lung CD4 T cell responses compared to transfer of BCG-DCs, including higher frequencies of antigen-specific, polyfunctional CD4 T cells contributing to better control of Mtb burden after challenge (Fig. 5). Thus, our studies suggest that deleting BCG *hip1* alone, or in concert with deleting additional immune evasion genes, is a feasible approach to enhance DC functions for the rational improvement of BCG as a vaccine. A growing number of studies indicate that vaccination through the mucosal route induces robust antigen-specific responses that confer better protection at mucosal surfaces, such as the lung, relative to parenteral routes (9, 13, 14, 43–48). Using an intratracheal DC installation model, we observed that transfer of DCs infected with BCG *hip1* into mouse lungs more effectively activated CD4 T cells, induced higher numbers of antigen-specific mucosal CD4 T cells secreting IFN- γ , IL-2, TNF- α , and IL-17 *in vivo*, and led to enhanced Mtb control after challenge compared to transfer of BCG-DCs. These studies provide proof of principle data and reveal insights into BCG interactions with DCs, but may not mirror mucosal vaccination using bacteria alone. Bacteria encounter a wider variety of myeloid cells in the lungs, including alveolar macrophages and lung DC subsets, that may differ from BMDCs. Therefore, further studies examining the effects of BCG *hip1* utilizing more traditional vaccination approaches are of interest.

In summary, our work supports a growing body of evidence that enhancing DC functions will improve BCG-induced immunity. Deletion of *hip1* in BCG augmented DC functions, improved antigen-specific CD4 T cell responses in the lungs, and promoted enhanced control of Mtb burden after challenge. These data indicate that strategies targeting BCG immune evasion genes, such as *hip1*, are a viable avenue for improving innate and adaptive immunity to provide enhanced control of Mtb.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS PAGE

APC	Antigen presenting cell
BCG	Bacille Calmette-Guérin
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
DC	Dendritic cell
Hip1	Hydrolase important for pathogenesis 1
ICS	Intracellular cytokine staining
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
Mtb	Mycobacterium tuberculosis
rmGM-CSF	Recombinant murine granulocyte macrophage colony-stimulating factor
TB	Tuberculosis
Th	T-helper
WCL	Whole cell lysate

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Summary sentence:

Deletion of *hip1* in BCG enhances DC maturation and antigen-specific CD4 T cell immunity in the lungs.

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Figure 1. Deletion of *hip1* from BCG does not affect growth of BCG in broth or in dendritic cells. (A) BCG Danish, BCG *hip1*, or complemented BCG *hip1* (comp) were inoculated into 7H9 liquid medium supplemented with OADC and glycerol at a starting OD₆₀₀ of 0.05, were incubated at 37°C shaking, and the absorbance of the cultures at OD₆₀₀, was recorded daily. (B) BMDCs from C57BL/6J mice were infected with BCG, BCG *hip1*, or complemented BCG *hip1* (comp) and grown at 37°C. DCs were collected and lysed at the indicated time points, and bacteria were plated on 7H10 plates for CFU determination. Results are representative of 3 independent experiments. Values are presented as means $\pm$ SD.

Figure 2. BCG *hip1* induces stronger pro-inflammatory cytokine production from innate immune cells.

(A) BMDCs from C57BL/6J mice were infected with BCG, BCG *hip1*, or BCG *hip1* complemented with *hip1* (BCG comp) at a multiplicity of infection (MOI) of 10 and incubated at 37°C. After 24 hours, the culture supernatants were assessed for levels of IL-6 and IL-12p40 via ELISA. (B) BMDCs were infected with varying MOIs (10 and 20) of BCG or BCG *hip1*, and culture supernatants were assayed for IL-6 and IL-12p40 at 24, 48, and 72 hours post-infection. Heat-killed BCG or BCG *hip1* were exposed to BMDCs (C) or

BMDMs (**D**) at MOIs of 5, 10, and 20 for 24 hours at 37°C. Culture supernatants were tested for IL-6 and IL-12p40 (**C**) or IL-6 and IL-1 β (**D**) via ELISA. Results are representative of 3 independent experiments. Statistical significance was determined by unpaired two-tailed student's t-test. Values are presented as mean $\pm$ SD. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001, ns= no significance, ui= uninfected

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Figure 3. BCG *hip1* enhances expression of costimulatory molecules on infected DCs
 BMDCs from C57BL/6J mice were either uninfected, infected with BCG or BCG *hip1* (MOI 10), or stimulated with LPS (1 μ g/ml) for 24 hours. BMDCs were then stained for maturation markers CD86 (top), CD40 (middle), and MHC class II (bottom). Representative histograms (left) and summary graphs (right) of median fluorescence intensities of each marker is shown. Cells were pre-gated on live, CD11c⁺CD11b⁺ singlets. Results are representative of 3 independent experiments. Statistical significance was determined by

unpaired two-tailed student's t-test. Values are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, ns= no significance.

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Figure 4. DCs infected with BCG Δ hip1 induce higher levels of IFN- γ and IL-17 production from CD4 T cells compared to BCG.

BMDCs from C57BL/6J mice were pulsed with OVA₃₂₃₋₃₃₉ peptide, and 6 hrs later, infected with BCG or BCG Δ hip1. After 24 hours, culture supernatants were removed and purified OT-II CD4 T cells were added to the adherent BMDC monolayer at a 4:1 T cell to DC ratio. 3 days after addition of CD4 T cells, culture supernatants were collected and IL-2, IFN- γ , and IL-17 levels were assessed via ELISA (A). Levels of IL-12p40, IL-12p70, IL-6, IL-1 β , and IL-23 from culture supernatants containing solely BMDCs were assessed via ELISA (B). Results are representative of 3 independent experiments. Statistical significance was determined by unpaired two-tailed student's t-test. Values are presented as mean $\pm$ SD.

*p<0.05, **p<0.01, ns= no significance.

Figure 5. Intratracheal instillation of DCs infected with BCG *hip1* enhances mucosal CD4 T cell responses and improves control of Mtb burden after aerosol challenge

(A) Diagram of experimental design. BMDCs were exposed to BCG or BCG *hip1* or were left uninfected for 24 hours prior to being intratracheally instilled (1×10^6 per mouse). Mice that did not receive any DCs were used as no transfer (NT) controls. Lung immune responses were assessed 1 week post-intratracheal instillation. Remaining animals were challenged 2 weeks post-intratracheal instillation with low dose aerosolized Mtb (H37Rv) and lung bacterial burden was assessed 4 weeks post-challenge. (B) Representative plots of the frequencies (left) and summary graph of the absolute counts (right) of CD4⁺CD44⁺ cells

in the lungs 1 week post-intratracheal instillation. (C) Lung IFN- γ^+ CD4 $^+$ cells (top) and IL-2 $^+$, TNF- α^+ , IL-17 $^+$ CD4 $^+$ cells (bottom) responding to whole cell lysate restimulation 1 week post-intratracheal instillation. (D) Summary graph of the frequency of lung polyfunctional (IFN- γ^+ IL-2 $^+$ TNF- α^+) CD4 $^+$ T cells responding to whole cell lysate restimulation 1 week post-intratracheal instillation. Cells were pre-gated on live, CD3 $^+$ CD8 $^-$ TCR $\gamma\delta^-$ singlets. (E) Lung bacterial burden at 4 weeks post-challenge from animals that received uninfected BMDCs or BMDCs stimulated with BCG or BCG *hip1*. Results are representative of 2 independent experiments. Statistical significance was determined by unpaired two-tailed student's t-test. Values are presented as mean $\pm$ SD. *p<0.05, **p<0.01, ***p<0.001, ns= no significance.