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Modulation of innate-adaptive immune crosstalk during *Mycobacterium tuberculosis* infection

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Modulation of innate-adaptive immune crosstalk during *Mycobacterium tuberculosis* infection

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Abstract

Modulation of innate-adaptive immune crosstalk during *Mycobacterium tuberculosis* infection

By Jonathan Kevin Sia

Mycobacterium tuberculosis, the etiological agent of tuberculosis (TB), is a successful pathogen that can strategically manipulate crosstalk between innate and adaptive immunity. Dendritic cells (DCs) are professional antigen-presenting cells that link innate immune recognition of *M. tuberculosis* to the development of antigen-specific CD4 T cells that combat infection. However, CD4 T cell immunity to infection is ultimately suboptimal. We investigate the role of DCs in the generation of CD4 T cell responses and methods to improve host immunity to infection and vaccination.

There is substantial evidence that IL-17 producing CD4 T cells (Th17) play an important role in protective immunity against *M. tuberculosis*. However, molecular mechanisms involved in the development of Th17 cells during infection are not fully defined. We show that DC co-stimulation through the CD40 pathway is required for the generation of antigen-specific Th17 cells. Further, we demonstrate that ligating CD40 on *M. tuberculosis* infected DCs augments Th17 polarization. Importantly, mucosal transfer of antigen-loaded, CD40-ligated DCs improves lung antigen-specific CD4 T cell responses and host control of *M. tuberculosis* infection. These results uncover a novel role for DC co-stimulation through CD40 in the development of Th17 cells during infection and provide evidence that engaging the CD40 pathway on DCs enhances immunity against *M. tuberculosis*.

We extend our studies to Bacillus Calmette-Guérin (BCG), an attenuated *Mycobacterium bovis* utilized as a TB vaccine with limited efficacy. We hypothesized that retention of immune subversion strategies by BCG limits its vaccine potential by hampering DC functions. Hip1 is an immune evasion protease in *M. tuberculosis* but its role in BCG was unknown. We demonstrate that DCs infected with BCG lacking *hip1* (BCGΔ*hip1*) are more mature, secrete increased levels of cytokines, and enhance CD4 T cells responses when compared to BCG. Notably, mucosal transfer of BCGΔ*hip1* infected DCs augments host control of lung bacterial burden following aerosol challenge with *M. tuberculosis*.

Our findings reveal that mycobacterial subversion of DCs attenuates the development of antigen-specific CD4 T cell immunity. We contribute insights into the role of DC co-stimulation during infection and improve host immunity in the context of infection and vaccination through DC-targeted strategies.

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"Don't be stupid."

Abbey Marie Jones, circa 2012

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Introduction

Mycobacterium tuberculosis, the etiologic agent of tuberculosis (TB), remains a significant global public health burden[1]. In 2016, there were 10.4 million new TB cases reported globally and nearly 1.7 million TB-related deaths[1]. Understanding the host response to *M. tuberculosis* infection is a key aspect of efforts to eradicate TB through the development of effective vaccines and immune therapeutics. Despite being developed nearly a century ago, Bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, remains the only licensed vaccine against TB. Vaccination with BCG provides protection against severe forms of disseminated TB in children, but has variable efficacy in preventing pulmonary disease in children and adults[2-4]. However, the immunological basis for the poor efficacy of BCG remains unclear. Moreover, long-held concepts regarding the nature of desired immune responses in an ideal TB vaccine, namely the induction of antigen-specific CD4 T cells producing IFN-γ, are being updated to reflect the expanding knowledge of host immunity to *M. tuberculosis* infection gathered from animal models and human cohort studies. Advances in imaging and single-cell technologies combined with high-throughput approaches and systems-based analyses are providing more information on the immune response to *M. tuberculosis* infection at increasingly higher resolutions. As understanding of the host response to *M. tuberculosis* infection grows, opportunities to leverage knowledge of the immunology of *M. tuberculosis* infection towards improving therapeutics and vaccines for TB are increasing.

This chapter will cover integral features of the innate and adaptive immune response to *M. tuberculosis* infection. Additionally, it will highlight recent findings on the hallmark granuloma and novel cellular players contributing to the host response to *M. tuberculosis* infection. Finally, it will provide an overview of the state of TB vaccine research, including a summary of BCG-based vaccines and the TB vaccine pipeline.

Immunopathogenesis of Tuberculosis in Humans and Animal Models

Overview of human TB disease

Transmission of *M. tuberculosis* occurs after inhalation of aerosolized droplets containing live bacteria into the lungs. Successful transmission is influenced by a variety of conditions, including proximity and duration of contact with an individual with active TB (ATB) disease, and the immune-competency of the individual infected with *M. tuberculosis*[5-7]. We now appreciate that in a clinical setting, *M. tuberculosis* infection presents as a continuum of diseased/infected states ranging from asymptomatic latent TB infection (LTBI) to ATB disease. This complexity, combined with remarkable heterogeneity in lesions within a single patient, has presented unique challenges to the eradication of TB[8]. While the majority of individuals exposed to *M. tuberculosis* are able to control infection in the form of LTBI, an estimated 5-10% of people exposed to *M. tuberculosis* develop ATB, which is characterized by persistent cough accompanied by sputum production, weight loss, weakness and night sweats[9]. TB diagnosis relies on evaluation of clinical symptoms and patient history combined with radiographic examination and detection of bacteria in sputum[9]. The presence of acid-fast bacilli (AFB) in sputum smears by microscopy does not specifically indicate infection with *M.*

tuberculosis; microbiological culture and nucleic acid amplification-based tests are required to confirm the presence of *M. tuberculosis* infection. Xpert MTB/RIF, a cartridge-based near-patient diagnostic assay utilizing real-time nucleic acid amplification of *M. tuberculosis* DNA, which also detects drug resistance to the first line drug, rifampicin, is recommended by the World Health Organization for TB diagnosis [10, 11]. Interferon gamma (IFN–γ) release assays (IGRAs), which leverage the specificity of the immune response to *M. tuberculosis*, are the basis of the QuantiFERON®-TB Gold In-Tube and T-SPOT.TB diagnostic assays. IGRAs measure IFN–γ produced by antigen-specific T cells in blood that recognize *M. tuberculosis* antigens (ESAT-6, CFP-10, TB7.7)[12]. IGRAs provide increased specificity over traditional Mantoux skin tests that depend on delayed type hypersensitivity reactions to purified protein derivative (PPD), which is not specific to *M. tuberculosis* infection and positive results may be due to BCG vaccination or exposure to environmental mycobacteria. However, IGRAs do not differentiate between active and latent TB and cannot be used to diagnose TB disease. Therefore, there is considerable interest in developing non-sputum based diagnostic approaches for TB with emphasis on biomarkers in blood and urine[13-17]. Applications of these assays to TB diagnosis in the field will need to adapt to complications due to a variety of co-infections and co-morbidities, including low CD4 T cell counts resulting in low frequencies of *M. tuberculosis*-specific responses in HIV-infected individuals.

Co-morbidities that modulate immune function can exacerbate TB disease or contribute to progression of LTBI individuals to ATB. HIV co-infection in latently infected

individuals increases the risk of developing TB from a 5-10% lifetime risk to a 10% annual risk and HIV infection is the single greatest risk factor for the development of TB[18-22]. The relevance of HIV co-infection to global TB mortality is highlighted by the fact that more than a fifth of all TB-related deaths in 2016 were in HIV-positive individuals[1]. Progressive depletion and dysfunction of CD4 T cells following HIV infection leads to immune suppression and negatively impacts immunity to *M. tuberculosis*. Specific depletion of *M. tuberculosis*-specific CD4 T cells has been reported in the peripheral blood[23, 24] and bronchoalveolar lavage (BAL) samples[25, 26] of HIV-infected individuals with LTBI. Studies have also described associations between TB and many other conditions or activities, including smoking, malnutrition, diabetes, helminth infections, chronic lung diseases, and cancer[27, 28]. Further investigations will be required to fully understand the basis of identified associations with other infections and morbidities.

Animal models of infection

Knowledge of the host response to *M. tuberculosis* infection has benefited greatly from the development of animal models of infection. The variable outcomes of *M. tuberculosis* infection in humans are challenging to model in a single animal model. Many experimental animals are susceptible to *M. tuberculosis* infection and can inform us about aspects of human disease. The mouse model for TB benefits from many advantages: ease of manipulation and housing, availability of well-characterized inbred strains, sophisticated techniques for the generation of mutant strains, availability of immunological and other reagents, and relatively low cost. Mice have been utilized to

model host responses to *M. tuberculosis* infection, to evaluate drug and vaccine candidates, and to study the immune response to mutant strains of mycobacteria. Experimental infection can be delivered through multiple routes: intravenously, intraperitoneally, intratracheally, or via aerosolized particles. The latter method, especially low-dose aerosol infection, is the most physiologically relevant and has become the preferred method. Different mouse strains have well-characterized lung pathologies and levels of susceptibility[29-33]. Typically, following bacterial deposition into the lungs, it takes approximately 2 weeks to begin priming adaptive immune responses in the lung-draining lymph nodes and a further 1-2 weeks for robust participation in the lungs by adaptive immune cells, but bacterial burdens continue to be maintained at a high level in the lungs of *M. tuberculosis* infected mice. There are limitations to what can be gleaned from mouse models of *M. tuberculosis* infection due to the differences in lung pathology between mice and humans. Further, true latent infection and significant immune control of infection are difficult to establish in the mouse model, though chemotherapeutically-induced models of paucibacillary disease in mice exist[34, 35]. The development of humanized mice that can recapitulate the heterogeneity of human lung pathology may extend the advantages of the mouse model, but humanized mice are also reported to display aberrant T cell responses and are unable to control bacterial burden[36, 37].

Other animal models of *M. tuberculosis* infection include guinea pigs, rabbits, fish, and non-human primates. Each has distinct advantages and disadvantages that make their use particularly suitable for different types of research questions. Following infection, guinea

pigs exhibit pathological features, such as the organization and development of caseous necrotic granulomas, that more accurately recapitulate the human granulomatous response compared to mice[38]. Further, guinea pigs are very susceptible to *M. tuberculosis* infection and, thus, are a good choice for testing candidate drugs and vaccines and studying dissemination dynamics. Similarly, rabbits develop a well-organized granuloma that can become necrotic following mycobacterial infection. However, rabbits are resistant to *M. tuberculosis* and high numbers of bacteria during inoculation or use of more virulent strains are needed[39-42]. Nevertheless, the rabbit model has been leveraged to study relatively rarer forms of TB, such as cutaneous and meningeal TB[43, 44]. However, the usefulness of both the guinea pig and rabbit models is hampered by the scarcity of immunologic reagents relative to mice. The establishment of the zebrafish model has provided novel insights into the establishment of the mycobacterial granuloma. Infection of transparent zebrafish larvae with the natural fish pathogen, *Mycobacterium marinum*, leads to the establishment of well-organized granulomas that become necrotic and can be visually monitored[45]. The primary advantage of the zebrafish model is the transparency of the zebrafish larvae, which, alongside facile manipulation of host and bacterial genetics, has been leveraged for insight into early innate immune events leading to the establishment of the granuloma as well as insights into human disease. Adaptive immunity is present in adult zebrafish and different populations of CD4 T cells have recently been described[46, 47], but these animals are no longer transparent and relevance of the adult zebrafish immune response to human TB have yet to be established.

The nonhuman primate (NHP) model of *M. tuberculosis* infection reflects much of the heterogeneity observed in human TB. Infection of NHPs is typically performed by aerosol or direct bronchoscopic deposition into the lungs of rhesus or cynomolgus macaques and, depending on dose of the inoculation and strain of bacteria utilized, leads to symptomatic ATB disease or asymptomatic infection in which bacteria persist at low levels akin to LTBI. The NHP model accurately recapitulates many of the hallmark granulomas seen in humans, including the heterogeneity of granulomas that can be present in the same animal[48], and presents similar clinical symptoms seen in humans[49-54]. The NHP is regarded as an important pre-clinical model for TB research and is an excellent model for studying immunity to *M. tuberculosis* and assessing candidate drug and vaccine efficacies[55-60]. Further, the NHP model can be used to study reactivation in the setting of SIV co-infection or other immune modulation, such as anti-TNF–α treatment, CD4 depletion, or inhibition of IDO[61-68].

Innate Immunity to *M. tuberculosis* Infection

The earliest encounter between host and pathogen in TB occurs at the interface between innate immune cells and *M. tuberculosis*. While innate immunity is critical for early anti-mycobacterial responses, it is also important for the progression of infection and long-term control of *M. tuberculosis* by continually priming and educating adaptive immune responses and by regulating inflammation. However, innate immune cells are often niches for bacterial replication and *M. tuberculosis* utilizes a variety of strategies that subvert innate immune responses to establish a chronic infection. Here, we will detail key features of the innate immune response to *M. tuberculosis* infection, starting from

recognition of the bacterium and phagosomal defenses within infected macrophages to priming of adaptive immune responses by professional antigen presenting cells (APC). In between, we will highlight how neutrophils and monocytes are mobilized after *M. tuberculosis* infection, the role that natural killer (NK) cells play during infection, how the balance of inflammation is regulated by the innate immune system, and how cell death affects the immune response. In each section, we will also highlight some of the myriad strategies that *M. tuberculosis* utilizes to subvert or evade the host innate immune response.

Recognition of M. tuberculosis by pattern recognition receptors

Pathogen associated molecular patterns (PAMPs) on *M. tuberculosis* are recognized via a variety of receptors to mediate opsonic and non-opsonic bacterial uptake: C-type lectins (e.g. Mannose receptors, DC-SIGN, Dectin-1, Dectin-2, Mincle), complement receptors (e.g. Complement receptor 3), collectins (e.g. Surfactant proteins A and D, Mannose-binding lectin), scavenger receptors (e.g. MARCO, SR-A1, CD36, SR-B1), Fc receptors (e.g. FcγR), glycophosphatidylinositol (GPI)-anchored membrane receptors (e.g. CD14), and toll-like receptors (TLRs) (e.g. TLR-2, TLR-4, TLR-9)[69-71]. Mannosylated lipoarabinomannan (ManLAM), phosphatidyl-inositol mannosides (PIM), phthiocerol dimycocerosates (PDIMs), phenolic glycolipids (PGLs), trehalose dimycolate (TDM), peptidoglycan and other mycobacterial components are recognized by an array of cell surface and intracellular receptors that mediate phagocytosis and/or antimicrobial defenses. *M. tuberculosis* DNA[72, 73] or bacterial second messengers[74] can be recognized by cytosolic pattern recognition receptors (PRRs), such as cGAS and

STING[75, 76], to induce downstream cytokine production and autophagy. Further, nucleotide oligomerization domain-like receptors (NLRs) are cytosolic PRRs that recognize *M. tuberculosis* PAMPs, such as muramyl dipeptide, to activate a multiprotein complex termed the inflammasome. Functional redundancies for many of the receptors are likely to exist due to promiscuous ligand binding by different receptors and the wide array of available ligands on *M. tuberculosis*. Indeed, single or double knockouts for canonical scavenger receptors and C-type lectin receptors did not modulate susceptibility or attenuate immune responses following *M. tuberculosis* infection[77]. However, increased susceptibility to *M. tuberculosis* infection in a variety of knockout mice demonstrate that a number of PRRs and their associated signaling pathways also play important, non-redundant roles in host defense against *M. tuberculosis* infection.

M. tuberculosis expresses a variety of known or putative TLR ligands and TLR-2, TLR-4, and TLR-9 have been implicated in host recognition of *M. tuberculosis* (reviewed in [70, 71]). Polymorphisms in specific TLRs or TLR signaling proteins have also been strongly associated with pulmonary TB in humans or have been shown to influence immunity against *M. tuberculosis*[78-81]. The contribution of individual TLRs to immunity against *M. tuberculosis* infection is variable, but the importance of the TLR signaling pathway to antimycobacterial immunity is evident in studies showing that mice lacking the common TLR adaptor protein, myeloid differentiation factor 88 (MyD88), quickly succumb to *M. tuberculosis* infection[82, 83]. Susceptibility of *MyD88-/-* mice to *M. tuberculosis* infection has been attributed to deficient expression of NOS2[83], impaired ability to activate the IL-1β or IL-1 receptor (IL1R) pathway[84, 85], impaired

receptivity of macrophages to IFN–γ signaling[86], and impaired IL-12 and TNF-α responses in macrophages and DCs[82]. Gene-deletion studies in single TLRs have revealed that innate immune responses to *M. tuberculosis* are likely the result of the complex activation of multiple signaling pathways. For instance, mice lacking both TLR-2 and TLR-9 are more susceptible to *M. tuberculosis* infection than mice lacking the ability to signal through either TLR by itself[87]. The susceptibility of *MyD88-/-* mice to *M. tuberculosis* infection is an example of the importance of common adaptor molecules that integrate signals from multiple PRRs and other innate immune pathways for the induction of antimycobacterial immunity. Further evidence for this concept is demonstrated by the increased susceptibility of *M. tuberculosis*-infected mice lacking CARD9, an adapter molecule integrating signals from C-type lectin receptors (CLRs), or PYCARD/ASC, an adapter molecule integrating signals from NLRs for the induction of the inflammasome[88, 89].

MyD88 signaling in innate immunity integrates signaling from TLR and interleukin-1 (IL-1) receptor families by bridging ligand-receptor binding to IL-1-receptor-associated kinases (IRAKs) and the activation of multiple downstream pathways, including NF-kB, mitogen activated protein kinases (MAPK), and activator protein 1 (AP1). The IL-1 signaling pathway is clearly required for resistance to *M. tuberculosis* infection in mouse models and is supported by human immunogenetics studies[90-93]. In mice, absence of IL-1 signaling led to severe susceptibility to *M. tuberculosis* infection. Both IL-1α and IL-1β, as well as their common receptor, IL-1R1, have been implicated in immunity to *M. tuberculosis*[84, 85, 94-98]. Secretion of the mature form of IL-1β requires cleavage

by the terminal inflammasome effector, caspase-1, but *M. tuberculosis*-infected mice lacking MyD88, ASC, or caspase-1 signaling do not display impaired IL-1β levels[84]. Further, mice deficient in IL-1β are considerably more susceptible to *M. tuberculosis* infection than mice lacking ASC or caspase-1[84]. These findings suggest that IL-1β is a key mediator of resistance to *M. tuberculosis* infection and alternative pathways exist for its cleavage during infection, but also indicate that the basis for resistance conferred by MyD88, CARD9, and PYCARD/ASC likely depend on additional factors beyond IL-1β.

While host recognition of *M. tuberculosis* leads to the activation of innate immunity, *M. tuberculosis* has also evolved strategies that evade innate immune responses mediated by PRRs. Strain-specific expression of cell envelope components may be associated with differential immune responses[99, 100]. Modulation of innate immune responses by *M. tuberculosis* is also accomplished through the presence of immune-inhibitory lipid components that compete with immune-activating mycobacterial components for the same receptors[101]. Lastly, *M. tuberculosis* can also impair innate immune responses to cell-envelope components through enzymatic means. For instance, previous work from our group on the *M. tuberculosis* serine-hydrolase, Hip1, demonstrated that Hip1 cleaves multimeric, cell-wall associated GroEL2 to a secreted monomeric form to mediate attenuated macrophage and dendritic cell (DC) responses[102-106]. Additionally, *M. tuberculosis* mutants lacking *hip1* or a putative mycobacterial metalloprotease, *zmp1*, display enhanced inflammasome activation[103, 107], suggesting that *M. tuberculosis* contain multiple strategies for dampening activation of the inflammasome.

Thus, in addition to the array of host receptors that mediate recognition of *M. tuberculosis*, innate immune responses to infection likely depend on the strain of *M. tuberculosis*, the presence of cell-wall components that can competitively inhibit the activation of PRRs, and the presence of *M. tuberculosis* enzymes that modify the immunogenicity of cell envelope components.

Phagosomal defense in macrophages

Macrophages are the first immune cells to encounter *M. tuberculosis* during infection and also represent the primary replicative niche for *M. tuberculosis*. Recognition of *M. tuberculosis* by macrophages leads to phagocytosis and sequestration of the bacterium in phagosomes, which typically eradicate pathogens via fusion with lysosomes and consequent acidification of the pathogen-containing phagolysosome. However, *M. tuberculosis* is able to survive and replicate in the phagosome by inhibiting phagosomal maturation and phagolysosomal generation through a variety of mechanisms (reviewed in [69, 108]). Further, transcriptional profiling of intraphagosomal bacteria indicated that *M. tuberculosis* readily counters the nitrosative, oxidative, hypoxic, and nutrient-poor phagosomal environment through the expression of stress-adaptive genes[109], though a genome-wide transposon site hybridization screen for *M. tuberculosis* survival in macrophages suggested that *M. tuberculosis* constitutively expresses genes required for its survival[110]. Nevertheless, it is clear that *M. tuberculosis* has adapted for a lifestyle inside the macrophage and employs many strategies to survive within these cells.

M. tuberculosis glycolipids can prevent accumulation of phosphatidylinositol 3-phosphate (PI3P) on phagosomal membranes and prevent phagolysosome biosynthesis[111]. *M. tuberculosis* also secretes phosphatases (SapM and PtpA) and serine/threonine kinases (PknG) that are proposed to interfere with phagosomal maturation[112-118]. There is also evidence that *M. tuberculosis* lipids, in particular phthiocerol dimycocerosates (PDIMs), can mediate escape from the phagosome and host cell death[119]. An *M. tuberculosis* secretion system, ESX-1, is also known for mediating disruptions in phagosomal integrity and preventing phagosome maturation. Promotion of aberrant phagosomal integrity and bacterial replication by *M. tuberculosis* ESX-1 is countered by IFN–γ-induced, Rab20-mediated phagosomal maturation[120]. ESX-1-mediated phagosomal escape of bacteria is hypothesized to work through disruption of the phagosome by ESAT-6 (6kDa early secretory antigenic target)[121-124], though recent evidence proposes a contact-dependent, ESAT-6-independent mechanism for ESX-1-mediated phagosomal permeabilization[125]. Nevertheless, ESX-1-mediated permeabilization of the phagosome exposes *M. tuberculosis* PAMPs to cytoplasmic nucleotide oligomerization domain 2 (NOD2) receptors to induce type I IFNs[126, 127]. *M. tuberculosis* ESX-3 has also been implicated in modulating intracellular trafficking of bacteria to avoid phagosomal maturation through inhibition of host endosomal sorting complex required for transport (ESCRT) [128-130].

M. tuberculosis entry into macrophages through different receptors can lead to distinct activation of pathways that can inhibit or promote bacterial replication. The overall effect of multiple receptors engaging distinct or overlapping *M. tuberculosis* ligands is a

complex and dynamic issue. TLR-2 recognition of mycobacterial ManLAM activates NF-κB and *NOS2* gene transcription that leads to antimycobacterial nitric oxide (NO) production[131]. NO production is strongly associated with resistance to *M. tuberculosis*, though evidence for the anti-mycobacterial effects of NO is stronger in the mouse model[132-141]. Although *in vitro* studies using human alveolar macrophages and primary monocytes did not find an anti-mycobacterial role for NO[142-144], specific staining for NOS2 in the BAL of TB patients reveals upregulation in infected individuals compared to healthy controls[145]. Nevertheless, *M. tuberculosis* has several strategies to cope with otherwise damaging reactive nitrogen and oxygen intermediates: *M. tuberculosis* KatG, a catalase-peroxidase, can inactivate phagosomal reactive oxygen[146] and the *M. tuberculosis* proteasome can mediate resistance to nitrosative stresses[147]. TLR-mediated recognition of *M. tuberculosis* is also reported to synergize with the vitamin D pathway to induce the antimicrobial peptide (AMP), cathelicidin, in human macrophages[148-152]. In addition to direct antimicrobial activity, cathelicidin has been shown to exert antimicrobial functions by activating transcription of host autophagy genes *Beclin-1* and *Atg5*[151].

Autophagy is the process whereby cytoplasmic constituents are degraded or recycled. A role for autophagy in anti-mycobacterial immunity in macrophages has been extensively characterized. Initial studies utilizing *M. bovis* suggested that autophagy plays a role in promoting phagosomal maturation to enhance bacterial killing[153-155]. Autophagy-related genes were revealed to be involved in regulating intracellular bacterial load of lab-adapted and clinical isolates of *M. tuberculosis* in a genome-wide siRNA screen of

infected human macrophage-like THP-1 cells[156]. Accumulating evidence indicates that autophagy is integrated into the host response to *M. tuberculosis* infection by synergizing with pathogen sensing, phagosomal maturation, and IFN-γ inducible pathways to mediate anti-mycobacterial immunity[72, 74, 75, 157-159]. Studies have demonstrated that *M. tuberculosis* employs several strategies to evade autophagy[160, 161]. It is also becoming clear that autophagy-related proteins are likely to perform multiple functions and care must be taken when interpreting specific knockouts or knockdowns of individual genes[162, 163]. Analysis of lung sections from *M. tuberculosis*-infected, *Atg5* knockout mice indicated that Atg5 may be involved in regulation of neutrophil responses during infection, suggesting autophagy-independent roles for Atg5. Further, a recently described role for Atg5 in LC3-associated phagocytosis (LAP) during *M. tuberculosis* infection supports the notion that specific components of autophagy can also overlap with other phagosomal pathways in immunity against mycobacteria[164].

Taken together, it is clear that macrophage recognition and phagocytosis of *M. tuberculosis* leads to a dynamic tug of war between anti-mycobacterial defenses and *M. tuberculosis* immune evasion. Macrophage defenses include antimicrobial peptides (AMPs), nitrosative stresses, phagolysosomal fusion and autophagy and may operate independently of or subsequent to IFN-γ signaling. On the other hand, *M. tuberculosis* can subvert macrophage defenses at the level of the bacterial cell wall components that limit phagosomal maturation and the bacterial genes that combat or allow adaptation to intracellular immune pressure.

Recruitment and function of neutrophils and monocytes following M. tuberculosis infection

Secretion of cytokines and chemokines early during infection recruits additional phagocytes to the site of infection. Early secretion of chemoattractants may be attributed to infected alveolar macrophages as well as lung epithelial cells[165-167]. Moreover, a recent study suggests that crosstalk between primary bronchial epithelial cells and infected macrophages may also promote secretion of chemokines[168]. Trafficking of additional monocytes and granulocytes to the lung exerts immune pressure on *M. tuberculosis* and is crucial for the initiation of adaptive immune responses, but it may also promote *M. tuberculosis* cell-to-cell transmission and dissemination.

Recruitment of neutrophils serves as an early line of defense against *M. tuberculosis* infection via secretion of antimicrobial molecules and inflammatory mediators, but neutrophils also serve as niches for bacterial replication and can impede immunity against *M. tuberculosis*. In humans with active pulmonary TB, neutrophils have been found to be a significant population of *M. tuberculosis*-infected phagocytes in the BAL and sputum[169]. Whole blood transcriptional profiling also identified a neutrophil signature in ATB patients associated with type I and type II IFN-inducible genes[170] and expression of the inhibitory molecule PD-L1[171], suggesting that neutrophils may be playing an immunomodulatory role in human TB. In mice, the kinetics and magnitude of neutrophil recruitment following *M. tuberculosis* infection depends on the strain of mouse infected. Evidence for a pathogenic role for neutrophils is shown in studies comparing neutrophil recruitment in resistant versus susceptible mouse strains[172, 173].

Depletion of neutrophils at the onset of *M. tuberculosis* infection specifically extended the lifespans of susceptible DBA/2 mice, suggesting that early neutrophil involvement was pathogenic in genetically susceptible mice[173]. Similarly, neutrophil depletion in susceptible I/St mice shortly after *M. tuberculosis* infection reduced lung pathology and bacterial growth and improved survival compared to C57BL/6 mice[174]. In a separate study, depletion of neutrophils five weeks after aerosol *M. tuberculosis* infection of resistant BALB/c mice enhanced the levels of lung IL-6 and IL-17 without impacting IFN-γ and modestly enhanced bacterial control[175]. Neutrophil depletion in the first four days following intravenous *M. tuberculosis* infection of BALB/c mice, however, led to enhanced bacterial growth at extrapulmonary sites, suggesting that anti-mycobacterial immunity conferred by neutrophils may be dependent on route of infection and the kinetics of neutrophil involvement[176]. Evidence for beneficial roles that neutrophils play in anti-mycobacterial defense focus on neutrophil secretion of AMPs such as cathelicidin and lipocalin-2 to restrict bacterial replication[177] or via uptake of AMP-containing apoptotic neutrophils by *M. tuberculosis*-infected macrophages[178]. Neutrophils can also release chromatin scaffolds that trap extracellular bacteria in an AMP-containing mesh. *M. tuberculosis* has been shown to induce the formation of neutrophil extracellular traps (NETs) *in vitro*[179] and levels of NETs detected in the plasma of ATB patients were associated with disease severity and decreased with antibiotic therapy[180]. Thus, the overall effect of neutrophil recruitment to the site of infection is determined by a combination of host genetics, context of infection (pulmonary versus extrapulmonary), and timing and duration of neutrophil activity.

In addition to neutrophils, monocytes are recruited to the site of *M. tuberculosis* infection. Similar to neutrophils, monocyte recruitment is important for innate immunity during *M. tuberculosis* infection but may also inadvertently promote *M. tuberculosis* dissemination. C-C chemokine receptor type 2 (CCR2) is a chemokine receptor expressed on monocytes and is responsible for CCL2-mediated recruitment of monocytes to sites of bacterial infection[181]. CCR2 was found to mediate immunity against *M. tuberculosis* depending on the dose of infection. CCR2 knockout mice were more susceptible to high dose intravenous *M. tuberculosis* infection[182], but not after low dose infection[183]. Monocytes transferred into *M. tuberculosis* infected mice were shown to be the predominant population of innate immune cells producing iNOS[184]. Additionally, monocyte delivery of *M. tuberculosis* to pulmonary lymph nodes can coordinate with DCs to prime CD4 T cells after infection[185]. Monocytes may therefore represent a recruited population of innate cells that combat *M. tuberculosis* infection through the production of RNI and priming of adaptive immunity. However, monocyte recruitment following *M. tuberculosis* infection may also be detrimental to the host by providing an environment full of permissive cells[186]. The recruitment of neutrophils and monocytes to the site of *M. tuberculosis* infection represents a host strategy to contain bacterial replication that is co-opted by the bacterium to facilitate its growth and dissemination.

Inflammation and cell death during M. tuberculosis infection

The regulation of inflammation is a critical factor that determines the outcome of *M. tuberculosis* infection. Over-exuberant inflammation impairs cellular immunity, damages lung tissue, and can lead to lung cavitation and enhanced transmission. Inversely, too

little inflammation can impair bacterial control by delaying the induction of innate and adaptive immunity. While neutrophil recruitment and activity during *M. tuberculosis* infection can help contain bacterial replication, sustained neutrophilic inflammation can mediate damaging inflammation and promote disease. Importantly, whole blood transcriptomics identified a neutrophil-driven type I IFN-inducible signature in human TB that decreased upon treatment[170]. Excessive type I IFN signaling has been shown to promote disease in mouse models and human samples. Mice lacking type I IFN signaling are more resistant to *M. tuberculosis* infection[187-190], though signaling through type I IFNs may play a protective role in the absence of IFN-γ[191, 192]. Mechanisms underlying the pathogenic role for type I IFNs during *M. tuberculosis* infection include inhibition of IL-1β production[193, 194], induction of IL-10[195], and loss of IFN-γ responsiveness in infected macrophages[195]. In addition to induction of type I IFNs, neutrophils have also been reported to drive lung destruction through the secretion of matrix metalloproteinase 8 (MMP8)[196].

Eicosanoids are lipid mediators of inflammation derived from the oxidation of arachidonic acid. The balance between pro-inflammatory prostaglandin E2 (PGE2) and anti-inflammatory lipoxin A4 (LXA4), two members of the eicosanoid family of signaling molecules, can determine the outcome of *M. tuberculosis* infection[197-199]. During *M. tuberculosis* infection, mice incapable of synthesizing PGE2 display increased susceptibility[198] and absence of the enzyme 5-lipoxygenase, which metabolizes arachidonic acid to LXA4, confers resistance[197]. Importantly, therapeutic correction of low PGE2 levels can confer enhanced survival in highly susceptible mice infected with

M. tuberculosis[97]. Leukotriene A4 hydrolase is an enzyme that catalyzes the production of pro-inflammatory leukotriene B4 from leukotriene A4, which can also be converted to anti-inflammatory lipoxin A4 as a counter-balance. In zebrafish, *LTA4H* mutants were found to be hypersusceptible to *M. marinum* infection due to dysregulation of the balance between leukotrienes and lipoxins; increased levels of lipoxin A4 in *LTA4H* mutants impaired TNF−α responses and promoted susceptibility[200]. The relevance of this finding to humans is highlighted in a TB meningitis cohort in Vietnam where heterozygosity for six *LTA4H* polymorphisms conferred a survival advantage over homozygosity[200]. Indeed, efficacy of anti-inflammatory glucocorticoid treatment in TB meningitis patients can be differentiated by a single nucleotide polymorphism in the *LTA4H* promoter controlling transcriptional activity, which suggests that the balance of inflammation is critical to disease progression and treatment outcomes in TB meningitis[201].

TNF-α is a critical pro-inflammatory cytokine in immunity against *M. tuberculosis* infection and can be secreted by a number of innate and adaptive immune cells. The importance of TNF-α in antimycobacterial immunity is clearly demonstrated by heightened susceptibility of TNF-α antibody-depleted animals or in animals lacking TNF receptor signaling following *M. tuberculosis* infection[202]. TNF-α is also a critical mediator of immunity against TB in humans. This is demonstrated by increased rates of progression to ATB in LTBI patients receiving anti-TNF treatment for inflammatory disorders[203], which can be recapitulated in the NHP model of infection[67]. The effects of anti-TNF treatment in humans and NHPs, as well as in mice[204-206], suggests

that TNF-α is critical for maintaining sequestration of *M. tuberculosis* in the granuloma. Histopathological evidence from gene-disrupted or antibody-depleted mice infected with *M. tuberculosis* also suggests that TNF-α signaling may be playing a role in modulating apoptotic or necrotic cell death following infection[204, 206].

Cell death can be a means of restricting bacterial replication by the host or a way to disseminate to secondary loci of infection for *M. tuberculosis*. Apoptosis of *M. tuberculosis*-infected cells leads to fewer viable bacteria and effective cross-presentation of bacterial antigens[199, 207, 208] whereas necrosis of *M. tuberculosis*-infected cells allows viable bacteria to exit and disseminate[198, 209, 210]. Pro-apoptotic *M. tuberculosis* mutants lacking *secA2*[211] or *nuoG*[212] were attenuated *in vivo* and mice infected with these strains displayed enhanced priming of adaptive immunity compared to infection with wild-type *M. tuberculosis*, suggesting that prevention of host cell apoptosis is an *M. tuberculosis* virulence strategy. Relatedly, *M. tuberculosis*-infected murine neutrophils can aid in dendritic cell trafficking to the draining lymph nodes to initiate antigen-specific CD4 T cell responses[213], but *M. tuberculosis* delays CD4 T cell priming by inhibiting neutrophil apoptosis[214]. Infection with the pro-apoptotic *nuoG* mutant *M. tuberculosis* resulted in earlier DC trafficking to lung draining lymph nodes and earlier priming of antigen-specific CD4 T cells, but enhanced priming was abrogated upon neutrophil depletion[214]. Additionally, uninfected macrophages performing a constitutive housekeeping function called efferocytosis can uptake *M. tuberculosis*-containing apoptotic bodies, which leads to delivery and killing of bacteria in lysosomes[215]. Taken together, apoptosis represents a strategy by the host to limit

infection through the combination of bacterial sequestration in apoptotic vesicles and the induction of adaptive immune responses, but *M. tuberculosis* may delay apoptosis or promote necrosis to facilitate replication and dissemination.

Initiation of adaptive immunity to M. tuberculosis by dendritic cells

An important function of innate immunity during *M. tuberculosis* infection is the priming of adaptive immune responses. Dendritic cells (DCs) are professional antigen presenting cells that initiate adaptive immunity by presenting *M. tuberculosis* antigens in the context of major histocompatibility complex (MHC), co-stimulatory molecules, and cytokines. Depletion of cells expressing the pan-DC marker, CD11c, following *M. tuberculosis* infection impaired bacterial control and delayed the initiation of adaptive immunity, illustrating the importance of DCs in mobilizing adaptive immune responses that can control bacterial replication[216]. There is abundant evidence that *M. tuberculosis* is able to infect murine[217-219] and human DCs[220-222]. In mice infected with GFP-expressing *M. tuberculosis*, DCs were found to be the major population of phagocytes infected by bacteria after 4 weeks[219]. Upon *M. tuberculosis* infection, DCs mature and migrate to the lung draining lymph nodes to initiate antigen-specific T cell responses, which depended on the chemokine receptor CCR7 and its corresponding chemokines CCL19 and CCL21[223-225]. Further, IL-12, a cytokine secreted by myeloid cells and important for the induction of IFN-γ responses, is required for DC migration during *M. tuberculosis* infection[226]. Priming of adaptive immune responses requires the transport of live bacteria to the lung draining lymph nodes[219, 223], but antigen-specific T cells can be primed by both the infected migratory DC and uninfected lymph node resident

DC. A study demonstrated that infected DCs migrate to the lung draining lymph nodes where they secrete soluble, unprocessed *M. tuberculosis* antigens that are summarily phagocytosed by uninfected lymph node resident DCs[227]. The exportation of *M. tuberculosis* antigens was initially proposed to circumvent inefficient antigen presentation by infected DCs. However, secretion of *M. tuberculosis* antigens by infected DCs may benefit the pathogen by diverting antigen away from MHC class II antigen presentation[228].

Effective interaction between DCs and T cells is dependent on appropriate function of antigen presentation machinery, including expression of MHC, co-stimulatory molecules, and cytokines following *M. tuberculosis* infection. However, there is abundant evidence that *M. tuberculosis* infection impairs antigen presentation to evade antigen-specific T cell responses. It is well recognized that *M. tuberculosis* infection leads to impaired MHC class II antigen presentation by macrophages (reviewed in [229]). *M. tuberculosis*-mediated inhibition of phagosomal maturation has been implicated in attenuating processing of *M. tuberculosis* antigen 85 (Ag85) and the MHC class II-associated invariant chain[230]. Multiple studies have also reported that *M. tuberculosis* infection impairs MHC class II expression in macrophages through inhibition of class II transactivator (CIITA), a master transcriptional regulator controlling expression of MHC class II molecules[231-234], although there is little evidence of similar inhibition of MHC class II in DCs. Nevertheless, *M. tuberculosis* infection of DCs leads to functional impairment of antigen presentation. *M. tuberculosis* infection has been shown to impair DC maturation of human (reviewed in [235]) and murine DC functions (reviewed in

[236, 237]). Studies examining proliferation of T cell receptor (TCR) transgenic CD4 T cells specific for *M. tuberculosis* antigen 85 (Ag85) as a proxy for functional antigen presentation have demonstrated that *M. tuberculosis* EsxH can impair antigen processing through inhibition of host endosomal sorting complex required for transport (ESCRT)[238]. Taken together, the initiation of the adaptive immune response requires the participation of DCs, which themselves are readily infected and subverted by *M. tuberculosis* infection. *M. tuberculosis* subversion of DC functions can interfere with antigen presentation and delay or impair the initiation of the adaptive immune response. Moreover, subversion of DC functions can influence the type of antigen-specific responses that develop. Improving DC functions during *M. tuberculosis* infection may improve innate and adaptive immunity and enhance immune control of bacterial burden. Indeed, mucosal transfer of Ag85B-loaded DCs following challenge with *M. tuberculosis* augmented the efficacy of BCG vaccination[239], suggesting that early antigen-presentation by DCs is an important component that determines the efficacy of vaccine-induced immunity. DCs are critical players that initiate adaptive immune responses to *M. tuberculosis* and determine the outcome of infection. Interventions or therapies that improve DC functions may provide benefits by augmenting crosstalk between DCs and antigen-specific T cells.

Adaptive Immunity Against *M. tuberculosis*

Protective immunity to *M. tuberculosis* and control of bacterial replication requires adaptive immune responses. This is best exemplified by the extreme susceptibility to mycobacterial infections of lymphopenic HIV patients and gene-deleted mice lacking

MHC class II or T cells in general. Cytokine secretion and direct antimicrobial actions of antigen-specific T cells are key features of the adaptive immune response against *M. tuberculosis* infection. Further, the long-lived nature of antigen-specific memory T cells provides the basis for developing vaccines that induce anti-mycobacterial immunity. There are also expanding roles for B-cells, γδ T cells, and CD1-restricted T cells that provide specific responses to a diverse set of *M. tuberculosis* antigens that complement antigens classically presented through MHC class I and II. However, adaptive immune responses can also become malignant by promoting excessive inflammation or rendered ineffective from chronic antigen exposure. Here we will cover the importance of timing, location, and quality of CD4 T cell responses during *M. tuberculosis* infection, how CD8 T cells contribute to immunity against *M. tuberculosis*, the roles that inhibitory receptors play during infection, the phenotypes and functions of memory T cells, and the roles that B-cells, γδ T cells, CD1-restricted lymphocytes, and mucosal associated invariant T (MAIT) cells play in immunity against *M. tuberculosis*.

Kinetics and homing of CD4 T cells after M. tuberculosis infection

In the mouse model of infection, CD4 T cell responses are absolutely required to control bacterial replication and animals lacking such responses succumb rapidly[240, 241]. MHC class II knockout mice or CD4 depletion led to abrupt mortality following *M. tuberculosis* infection[240, 241]. CD8 T cells play a key role in immunity against *M. tuberculosis*, but cannot compensate for CD4 deficiency[240]. Similarly, antibody depletion of CD4 in cynomolgus macaques severely compromised control of *M. tuberculosis* and led to reactivation in latently infected animals[66]. Thus, the initiation of

the CD4 T cell response is a key feature defining the outcome of *M. tuberculosis* infection. There is a widely recognized delay in the initiation of antigen-specific CD4 T cell responses following low dose aerosol infection of mice[223, 242-245] and nonhuman primates[246]. *M. tuberculosis* infected cynomolgus macaques had detectable antigen-specific responses 4 weeks post-infection[246]. In mouse models of infection, antigen-specific CD4 T cell responses are first detected in the lung draining lymph nodes 2 weeks after infection. Significant antigen-specific lung CD4 T cell responses are subsequently detected in the lungs 3 weeks after infection. This is in stark contrast to antigen-specific responses to other bacterial[247] or viral[248] pathogens, which are detected swiftly after infection. Adoptive transfer of ESAT-6-specific CD4 T cells prior to aerosol *M. tuberculosis* infection have demonstrated an apparent kinetic bottleneck whereby lung antigen-specific activation occurs only 7 days after infection despite the presence of antigen-specific T cells[249], suggesting that antigen-specific responses are delayed by mechanisms other than trafficking of CD4 T cells from the mediastinal lymph nodes to the lungs. Delay in the initiation of adaptive immune responses to *M. tuberculosis* infection may be due to a variety of factors, including slow-growth of the bacterium, inhibited apoptosis of infected macrophages and neutrophils, and delayed activation and migration of DCs, which cumulatively allow *M. tuberculosis* to establish a persistent infection in the lung.

CD4 T cells interact with infected macrophages to restrict intracellular *M. tuberculosis* replication. Thus, the effectiveness of the CD4 T cell response depends on proper homing of antigen-specific CD4 T cells from lymphoid tissues to *M. tuberculosis* infected cells in

the lung. In *M. tuberculosis*-infected mice, antigen-specific CD4 T cells expressing CXCR3 localized to the lung parenchyma and were more efficient at controlling bacteria following *M. tuberculosis* infection when compared to vasculature-restricted CD4 T cells that expressed CX3CR1[250]. Interestingly, cells retained in the lung vasculature secreted the highest amount of IFN–γ during infection[250]. Adoptive transfer studies demonstrated that IFN–γ accounted for greater control of bacterial burden in the spleen over the lung and drove immunopathology when overexpressed[251], suggesting that the function of IFN–γ may be to mediate control of bacterial dissemination to extrapulmonary sites and that IFN–γ may be detrimental when unrestrained. The distinction between vasculature-restricted and parenchyma-localizing CD4 T cells seems less important in rhesus macaques[252], where the majority of antigen-specific CD4 T cells can be found in the lung parenchyma but are restricted to the outer lymphocytic cuff of granulomas. Notably, studies have demonstrated that expression of indoleamine 2,3-dioxygenase (IDO) by cells in the granulomas of *M. tuberculosis* infected rhesus macaques can mediate inhibition of T cell entrance into granuloma and biochemical inhibition of IDO led to reorganization of the granuloma to include T cells localizing into the macrophage core[63, 253]. Taken together, there is strong evidence that localization of antigen-specific CD4 T cells into the lung tissues where *M. tuberculosis* infected myeloid cells reside is an important feature of protective immunity to *M. tuberculosis*.

Quality and specificity of the CD4 T cell response to M. tuberculosis

The quality of the T cell response is an important feature determining the outcome of *M. tuberculosis* infection. Canonically, the production of IFN–γ by Th1 cells, CD8 T cells,

and other lymphocytes is considered essential for protection against mycobacterial infections. In human immunogenetics studies, Mendelian susceptibility to mycobacterial disease (MSMD) describe a spectrum of genetic mutations in seven autosomal genes (*IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *IRF8*, *ISG15*) and two X-linked genes (*IKBKG*, *CYBB*) that confer susceptibility to avirulent environmental mycobacteria and BCG[254]. Deficiencies related to IFN–γ signaling in young patients with mutations in *IFNGR1* and *IFNGR2* confer fatal susceptibility to mycobacterial infections[255-258]. STAT1 is an intracellular molecule important for IFN–γ signaling and individuals with heterozygous germline *STAT1* mutations lose gamma-interferon activating factor (GAF) expression[259]. GAF is an important transcription factor that facilitates IFN–γ induced gene expression. Individuals with heterozygous *STAT1* mutations have impaired nuclear accumulation of GAF and suffer from recurrent mycobacterial infections[259]. Additionally, mutations affecting IL-12 expression levels and signaling also confer susceptibility to mycobacterial infections. Two mutations in the leucine zipper domain of NEMO, an intracellular protein involved in NF-kB activation, impairs CD40-mediated IL-12 production in monocytes and DCs[260] and leads to recurrent mycobacterial infections. Similarly, defects that impair IL-12p40 leads to decreased IFN–γ levels and confers susceptibility to mycobacterial infections[261-264]. Mutations in *IL12RB* are the most frequent genetic factors associated with MSMD, but recurrent mycobacterial susceptibility in individuals with *IL12RB* mutations can be mitigated with BCG vaccination or primary BCG disease[261, 262, 265-268], suggesting that IL-12/IL-23 signaling may not be completely required for secondary immunity. IFN–γ is readily detected in human BAL in patients with TB disease and decrease following therapy[269],

which is likely a consequence of decreasing bacterial loads. In contrast, studies on human PBMCs show a decrease in IFN–γ responses in ATB patients compared to controls[270-275]. Lower frequencies of *M. tuberculosis*-specific IFN–γ responses in ATB patients may reflect trafficking of these cells to the lungs resulting in specific depletion from the periphery. IFN–γ secretion is also an important tool leveraged for the detection of *M. tuberculosis*-specific CD4 T cell responses in humans and in animal models. Genome-wide analysis of *M. tuberculosis*-specific CD4 T cell epitopes in LTBI individuals revealed three broadly immunodominant antigenic islands related to bacterial secretion systems recognized by IFN–γ secreting CD4 T cells[276]. Animal models of TB also demonstrate a key role for IFN–γ in immunity against *M. tuberculosis* infection. Mice deficient in IFN–γ succumb to low dose *M. tuberculosis* infection[277, 278]. Correspondingly, mice lacking IL-12 are also unable to control *M. tuberculosis* infection[226, 279, 280]. The antimycobacterial effects of IFN–γ in mouse models are broadly related to the induction of antimicrobial peptides, iNOS, and cytokines that activate infected macrophages to restrict intracellular bacterial replication, though other mechanisms underlying IFN–γ mediated immunity to *M. tuberculosis* infection are still being elucidated. IL-10 deficient mice are less susceptible to *M. tuberculosis* infection due to an enhanced Th1 response[281], suggesting that IL-10 limits Th1 immunity during *M. tuberculosis* infection. However, Th1 cells secreting IL-10 can also impair host control of *M. tuberculosis* infection[282] and CD4 T cells producing both IFN–γ and IL-10 are detected in the BAL of ATB patients[283]. Given that IL-10 secretion by Th1 cells has been shown to be a result of high antigen-dose[284], it is possible that adaptive immunity at stages of infection when bacterial burden is high may be compromised by T

cell derived IL-10. Interestingly, adoptive transfer of T-bet knockout ESAT-6 specific TCR-transgenic CD4 T cells skewed towards Th1 *in vitro* retains the capacity for early protection against *M. tuberculosis* infection[285], suggesting that protection conferred by Th1 cells may be independent of T-bet or IFN–γ production. Taken together, these studies demonstrate a clear requirement for the IL-12/IFN–γ axis in immunity against *M. tuberculosis* infection in humans and animal models. Further studies delineating the mechanisms underlying IFN–γ and Th1 mediated immunity against *M. tuberculosis* are warranted.

Although Th1 responses are important for immunity against TB, studies have also demonstrated that CD4 T cell subsets secreting IL-17 (Th17) and FoxP3+ regulatory CD4 T cells contribute to the response against *M. tuberculosis* infection. There are context-dependent beneficial or detrimental roles for Th17s during infection with *M. tuberculosis*. Infection with a W-Beijing lineage strain of *M. tuberculosis*, HN878, induce Th17 responses and mice deficient in IL-17 display increased bacterial burden following infection[286]. IL-17 receptor A subunit (IL17RA) knockout mice[287] and IL-17A knockout mice[288] also displayed impaired long-term control of high-dose infection with H37Rv. Transfer of BCG-specific, IFN–γ knockout Th17 cells into *M. tuberculosis* infected, T cell deficient mice conferred enhanced protection and prolonged survival compared to transfer of naïve IFN–γ knockout CD4 T cells[289], suggesting that Th17 cells can mediate protection independently of IFN–γ. In humans, significant frequencies of IL-17-producing CD4 T cells were found in the PBMC and BAL of BCG-vaccinated healthy individuals and declined in patients with active disease[290]. Further, individuals

with bi-allelic *RORC* loss-of-function mutations displayed impaired IL-17 and IFN–γ responses and were susceptible to mycobacterial disease and candidiasis[291]. The development of antigen-specific Th17 immunity during infection is poorly understood. Furthermore, the precise role of Th17 cells in protective immunity to *M. tuberculosis* remains unclear but may be related to their role in the development of less hypoxic granulomas[292], in the recruitment of Th1 cells[293], or in the induction of CXC-chemokines and B-cell follicles[294]. However, unrestrained IL-17 responses have also been shown to promote detrimental immunopathology, typically through pathological neutrophilia. IFN–γR1 knockout animals[295] or IFN–γR1 chimeras selectively lacking the receptor in nonhematopoietic cells[296] display amplified Th17 responses following *M. tuberculosis* infection that lead to a pathogenic accumulation of neutrophils detrimental to the host, suggesting that IFN–γ signaling serves a regulatory role by limiting excessive IL-17-mediated neutrophilia.

FoxP3+ CD4 T cells, or T-regulatory cells (T-regs), can impair anti-mycobacterial T cell responses and contribute to disease but can also limit overt inflammation. FoxP3+ T-regs can be found in the peripheral blood and airways of *M. tuberculosis*-infected macaques[297] and humans[298-303]. In mice, T-regs accumulate in the lung draining lymph nodes and the lungs following low-dose aerosol *M. tuberculosis* infection[304]. Importantly, FoxP3+ T-regs localized to pulmonary areas adjacent to effector CD4 T cells and depletion of T-regs before and early after infection enhanced control of bacterial burden[304]. Further, *M. tuberculosis*-specific T-regs delay the expansion of anti-mycobacterial CD4 and CD8 T cells and, consequently, transfer of *M. tuberculosis*-

specific T-regs confers increased susceptibility to infection[305]. Regulation of T-regs during *M. tuberculosis* infection may be mediated by Th1 responses since *M. tuberculosis*-specific T-regs are selectively eliminated following IL-12 driven T-bet expression[306]. The functional properties of T-regs responsible for limiting anti-mycobacterial CD4 and CD8 responses remains unclear. IL-10 was not found to be secreted by T-regs in mice infected with H37Rv[304]. In contrast, T-regs from mice infected with the W-Beijing strain, HN878, were found to secrete IL-10, express inhibitory receptors, and expand to greater degrees compared to infection with H37Rv[189], suggesting that IL-10 secretion by T-regs may be dependent on bacterial strain. Notably, the expansion of T-regs in the lungs of mice and outbred guinea pigs infected with W-Beijing strains occurred concurrent with a loss of Th1 responses and is associated with severe pulmonary pathology[189, 307]. However, progressive loss of T-regs in chronically infected TLR-2 knockout mice was associated with increased pulmonary inflammation[308], highlighting a role for TLR-2 mediated recruitment of T-regs in limiting tissue pathology at chronic stages of disease. Taken together, these results suggest that the functional contribution of T-regs to immunity against *M. tuberculosis* infection and outcome of disease may be dependent on multiple factors, including strain of bacteria and stage of infection.

In humans and animal models, *M. tuberculosis* establishes a persistent infection despite the induction of adaptive immune responses. Persistent inflammation and chronic antigen exposure precedes functional exhaustion due to chronic antigenic stimulation. In contrast to the expression of Ag85B, which decreases early following infection, ESAT-6 is

expressed by *M. tuberculosis* throughout infection[309, 310]. Multiple studies examining CD4 T cell responses to ESAT-6 and Ag85B have suggested that antigen-specific responses are dictated by bacterial expression of those antigens throughout infection. CD4 T cells specific for ESAT-6 display a terminally differentiated phenotype with evidence for functional exhaustion, which runs in contrast to Ag85B-specific CD4 T cells that appear functional but are quickly diminished[242, 311-316]. Indeed, a vaccine that contains ESAT-6, Ag85B, and Rv2660c, which is expressed at late stages of infection, demonstrated enhanced efficacy compared to BCG or to a vaccine containing ESAT-6 and Ag85B[317], suggesting that rational incorporation of antigens present at different stages of infection may improve vaccine efficacy. A clearer understanding of protective CD4 T cell immunity will require further studies on the spectrum of antigens recognized by CD4 T cells following infection with *M. tuberculosis* in animal models and in humans.

The role of CD8 T cells in M. tuberculosis infection

Mice with gene deletion of β2 microglobulin, which abrogates MHC class I antigen presentation, or mice depleted of CD8 T cells, live longer than corresponding disruptions to the MHC class II pathway or CD4 T cell responses following *M. tuberculosis* infection[240]. Regardless, CD8 T cells contribute significantly to immunity against *M. tuberculosis* infection. Mice lacking TAP-1 (transporter associated with antigen processing 1) antigen presentation molecules have deficient CD8 T cell responses and succumb more rapidly following *M. tuberculosis* infection compared to wild-type controls[318, 319]. Depletion of CD8 T cells in rhesus macaques compromises protective immunity from BCG vaccination or chemotherapeutic interventions[54], suggesting that

CD8 T cells are important components of recall responses to *M. tuberculosis* infection. Similarly, in a mouse model of latency induced by antibiotic treatment, CD8 T cell responses were found to be important in preventing reactivation[320]. The importance of CD8 T cells during *M. tuberculosis* infection is related to their secretion of cytokines and cytolytic effector molecules that can limit bacterial replication. In addition to IFN–γ and TNF–α, CD8 T cells secrete perforin to lyse *M. tuberculosis*-infected macrophages[321]. CD8 T cells can also release granulysin in cytotoxic granules to directly kill intracellular *M. tuberculosis*[322, 323]. The use of anti-TNF–α therapy in patients with rheumatoid arthritis depletes a subset of effector memory CD8 T cells that secrete granulysin and express cell surface TNF[324], which may partially explain the increased progression from LTBI to ATB in patients undergoing anti-TNF–α therapy. Human CD8 T cells respond to epitopes in CFP10[325], ESAT-6[326, 327], and the Ag85 complex[328, 329]. A variety of human CD8 T cell clones tested against a panel of synthetic peptides derived from immunodominant *M. tuberculosis* antigens revealed that CD8 T cell responses are concentrated towards a limited set of epitopes and are generally restricted by the HLA-B allele[330, 331]. *M. tuberculosis* escape from the phagosome and induction of apoptosis by *M. tuberculosis*-infected macrophages can promote cross-presentation of *M. tuberculosis* antigens to CD8 T cells. However, as previously discussed, virulent *M. tuberculosis* has been shown to inhibit host apoptosis and favor necrosis in order to circumvent efficient induction of CD8 T cell responses. Taken together, CD8 T cells are a critical component of adaptive immunity to *M. tuberculosis* infection and play an important role in different disease contexts by limiting reactivation during latency and by directly participating in antimicrobial functions during active infections.

Inhibitory receptors during M. tuberculosis infection

Chronic viral infections, such as HIV, induce the expression of co-inhibitory receptors on the surface of T cells that can dampen T cell functionality. Abrogation of inhibitory receptor ligation has been shown to be a viable strategy to revitalize functionally exhausted virus-specific T cell responses. The evidence for the importance of co-inhibitory receptors during *M. tuberculosis* infection in animal models and in human samples vary between human and small animal models and between the specific inhibitory receptor studied. Expression of inhibitory molecules, including PD-1 and CTLA-4, among *M. tuberculosis*-specific CD4 T cells has been shown to decrease following treatment[332, 333]. Importantly, expression of PD-1 on antigen-specific CD4 T cells from LTBI was not associated with decreased effector functions and these cells proved to be polyfunctional upon antigen restimulation[334], suggesting that PD-1 may be an indicator of bacterial burden and CD4 T cell activation rather than functional exhaustion. There is evidence that T cell responses during ATB disease are less polyfunctional and have limited proliferative capacity compared to LTBI individuals[335, 336], but whether this functional impairment is mediated by inhibitory receptors such as PD-1 remains unclear. PD-1 deficient mice infected with *M. tuberculosis* have increased bacterial burden, neutrophilic infiltration, overt inflammation, tissue necrosis, and diminished lifespan compared to wild-type mice[337], suggesting that PD-1 is required to prevent aberrant inflammation during *M. tuberculosis* infection. Further, adoptive transfer studies demonstrated that PD-1 expressing CD4 T cells are highly proliferative[311, 315] and CD4 T cells lacking PD-1 can drive pathology and mortality following *M.*

tuberculosis infection[338], together suggesting that PD-1 may mark functional CD4 T cells with intrinsic receptivity for immunoregulation. T cell immunoglobulin and mucin domain-containing 3 (Tim-3) is another inhibitory receptor shown to play a role in mediating antimicrobial responses by binding to one of its ligands, galectin-9[339], and inducing the production of IL-1β by human and murine macrophages infected with *M. tuberculosis*[339, 340]. In contrast to PD-1, Tim-3 deficient mice were less susceptible to *M. tuberculosis* infection and Tim-3 blockade was shown to improve antigen-specific CD4 and CD8 T cell cytokine expression[341], suggesting that Tim-3 may be playing a role in limiting T cell responses by promoting functional exhaustion. However, evidence from human samples does not support a clear role for Tim-3 in attenuating T cell responses[342]. The mechanisms underlying the roles of receptors such as PD-1 and Tim-3 require further study and may deviate from their role in viral immunity. The evidence accumulated thus far suggest that these molecules mark functional T cells that play important roles in antimicrobial activity and prevention of uncontrolled inflammation following *M. tuberculosis* infection.

Memory T cell responses

In humans, antigen-specific memory T cell responses have been detected in individuals with LTBI and in TB patients following successful treatment and cure. Memory T cell subsets can be identified according to their cell surface phenotype and functional properties and distinct populations of antigen-specific memory T cells can be categorized based on their expression of a panel of cell surface activation markers and chemokine receptors[343]. Characterization of *M. tuberculosis*-specific memory CD4 T cells in

LTBI indicated that these cells did not express activation markers and were largely of a CD45RA-CCR7- phenotype descriptive of T effector memory (TEM) cells[334, 344]. In contrast, analysis of LTBI individuals using MHC class II tetramers revealed a population of tetramer+CD45RA-CCR7+ central memory CD4 T cells that further expressed CXCR3+CCR6+[276], highlighting the heterogeneity of memory CD4 T cell phenotypes that can vary based on antigen-specificity, disease status, and manner in which specific responses are identified. Human memory CD8 T cells are predominantly terminally differentiated effector memory T cells (TEMRA) in individuals with LTBI[345, 346]. Memory T cell responses have also been studied in the context of "memory-immune" mice, which are *M. tuberculosis* infected mice that subsequently receive antibiotic treatment. In this context, both memory CD4[347, 348] and CD8[349, 350] T cells play a role in immunity against *M. tuberculosis* infection. T cells from memory-immune mice expanded rapidly, secreted IFN–γ, and conferred a significant level of protection at early timepoints after infection[348, 351-353] but are ultimately unable to confer long-term protection[354], suggesting that memory T cells generated after primary *M. tuberculosis* infection have limited capacity to protect from re-infection.

B-cell and antibody responses during M. tuberculosis infection

There is a body of evidence suggesting that humoral immunity plays a role in defense against *M. tuberculosis* infection (reviewed in [355]). B-cells can be found alongside T cells in the lymphocytic cuff in human granulomas[356-358] and whole blood gene expression analysis revealed significant changes in B-cell associated genes in TB patients after initiation of TB treatment[359]. Notably, antibodies to *M. tuberculosis* proteins have

been reported in the sera of TB patients[360] and antibodies identified in a subset of healthcare workers exposed to *M. tuberculosis* provide modest protection *in vitro* and in a mouse model of infection[361]. Utilization of a high throughput approach to identifying antibody targets in the *M. tuberculosis* proteome revealed a set of extracellular antigens recognized by antibodies in the plasma of patients with ATB[362], suggesting that B-cells are active participants in immunity to *M. tuberculosis* infection. B-cell deficient mice have elevated neutrophilic recruitment and exacerbated lung immunopathology following *M. tuberculosis* infection[363], which is mediated through enhanced IL-17 responses in *M. tuberculosis* infected B-cell deficient or B-cell depleted animals[364]. These studies suggest that B-cells can influence the outcome of *M. tuberculosis* infection by moderating inflammatory responses. Antibody production by B-cells can promote divergent outcomes[365]. Binding of antibody to the inhibitory Fc gamma receptor II B attenuates macrophage IL-12 production and negatively impacts Th1 responses[366] while passive transfer of monoclonal antibodies specific for *M. tuberculosis* cell wall components can improve the outcome of infection in mice[355]. B-cell secretion of cytokines can also influence *M. tuberculosis* infected macrophages. Type I IFN expression by murine B-cells and B-cells from pleural effusion of TB patients altered macrophage polarization towards an anti-inflammatory phenotype[367]. Taken together, these studies highlight a role for B-cells, which constitute a significant population of lymphocytes around lung granulomas, in the adaptive immune response to *M. tuberculosis* infection by modulating inflammation through the secretion of antibodies and cytokines.

γδ, CD1-restricted T cells, and MAIT cells in immunity against M. tuberculosis

γδ T cells are a population of T cells expressing a restricted repertoire of TCR genes, recognize non-peptide antigens such as microbial metabolites and phosphoantigens[368], and can be found at mucosal surfaces including the lung[369]. γδ T cells proliferate when exposed to *M. tuberculosis*-infected monocytes[370]. Multiple *M. tuberculosis* metabolites, including pyrophosphate, prenyl pyrophosphate derivatives[371, 372], and triphosphorylated thymidine containing compounds[373], are recognized by human γδ T cells. Human γδ T cells can also respond to mycobacterial heat shock proteins[374], though this response may be dependent on BCG immunization[375]. Vγ9Vδ2 expressing γδ T cells represent a significant proportion of *M. tuberculosis*-reactive T cells in peripheral blood[376-378] and can restrict intracellular *M. tuberculosis* replication in macrophages[379]. Interestingly, Vγ9Vδ2 T cells can function as antigen presentation cells via provision of CD40 co-stimulation to promote the expansion of αβ T cells with enhanced capacity to restrict intracellular BCG replication[380]. Additionally, human Vγ2Vδ2 T cells recognize *M. tuberculosis*[381] and, in nonhuman primates, Vγ2Vδ2 T cells are expanded by phosphoantigen and IL-2 administration[382]. Adoptively transferred Vγ2Vδ2 T cells into naïve animals confers protection against *M. tuberculosis* infection[383]. γδ T cells have been shown to mediate direct killing of *M. tuberculosis* via secretion of granulysin and perforin[384] or through the induction of TNF−α by monocytes[385]. There is also evidence that γδ T cells can influence DC crosstalk with T cells by promoting DC maturation and expression of co-stimulatory molecules[386]. In mice, γδ T cells accumulate in the lung draining lymph nodes, are responsive to *M.*

tuberculosis antigen independent of MHC class II[387], and are significant sources of early IL-17 production following *M. tuberculosis* infection[388].

Due to the large repertoire of glycolipids present on the mycobacterial cell wall, a significant T cell response is directed at glycolipid antigens presented by the CD1 family of molecules. CD1 molecules are a family of MHC class I-like antigen presentation molecules that present glycolipid antigens to T cells. There are five CD1 family members in humans, split into 2 groups based on sequence homology. Group 1 molecules include CD1a, CD1b, CD1c, and CD1e. CD1d is the sole inclusion in group 2[389]. Mycobacterial lipids are readily presented by CD1 molecules in human cells, but mechanistic studies of this family of molecules is limited because mice only express two orthologs of CD1d and do not express group 1 molecules. Nevertheless, studies in human cells revealed that mycobacterial lipids presented by group 1 CD1 molecules promote T cell proliferation and cytokine production[390-397]. Mycobacterial glycerol monomycolate, glucose monomycolate, sulphoglycolipids, and mycolic acid can be presented through CD1b[391-393, 398]. CD1b-restricted T cells expand and secrete IFN–γ and IL-2 upon interaction with cognate antigen and contract following anti-TB therapy[395]. *M. tuberculosis* lipids presented through CD1a and CD1c have also been identified. A family of *M. tuberculosis* lipopeptides called didehydroxymycobactins are presented by CD1a[399] and a variety of phospholipid antigens are presented by CD1c[390]. The precise role of CD1-restricted T cells in immunity during *M. tuberculosis* infection remains unclear and further studies on their function in the

periphery and especially in the BAL would inform their potential as targets for TB vaccines.

Mucosal-associated invariant T (MAIT) cells are a subset of T cells with innate-like qualities enriched in mucosal tissues, including the intestinal mucosa, lung, and liver[400-402]. These cells recognize antigen through a non-polymorphic MHC class I-related molecule 1 (MR1)[403] presenting pterin-containing byproducts of riboflavin synthesis in bacteria and fungi[404]. In humans, MAIT cells express a semi-invariant Vα7.2 and CD161 and can either be double negative for CD4 and CD8 or CD4-CD8+[400, 405]. MAIT cells have been described in the peripheral blood of healthy individuals and are depleted in ATB patients[406], possibly reflecting migration into the lung. These cells produce IFN–γ and TNF–α upon activation[406, 407], but their contribution to the immune response to *M. tuberculosis* infection require further studies.

Initiation and heterogeneity of the granuloma

The granuloma is a hallmark histopathological structure in TB. It represents host sequestration of bacteria in order to limit dissemination as well as a niche for long term persistence of *M. tuberculosis*. Further, selectively drug permeable nature of the TB granuloma can diminish the efficacy of drugs meant to treat persistent bacteria but cannot penetrate the granuloma[408]. The granuloma is composed of an aggregate of *M. tuberculosis* infected and uninfected macrophages in varying stages of maturation and differentiation[409-411]. Macrophages in the granuloma can undergo an epithelioid transformation, become lipid-filled foamy macrophages, or merge into multinucleated

giant cells. This central core of macrophages is accompanied by neutrophils, dendritic cells, and fibroblasts circumscribed by T and B lymphocytes and progressively becomes a hypoxic environment where many cells undergo necrotic death to form an acellular core termed the caseum[412]. The granuloma is a hallmark structure in human TB that is modeled variably amongst available animal models. C57BL/6 and BALB/C mice do not naturally recapitulate the human granuloma in that lung lesions are rarely necrotic and caseating. The animal models that most closely recapitulates the heterogeneity of human granulomas include certain susceptible inbred mouse strains that present with necrotizing granulomas (C3HeB/FeJ, DBA/2, CBA/J, I/St) and the nonhuman primate model. Additionally, the zebrafish model has also yielded fundamental insights into the initiation and dynamics of the tuberculous granuloma.

The transparency of zebrafish larvae has made direct visualization of the initiation of the granuloma possible following infection with *M. marinum*[45]. Studies based on this model have revealed that the innate immune response is sufficient to initiate the granuloma following infection. Recruitment of additional macrophages mediated, in part, by mycobacterial ESX-1 proteins initiates a cascade of events that leads to the establishment of the mycobacterial granuloma[413, 414]. Importantly, recruited macrophages can traffic through the initial granuloma to phagocytose apoptotic infected macrophages and egress to form distal secondary granulomas[415]. Mycobacterial lipids play a key role in establishing the granuloma by limiting macrophage effector functions and promoting the recruitment of additional macrophages to facilitate dissemination. In particular, mycobacterial PDIM can mask TLR-signaling and prevent induction of

nitrosative stresses[416] and mycobacterial phenolic glycolipid (PGL) can induce macrophage production of CCL2 to recruit CCR2+ monocytes that permit bacterial dissemination[417]. These studies collectively indicate that the initiation of the mycobacterial granuloma is dependent on recruitment of bacteria-permissive macrophages and monocytes following initial infection and can be mediated by mycobacterial secreted factors and membrane lipids.

TB granulomas can vary in their cellular composition, oxygenation levels, inflammatory milieu, and bacterial burden. This heterogeneity can exist between and within infected hosts. Infection of cynomolgus macaques with a panel of *M. tuberculosis* isolates that differed by a single nucleotide polymorphism revealed that individual granulomas can be founded by a single bacterium and can vary in their bacterial burden compared to other granulomas within the same host[48]. Analysis of T cell functionality between sterile and non-sterile granulomas revealed a modest association between IL-10 and IL-17 responses and clearance of *M. tuberculosis* in sterile granulomas[418]. However, in the context of TNF−α neutralization in latently infected macaques, IL-10 and IL-17 responses were associated with animals at higher risk of reactivation[68]. Proteome analysis of laser-capture microdissected human and rabbit lung lesions suggests that inflammatory responses typical of the center of the TB granuloma are physically segregated from anti-inflammatory responses in adjacent lung tissue[419]. T cell functionality in the granuloma may therefore be a function of disease status and proximity to the bacteria-containing, hypoxic, and necrotic core of the TB granuloma. Additionally, T cells near the granuloma can be negatively impacted by the depletion of key amino acids required

for proper function. As mentioned previously, IDO, an enzyme that functions in the catabolism of tryptophan, is expressed by cells in the core of the granulomas of rhesus macaques infected with *M. tuberculosis* [253] and inhibition of IDO promoted granuloma reorganization and attenuated disease [63]. The functionality of T cells within granulomas may also be regulated by direct crosstalk with infected myeloid cells, including macrophages and DCs. Intravital imaging of mycobacteria-induced liver granulomas revealed limited antigen-specific T cell migration arrest in response to infected myeloid cells[314], suggesting that T cells do not interact meaningfully with infected cells in granulomas. Taken together, these studies highlight the vast complexity and heterogeneity of the TB granuloma.

TB Vaccines

The only currently licensed vaccine against TB is Bacillus Calmette-Guérin (BCG), an attenuated strain of *M. bovis*[420, 421]. BCG confers protection against severe forms of TB, including miliary TB and TB meningitis[422], but does not reliably protect against pulmonary TB in children or adults[3, 4, 423]. Lack of validated correlates of protection against TB represents a severe limitation to TB vaccine development. Despite the importance of IFN–γ responses in resistance against *M. tuberculosis* infection in humans and animal models, accumulating evidence suggests that induction of enhanced IFN–γ responses is not sufficient to obtain a more efficacious TB vaccine. Indeed, the frequency and functional profile of BCG-specific CD4, CD8, and γδ T cells from whole blood, including IFN–γ producing T cells, did not correlate with protection against TB in newborns[424]. As of 2017, there are 14 TB vaccine candidates in varying phases of

clinical development representing three broad strategies: subunit vaccines pairing *M. tuberculosis* antigens with adjuvants; viral-vectored vaccines utilizing an attenuated virus for antigen delivery; whole-cell vaccines utilizing attenuated *M. tuberculosis* or related mycobacterial species. Protein subunit vaccines currently under clinical development include M72/AS01E[425], H4:IC31[426], H56:IC31[427], and ID93/GLA-SE[428]. Amongst viral-vectored vaccines, results from the MVA85A phase IIb clinical trial has prompted reevaluation of immune correlates to aim for in a TB vaccine. MVA85A is a modified vaccinia Ankara virus expressing Ag85A from *M. tuberculosis* that was utilized as a booster vaccine in infants previously vaccinated with BCG[429]. Notably, vaccination with MVA85A enhanced frequencies of antigen-specific, polyfunctional CD4 T cells co-expressing IFN–γ, IL-2, and TNF–α[429]. Although MVA85A vaccination enhanced antigen-specific CD4 T cell responses, it did not provide added protection against TB disease in infants[429]. Other viral-vectored vaccines in various stages of development include Ad5Ag85A[430], ChAdOx1.85A + MVA85A[431], MVA85A-IMX313[432], and TB/FLU-04L. Additionally, a recent study utilizing a recombinant cytomegalovirus (CMV) demonstrated protection in rhesus macaques[433]. While viral vectors do not require the use of adjuvants, previous exposure to the vector may attenuate vaccine-induced responses and represents a potential complication to the use of viral vectors. Whole-cell vaccines currently under development include killed *M. vaccae*, DAR-901[434], VPM1002, MTBVAC, and RUTI. VPM1002 represents an approach to improve BCG immunogenicity and vaccine potential by engineering BCG to express lysteriolysin from *Listeria monocytogenes* to escape the phagosome and carry a urease deletion mutation that facilitates phagosomal acidification thereby enhancing

MHC class I antigen presentation to CD8 T cells[435]. MTBVAC is a genetically attenuated *M. tuberculosis* strain lacking *phoP* and *fadD26* that abrogates synthesis of various surface lipids[436]. Lastly, the therapeutic vaccine candidate RUTI was developed by growing *M. tuberculosis* under stress prior to fragmentation, detoxification, and delivery in liposomes to LTBI individuals in order to prevent progression to ATB[437-439].

There have been substantial advances in our understanding of immunity against *M. tuberculosis* from the days of Drs. Calmette and Guérin. Nevertheless, the absence of suitable alternatives to BCG highlights the challenges before us. *M. tuberculosis* is adept at subverting the crosstalk between innate and adaptive immunity and it will be important to understand that crosstalk for the rational development of better vaccines. Even in the absence of protective correlates and in the face of disappointing preliminary results for MVA85A, the state of TB vaccine development is resurgent now more than ever and provides cause for hopeful optimism for more efficacious vaccines and therapeutics against TB.

Thesis overview

Considerable evidence supports the hypothesis that *M. tuberculosis* manipulates CD4 T cell responses by subverting DC functions. However, the consequences of DC subversion by *M. tuberculosis* and the specific contributions of different DC functions during crosstalk with CD4 T cells is not well understood. Although there is substantial evidence suggesting that Th17 cells play a role in immunity against *M. tuberculosis*, there is a

knowledge gap regarding how antigen-specific Th17 cells are generated following infection. Further, there is controversy regarding the role that Th17 cells play during *M. tuberculosis* infection that is clouded by the fact that infection does not substantively elicit such cells without ablation of IFN-γ signaling pathways or use of a hypervirulent strain. Lastly, efforts towards the rational improvement of BCG as a vaccine are undercut by a poor grasp of the mechanisms underlying its limited efficacy. Understanding the DC-CD4 T cell crosstalk in the context of infection with *M. tuberculosis* and vaccination with BCG can provide important insights that can be applied towards the development of novel vaccines and host-directed therapies for TB.

In this dissertation, we provide evidence that *M. tuberculosis* subversion of DC functions through Hip1 attenuates antigen-specific CD4 T cell responses (Chapter II). We identify the CD40-CD40L pathway to be required for the generation of Th17 cells during *M. tuberculosis* infection and demonstrate that augmenting antigen-specific Th17 cells, via ligation of CD40 on DCs, improves host immunity against *M. tuberculosis* (Chapter III). We apply our findings to the context of vaccine-induced immunity and find that removal of *hip1* from BCG enhances DC functions and subsequently improves lung CD4 T cell responses against *M. tuberculosis* challenge (Chapter IV). Finally, we summarize the findings of this dissertation, outline future considerations, and discuss the relevance of our work to the broader context of the global TB challenge (Chapter V).

Chapter II

Mycobacterium tuberculosis impairs dendritic cell functions through the serine hydrolase Hip1

Chapter adapted from:

Madan-Lala R., **Sia J.K.**, King R., Adekambi T., Monin L., Khader S.A., Pulendran B., and Rengarajan J. (2014). *Mycobacterium tuberculosis* impairs dendritic cell functions through the serine hydrolase Hip1. *Journal of Immunology*, 192:4263-72. PMCID: PMC3995873; PMID: 24659689

Attributions for collaborations:

Figure 5 and 6 were contributed by myself. Data contained within Figure 7 were partially contributed by myself in close collaboration with R. Madan-Lala and T. Adekambi and with technical assistance from R. King. All other figures are derived from the work of R. Madan-Lala.

Abstract

Mycobacterium tuberculosis (*M. 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. While 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 antigen presenting cells (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 antigen 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 T helper 1 (Th1)-inducing cytokine IL-12, as well as other pro-inflammatory cytokines (IL-23, IL-6, TNF-α, IL-1β, 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 co-stimulatory molecules, CD40 and CD86, indicating that *M. tuberculosis* impairs DC maturation through Hip1. Further, we show that *M. tuberculosis* promotes sub-optimal antigen presentation, as DCs infected with the *hip1* mutant showed increased capacity to present antigen to OT-II- and early secreted antigenic target 6 (ESAT-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 antigen-specific T cell responses, with important implications for vaccination strategies.

Introduction

The immense success of *M. tuberculosis* as a pathogen can be largely attributed to its ability to subvert host innate and adaptive immune responses [108, 440-444]. Upon infection with *M. tuberculosis*, the majority of infected individuals mount robust CD4 T cell responses involving T helper 1 (Th1) cytokines such as IFN-γ and TNF-α, which are critical for activating macrophages and inducing microbicidal responses. Several studies have shown increased susceptibility to mycobacterial diseases in IFN-γ-deficient mice and in humans with IL-12 or IFN-γ-receptor abnormalities [264, 278, 445]. While Th1 responses are required to control *M. tuberculosis* infection, they are not sufficient for eradicating the pathogen from the host. This is because *M. tuberculosis* has evolved multiple strategies to resist host defenses. These include interfering with the ability of IFN-γ to effectively activate antimicrobial responses in *M. tuberculosis*-infected macrophages, inhibition of phagosome acidification and maturation, resistance to reactive oxygen and nitrogen intermediates (ROI and RNI), impairing antigen presentation [108, 446] and preventing optimal activation of pattern recognition receptor (PRR)-dependent pathways in macrophages [99, 103, 447-452]. *M. tuberculosis* has been shown to inhibit macrophage activation and cytokine induction through secreted and cell envelope associated factors [99, 447-449, 452, 453]. We have shown that the cell-envelope associated serine hydrolase, Hip1 (Hydrolase Important for Pathogenesis 1), a protein critical for *M. tuberculosis* virulence, hinders optimal TLR2- and inflammasome-dependent activation in macrophages and promotes dampening of pro-inflammatory responses [102, 103, 110, 454, 455]. Thus, Hip1 prevents robust macrophage responses to *M. tuberculosis* infection.

In addition to macrophages, it is increasingly appreciated that dendritic cells (DCs) also serve as an important intracellular niche for *M. tuberculosis* [217, 218, 220, 456, 457]. DCs are the primary antigen presenting cells (APCs) of the immune system and are strategically located at sites of pathogen entry. Immature DCs recognize pathogen associated molecular patterns (PAMPs) via PRRs and concomitant with phagocytosis and internalization of microbes, these events lead to a process of maturation. Mature DCs are characterized by high surface expression of major histocompatibility class II (MHC class II), co-stimulatory molecules such as CD40, CD80 and CD86 and secretion of key cytokines, such as the Th1-polarizing cytokine IL-12 [458]. Mature DCs can migrate into secondary lymphoid organs, where they present pathogen-derived antigens to naïve T cells, initiate activation and differentiation of these T cells and play a critical role in determining the types of Th subsets that are generated in response to infection [457, 459-461]. Thus, DCs play a central role in immunity to microbial pathogens by effectively linking innate and adaptive immune responses [460, 462]. Recent studies have demonstrated that *M. tuberculosis* infects human and mouse dendritic cells at high frequencies *in vitro* and *in vivo*, and there is growing evidence that DCs play a critical role in immunity to TB [218, 219, 222, 463, 464]. In the aerogenic mouse model of TB, *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 [219, 244, 464]. Depletion of $CD11c^{+}$ cells in mice, which includes DCs, caused a delay in CD4 T cell responses and impaired control of *M. tuberculosis* [216]. However, *M. tuberculosis* has also been shown to interfere with DC

migration and antigen presentation *in vivo* [219], which likely impact the priming of Th1 responses. Thus, interactions between *M. tuberculosis* and DCs during early stages of infection will directly influence the onset and development of adaptive immunity. While *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 *M. tuberculosis* infection impairs key aspects of DC functions through Hip1 (Rv2224c) and thereby impacts adaptive immune responses. Infection of DCs by a *hip1*-deficient mutant induced significantly higher levels of IL-12 and other pro-inflammatory cytokines compared to wild type *M. tuberculosis*, and enhanced surface expression of MHC class II, CD40 and CD86. This enhanced DC maturation induced by the *hip1* mutant was dependent largely on MyD88 and partially on TLR2/9 pathways. Further, we provide evidence that DCs matured by the *hip1* mutant were more efficient in presenting antigens to CD4 T cells and priming Th1 and Th17 responses. Overall, our data demonstrate that *M. tuberculosis* Hip1 impairs DC functions and modulates the nature of antigen-specific T cell responses. Enhancing adaptive immune responses by boosting DC activation and antigen presentation has important implications for developing better vaccines for TB.

Results

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

While *M. tuberculosis* has been shown to infect DCs and impair their functions *in vivo* [219], 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 the *hip1* mutant strains of *M. tuberculosis* to induce IL-12, a cytokine that is critical for inducing the differentiation of naïve T cells into the IFN-γ-secreting Th1 phenotype. We infected bone marrow-derived dendritic cells (BMDCs) from C57BL/6J mice with the wild type or *hip1* mutant strains of *M. tuberculosis* at an MOI=5 and measured the levels of IL-12p40 and p70 subunits at 24 hours post-infection in the cell free supernatants. The intracellular bacterial counts in wild type and *hip1* mutant- infected BMDCs at 4 hours and 8 days post-infection were comparable (data not shown). The *hip1* mutant induced significantly higher levels of IL-12p40 and IL-12p70 in infected DCs compared to 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 to 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 and B), confirming that *M. tuberculosis* limits IL-12 production through Hip1. While IL-12 is a major Th1-polarizing cytokine secreted by myeloid DCs upon microbial stimulation, DCs

also secrete other pro-inflammatory 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 to wild type and these levels were restored to wild type levels by the complemented strain (Fig. 1C and 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 pro-inflammatory cytokines in infected DCs, in a Hip1-dependent manner.

M. tuberculosis impairs DC maturation through Hip1

Following phagocytosis and antigen capture at the site of infection by immature DCs, interactions between PAMPs and PRRs induce maturation of DCs and migration into the local draining lymph nodes where they prime T cells through cell surface expression of co-stimulatory molecules, MHC class 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=5 for 24 hours, and monitored the surface expression of CD40, CD86 and MHC class II by flow cytometry. While wild type *M. tuberculosis* induced all three markers on $CD11c^+$ BMDCs, the expression levels were much lower than that induced by lipopolysaccharide (LPS) from *Salmonella* (Fig. 2). In contrast, *hip1* mutant induced higher surface expression of CD40, CD86 and MHC class 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=5, either independently or together, and measured the surface expression of CD40 by flow cytometry after 24 hours (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 while 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 pro-inflammatory responses by limiting interactions between TLR2 agonists on *M. tuberculosis* and TLR2 on macrophages,

leading to sub-optimal TLR2 activation. Since studies have shown that *M. tuberculosis* engages different TLRs on macrophages and DCs [465], 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 and MyD88$^{-/-}$ mice with the wild type or *hip1* mutant and assayed the supernatants for IL-12p40, IL-12p70 and IL-6 by ELISA. As seen in Fig. 4A, production of all three cytokines was largely abolished in the MyD88$^{-/-}$ BMDCs. Next, we infected BMDCs derived from TLR2$^{-/-}$ mice with wild type or *hip1* mutant and assayed the supernatants for cytokines. As seen in Fig. 4B, IL-6 and IL-12p70 levels are largely abolished in TLR2$^{-/-}$ BMDCs and IL-12p40 levels are significantly reduced compared to BMDCs from C57BL/6J mice. Since engagement of TLR9 on DCs has been implicated in IL-12 production, we tested the involvement of TLR9 in the enhanced IL-12 production induced by 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$^{-/-}$/TLR9$^{-/-}$ BMDCs. These results indicate that the enhanced cytokine secretion in the absence of Hip1 is dependent on activation of TLR2 and TLR9 pathways.

We next examined whether *M. tuberculosis* regulates the cell surface expression of co-stimulatory markers in a MyD88-TLR dependent manner. For this we infected BMDCs with wild type or *hip1* mutant at MOI=5 for 24 hours, and monitored the surface expression of CD40 by flow cytometry. As seen by the median of fluorescence intensity of CD11c^{+} cells, CD40 expression was largely abolished in MyD88$^{-/-}$ DCs while being

mostly independent of TLR2 and TLR9 (Fig 4D). Overall, these data show that Hip1 mediated enhanced DC maturation is dependent on MyD88-TLR2/9 pathways.

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

DCs are the most effective antigen-presenting cell for activating naïve CD4 T cells. Expression of high levels of co-stimulatory molecules and MHC class II on the cell surface is essential for efficient antigen presentation and T cell activation. We therefore hypothesized that enhanced expression of co-stimulatory molecules and MHC class II in DCs infected with the *hip1* mutant would impact antigen presentation to naïve CD4 T cells. To test this hypothesis, we infected BMDCs with wild type or *hip1* mutant at MOI=10 for 24 hours followed by a co-culture with naïve TCR transgenic CD4 T cells that were specific for the *M. tuberculosis* ESAT-6_{1-20} peptide, in the presence of ESAT-6_{1-20} peptide. Supernatants were collected 72 hours after co-culture and assayed for IFN-γ and IL-2 by ELISA. We found that wild type infected DCs elicited significantly lower IFN-γ and IL-2 from ESAT-6_{1-20}-specific CD4 T cells as compared to 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 antigen. Co-culture of *hip1* mutant-infected DCs with naïve TCR transgenic CD4 T cells isolated from OT-II mice and $OVA_{323-339}$ showed enhanced induction of IL-2 and the Th1 cytokine IFN-γ compared to their wild type counterparts (Fig. 5B). Thus, the absence of Hip1 enhanced the capacity of DCs to present antigen to CD4 T cells and induce Th1 cytokine responses. These data show that

suboptimal DC maturation and antigen 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 co-stimulatory molecules like CD40 to induce Th1 differentiation. Since 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 MOI=10 for 24 hours followed by a co-culture with purified CD4 T cells from uninfected C57BL/6J mice. After 72 hours, 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 to wild type *M. tuberculosis*, indicating that Hip1 controls Th cell differentiation by dendritic cells.

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 three weeks post infection, since at this time point, antigen-specific IFN-γ producing CD4 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 10μg/ml ESAT-6_{1-20} peptide for 48 hours 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-6_{1-20} peptide stimulation compared to 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 peripheral blood monocytes (PBMCs) from healthy donors and differentiated them *in vitro* in the presence of GM-CSF and IL-4. These monocyte derived DCs (MDCs) were infected with the wild type or *hip1* mutant at MOI=10 for 24 hours. 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 to 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 co-cultured with autologous lymphocytes from the respective donors for three days, 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. While 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 antigen presentation to CD4 T cells. Thus, we show that the *M. tuberculosis* serine 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 pro-inflammatory 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 [219, 466, 467]. A study using human MDCs showed that *M. tuberculosis* induces minimal up-regulation of surface maturation markers as compared to a potent cytokine-maturation cocktail, and *M. tuberculosis*-infected DCs were compromised in their ability to induce allogeneic lympho-proliferation [466]. *M. tuberculosis* has also been shown to interfere with DC migration and antigen presentation *in vivo* [219]. Our finding that *M. tuberculosis* prevents optimal expression of IL-12 and CD40 has important implications for the initiation and amplification of *M. tuberculosis*-specific adaptive immune responses. Our studies support the idea that down-modulation of CD40 expression on DCs and

restricting IL-12 production through Hip1 is an important strategy employed by *M. tuberculosis* to restrict the delivery of DC-derived signals required for inducing optimal Th1 responses.

Our studies implicating a role for the serine hydrolase Hip1 in preventing optimal DC maturation and IL-12 production extend our previous results showing that *M. tuberculosis* prevents robust pro-inflammatory cytokine and chemokine responses in macrophages through Hip1. Our previous data suggested that Hip-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 hypo-immune response that delays detection of *M. tuberculosis* by the host [103]. In contrast, *hip1*-deficient *M. tuberculosis* induced robust MyD88- and TLR2-dependent activation of macrophages and enhanced pro-inflammatory responses. In this study, we show that the enhanced IL-12 produced by DCs infected with the *hip1* mutant is dependent on TLR2 as well as TLR9 pathways (Fig. 4). The additional requirement for TLR9 is consistent with the increased engagement of TLR9 reported in DCs relative to macrophages [87, 465]. While 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. While 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 antigen presentation as a strategy for improving vaccination against TB. Recent data showing that nanoparticles containing TLR4 and TLR7 ligands boost the magnitude and persistence of vaccine elicited antibody responses, improving vaccine mediated protection against influenza virus, demonstrate that these approaches are feasible and efficacious in the setting of infectious diseases [468].

To test how the enhanced DC maturation, MHC class II expression and IL-12 production induced by the *hip1* mutant affect *M. tuberculosis*-specific CD4 T cell responses, we studied the antigen 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 co-culture 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 Th1 cell responses to *M. tuberculosis* (Fig. 5A). Several studies have shown that TLR2 signaling induces DCs to stimulate a Th2 or T regulatory pathway [469-473]. The present results indicating a role for Th1 induction by enhanced TLR2 signaling in the *hip1* mutant infected DCs suggests that the context in which TLR2 signaling occurs may play a role in the outcome.

Higher levels of IFN-γ were also observed *in vivo* within the lungs of *hip1* mutant-

infected mice at 3 weeks post-infection (Fig. 6B). One of the hallmarks of *M. tuberculosis* infection is a delayed Th1 response in the lungs, which is in part due to delayed DC migration and antigen presentation to T cells [223, 225, 242, 244, 249]. This delay, in combination with inadequate tempering of the ensuing inflammatory response, contributes to the damage sustained by the host in TB. Our studies suggest that the presence of Hip1 contributes to this delay by hindering antigen presentation and is an immune evasion mechanism employed by *M. tuberculosis* to manipulate the onset 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 co-culture experiments *in vitro,* as well as *in vivo* in *M. tuberculosis*-infected mice (Figs. 6 and 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. While the significance of earlier 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; intra-tracheal *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 [288, 290]. However, IL-17 production during chronic infection or unchecked Th17 responses may be detrimental by mediating immune pathology [474]. Thus, a finely tuned balance between Th1 and Th17 subsets is likely to be required for protective immunity to *M. tuberculosis* infection. Since mice infected with the *hip1* mutant exhibit severely reduced

lung pathology relative to wild type despite high bacterial burdens [102, 454, 455], we speculate that robust pro-inflammatory responses and more efficient antigen 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* antigens have been implicated in inhibiting DC maturation and functions. The *M. tuberculosis* antigen ESAT-6 inhibited LPS/CD40L-induced maturation of human PBMC-derived DCs and reduced IFN-γ production from T cells [475], and the *M. tuberculosis* mannose capped cell wall component ManLAM inhibited LPS-induced DC maturation by targeting DC SIGN [476]. 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*, ΔfbpA, induced higher expression of MHC class II on murine BMDCs as well as higher levels of IL-12p70; these DCs primed T cells to produce more IFN-γ as compared to wild type *M. tuberculosis* [467]. Further, ΔfbpA vaccinated mice showed better protection against *M. tuberculosis* challenge compared to those vaccinated with BCG. Similar studies are ongoing with the *hip1* mutant to assess whether the *hip1* mutant in *M. tuberculosis* or BCG has potential as a vaccine candidate.

In summary, we have shown that *M. tuberculosis* serine hydrolase Hip1 impairs dendritic cell 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 pro-inflammatory cytokines. This inhibition of DC maturation and cytokine secretion compromises antigen presentation to CD4 T cells and results in lower IFN-γ and IL-17 compared to the *hip1* mutant. Overall these findings show that optimal activation of DCs should result in a more efficient T cell response against *M. tuberculosis* and have important implications for vaccine design.

Acknowledgements

We gratefully acknowledge Dr. Padmini Salgame for TLR2/9 double KO bone marrow, Paul Hakimpour for breeding and maintaining several knockout mice strains, Dr. Chris Ibegbu for help with human MDC experiments and Dr. David Weiss for helpful discussions.

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 of the National Institutes of Health.

Bacterial strains and media

M. tuberculosis H37Rv (wild type), *hip1::tn* (*hip1* mutant) and *hip1* mutant-complemented strains were grown at 37°C in Middlebrook 7H9 broth or 7H10 agar

supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC), 0.5% glycerol, 0.05% Tween 80 (for broth), with the addition of 25 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO) for the *hip1* mutant and 10μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO) for the *hip1* mutant-complemented strain. For inactivation of *M. tuberculosis* strains, bacteria were grown in Middlebrook 7H9 until mid-log phase, washed twice with PBS and heat killed by incubating at 80°C for 2 hours.

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 Jackson Laboratory. MyD88$^{-/-}$and TLR2$^{-/-}$ mice originally generated in the laboratory of Dr. S. Akira (Osaka University, Japan), and OTII transgenic mice specific for $OVA_{323-339}$ peptide originally generated in the laboratory of Dr. F. Carbone (University of Melbourne, Australia) were bred at the Yerkes animal facility; bone marrow cells from TLR2/9$^{-/-}$ mice were a kind gift from Dr. Padmini Salgame (University of Medicine and Dentistry of New Jersey, Newark, NJ); TCR transgenic mice specific for ESAT-6_{1-20}/I-A^b epitope were obtained from Dr. Andrea Cooper (Trudeau Institute, NY).

Murine dendritic cell infection and cytokine assays

For generating murine bone marrow derived dendritic cells (BMDCs), bone marrow cells from C57BL/6J mice were grown in RPMI 1640 medium (Lonza, Walkersville, MD) with 10% heat inactivated Fetal bovine serum (FBS; HyClone, Logan, UT), 2mM

glutamine, 1X β mercaptoethanol, 10mM HEPES, 1mM sodium pyruvate, 1X non-essential 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 AutoMACS column as per manufacturers' instructions, where indicated (Miltenyi Biotec, Auburn, CA). For all experiments cells were throughout maintained in medium containing GM-CSF. For infection, BMDCs were plated onto 24-well plates ($3X10^5$ per well). Bacteria were filtered through 5μM filters, re-suspended in complete medium containing 20ng/ml GM-CSF and sonicated twice for 5 seconds each before addition to the adherent monolayers. Each bacterial strain was used for infection (in duplicate or triplicate) at an MOI=5 or as indicated. Infection of BMDCs was carried out for 4 hours, after which monolayers were washed 4X with PBS before replacing with RPMI medium containing 20ng/ml GM-CSF. To determine intracellular CFU, one set of dendritic cells was lysed in PBS containing 0.5% Triton X, and plated on 7H10 agar plates containing the appropriate antibiotics. Alternatively, BMDCs were infected with heat-killed *M. tuberculosis* at MOI=5 or as indicated in RPMI medium containing 20ng/ml GM-CSF. Cell-free supernatants from dendritic cells 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 Corporation, Woburn, MA).

Assays were carried out according to manufacturers' instructions. Uninfected BMDCs were used as controls for each experiment.

Flow cytometry and antibodies

Murine anti-CD11c APC (clone N418) and anti-CD11b FITC (clone M1/70) were obtained from Biolegend (San Diego, CA); anti-CD40 PE (clone 3//23), anti-CD86 PE (clone GL1) and anti-MHCII PE (clone M5/114.15.2) were purchased from BD Biosciences, San Jose, CA. Staining for cell surface markers was done by re-suspending ~1x10^6 cells in 200µl PBS with 2% FBS containing the antibody cocktail. Cells were incubated at 4^{o}C for 30 minutes and then washed with PBS containing 2% FBS. Data were immediately acquired using FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, San Carlos, CA).

Antigen specific CD4 T cell antigen presentation assays

CD4 T cells were purified from single cell suspensions of spleen and lymph nodes of 6-8 weeks old ESAT-6 transgenic and OTII transgenic mice using CD4 T cell isolation kit and AutoMACS column as per manufacturers' instructions (Miltenyi Biotec, Auburn, CA). BMDCs were incubated in 24 well plates (3X10^5 per well) with 10µg/ml ESAT-6_{1-20} peptide or $OVA_{323-339}$ peptides for 6 hours, washed with PBS and infected with heat killed wild type, *hip1* mutant, or medium alone for 24 hours. Infected DCs were washed twice with PBS and co-cultured with antigen specific CD4 T cells at 1:4 ratio for 72 hours. Supernatants collected from these cells were analyzed for IFN-γ (Mabtech, Cincinnati, OH) and IL-2 (BD Biosciences, San Jose, CA) by ELISA according to

manufacturers' instructions.

CD4 T cell polarization assays

CD4 T cells were purified from single cell suspensions of spleen and lymph nodes of 6-8 week old C57BL/6J mice as described above. BMDCs infected with wild type, *hip1* mutant, or medium alone for 24 hours as described above were co-cultured with CD4 T cells at 1:4 ratio for 72 hours. Cell free supernatants collected from these cells were analyzed for IFN-γ (Mabtech, Inc. Cincinnati, OH) and IL-17 (eBioscience, San Diego, CA) by ELISA according to manufacturers' instructions.

Aerogenic infection of mice with *M. tuberculosis* strains

M. tuberculosis strains, H37Rv and *hip1* mutant were grown to early log phase (OD600 of ~0.6-0.8), washed 2X in 1X 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 of H37Rv or the *hip1* mutant into 8-10 week old C57BL/6J mice using an aerosol apparatus manufactured by InTox Products, 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 three weeks post infection and digested with 1mg/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 70-µm-cell strainer (BD Biosciences, San Jose, CA), treated with RBC

lysis buffer for 3-5 minutes and washed twice with cell culture media. Cells were counted and stimulated with 10μg/ml ESAT-6_{1-20} peptide for 48 hours. Cell free supernatants were isolated and assayed for IFN-γ and IL-17 by ELISA.

Human dendritic cell infection and Th cell differentiation assays

PBMCs were isolated from the blood of healthy donors by centrifugation in CPT tubes (BD Biosciences, San Jose, CA). $CD14^+$ monocytes were purified from PBMCs by positive selection using $CD14^+$ micro beads (Miltenyi Biotec, Auburn, CA). Cell purity was >80% as assessed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA). To generate immature monocyte derived DCs (MDCs), $CD14^+$ cells were cultured at $1X10^6$ cells/ml in RPMI 1640 (Lonza, Walkersville, MD), supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 1X nonessential amino acids, and 20ng/ml human recombinant GM-CSF (Pepro Tech, New Jersey) and 40 ng/ml IL-4 (PeproTech, New Jersey). 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 ($3X10^5$ per well), and were infected with heat-killed H37Rv or *hip1* mutant at MOI=10. Cell-free supernatants from dendritic cells monolayers were isolated at 24 hours post infection and assayed for cytokines by ELISA using duo set kits for IL-12p40 and IL-6 (R&D, Minneapolis, MN; BD Biosciences, San Jose, CA). Assays were carried out according to manufacturers' instructions. For T cell polarization assays, infected DCs were co-incubated with

autologous lymphocytes at a ratio of 1:5 at 37°C with 5% CO2. Cell free supernatants were isolated at day 3 and assayed for IFN-γ and IL-17 by ELISA (BD Biosciences, San Jose, CA; R&D, Minneapolis, MN).

Statistical analysis

The statistical significance of data was analyzed using the Student's unpaired t-test (GraphPad Prism 5.0). Data are shown as mean ± S.D. of one representative experiment from two to five independent experiments.

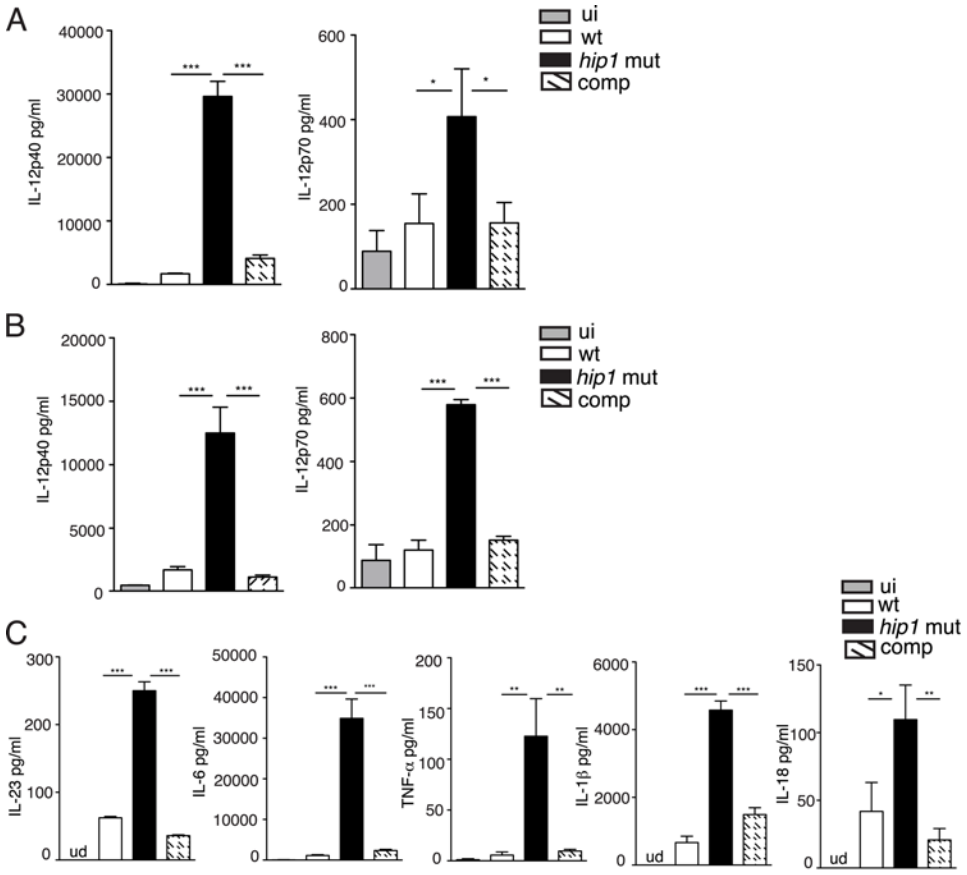

Figure 1. Enhanced inflammatory response in *hip1* mutant-infected DCs.

(A) Purified BMDCs derived from C57BL/6J mice were exposed to medium alone or infected with the wild type, *hip1* mutant or *hip1* mutant complemented with Hip1 (comp) *M. tuberculosis* at MOI=5. 24 hours post-infection, cell free supernatants were assayed for IL-12p40 and IL-12p70 by ELISA. Purified C57BL/6J BMDCs were infected with heat-killed wild type, *hip1* mutant or comp strain at MOI=5. 24 hours post-infection, cell free supernatants were assayed for IL-12p40, 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. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

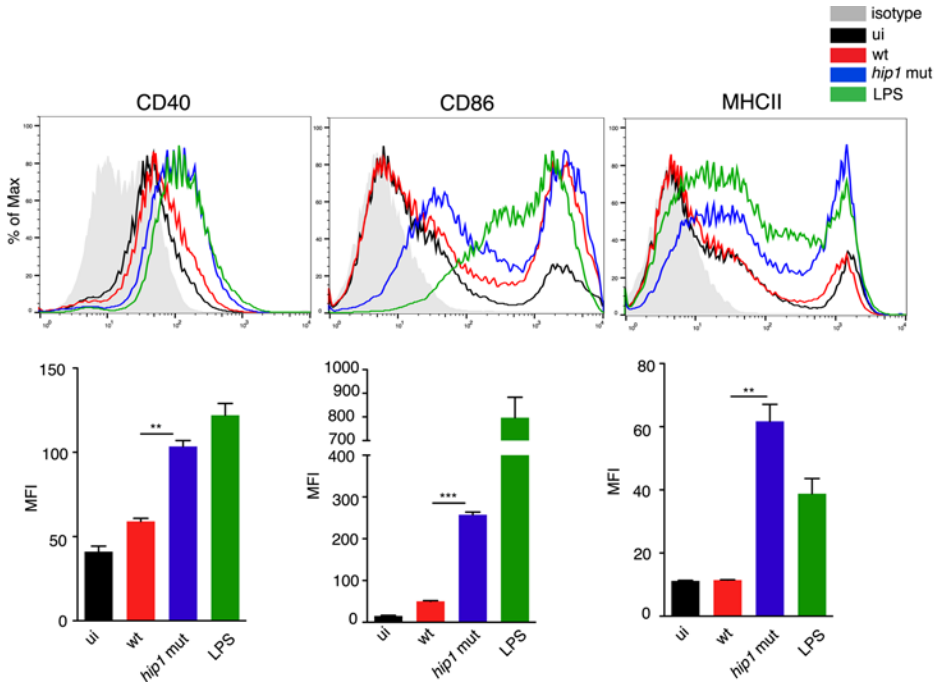

Figure 2. Enhanced surface expression of CD40, CD86 and MHC class II on *hip1* mutant-infected DCs.

C57BL/6J BMDCs were exposed to medium alone, heat-killed wild type or *hip1* mutant at MOI=5 or 1 µg/ml LPS for 24 hours. DCs were labeled with anti-CD11c-APC and anti-CD40-PE, anti-CD86-PE or anti-MHC class II-PE. Representative histograms and median PE fluorescence intensity 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, **, $p<0.01$; ***, $p<0.001$.

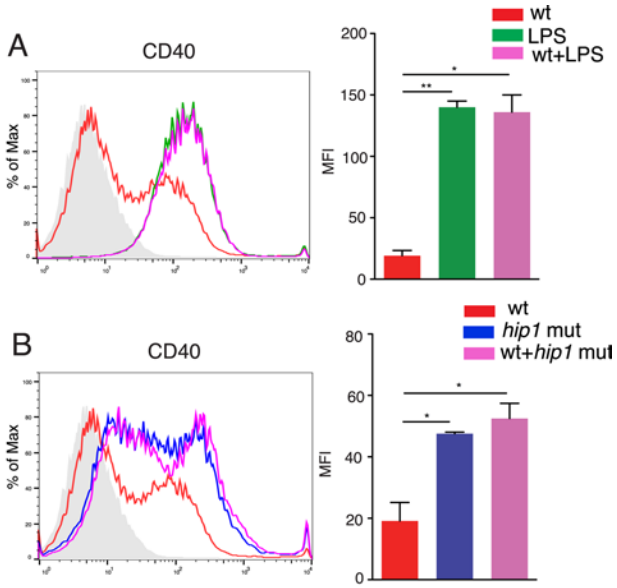

Figure 3. Wild type *M. tuberculosis* does not block LPS- or *hip1* mutant-induced DC maturation.

C57BL/6J BMDCs were exposed to medium alone or 1 μg/ml LPS in the presence or absence of heat-killed wild type *M. tuberculosis* at MOI=5 (A) or infected with heat-killed wild type, *hip1* mutant or wild type+*hip1* mutant (1:1) at MOI=5 for 24 hours (B). DCs were labeled with anti CD11c-APC and anti CD40-PE. Representative histograms and median PE fluorescence intensity 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, *, $p<0.05$; **, $p<0.01$.

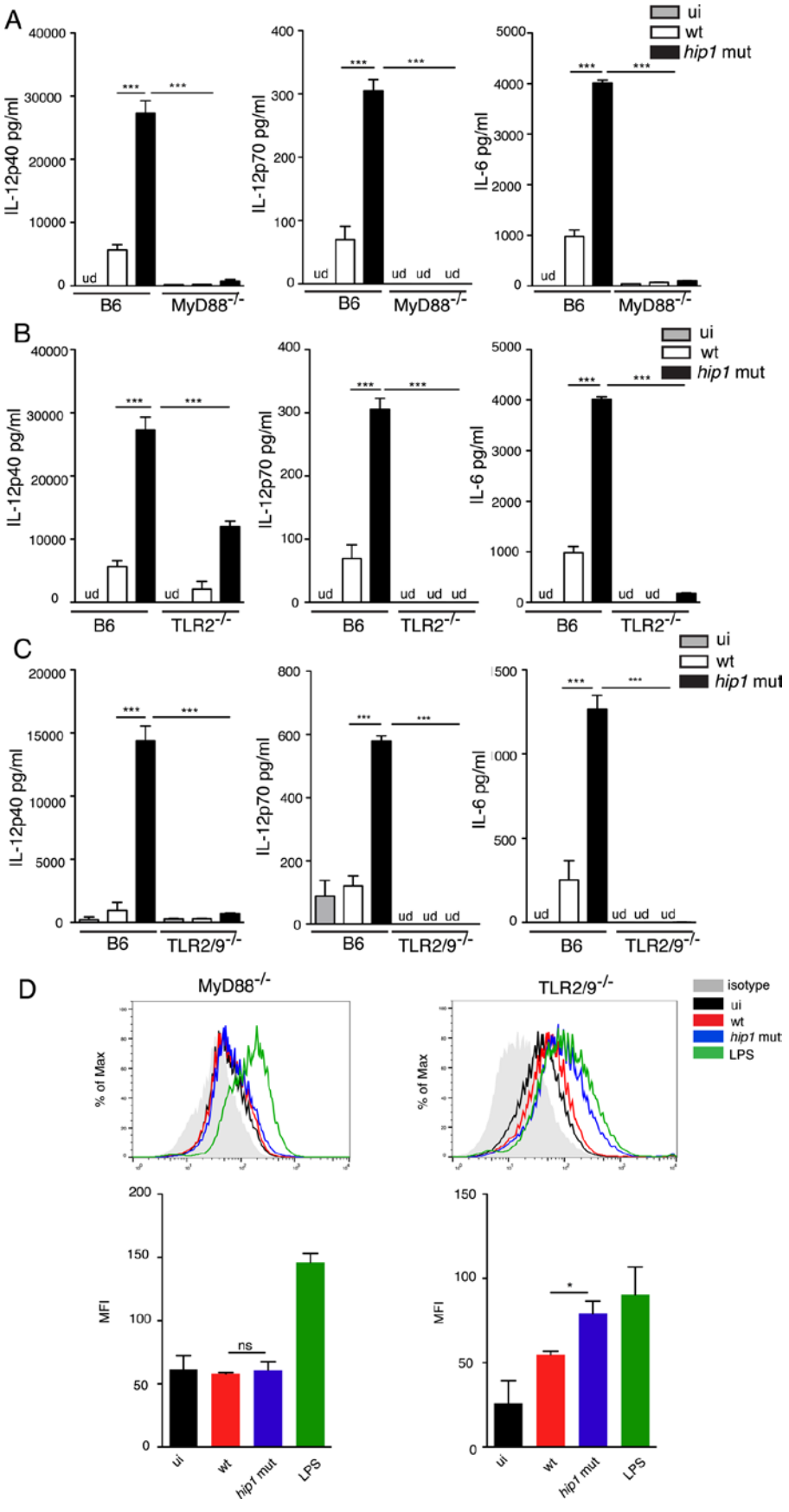
A
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Figure 4. *M. tuberculosis* impairment of DC activation and maturation requires MyD88- and TLR2/9-dependent pathways.

Purified BMDCs from C57BL/J6 and MyD88$^{-/-}$ (A), TLR2$^{-/-}$ (B) *or* TLR2/9$^{-/-}$ (C) mice were infected with heat-killed wild type or the *hip1* mutant at MOI=5 for 24 hours and cell-free supernatants were assayed for IL-6, IL-12p40 and IL-12p70 by ELISA. (D) Infected BMDCs from MyD88$^{-/-}$ and TLR2/9$^{-/-}$ were labeled with anti-CD11c-APC and anti-CD40-PE. Representative histograms and median PE fluorescence intensity for CD11c^{+} cells are shown. Data are representative of three (A and B) or two (C and D) independent experiments. Values are presented as mean +/- SD, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

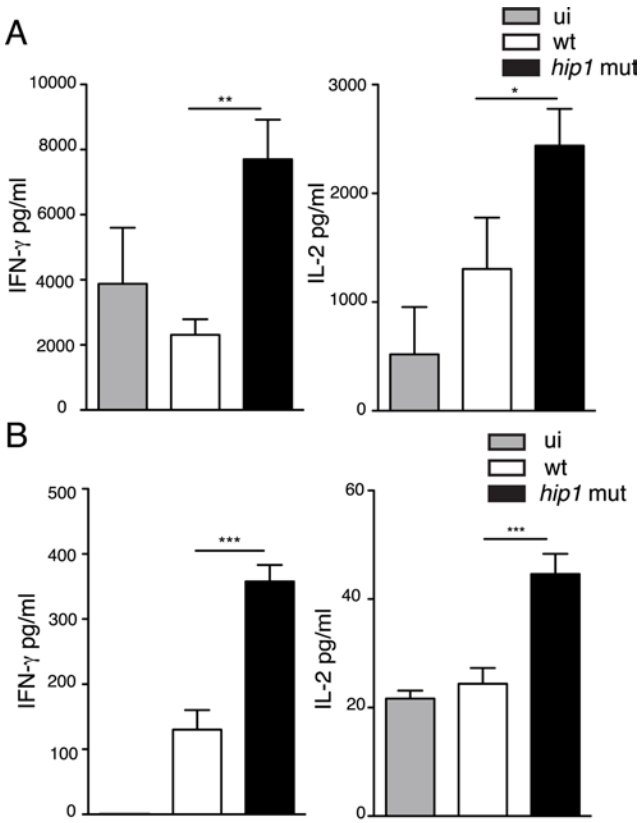


Figure 5. *hip1* mutant augments antigen presentation by BMDCs.

Purified C57BL/6J BMDCs in medium alone or infected with heat-killed wild type or *hip1* mutant at MOI=10 for 24 hours and then co-cultured with ESAT-$6_{1\text{-}20}$ peptide and ESAT-6 specific transgenic CD4 T cells (A) or $OVA_{323\text{-}339}$ peptide and OT-II specific transgenic CD4 T cells for 3 days (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. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

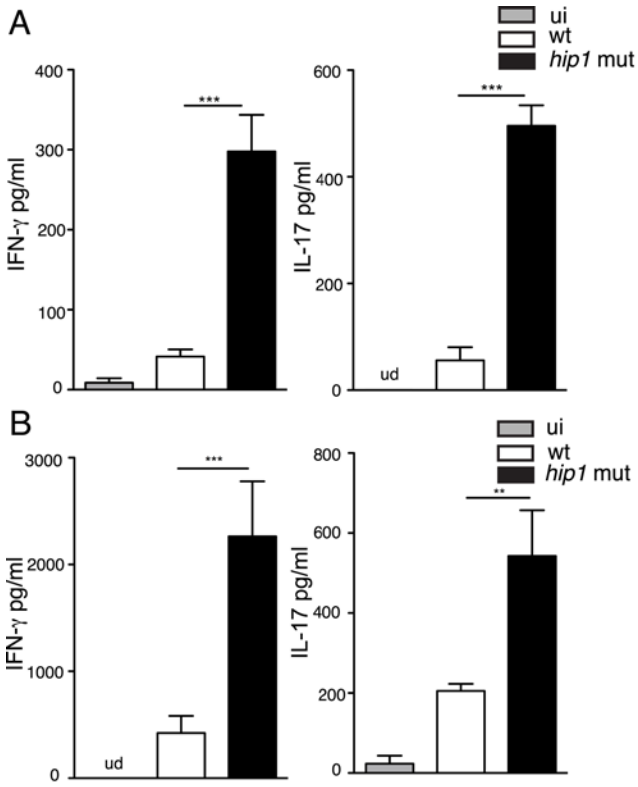


Figure 6. *M. tuberculosis*-DC interactions modulate CD4 T cell differentiation *in vitro* and *in vivo*.

(A) Purified C57BL/6J BMDCs in media alone or infected with heat-killed wild type or the *hip1* mutant at MOI=10 for 24h were co-cultured with CD4 T cells from C57BL/6J mice for 3 days. 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 wild type or the *hip1* mutant at three weeks post infection, and cells were stimulated with 10μg/ml ESAT-6_{1-20} peptide for 48 hours. 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. **, $p<0.01$; ***, $p<0.001$.

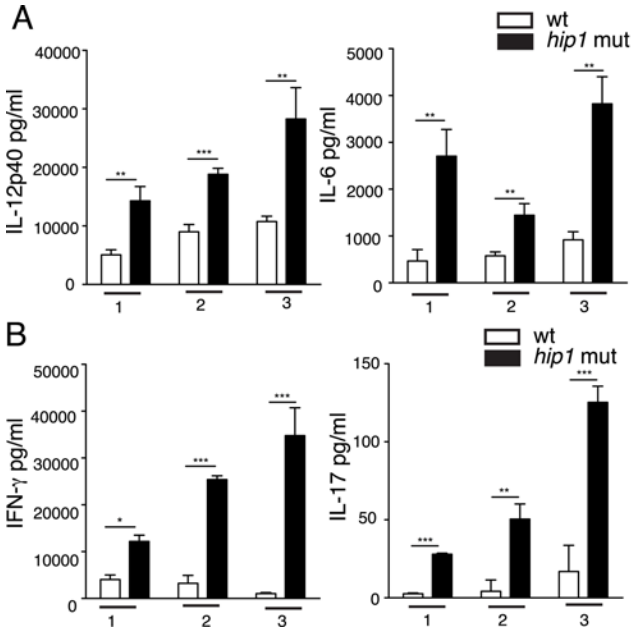
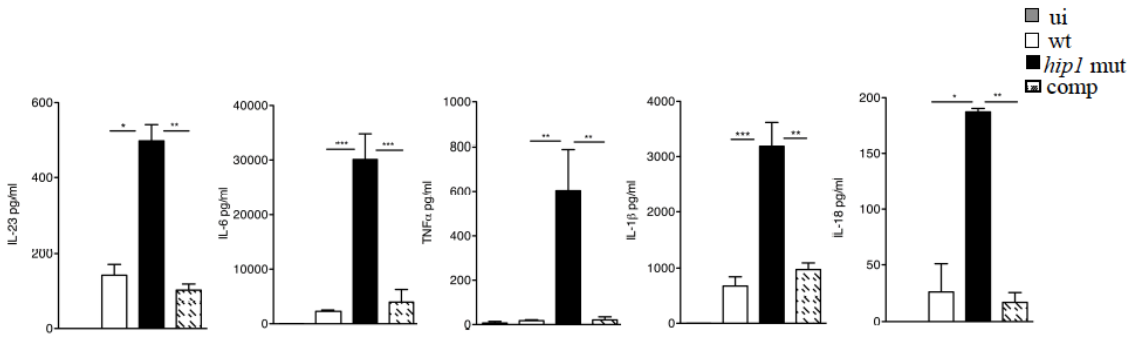


Figure 7. ***M. tuberculosis*** **interacts with human DCs to impair T cell differentiation.**

(A) Human MDCs were infected with heat-killed wild type or *hip1* mutant *M. tuberculosis* at MOI=10 for 24 hours. Cell free supernatants were assayed for IL-12p40 and IL-6 by ELISA (B) MDCs infected with heat-killed wild type or *hip1* mutant were co-cultured with autologous lymphocytes isolated from the corresponding donors for 3 days. Cell free supernatants were assayed for IFN-γ and IL-17 by ELISA. Data from 3 healthy donors are represented. Values are presented as mean +/-SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



Supplemental Figure 1. **Enhanced IL-23, IL-6, TNF-α, IL-1β, and IL-18 responses from DCs infected with live *hip1* mutant.**

C57BL/6J BMDCs were exposed to medium alone or infected with live wild type, *hip1* mutant, or *hip1* mutant complemented with Hip1 (comp) at MOI 5. Cell free supernatants were collected 24 hours post infection and assayed for IL-23, IL-6, TNF-α, IL-1β, and IL-18 by ELISA. Data are representative of two independent experiments. Values are presented as mean plus SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Chapter III

Engaging the CD40-CD40L pathway augments T-helper cell responses and improves control of *Mycobacterium tuberculosis* infection

Chapter adapted from:

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Abstract

Mycobacterium tuberculosis (*M. tuberculosis*) impairs dendritic cell (DC) functions and induces suboptimal antigen-specific CD4 T cell immune responses that are poorly protective. Mucosal T-helper cells producing IFN-γ (Th1) and IL-17 (Th17) are important for protecting against tuberculosis (TB), but the mechanisms by which DCs generate antigen-specific T-helper responses during *M. tuberculosis* infection are not well defined. We previously reported that *M. tuberculosis* impairs CD40 expression on DCs and restricts Th1 and Th17 responses. We now demonstrate that CD40-dependent co-stimulation is required to generate IL-17 responses to *M. tuberculosis*. CD40-deficient DCs were unable to induce antigen-specific IL-17 responses after *M. tuberculosis* infection despite the production of Th17-polarizing innate cytokines. Disrupting the interaction between CD40 on DCs and its ligand CD40L on antigen-specific CD4 T cells, genetically or via antibody blockade, significantly reduced antigen-specific IL-17 responses. Importantly, engaging CD40 on DCs with a multimeric CD40 agonist (CD40LT) enhanced antigen-specific IL-17 generation in *ex vivo* DC-T cell co-culture assays. Further, intratracheal instillation of *M. tuberculosis*-infected DCs treated with CD40LT significantly augmented antigen-specific Th17 responses *in vivo* in the lungs and lung-draining lymph nodes of mice. Finally, we show that boosting CD40-CD40L interactions promoted balanced Th1/Th17 responses in a setting of mucosal DC transfer, and conferred enhanced control of lung bacterial burdens following aerosol challenge with *M. tuberculosis*. Our results demonstrate that CD40 co-stimulation by DCs plays an important role in generating antigen-specific Th17 cells and targeting the CD40-CD40L pathway represents a novel strategy to improve adaptive immunity to TB.

Introduction

Critical to the success of *Mycobacterium tuberculosis* (*M. tuberculosis*) as a pathogen is its ability to manipulate host innate and adaptive immune responses to its benefit. Despite the development of antigen-specific T cell responses following infection, *M. tuberculosis* is able to persist within the host, indicating that *M. tuberculosis*-specific T cell immunity is suboptimal and ineffective at eliminating the pathogen [444, 477]. Indeed, several studies have shown that mice infected with *M. tuberculosis* exhibit delayed initiation of antigen-specific CD4 T cell responses, which is preceded by delayed migration of *M. tuberculosis*-containing dendritic cells (DCs) from the lung to draining lymph nodes [223, 242, 243]. Moreover, although IFN-γ and T-helper 1 (Th1) responses are important for controlling infection, they are not sufficient to eradicate bacteria and do not protect against developing tuberculosis (TB) [429, 478, 479]. Recently, IL-17 and Th17 responses have emerged as important for protective immunity to TB [286-290, 293, 294, 480]. Studies in mice suggest that early induction of IL-17 in the lung promotes control of mycobacterial growth, and balanced Th1/Th17 responses in the lung have been reported to be more effective [481-483]. We previously reported that an avirulent *hip1* (Hydrolase important for pathogenesis 1; Rv2224c) mutant *M. tuberculosis* strain induced significantly higher IL-17 and IFN-γ responses compared to infection with wild type *M. tuberculosis* due to enhanced functions of infected DCs [102, 104]. Together, these studies suggest that wild type *M. tuberculosis* subverts DCs to prevent optimal T-helper responses and that augmenting DC functions during infection may be beneficial for improving protective immunity. While several studies have reported that *M. tuberculosis* manipulation of DC functions leads to suboptimal Th1 responses [104, 219,

227, 484], we lack insights into Th17 generation during *M. tuberculosis* infection. To gain insight into host pathways involved in generating Th17 responses during *M. tuberculosis* infection, we sought to define the molecular mechanisms underlying Th17 responses following *M. tuberculosis* infection of DCs.

As the primary antigen-presenting cells in the immune system, DCs are instrumental in shaping adaptive immunity and determining the types of antigen-specific T-helper subsets that are generated in response to infection. Upon phagocytosis of the pathogen, DCs present pathogen-derived antigens to naïve CD4 T cells, provide critical co-stimulatory signals and produce cytokines; these signals initiate antigen-specific T-helper cell activation and polarization towards specific subsets [235, 237, 485]. However, beyond the role of cytokines such as IL-1β, IL-6, and IL-23 in polarizing and committing antigen-specific CD4 T cells towards a Th17 phenotype, the DC-T cell interactions underlying Th17 polarization during *M. tuberculosis* infection are poorly defined. We previously showed that eliminating Hip1-dependent immune evasion mechanisms in *M. tuberculosis* enhanced the capacity of DCs to induce Th17 responses and was accompanied by significantly higher expression of the co-stimulatory molecule, CD40, on infected DCs [104]. Because co-stimulation of naïve T cells in the context of cognate interactions between DCs and T cells is critical for optimal activation and differentiation of antigen-specific T cells, these data suggested that impaired CD40-dependent co-stimulation during wild type *M. tuberculosis* infection may lead to suboptimal Th17 responses in TB. CD40 has previously been implicated in generating Th1 responses during *M. tuberculosis* infection [486], but its role in the polarization of the Th17 subset

during infection is not defined. We therefore sought to investigate the contribution of the CD40 co-stimulatory pathway in Th17 generation during *M. tuberculosis* infection and determine the effects of augmenting CD40 co-stimulation on bacterial control. In this study, we show that CD40 expression on DCs is required for the generation of IL-17 responses to *M. tuberculosis* infection and that interaction between CD40 on DCs and CD40L on CD4 T cells is critical for generating antigen-specific IL-17 responses. Importantly, we found that engaging CD40 on DCs via crosslinking with a multimeric CD40 agonist reagent (CD40LT) significantly enhanced antigen-specific IL-17 responses to *M. tuberculosis*. Further, intratracheal instillation of *M. tuberculosis*-infected DCs treated with CD40LT led to significant enhancement of antigen-specific Th17 responses in the lungs and mediastinal lymph nodes (MLN) of mice, showing that engaging the CD40-CD40L pathway can overcome suboptimal Th17 responses to *M. tuberculosis in vivo*. Finally, we show that CD40 engagement in the setting of a DC transfer model enhances control of *M. tuberculosis* following aerosol challenge. Our results demonstrate that the CD40-CD40L pathway is critical for generating IL-17 responses and that targeting this co-stimulatory pathway represents a novel strategy to potentially improve protection against TB.

Results

CD40 on DCs is required for the generation of antigen-specific IL-17 responses during *M. tuberculosis* infection.

To test whether CD40 expression is required for differentiation of naïve CD4 T cells into IL-17-producing cells in response to *M. tuberculosis* infection, we used DC-T cell co-

culture assays as previously described [104]. We infected bone marrow derived DCs from wild type C57BL/6 mice (B6) or from *CD40*$^{-/-}$ mice for 24 hours, followed by co-culture with purified naïve TCR-transgenic (Tg) CD4 T cells isolated from OT-II mice in the presence of $OVA_{323–339}$ peptide (Fig 1A). Supernatants were harvested 72 hours after co-culture and assayed for IL-17, IL-2 and IFN-γ by ELISA. *M. tuberculosis*-infected DCs from B6 mice induced increasing levels of IL-17, IL-2 and IFN-γ cytokines with increasing concentrations of peptide. In contrast, *CD40*$^{-/-}$ DCs were significantly impaired in their ability to induce IL-17-producing cells in response to *M. tuberculosis*, but retained the capacity to induce IFN-γ and IL-2 (Fig 1A). These data indicate that CD40 is specifically required to generate antigen-specific IL-17 responses.

To assess whether the defect in IL-17 production was specific for CD40 deficiency, we examined the contribution of the co-stimulatory molecules CD80 and CD86, which are known to be essential for IL-2 production and are required for optimal T cell proliferation [487, 488]. While DCs that were doubly-deficient in CD80 and CD86 were severely impaired in IL-2 production, their ability to induce antigen-specific IL-17 responses were comparable to DCs from B6 mice (Fig 1B) and did not exhibit the defective IL-17 responses observed in *CD40*$^{-/-}$ DCs. These data indicate that CD40-dependent co-stimulation plays an essential and specific role in the generation of IL-17 responses to *M. tuberculosis*.

Cytokines produced by infected DCs are known to be critical for polarizing antigen-specific CD4 T cell subsets [481, 489]. Since IL-6, IL-1β, and TGF-β have been shown

to induce Th17 polarization, we sought to assess whether defective IL-17 responses seen in *M. tuberculosis*-infected *CD40*$^{-/-}$ DCs was due to defects in their ability to produce innate cytokines following *M. tuberculosis* infection. However, levels of IL-6, IL-1β, and IL-12 produced by DCs from *CD40*$^{-/-}$ mice were comparable to the levels seen in DCs from B6 mice (Fig 1C) and bioactive TGF-β was undetectable in all culture conditions. Thus, the inability of *CD40*$^{-/-}$ DCs to induce IL-17 responses is not due to impaired innate cytokine responses, suggesting that interaction of CD40 expressed on DCs with its ligand, CD40L (CD154), may be necessary for production of IL-17 by CD4 T cells following *M. tuberculosis* infection.

CD40-CD40L interaction is critical for inducing antigen-specific IL-17 responses to *M. tuberculosis* infection.

CD40L is expressed on antigen-activated T cells and binding of CD40 with CD40L provides accessory co-stimulatory signals that are necessary for optimal activation and differentiation of antigen-specific T cells. In order to determine whether interaction of CD40 with CD40L was required for IL-17 generation, we carried out co-culture assays using antigen-specific CD4 T cells isolated from OT-II mice crossed to mice lacking CD40L (*CD40lg*$^{-/-}$ x OT-II). This allowed us to test whether CD40L on T cells was required for IL-17 generation in a setting where CD40 expression on DCs remained intact. We found that *CD40lg*$^{-/-}$ CD4 T cells were attenuated in their ability to generate IL-17 responses after co-culture with *M. tuberculosis*-infected DCs (Fig 2A), concordant with the defective IL-17 response seen with *CD40*$^{-/-}$ DCs (Fig 1). Interestingly, *CD40lg*$^{-/-}$ T cells also displayed attenuated IFN-γ and IL-2 responses (S1 Fig), which suggests that

lack of CD40L leads to broader defects in T cell responses compared to the absence of CD40. These results show that both CD40 and CD40L are required for optimal IL-17 generation. To further extend our genetic knockouts studies, we carried out co-culture assays in which we blocked CD40-CD40L interactions using saturating doses of a non-agonistic, anti-CD40L monoclonal antibody (clone MR1). This antibody has been shown to successfully block CD40-CD40L interactions *in vitro* (S2 Fig) and *in vivo* [490]. Blockade of CD40-CD40L interaction between *M. tuberculosis*-infected DCs and CD40L-replete antigen-specific CD4 T cells significantly reduced antigen-specific IL-17 responses (Fig 2B). Together, these data show that interaction between CD40 and CD40L is critical for production of IL-17 by CD4 T cells during *M. tuberculosis* infection.

Engaging CD40 on DCs enhances antigen-specific IL-17 responses.

The requirement for the CD40-CD40L pathway in IL-17 generation suggested that boosting interactions between CD40 and CD40L could serve as a tool to augment IL-17 responses. To exogenously engage CD40 on *M. tuberculosis*-infected DCs, we utilized a multimeric CD40 agonist in which two trimeric CD40L constructs are artificially linked (CD40L trimers; CD40LT). The CD40LT reagent effectively aggregates and activates CD40 independently of T cells. Addition of CD40LT to *M. tuberculosis*-infected B6 DCs produced enhanced levels of IL-12 (Fig 3A), consistent with previous reports showing IL-12 induction after CD40 engagement [491]. Importantly, treatment with CD40LT significantly enhanced production of IL-6 and IL-23, which are key cytokines for Th17 polarization and expansion (Fig 3A). IL-1β, which also promotes Th17 differentiation in combination with IL-6 and IL-23, was not altered by treatment with CD40LT (Fig 3A).

Moreover, CD40LT-treated *M. tuberculosis*-infected DCs induced significantly higher levels of IL-17 from co-cultured ESAT-6 TCR-Tg CD4 T cells compared to *M. tuberculosis*-infected DCs lacking CD40 engagement (Fig 3B). In contrast, CD40LT-treatment did not alter production of IFN-γ from antigen-specific CD4 T cells *in vitro* (Fig 3B). These data show that CD40 engagement augments antigen-specific IL-17 generation.

Co-stimulatory signals synergize with antigen-specific signals downstream of T cell receptor (TCR) ligation to promote full activation of T cells. Absence of signaling through the CD80/86-CD28 co-stimulatory pathway, for example, results in suboptimal T cell activation and anergic responses [487, 488, 492, 493]. CD28 signaling is thought to function by lowering the T cell activation threshold, thus facilitating optimal T cell activation and IL-2 production. To investigate whether CD40 engagement on DCs similarly impacts the activation threshold of *M. tuberculosis*-specific T cells and whether this, in turn, influences IL-17 production, *M. tuberculosis*-infected DCs were either treated with CD40LT or left untreated, pulsed with increasing concentrations of ESAT-$6_{1–20}$ peptide, and co-cultured with ESAT-6 TCR-Tg CD4 T cells. We found that CD40LT-treated DCs displayed an enhanced capacity to induce IL-17 responses at all antigen doses compared to untreated conditions (Fig 3C). The ability of *M. tuberculosis*-infected DCs to induce IL-17 at lower concentrations of peptide after CD40LT treatment suggests that signals induced by CD40 engagement lowers the threshold for antigen-specific production of IL-17. Thus, CD40-dependent co-stimulation may serve to

overcome suboptimal generation of IL-17 responses elicited early in *M. tuberculosis* infection when antigen levels are low.

In order to dissect the relative contributions of Th17-polarizing cytokines and CD40-CD40L interaction on IL-17 responses, *M. tuberculosis*-infected DCs were treated with or without CD40LT and then co-cultured with ESAT-6 TCR-Tg CD4 T cells in the presence of the MR1 CD40L-blocking antibody as described in Fig 2B. Interestingly, antibody blockade of CD40-CD40L interaction significantly decreased antigen-specific IL-17 responses even in the presence of CD40LT (Fig 3D). These data suggest that exogenous engagement of CD40 on DCs that results in enhanced production of Th17-polarizing cytokines is not sufficient for generating antigen-specific IL-17 responses in a setting where CD40 cannot interact with CD40L on antigen-specific CD4 T cells.

CD40 engagement of *M. tuberculosis*-infected DCs induces antigen-specific Th17 *in vivo*.

Induction of early IL-17 responses on mucosal surfaces of the lung is thought to be important for immunity to *M. tuberculosis* and inducing balanced *M. tuberculosis*-specific Th1/Th17 responses may enhance protective immunity. To determine whether CD40 engagement on DCs can enhance the induction of *M. tuberculosis*-specific lung Th17 responses *in vivo*, we utilized a mucosal transfer approach via intratracheal instillation of DCs. This approach allows for targeted manipulation of *M. tuberculosis*-infected DCs without potential confounding from off-target effects such as CD40 engagement of alveolar macrophages. Transferred DCs have been shown to prime

adoptively transferred *M. tuberculosis*-antigen-specific T cells in lymph nodes and lungs of mice by 3 days post-intratracheal instillation [227].

We adoptively transferred naïve CD45.2$^+$ ESAT-6 TCR-Tg CD4 T cells into CD45.1$^+$ congenic hosts. The next day, we transferred DCs infected with *M. tuberculosis* in the presence or absence of CD40LT by intratracheal instillation (Fig 4A). At 6 and 12 days after DC transfer, we assessed Th17 responses in the lungs and MLN by determining the expression of IL-17 and RORγt in CD45.2$^+$ ESAT-6-specific CD4 T cells by intracellular cytokine staining (ICS) and flow cytometry. Engaging CD40 on *M. tuberculosis*-infected DCs using CD40LT enhanced the frequency of ESAT-6-specific RORγt$^+$IL-17$^+$ T cells in the lungs (Fig 4B) and MLN (Fig 4C). Notably, the majority of IL-17$^+$ cells expressed RORγt, the transcription factor that determines Th17 lineage commitment [494], indicating that CD40LT-treated *M. tuberculosis*-infected DCs polarized CD4 T cells into Th17 cells.

Enhanced antigen-specific Th17 responses in the lungs and MLN of mice following transfer of CD40LT-engaged *M. tuberculosis*-infected DCs or *hip1* mutant *M. tuberculosis*-infected DCs

To determine Th1 and Th17 responses in the lungs and MLN of mice at 6 and 12 days after intratracheal instillation of DCs, we assessed IFN-γ and IL-17 production in CD45.2$^+$ ESAT-6 TCR-Tg T cells by flow cytometry (Fig 5A). Transfer of *M. tuberculosis*-infected DCs that were treated with CD40LT resulted in a greater expansion of ESAT-6 TCR-Tg CD4 T cells compared to *M. tuberculosis* DCs that did not receive

exogenous CD40LT, and was comparable to the expansion of ESAT-6 TCR-Tg CD4 T cells in response to *hip1* mutant *M. tuberculosis*-infected DCs (Fig 5B). Moreover, transfer of CD40LT-treated, *M. tuberculosis*-infected DCs significantly enhanced the frequencies of antigen-specific Th17 cells in lungs and MLN compared to *M. tuberculosis*-infected DCs alone and was comparable to the Th17 frequencies elicited by *hip1* mutant *M. tuberculosis*-infected DCs (Fig 5C). We also observed higher frequencies of antigen-specific IFN-γ^+ CD4 T cells in the lung, but not MLN, on day 6 post-intratracheal transfer of either *M. tuberculosis*-infected CD40LT-treated DCs or *hip1* mutant *M. tuberculosis*-infected DCs compared to their untreated counterpart (Fig 5D). 12 days after intratracheal instillation of DCs, CD45.2$^+$ ESAT-6-specific IFN-γ responses in the lungs were comparable, suggesting that DCs that did not receive CD40LT were delayed in inducing Th1 responses relative to *M. tuberculosis*-infected CD40LT-treated and *hip1* mutant *M. tuberculosis*-infected DCs. Interestingly, antigen-specific CD4 T cells producing IL-17 and IFN-γ were mutually exclusive populations and double producing cells were not detected. These data demonstrate that engagement of the CD40 pathway can overcome deficits in Th17 generation during *M. tuberculosis* infection and leads to enhanced antigen-specific Th1 and Th17 responses *in vivo*.

CD40 engagement of DCs enhances control of *M. tuberculosis* infection.

DCs loaded with *M. tuberculosis* antigens have been previously shown to confer better anti-mycobacterial immunity than BCG (Bacillus Calmette-Guérin) vaccination in mouse models [495, 496]. Therefore, DC-based vaccination provides a useful model to study the impact of boosting CD40-engagement on priming of antigen-specific T cell pools and on

the control of *M. tuberculosis* infection *in vivo*. We exposed DCs to heat-killed (HK) *M. tuberculosis* followed by treatment with CD40LT. DCs stimulated with HK *M. tuberculosis* and CD40LT were equivalent to *ex vivo* assays using live *M. tuberculosis* (S3 Fig). Comparison groups included transfer of uninfected DCs, DCs stimulated with HK wild type *M. tuberculosis* or with HK *hip1* mutant *M. tuberculosis*. Upon transfer of antigen-loaded DCs into mouse lungs by intratracheal instillation, we assessed immune responses generated by transferred DCs by measuring the activation of endogenous CD4 T cell responses and frequencies of Th17 and Th1 cells in the lungs of mice 6 and 12 days after DC transfer. 15 days after DC transfer, we challenged mice with low-dose aerosolized *M. tuberculosis*. At 5 weeks post-challenge (day 50), we determined lung bacterial burden and *M. tuberculosis*-specific Th1 and Th17 responses (Fig 6A).

CD40LT treatment induced significantly higher frequencies of activated $CD44^+$ CD4 T cells (Fig 6B) and higher frequencies of lung IL-17^+ CD4 T cells 6 and 12 days after DC transfer (Fig 6C). IFN-γ^+ CD4 T cell frequencies were higher on day 6 in mice receiving CD40LT-treated DCs compared to untreated *M. tuberculosis*-DCs, but were comparable by day 12. As expected, transfer of *hip1* mutant *M. tuberculosis*-stimulated DCs induced robust Th17 and Th1 responses in the lungs of mice on day 6 and day 12 post-DC transfer. Following aerosol challenge with low dose *M. tuberculosis* 15 days after intratracheal transfer of DCs, we assessed bacterial burden in the lungs of mice 5 weeks after challenge by plating for CFU. As shown in Fig 6D, transfer of DCs stimulated with HK *M. tuberculosis* resulted in significant reductions in lung bacterial burden at day 50 compared to transfer of DCs alone. Interestingly, CD40LT treatment reduced bacterial

burden even further, showing that boosting CD40-CD40L interactions could overcome pathogen-mediated impairment of CD40 co-stimulation and promote enhanced anti-mycobacterial immunity. Notably, transfer of DCs exposed to *hip1* mutant *M. tuberculosis* also showed comparable reductions in bacterial burden. These results are consistent with our previous report showing that *hip1* mutant *M. tuberculosis* inherently induces superior DC responses compared to wild type *M. tuberculosis*, i.e., significantly higher induction of Th1- and Th17-polarizing cytokines, higher expression of CD40, enhanced antigen presentation and balanced Th1/Th17 responses [104]. To assess *M. tuberculosis*-specific Th1 and Th17 responses in the lungs of mice post-challenge, we stimulated lung cells *ex vivo* with *M. tuberculosis* whole cell lysate (WCL) and determined IFN-γ^+ and IL-17^+ CD4 T cell frequencies by flow cytometry. We found significantly enhanced Th17 responses in mice that intratracheally received CD40LT-treated DCs or *hip1* mutant *M. tuberculosis*-stimulated DCs compared to those that received *M. tuberculosis*-stimulated DCs. However, there was no discernible difference between the groups in terms of lung CD4 IFN-γ responses (Fig 6E). Importantly, lung IL-17 responses inversely correlated with bacterial burden, while there was no significant correlation between IFN-γ responses and lung bacterial burden (Fig 6F). Our data show that we can improve protection against *M. tuberculosis* challenge by overcoming *M. tuberculosis*-mediated impairments in CD40 co-stimulation.

Discussion

The findings in this study identify the CD40-CD40L pathway as a critical mechanism for the generation of antigen-specific Th17 responses and highlight the importance of DC-T

cell crosstalk in immunity to *M. tuberculosis* infection. Importantly, we provide insights into improving adaptive immunity to TB by augmenting the functions of DCs and show that exogenously engaging CD40 on DCs significantly enhances control of *M. tuberculosis* burden in the lungs of infected mice.

Co-stimulatory signals provided by antigen presenting cells such as macrophages and DCs are critical for full activation of naïve antigen-specific CD4 T cells and promote their rapid expansion into cytokine-producing effector cells, which exert their antimicrobial functions at the site of infection. While differentiation of activated CD4 T cells into IFN-γ^+ Th1 subsets is relatively well understood, the molecular mechanisms underlying the generation of Th17 cells, particularly during *M. tuberculosis* infection, are less clear. Moreover, the mechanisms by which *M. tuberculosis* induces delayed, suboptimal T cell immunity, which enables the pathogen to successfully evade adaptive immunity and persist within the host, remain poorly understood. Several studies, including our own, have shown that *M. tuberculosis* impairs antigen presentation in infected DCs and dampens production of Th17-polarizing cytokines, such as IL-6, IL-23 and IL-1β [103-105, 219, 466, 484]. However, very little was known about the role of co-stimulatory pathways in driving Th17 development in TB prior to our study. Our work shows that innate cytokines are important for the generation of IL-17 responses (Fig 1 and 3) and is consistent with other studies showing a critical role for CD40-dependent IL-6 and IL-23 in the induction and expansion of Th17 cells [497-499]. Interestingly, our results show that blocking CD40-CD40L interaction with MR1 attenuates IL-17 responses to *M. tuberculosis*-infected DCs despite treatment of DCs with CD40LT,

which suggests that optimal induction of IL-17 to *M. tuberculosis* infection requires CD40-CD40L interaction (Fig 3D). However, studies have shown that exogenous addition of supraphysiological levels of Th17-polarizing cytokines can drive *CD40*$^{-/-}$ DCs to induce IL-17 [497]. Our data suggest that co-stimulatory interactions between *M. tuberculosis*-infected DCs and T cells are required for optimal generation of IL-17 responses. In addition, localization of CD4 T cells in close proximity to infected DCs is likely to be an important determinant of the type of antigen-specific CD4 T cells mobilized after infection. Recent work has demonstrated that uninfected MLN-resident DCs acquire antigen from infected lung DCs and can prime *M. tuberculosis*-specific CD4 T cells to produce IFN-γ [227]. It is possible that while MLN-resident DCs acquire antigen, their maturation status and co-stimulatory capacity may be suboptimal without *M. tuberculosis* infection and, thus, not amenable to generating CD4 T cell responses beyond IFN-γ. Moreover, within the Th1 subset, studies have shown distinct IFN-γ-producing CD4 T cells in the vasculature and parenchyma of *M. tuberculosis*-infected mice [250]. However, localization of Th17 cells within lung compartments and the role of lung-specific DC subsets in driving the polarization of Th1 and Th17 during *M. tuberculosis* infection are poorly understood. Our study uses bone marrow derived DCs and therefore the extent to which our experiments model *in vivo*-generated lung DCs needs to be investigated further. Overall, our data showing that CD40-CD40L interaction is required for optimal Th17 generation in response to *M. tuberculosis* and that boosting CD40-CD40L interactions augments Th1 and Th17 responses suggests that restriction of co-stimulatory pathways is an important virulence mechanism used by *M. tuberculosis* for inducing suboptimal T-helper responses that benefits the pathogen.

Our finding that exogenous induction of CD40-mediated co-stimulation, via CD40LT treatment, is able to elicit IL-17 production at lower concentrations of peptide stimulation (Fig 3) than by *M. tuberculosis*-infected DCs alone leads to an interesting speculation. In early stages of *M. tuberculosis* infection, low levels of antigen in the lung, combined with impaired CD40 induction on *M. tuberculosis*-infected DCs, likely results in suboptimal co-stimulation of naïve CD4 T cells and therefore suboptimal and delayed induction of Th17 responses. However, engaging the CD40-CD40L pathway and promoting interactions between these two molecules likely facilitates better Th17 generation, even when lung antigen levels are low during early stages of infection. It has been reported that higher peptide concentrations are required for inducing Th17 polarization compared to Th1 in a study that examined activation of Smarta-2 TCR-Tg T cells [497]. Efficient CD40-mediated co-stimulation may serve to lower the threshold for T cell activation and Th17 polarization, and overcome the need for high antigen loads. Interestingly, *hip1* mutant *M. tuberculosis*-loaded DCs induced higher Th17 responses compared to wild type *M. tuberculosis*, even without CD40LT treatment (Fig 5), and enhanced protection (Fig 6). We have previously shown that *hip1* mutant *M. tuberculosis* induces high levels of CD40 and Th17 responses [104]. Therefore, it is likely that elimination of Hip1 results in efficient CD40-dependent co-stimulation, and bypasses the need for exogenous engagement of the CD40-CD40L pathway. However, we do not rule out the possibility that *hip1* mutant *M. tuberculosis* activates alternate DC pathways that promote robust T cell immunity and further investigation into the common and exclusive immune pathways activated by CD40LT and *hip1* mutant *M. tuberculosis* is of interest.

Previous work by Demangel et al demonstrated that lung Th1 responses can be augmented by transferring BCG-infected DCs in conjunction with agonistic anti-CD40 mAb [491]. However, this approach did not significantly restrict *M. tuberculosis* lung burdens following challenge compared to BCG-infected DCs alone. We speculate that the use of heat killed *M. tuberculosis* in our study as well as the timing of the aerosol challenge at 2 weeks after intratracheal instillation of DCs (in contrast to 2 days post-DC transfer in the Demangel et al study) likely established higher frequencies of antigen-specific Th17 and Th1 precursors, leading to better control of *M. tuberculosis*. Additionally, recent work by Griffiths et al showed that mice vaccinated with BCG followed by intratracheal delivery of Ag85B peptide loaded DCs, one day before and four days after challenge with *M. tuberculosis* HN878, had enhanced bacterial control [239]. Interestingly, they achieved similar reductions in bacterial burden after administration of TLR-9 and CD40 agonists together with Ag85B peptide and also showed higher levels of lung IFN-γ and IL-17 responses. The study by Griffiths et al complements our results, which provide mechanistic evidence that the CD40-CD40L pathway is critical for the generation of *M. tuberculosis*-specific lung Th17 responses. While IL-17 responses appear to be required for resistance against infection with the hypervirulent *M. tuberculosis* HN878 strain, IL-17 may also be important for generating efficacious vaccine-induced immunity. Our data show an association between enhanced IL-17 responses and lower bacterial burden after aerosol *M. tuberculosis* challenge (Fig 6F), but do not directly link Th17 responses with increased protection. While we have demonstrated that engaging CD40 on DCs confers enhanced Th17 responses in the lungs

in a setting of mucosal DC transfer, we also observed augmented Th1 responses *in vivo* prior to challenge (Fig 5 and 6). Therefore, our data demonstrate that CD40 engagement on DCs improves adaptive immunity to TB, likely due to induction of a balanced Th1/Th17 response. Although we have not shown that the Th17 cells generated in the lung following transfer of DCs stimulated with HK *M. tuberculosis* + CD40LT or HK *hip1* mutant *M. tuberculosis* are directly responsible for the increased protection seen in Fig 6, our studies provide a platform to further investigate the potential of designing vaccination strategies that overcome *M. tuberculosis* immune evasion, either by augmenting CD40 co-stimulation and/or deletion of immunomodulatory factors such as *hip1* (in BCG or other live attenuated *M. tuberculosis* vaccine strains) that impair DC functions.

Our studies on understanding the role of CD40 co-stimulation in Th17 responses significantly extend our understanding of the CD40-CD40L pathway during infection, as previous investigations studying this pathway in TB as well as in other infections have focused on Th1 responses. CD40 has been shown to promote Th1 responses by synergizing with TLR signaling to induce high levels of IL-12 production from antigen presenting cells in several infections [491, 500, 501]. While our own data show that *CD40*$^{-/-}$ DCs and CD40LT-treated DCs infected with *M. tuberculosis* do not affect IFN-γ responses in a closed system *in vitro* (Fig 1 and 3), treatment of *M. tuberculosis*-infected DCs with CD40LT does augment IFN-γ responses in the lungs 6 days after intratracheal instillation of DCs (Fig 5 and 6), suggesting that engaging the CD40-CD40L pathway enhances both Th1 and Th17 responses *in vivo* and may lead to a more balanced

Th1/Th17 immunity to TB. Engagement of CD40 is not uniquely important for Th17 generation, as previous investigations on the role of CD40 in mycobacterial diseases have supported the importance of CD40 in the amplification of Th1 responses. *CD40*$^{-/-}$ mice were shown to be susceptible to aerosol infection with *M. tuberculosis* due to a defective Th1 response [486], but *CD40lg*$^{-/-}$ mice were reported to be resistant to *M. tuberculosis* infection and capable of establishing Th1 immunity [486, 502]. Together with our data showing that *M. tuberculosis* poorly induces CD40 expression on infected DCs [104], these studies suggest that, while CD40L may be dispensable for generating Th1 responses that control bacterial burden, engaging the CD40-CD40L pathway may be important for generating balanced Th1/Th17 responses that may better control *M. tuberculosis* infection. Moreover, while IL-17 responses were not examined in those studies, mucosal Th17 cells are also likely to contribute to controlling *M. tuberculosis* in *CD40*$^{-/-}$ mice *in vivo;* this may be dependent on antigen load as the reported susceptibility of *CD40*$^{-/-}$ mice disappeared after high dose aerosol challenge [486]. Our work showing that promoting CD40-CD40L interaction augments early Th17 responses in the lung (Fig 5 and 6) is consistent with several previous reports showing an important role for Th17 cells in protection at mucosal surfaces such as in the lung and intestine [482, 483, 503, 504]. In TB, it has been suggested that Th17 cells in the lung may act directly on infected cells or by recruiting additional immune cells, such as IFN-γ^+ Th1, to combat infection. Notably, in Figure 5 and 6, we show that intratracheal instillation of *M. tuberculosis*-infected DCs treated with CD40LT is associated with an earlier IFN-γ response in the lungs compared to *M. tuberculosis*-DC, which supports the idea that induction of early antigen-specific Th17 can serve to recruit antigen-specific Th1 cells. Our work highlights

the importance of augmenting DC co-stimulation in order to improve adaptive immunity to TB and provides evidence that specifically augmenting DCs through CD40 can enhance antigen-specific mucosal immunity.

The generation of robust antigen-specific immunity that goes beyond IFN-γ-producing Th1 responses is an important consideration for vaccines and host-directed therapeutics for TB. The IL-12/STAT-1/IFN-γ axis is important for the control of *M. tuberculosis*, but robust induction of IFN-γ alone does not correlate with enhanced protection against developing TB disease in a variety of vaccine trials [429, 478], and there is mounting evidence for IFN-γ independent and Th17-mediated mechanisms of *M. tuberculosis* control [285, 289, 505]. In fact, recent work in mice has demonstrated that IFN-γ plays a more important role in control of bacterial burden at extra-pulmonary sites such as the spleen and must be restrained to prevent lung pathology [251]. In humans, bi-allelic mutations in *RORC,* leading to abrogated IL-17 responses, is associated with susceptibility to mycobacteria, suggesting a role for IL-17 responses in human TB [506]. In addition, the emerging importance of mucosal Th17 responses in protective and vaccine-induced immunity to *M. tuberculosis* [482, 483, 504] highlights the need to design and evaluate candidate vaccines that induce robust early Th17 responses. It is important to keep in mind, however, that unbalanced production of IL-17 can be pathogenic [507]. Over-exuberant induction of IL-17 at non-mucosal sites via repeated subcutaneous BCG exposure can lead to worsening of disease [474] and damaging neutrophilia, while IFN-γ receptor signaling limits excessive Th17-mediated neutrophilia

[295]. In this context, future studies aimed at augmenting CD40 co-stimulation would benefit from studying how augmenting this pathway impacts neutrophil responses.

In summary, our studies demonstrate a novel role for CD40 co-stimulation in generating Th17 responses in TB and show that augmenting the CD40-CD40L pathway, either through DC-targeted strategies or deletion of immune-evasion genes in the pathogen, can bolster adaptive immunity in TB. Our results indicate that targeting DC co-stimulatory pathways in the context of subunit vaccines or live attenuated vaccines represents a novel strategy to induce balanced Th1/Th17 immunity and improve control of *M. tuberculosis* infection.

Material and Methods

Ethics statement

All experiments using animals or tissue derived from animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University (Protocol number YER-2003476-060919GN). Experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Bacterial strains

M. tuberculosis H37Rv was grown as described previously [104, 105]. Briefly, bacteria were grown at 37°C in Middlebrook 7H9 broth or 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson, Franklin Lakes, NJ),

0.5% glycerol, and 0.05% Tween 80 (for broth), with the addition of 25 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO) for *hip1* mutant *M. tuberculosis*. For heat inactivation, bacterial stocks in 7H9 were grown to mid-log phase, sonicated, washed twice with PBS and inactivated at 80° C for 2 hours.

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/6, and C57BL/6 CD45.1^{+} congenic mice, *CD80*$^{-/-}$*CD86*$^{-/-}$, and *CD40*$^{-/-}$ mice were purchased from The Jackson Laboratory. OT-II TCR Tg mice specific for $OVA_{323–339}$ peptide were obtained from Dr. Bali Pulendran (originally generated in the laboratory of Dr. F. Carbone, University of Melbourne), and TCR-Tg mice specific for early secreted antigenic target 6 $(ESAT\text{-}6)_{1–20}$/I-A^{b} epitope were obtained from Dr. Andrea Cooper (Trudeau Institute) and were bred at the Yerkes animal facility. *CD40lg*$^{-/-}$ x OT-II Tg mice were obtained from Dr. Mandy Ford (Emory University) and bred at the Yerkes animal facility.

Generation of bone marrow derived dendritic cells

For generating murine bone marrow derived DCs, bone marrow cells from indicated strains of mice were flushed from excised femurs and tibias and grown in RPMI 1640 medium (Lonza, Walkersville, MD) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM glutamine, 1x β-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 1x nonessential amino acids, and 20 ng/ml murine recombinant GM-

CSF (R&D Systems, Minneapolis, MN). Incubations were carried out at 37℃ with 5% CO_2. Fresh medium with GM-CSF (20ng/ml) was added on days 3 and 6, and cells were used on day 8 for all experiments. We routinely obtained >85% $CD11c^+$ $CD11b^+$ $MHCII^+$ cell purity by flow cytometry. DCs were further purified using CD11c microbead kits as per the manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

***M. tuberculosis* infection of DCs**

$3x10^5$ CD11c-purified bone marrow derived DCs were plated onto 24-well plates overnight prior to infection. For live infections, bacteria were filtered through 5 μm filters, resuspended in complete medium, and sonicated twice for 5 seconds before addition to the adherent monolayers. Bacteria were used for infection (in triplicate) at a multiplicity of infection (MOI) of 10 or as indicated. Infection of DCs was carried out for 4 hours, after which monolayers were incubated with amikacin (200 μg/ml; Sigma-Aldrich) for 45 minutes to kill extracellular bacteria and then washed four times with PBS before incubating in complete medium. To determine bacterial input, a set of wells was lysed in PBS containing 0.5% Triton X-100 and plated onto 7H10 agar plates for CFU enumeration after 21 days. For stimulation of DCs with heat killed *M. tuberculosis*, DC were exposed to heat-killed *M. tuberculosis* at an MOI of 10 in complete medium as determined by CFU enumerated from bacterial stocks prior to heat killing. Uninfected DCs were used as controls for each experiment. For some experiments, DCs were treated with multimeric CD40LT reagent (Adipogen) concurrent with infection or stimulation. Cell free supernatants were collected after 24 hours to assay for cytokines: IL-12p40, IL-

12p70, IL-6, IL-1β (BD OptEIA, San Jose, CA) and IL-23 (Biolegend, San Diego, CA) by ELISA according to manufacturers' instructions.

DC-T cell co-culture assays

CD4 T cells were purified from single-cell suspensions of spleen and lymph nodes from 6–8 week old transgenic mice (Naïve CD4 negative selection kit, Stemcell Technologies) of the indicated strain. Purified CD4 T cells show ≥ 99% purity by FACS analysis. DCs were incubated with 10 μg/ml (or as indicated) of $OVA_{323–339}$ or ESAT-6_{1-20} peptide for 6 hours, washed with PBS, and infected with *M. tuberculosis* with or without CD40LT for 24 hours. DCs were then washed twice with PBS and co-cultured with antigen-specific CD4 T cells to achieve a 1:4 DC:T cell ratio for 72 hours. Cell free supernatants collected from co-cultured cells were analyzed for IFN-γ (Mabtech, Cincinnati, OH), IL-17 (ELISA Ready-Set-Go, eBioscience), and IL-2 (BD Biosciences) by ELISA according to the manufacturers' instructions.

Blockade of CD40-CD40L pathway

$2x10^4$ CD11c-purified DCs were seeded in 96-well plates overnight, pulsed with relevant peptide and treated with the indicated conditions for 24 hours. Afterwards, purified antigen-specific CD4 T cells were incubated with 20 μg/ml anti-CD40L (clone MR1) blocking antibody and co-cultured with DCs to achieve a 1:10 DC:T cell ratio. Co-cultured cells were incubated at 37℃ with 5% CO_2 for 72 hours prior to harvest of supernatants for ELISAs.

Intratracheal instillation of DCs and tissue harvest

CD11c-purified DCs were stimulated with indicated conditions for 24 hours. DCs were then washed twice and resuspended in sterile PBS at $1x10^6$/50 ul and injected intratracheally into isoflurane-anesthetized mice. For some experiments, recipient mice ($CD45.1^+$) received purified $1x10^6$ ESAT-6_{1-20} TCR-Tg CD4 T cells ($CD45.2^+$) one day prior to DC instillation by tail-vein injection. 6 and 12 days post-intratracheal instillation, lungs and mediastinal lymph nodes were harvested. Lungs were digested with 1 mg/ml collagenase D (Worthington) at 37°C for 30 min. For some experiments, the upper right lobe of the lung was used for determining CFU and the rest of the lung was used for cellular assays. Homogenized single-cell lung suspensions were obtained through mechanical disruption and 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 used to set up stimulations for intracellular cytokine staining and flow cytometry. Single cell suspensions were stimulated with media, ESAT-6_{1-20} (10 μg/ml), *M. tuberculosis* whole cell lysate (10 μg/ml), or PMA (80 ng/ml) and ionomycin (500 ng/ml) as indicated. BFA (5 μg/ml) and monensin (1:1500) were added to the stimulated cells after 1.5 hours and cells were cultured for an additional 4.5 hours, or 16 hours for whole cell lysate stimulations, and then stained for flow cytometry.

Flow cytometry

Live cells were discriminated by a live/dead fixable aqua dead cell stain (Molecular Probes). For staining DCs, murine anti-CD11c PE-Cy7 (clone N418, eBioscience), anti-

CD11b APC-Cy7 (clone M1/70, Biolegend), anti-CD40 PE-Cy5 (clone 1C10, eBioscience), anti-CD86 APC (clone GL1, eBioscience), and anti–MHC II PE (clone M5/114.15.2, BD) were utilized. For staining T cells, murine anti-CD3 V450 (clone 500A2, BD), anti-CD4 Alexa700 (clone RM4-5, BD), anti-CD8 PerCP (clone 53-6.7, BD), anti-TCR γδ BV605 (clone GL3, Biolegend), anti-CD44 APC-Cy7 (clone IM7, BD), anti-CD45.1 BV785 (clone A20, BioLegend), and anti-CD45.2 BV650 (clone 104, BioLegend) were utilized to stain for surface markers. Murine anti-RORγt PE (clone B2D, eBioscience), anti-TNF-α PE-Cy7 (clone MP6-XT22, BD), anti-IFN-γ APC (clone XMG1.2, eBioscience), anti-IL-2 FITC (clone JES6-5H4, BD), and anti-IL-17 PE-CF594 (clone TC11-18H10, BD) were stained intracellularly with the BD Cytofix/Cytoperm or BD Transcription Factor kit as per manufacturer's instructions. Staining for cell-surface markers was done by resuspending ~1-2x10^6 cells in 100 ml PBS with 2% FBS containing the antibody mixture at 4℃ for 30 min and then washing with PBS containing 2% FBS. Data were immediately acquired using an LSRII flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (FlowJo LLC, Ashland, OR).

Aerogenic infection of mice with *M. tuberculosis*

M. tuberculosis H37Rv was grown to OD_{600} of ~0.6–0.8, washed two times in 1× PBS. 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 into 8-10 week old C57BL/6J 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 (for entry) or as indicated. CFU was enumerated after 21 days.

Statistical analyses

The statistical significance of data was analyzed using the Student's unpaired t-test for comparisons between two groups or one-way analysis of variance (ANOVA) with a Tukey posttest correction for multiple comparisons for analysis of two or more groups (GraphPad Prism 6.0h). In order to calculate correlation, a linear regression was utilized to generate a best-fit line and Spearman's correlation coefficient calculated (GraphPad Prism 6.0h). Data are shown as mean ±S.D. of one representative experiment from multiple independent experiments.

Acknowledgements

We gratefully thank Dana Tedesco and Dr. Arash Grakoui for anti-CD40L blocking antibody (MR1) and helpful input on CD40L blockade assays; Drs. David Pinelli and Mandy Ford for *CD40lg*$^{-/-}$ x OT-II TCR transgenic mice and input on breeding strategy; Dr. Joel Ernst for aid with intratracheal instillation technique. We also thank Melanie Quezada and members of the Rengarajan lab for helpful discussions.

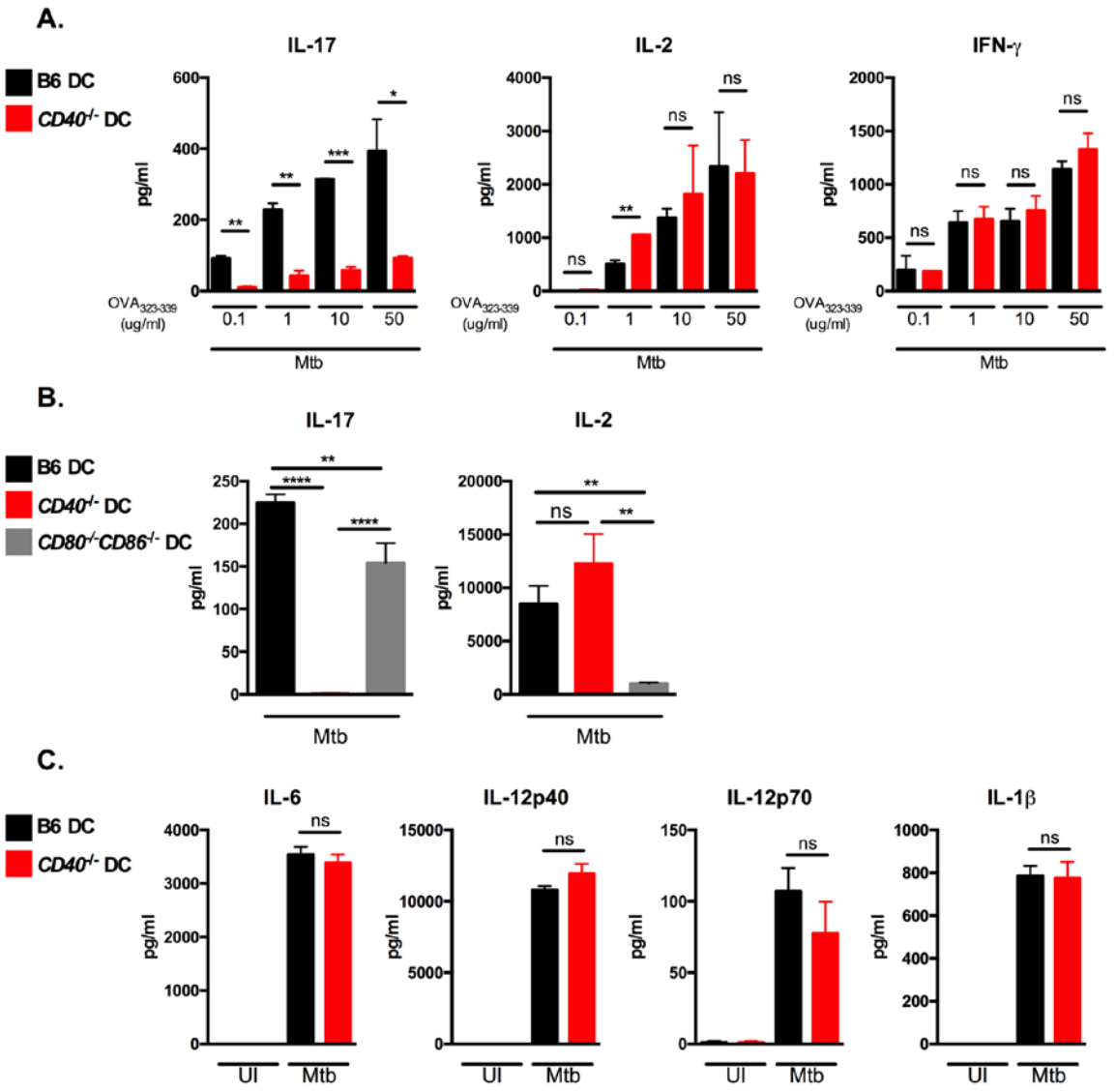

A.
IL-17
IL-2
IFN-γ
B6 DC
CD40-/- DC
pg/ml
OVA323-339 (ug/ml)
0.1
1
10
50
Mtb
B.
IL-17
IL-2
B6 DC
CD40-/- DC
CD80-/-CD86-/- DC
pg/ml
Mtb
C.
IL-6
IL-12p40
IL-12p70
IL-1β
B6 DC
CD40-/- DC
pg/ml
UI
Mtb
ns

Figure 1. CD40 on DCs is required for the generation of antigen-specific IL-17 responses during *M. tuberculosis* infection.

(A) DCs derived from B6 or *CD40*$^{-/-}$ mice were pulsed with $OVA_{323-339}$ at the indicated concentrations and infected with *M. tuberculosis* for 24 hours followed by co-culture with purified OT-II TCR-Tg CD4 T cells for 72 hours. Supernatants were assayed for the indicated cytokines by ELISA. (B) DCs from B6, *CD40*$^{-/-}$, or *CD80*$^{-/-}$*CD86*$^{-/-}$ mice were pulsed with 10 μg/ml $OVA_{323-339}$, infected with *M. tuberculosis* and co-cultured with OT-II TCR-Tg CD4 T cells. Cell-free supernatants were harvested after 72 hours and assessed for the indicated cytokines by ELISA. (C) B6 or *CD40*$^{-/-}$ DCs were left uninfected (UI) or infected with *M. tuberculosis*. After 24 hours, cell-free supernatants were collected and assessed for the indicated cytokines by ELISA. Data are representative of 3-4 independent experiments. Values are presented as mean ± SD. Statistical significance was determined using a 2-tailed unpaired T-test. * $p<0.05$; ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$, ns = not significant

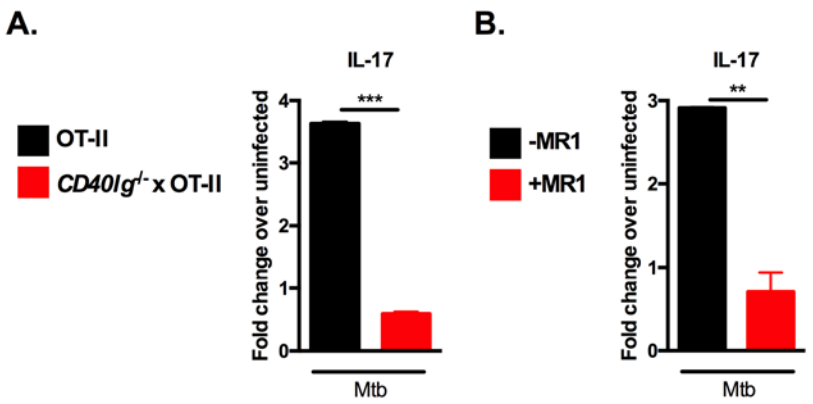


Figure 2. CD40-CD40L interaction is critical for inducing antigen-specific IL-17 responses to *M. tuberculosis* infection.

(A) B6 DCs were pulsed with $OVA_{323-339}$ at 10 µg/ml and infected with *M. tuberculosis* for 24 hours followed by co-culture with purified CD4 T cells from OT-II or *CD40lg*$^{-/-}$ x OT-II TCR-Tg mice or (B) with purified OT-II TCR-Tg T cells in the presence or absence of 20 µg/ml anti-CD40L blocking antibody (clone MR1). Cell-free supernatants were collected after 72 hours and IL-17 assessed by ELISA. Data are representative of two independent experiments. Values are presented as mean fold change over uninfected ± SD. Statistical significance was determined using a 2-tailed unpaired T-test. ** $p<0.005$, *** $p<0.0005$

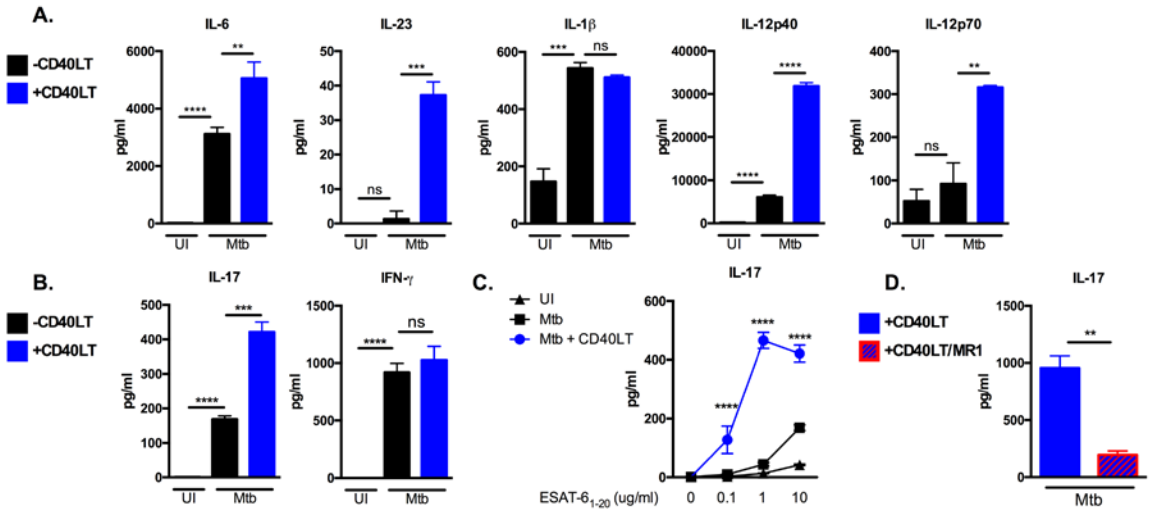

A.
-CD40LT
+CD40LT
IL-6
IL-23
IL-1β
IL-12p40
IL-12p70
pg/ml
UI
Mtb
ns
B.
IL-17
IFN-γ
C.
UI
Mtb
Mtb + CD40LT
ESAT-6$_{1-20}$ (ug/ml)
0
0.1
1
10
D.
+CD40LT
+CD40LT/MR1

Figure 3. Engaging CD40 on DCs enhances antigen-specific IL-17 responses.

(A) B6 DCs were left uninfected or infected with *M. tuberculosis* in the presence or absence of 1 μg/ml multimeric CD40LT reagent (CD40LT) for 24 hours. Cell-free supernatants were collected after 24 hours and the indicated innate cytokines were measured by ELISA. (B) DCs from (A) were pulsed with ESAT-6_{1-20} at 10 μg/ml in the presence or absence of CD40LT and co-cultured with ESAT-6 TCR-Tg T cells for 72 hours. Supernatants were assayed for IL-17 and IFN-γ by ELISA. (C) B6 DCs were pulsed with increasing concentrations of ESAT-6_{1-20} peptide (0, 0.1, 1.0 and 10 μg/ml) either left uninfected (UI) or infected with *M. tuberculosis* in the presence or absence of 1 μg/ml CD40LT for 24 hours followed by co-culture with purified ESAT-6 TCR-Tg CD4 T cells for 72 hours. Supernatants were assayed for IL-17 by ELISA. (D) B6 DCs were pulsed with ESAT-6_{1-20} peptide at 10 μg/ml and infected with *M. tuberculosis* in the presence or absence of 1 μg/ml CD40LT for 24 hours. Co-culture with ESAT-6 TCR-Tg CD4 T cells was done in the presence or absence of 20 μg/ml anti-CD40L blocking antibody (clone MR1). Cell-free supernatants were collected after 72 hours and IL-17 levels determined by ELISA. Data are representative of 3-4 independent experiments. Values are presented as mean ± SD. Statistical significance was determined using a 2-tailed unpaired T-test. ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$, ns = not significant

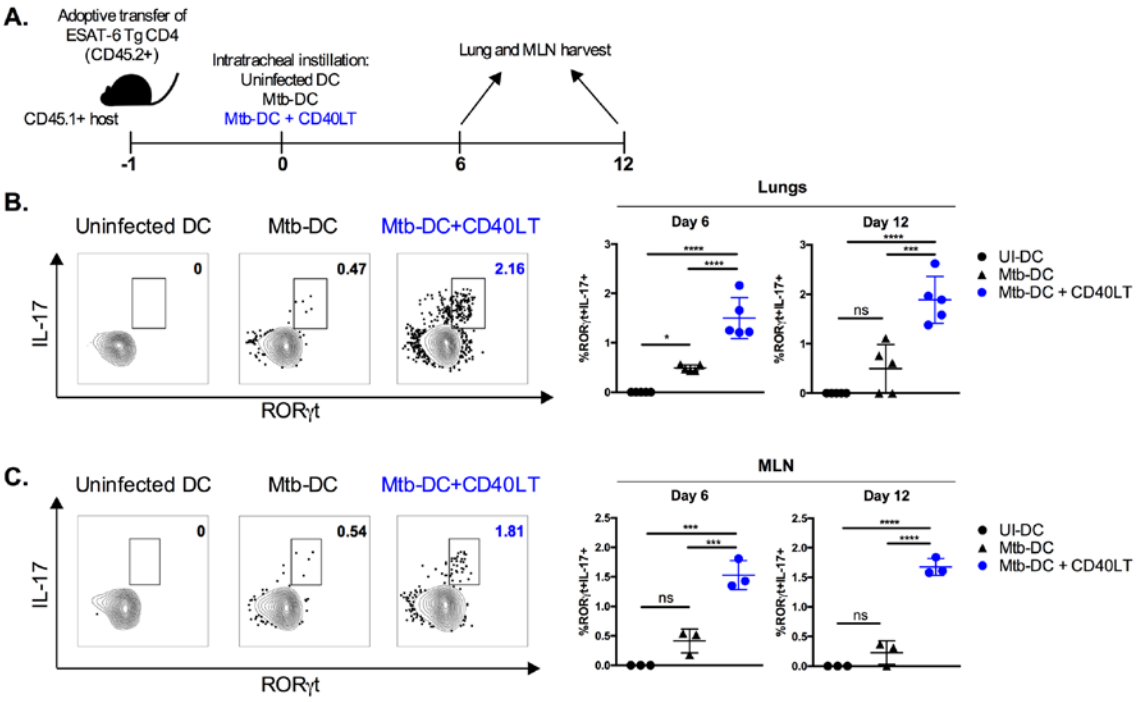

A.
Adoptive transfer of
ESAT-6 Tg CD4
(CD45.2+)
CD45.1+ host
Intratracheal instillation:
Uninfected DC
Mtb-DC
Mtb-DC + CD40LT
Lung and MLN harvest
-1
0
6
12
B.
Uninfected DC
Mtb-DC
Mtb-DC+CD40LT
IL-17
RORγt
0
0.47
2.16
Lungs
Day 6
Day 12
%RORγt+IL-17+
ns
UI-DC
Mtb-DC
Mtb-DC + CD40LT
C.
Uninfected DC
Mtb-DC
Mtb-DC+CD40LT
IL-17
RORγt
0
0.54
1.81
MLN
Day 6
Day 12
%RORγt+IL-17+
ns
UI-DC
Mtb-DC
Mtb-DC + CD40LT

Figure 4. CD40 engagement of *M. tuberculosis*-infected DCs induces antigen-specific Th17 *in vivo*.

(A) Diagram of experimental design. CD45.2$^+$ ESAT-6 TCR Tg CD4 T cells were purified from spleen and lymph nodes and intravenously transferred (1x10^6 per mouse) into congenic (CD45.1$^+$) hosts. Animals were rested for 1 day after which DCs (1x10^6 per condition) were transferred into recipient hosts by intratracheal instillation: uninfected DCs (UI-DC), *M. tuberculosis*-infected DCs (Mtb-DC) or *M. tuberculosis*-infected DCs with CD40L trimer treatment (Mtb-DC + CD40LT). Lungs and MLN were harvested 6 and 12 days post-intratracheal instillation and CD4 T cell responses assessed. MLN were pooled to attain sufficient cells for restimulation. Representative flow plots (left; day 6 values) and summary graph (right) of the lung (B) and pooled MLN (C) frequencies of RORγt$^+$IL-17$^+$ cells after ESAT-6$_{1-20}$ restimulation 6 and 12 days after DC transfer. Populations shown have been pre-gated on live CD3$^+$CD8$^-$γδ TCR$^-$CD45.2$^+$ singlets. 5 mice were used for each group. Statistical significance was determined using one-way analysis of variance (ANOVA) correcting for multiple comparisons. * $p<0.05$, *** $p<0.0005$, **** $p<0.0001$, ns = not significant

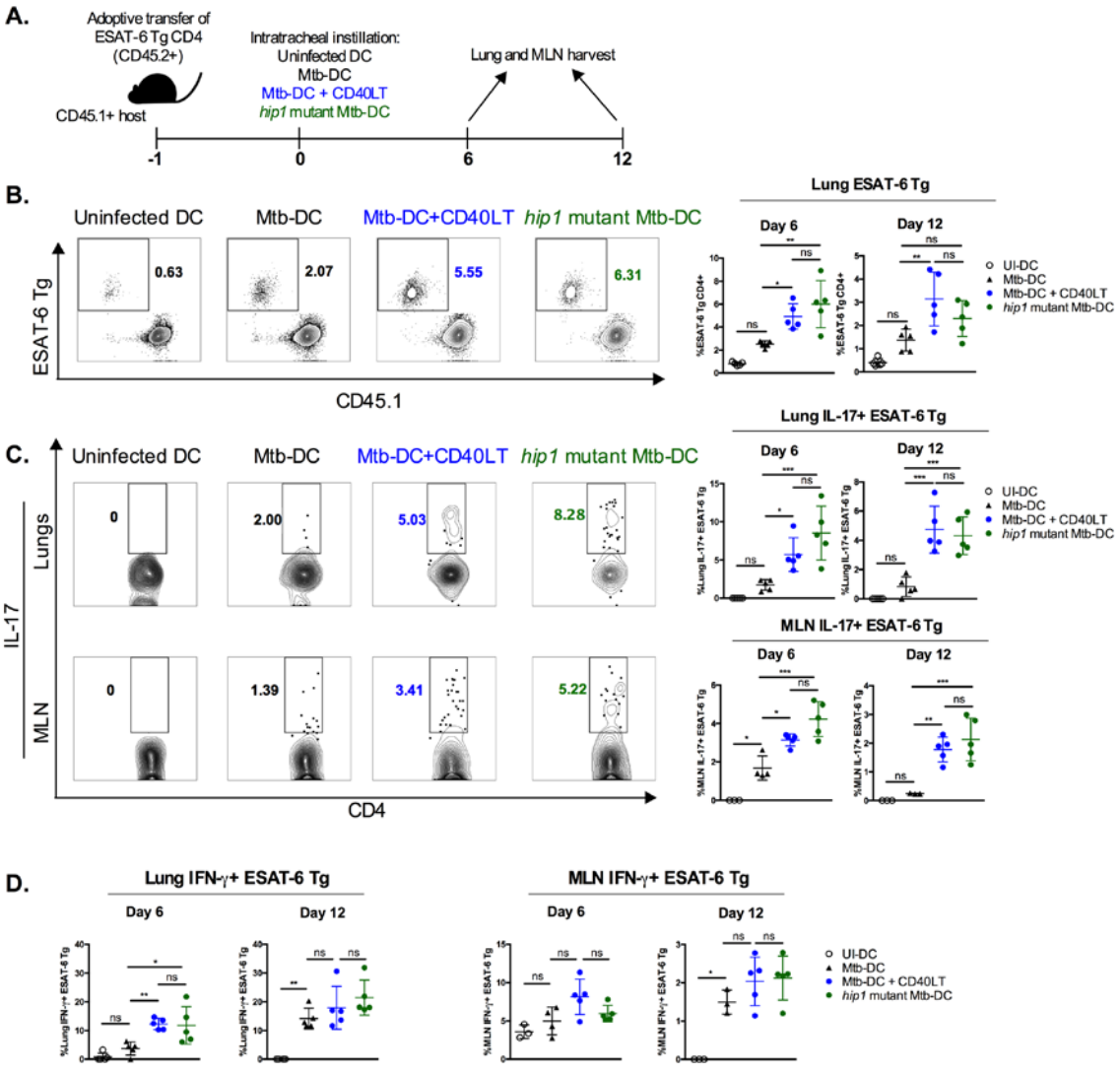

A.
Adoptive transfer of ESAT-6 Tg CD4 (CD45.2+)
CD45.1+ host
Intratracheal instillation:
Uninfected DC
Mtb-DC
Mtb-DC + CD40LT
hip1 mutant Mtb-DC
Lung and MLN harvest
-1
0
6
12
B.
Uninfected DC
Mtb-DC
Mtb-DC+CD40LT
hip1 mutant Mtb-DC
ESAT-6 Tg
CD45.1
0.63
2.07
5.55
6.31
Lung ESAT-6 Tg
Day 6
Day 12
UI-DC
Mtb-DC
Mtb-DC + CD40LT
hip1 mutant Mtb-DC
C.
Uninfected DC
Mtb-DC
Mtb-DC+CD40LT
hip1 mutant Mtb-DC
IL-17
Lungs
MLN
CD4
0
2.00
5.03
8.28
0
1.39
3.41
5.22
Lung IL-17+ ESAT-6 Tg
MLN IL-17+ ESAT-6 Tg
Day 6
Day 12
D.
Lung IFN-γ+ ESAT-6 Tg
MLN IFN-γ+ ESAT-6 Tg
Day 6
Day 12
UI-DC
Mtb-DC
Mtb-DC + CD40LT
hip1 mutant Mtb-DC

Figure 5. Enhanced antigen-specific Th17 responses in the lungs and MLN of mice following transfer of CD40LT-engaged *M. tuberculosis*-infected DCs or *hip1* mutant-infected DCs

(A) Diagram of experimental design. As before, purified CD45.2$^+$ ESAT-6 TCR Tg CD4 T cells were adoptively transferred 1 day before intratracheal instillation of DCs: uninfected DCs (UI-DC), *M. tuberculosis*-infected DCs (Mtb-DC), *M. tuberculosis*-infected DCs with CD40L trimer treatment (Mtb-DC + CD40LT), or *hip1* mutant *M. tuberculosis*-infected DC (*hip1* mutant-DC). Lungs and MLN were harvested 6 and 12 days post-intratracheal instillation and CD4 T cell responses assessed. (B) Representative flow plots (left; day 6 values) and summary graph (right) of the frequencies of CD45.2$^+$ ESAT-6 TCR-Tg CD4 T cells in the lungs 6 and 12 days post-instillation. (C) Representative flow plots (left; day 6 values) and summary graphs (right) of the frequencies of IL-17$^+$ ESAT-6 TCR-Tg CD4 T cells in the lungs (top) and MLN (bottom) after stimulation with ESAT-6$_{1-20}$ peptide (10 μg/ml). (D) Summary graphs of the frequencies of IFN-γ^+ ESAT-6 TCR-Tg CD4 T cells in the lungs (left) and MLN (right) after ESAT-6$_{1-20}$ restimulation (10 μg/ml). Populations shown have been pre-gated on live CD3$^+$CD8$^-$γδ TCR$^-$CD45.2$^+$ singlets. 5 mice were used for each group. Statistical significance was determined using one-way analysis of variance (ANOVA) correcting for multiple comparisons. * $p<0.05$; ** $p<0.005$, *** $p<0.0005$, ns = not significant

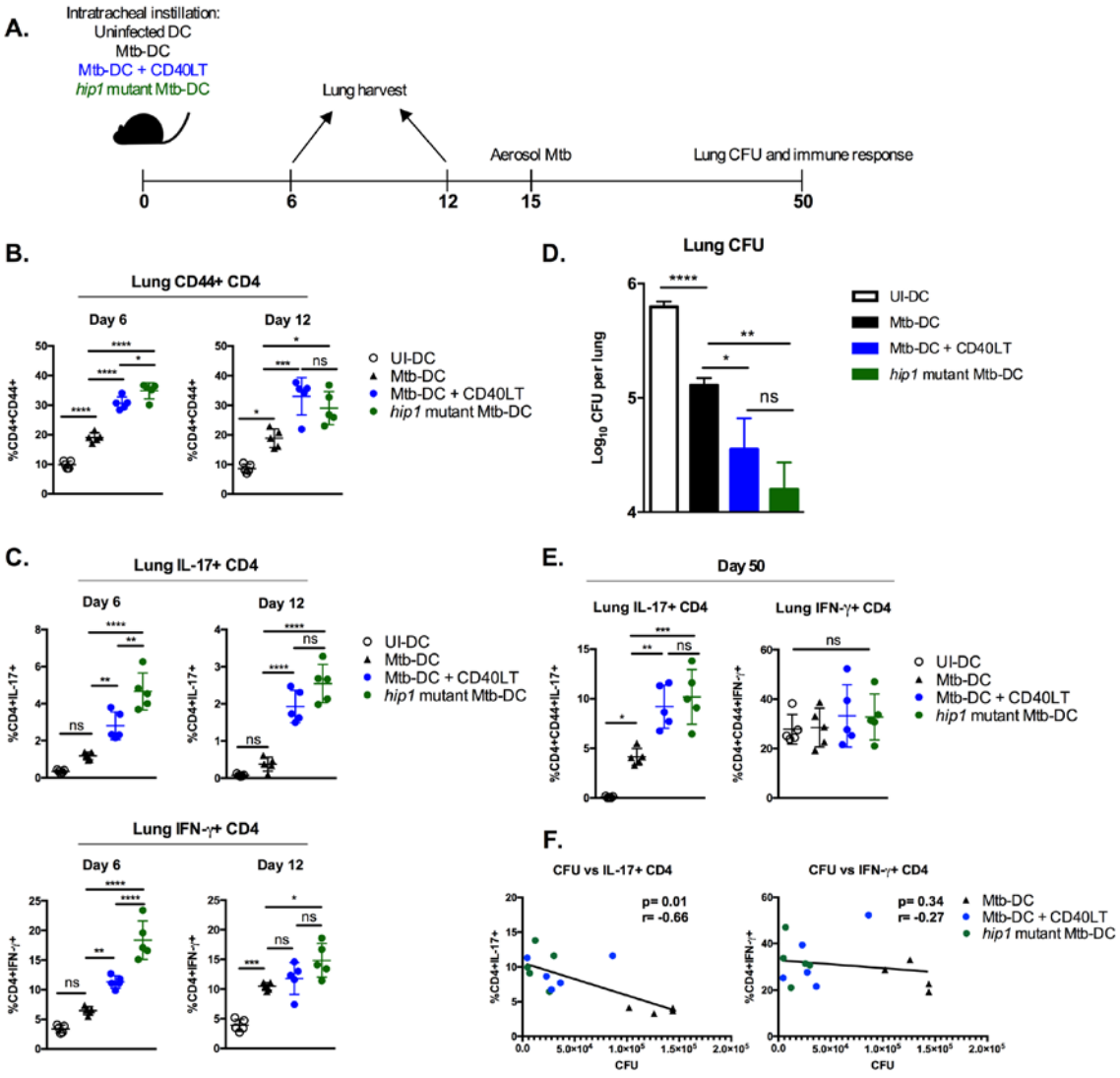
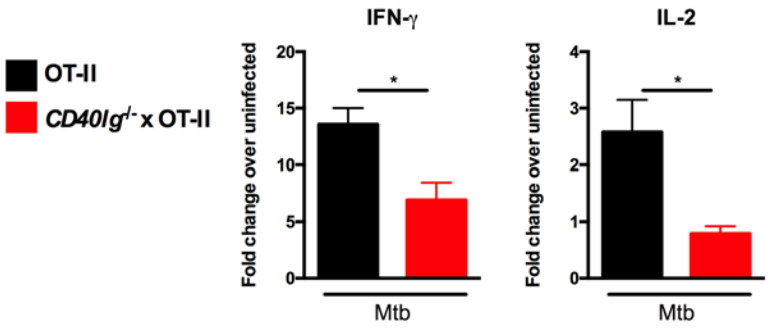
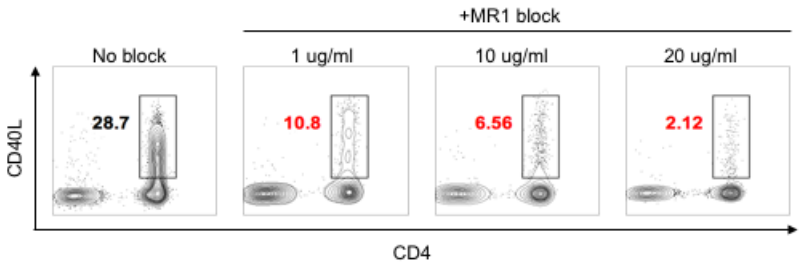

A.
Intratracheal instillation:
Uninfected DC
Mtb-DC
Mtb-DC + CD40LT
hip1 mutant Mtb-DC
Lung harvest
Aerosol Mtb
Lung CFU and immune response
0
6
12
15
50
B.
Lung CD44+ CD4
Day 6
Day 12
%CD4+CD44+
UI-DC
Mtb-DC
Mtb-DC + CD40LT
hip1 mutant Mtb-DC
C.
Lung IL-17+ CD4
Day 6
Day 12
%CD4+IL-17+
Lung IFN-γ+ CD4
%CD4+IFN-γ+
D.
Lung CFU
Log10 CFU per lung
E.
Day 50
Lung IL-17+ CD4
%CD4+CD44+IL-17+
Lung IFN-γ+ CD4
%CD4+CD44+IFN-γ+
F.
CFU vs IL-17+ CD4
p= 0.01
r= -0.66
CFU vs IFN-γ+ CD4
p= 0.34
r= -0.27
CFU

Figure 6. CD40 engagement of DCs enhances control of *M. tuberculosis* infection. B6 DCs were left uninfected or infected with heat-killed *M. tuberculosis* in the presence or absence of CD40LT treatment (1 μg/ml), or infected with heat-killed *hip1* mutant *M. tuberculosis*, each for 24 hours. Cells were washed twice and reconstituted in PBS to deliver $1x10^6$ DC per mouse intratracheally. (A) Diagram of experimental design. Lung responses were assessed 6 and 12 days post-instillation. Mice were infected through the aerosol route with ~100 CFU *M. tuberculosis* 15 days post-instillation and bacterial burden was assessed 35 days (5 weeks) post-challenge. (B) Frequencies of $CD44^+$ CD4 T cells in the lungs 6 and 12 days after intratracheal instillation of DCs. (C) Frequencies of IL-17^+ (top) and IFN-γ^+ (bottom) $CD4^+$ cells in the lungs 6 and 12 days after intratracheal instillation of DCs. Cells were stimulated with PMA (80 ng/ml) and ionomycin (500 ng/ml). (D) Lung bacterial burden 35 days post-challenge (overall day 50 post-DC intratracheal instillation). Bacterial burden was assessed by plating homogenized lungs on 7H10 agar plates and counting CFU. (E) Lung $CD4^+$ IL-17^+ and IFN-γ^+ frequencies at day 50 following *M. tuberculosis* whole cell lysate (10 μg/ml) restimulation. (F) Correlation plots showing association between lung bacterial burden and IL-17 (left) or IFN-γ (right) responses to WCL restimulation. A linear regression was utilized to generate a best-fit line and Spearman's correlation coefficient calculated. 4-5 mice were used for each group. Statistical significance (B-E) was determined using one-way analysis of variance correcting for multiple comparisons. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$, ns = not significant.



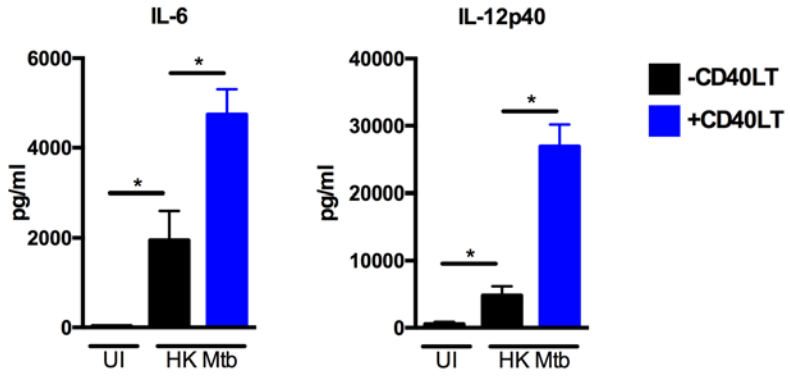
Supplemental Figure 1. CD40L is required for IFN-γ and IL-2 responses from antigen-specific CD4 T cells *in vitro*

DCs from C57BL/6 (B6) were pulsed with $OVA_{323-339}$ at 10 μg/ml and infected with *M. tuberculosis* for 24 hours followed by co-culture with purified OT-II or *CD40lg*$^{-/-}$ x OT-II TCR-Tg CD4 T cells. Cell-free supernatants were collected after 72 hours and assessed for the indicated cytokines by ELISA. Values are presented as mean ± SD. Statistical significance was determined using a 2-tailed unpaired T test. * $p<0.05$



Supplemental Figure 2. CD40-CD40L interaction can be blocked using non-agonistic anti-CD40L antibody MR1.

To determine optimal concentrations of blocking antibody, $1x10^6$ splenocytes from OT-II TCR-Tg mice were plated with 5 µg/ml anti-CD16/32 (Fc Block) and pulsed with 10 µg/ml $OVA_{323-339}$ peptide for 6 hours in the presence or absence of non-agonistic anti-CD40L antibody (clone MR1) at the indicated concentrations. After 6 hours, PE-conjugated anti-CD40L antibody (clone MR1, 1:100) was spiked into the sample and left in the dark at 37℃ for 18 hours. Cells were then washed, stained for viability, CD3 and CD4, and acquired immediately. Representative flow plots of recovered CD40L expression on live $CD3^+$ cells are shown demonstrating titratable blockade of CD40L by MR1.



Supplemental Figure 3. CD40 engagement enhances cytokine production from DCs exposed to heat-killed *M. tuberculosis*

B6 DCs were left uninfected or exposed to heat-killed *M. tuberculosis* in the presence or absence of 1 μg/ml multimeric CD40LT reagent (CD40LT) for 24 hours. Cell-free supernatants were collected after 24 hours and the indicated innate cytokines were measured by ELISA. Data are representative of 3 independent experiments. Values are presented as mean ± SD. Statistical significance was determined using a 2-tailed unpaired T-test. * $p<0.05$

Chapter IV

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

Chapter adapted from:

Bizzell E.*, **Sia J.K.***, Quezada M., Enriquez A., and Rengarajan J. (2018). Deletion of BCG Hip1 protease enhances dendritic cell and CD4 T cell responses. *Journal of Leukocyte Biology*. Epub 2018/01/19. doi: 10.1002/JLB.4A0917-363RR. PMID: 29345365

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Attributions for collaborations:

Figures 3, 4, and 5 were contributed by myself in close collaboration with E. Bizzell. All other figures are derived from the work of E. Bizzell.

Abstract

Dendritic cells (DCs) play a key role in the generation of CD4 T cell responses to pathogens. *Mycobacterium tuberculosis* (*M. tuberculosis*) harbors immune evasion mechanisms that impair DC responses and prevent optimal CD4 T cell immunity. The vaccine strain *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) shares many of the immune evasion proteins utilized by *M. tuberculosis*, but the role of these proteins in DC and T cell responses elicited by BCG is poorly understood. We previously reported that the *M. tuberculosis* 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* (BCGΔ*hip1*) and show that it has superior capacity to induce DC maturation and cytokine production compared to parental BCG. Furthermore, BCGΔ*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 BCGΔ*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, BCGΔ*hip1*-infected DCs enhanced control of pulmonary bacterial burden following *M. tuberculosis* 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 *M. tuberculosis* burden after challenge.

Introduction

Critical to the success of *Mycobacterium tuberculosis* (*M. tuberculosis*) as a pathogen is its ability to evade host innate and adaptive immunity. *M. tuberculosis* 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 *M. tuberculosis* promote sub-optimal DC maturation, cytokine production and antigen presentation to CD4 T cells, which adversely affects T cell immunity and impedes control of *M. tuberculosis* infection [235, 508]. 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 [4, 509]. 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 *M. tuberculosis* genome [510] and BCG retains many of the genes shown to encode immune evasion proteins in *M. tuberculosis*. 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 *M. tuberculosis* cell wall-associated serine protease, Hip1 (Hydrolase important for pathogenesis 1, Rv2224c), is involved in impairing DC functions [104]. Since Hip1 from BCG and *M. tuberculosis* 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* (BCGΔ*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 BCGΔ*hip1* produce significantly enhanced levels of pro-inflammatory cytokines and express higher levels of major histocompatibility complex (MHC) class II and co-stimulatory 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 BCGΔ*hip1*-infected DCs improved antigen-specific CD4 T cell responses in the lungs and enhanced control of *M. tuberculosis* 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.

Results

Construction of a BCGΔ*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 BCGΔ*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, BCGΔ*hip1*, or a BCGΔ*hip1* strain complemented with *hip1* (BCGΔ*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).

BCGΔ*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 BCGΔ*hip1*. We infected BMDCs from C57BL/6J mice with BCG, BCGΔ*hip1* or BCGΔ*hip1* comp and measured DC cytokine production by ELISA. DCs infected with BCGΔ*hip1* produced significantly higher levels of IL-6 and IL-12p40 than DCs infected with BCG. Importantly, cytokine levels induced by BCGΔ*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 BCGΔ*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 BCGΔ*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 BCGΔ*hip1* also resulted in significantly higher levels of cytokines compared to heat-killed BCG, indicating that enhanced cytokine production by DCs infected with BCGΔ*hip1* was not dependent on viability of the bacteria (Fig. 2C). Further, we found that bone marrow-derived macrophages (BMDMs) infected with BCGΔ*hip1* produced higher levels of IL-6 and IL-1β 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.

BCGΔ*hip1* enhances expression of co-stimulatory 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 co-stimulatory 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 co-stimulatory molecules CD40 and CD86 on DCs infected with BCG or BCGΔ*hip1* by flow cytometry. DCs infected with BCGΔ*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 co-stimulatory molecules.

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

IL-12 is known to drive the polarization of IFN-γ-producing Th1 subsets while IL-6, IL-1β, TGF-β, and IL-23 drive the polarization and expansion of IL-17-producing Th17 subsets [511]. Since DCs infected with BCGΔ*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 BCGΔ*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 BCGΔ*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 BCGΔ*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 BCGΔ*hip1*-infected DCs as well as higher levels of Th17-polarizing cytokines IL-6 and IL-1β (Fig. 4B). We do not detect significant levels of TGF-β under any of our culture conditions. Further, compared to BCG-infected DCs, BCGΔ*hip1*-infected DCs produced higher levels of IL-23, which is a cytokine known to maintain Th17 lineage commitment [512]. These data demonstrate that BCGΔ*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 BCGΔ*hip1* enhances lung CD4 T cell responses *in vivo* and improves control of *M. tuberculosis* 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 [227], and antigen-loaded DCs have previously been shown to confer protection against *M. tuberculosis* challenge [239, 495, 496, 513]. Therefore, we used a tractable DC intratracheal instillation model to assess antigen-specific CD4 T cell responses *in vivo* following transfer of BCG or BCGΔ*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 *M. tuberculosis* whole cell lysate (WCL). We measured the number of activated $CD44^+$ CD4 T cells in the lungs following intratracheal instillation of BCG or BCGΔ*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 BCGΔ*hip1* compared to mice that received BCG (Fig. 5B), indicating that BCGΔ*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 BCGΔ*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 [514]. Interestingly, we observed higher frequencies of CD4 T cells producing IFN-γ, IL-2, and TNF-α in animals that received BCGΔ*hip1* DCs compared to BCG DCs (Fig. 5D). To investigate whether mucosal administration of DCs exposed to BCGΔ*hip1* would provide enhanced bacterial control

compared to BCG after low dose aerosol *M. tuberculosis* challenge, we intratracheally instilled BCG DCs or BCGΔ*hip1* DCs, rested mice for 2 weeks prior to aerogenic challenge with low-dose *M. tuberculosis* H37Rv, and determined lung *M. tuberculosis* bacterial burden 4 weeks post-challenge. As shown in Fig. 5E, mice that received BCGΔ*hip1*-infected DCs harbored significantly less *M. tuberculosis* CFU post-challenge compared to mice that received BCG-infected DCs (Fig. 5E). These results demonstrate that mucosal-targeted approaches using BCGΔ*hip1* can augment antigen-specific CD4 T cell responses compared to BCG and lead to enhanced control of *M. tuberculosis* 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 BCGΔ*hip1*-infected DCs produce higher levels of cytokines, express elevated levels of co-stimulatory 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 *M. tuberculosis* 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 [515], and diminish activation of antigen-specific CD8 T cells by inducing DC death [516]. Furthermore, BCG-infected DCs produce significantly lower levels of IL-23, IL-1β, TNF-α, and IL-12 than *M. tuberculosis*-infected DCs [517], indicating that BCG stimulates weaker innate immune responses than *M. tuberculosis*. Interestingly, a study by Satchidanandam *et al* showed that overexpression of an *M. tuberculosis* glycosylated protein, Rv1860, in BCG impaired DC maturation, attenuated Th1 and Th17 polarization, and led to subsequent loss of protection against *M. tuberculosis* challenge conferred by BCG vaccination [518], suggesting that *M. tuberculosis* 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 *M. tuberculosis* after challenge.

Several avenues have been explored for improving BCG immunogenicity and efficacy, including introduction of immunodominant proteins from *M. tuberculosis* [518-521], and expression of host proteins [522-528]. The majority of studies utilizing recombinant or mutant strains of BCG are primarily focused on enhancing macrophage driven responses and functions, such as phagasomal maturation, apoptosis of infected macrophages, and

bacterial escape from phagasomal compartments [107, 529-533]. For instance, a recombinant strain of BCG lacking urease production and expressing listeriolysin from *Listeria monocytogenes* (BCGΔ*ureC::hly)* was shown to enhance apoptosis of infected macrophages [530], leading to increased central memory T cell responses [534], enhanced Th1 and Th17 immunity [535], and cross-presentation to CD8 T cells [531]. Interestingly, deletion of anti-apoptotic gene *nuoG* in BCGΔ*ureC::hly* showed enhanced efficacy over BCGΔ*ureC::hly* [536]. Notably, we show evidence that BCGΔ*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 BCGΔ*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 *M. tuberculosis*-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 [317], and utilizes a liposome-based adjuvant (CAF01) that targets DCs [537]. Furthermore, improving DC antigen-presentation by induction of autophagy has been shown to improve BCG immunogenicity and improve control of *M. tuberculosis* burden after challenge [538]. Our data show that transfer of BCGΔ*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 *M. tuberculosis* 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 [239, 496, 504, 513, 539-543]. Using an intratracheal DC installation model, we observed that transfer of DCs infected with BCGΔ*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 *M. tuberculosis* 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 BCGΔ*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 *M. tuberculosis* 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 *M. tuberculosis*.

Materials and Methods

Bacterial strains and culture conditions

BCG (Danish), BCGΔ*hip1*, and BCGΔ*hip1* complemented with *hip1* (BCGΔ*hip1* comp) were grown at 37ºC 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 BCGΔ*hip1* was supplemented with 20μg/ml of streptomycin (Sigma-Aldrich, St. Louis, MO), and media for BCGΔ*hip1* was supplemented with 50μg/ml of hygromycin (Roche Diagnostics, Indianapolis, IN). For growth curves, bacterial strains were inoculated into supplemented 7H9 medium at OD_{600} 0.05, and the OD_{600} measurements were taken daily.

Construction of BCGΔ*hip1* and complemented strains

BCG was transformed via electroporation with 3μg of pEBOP-2 (pYUB657 suicide vector containing a Δ*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 [544]. 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'-CGGCCACCCGCTCACCGCCCTCG-3') and downstream (reverse primer 5'-GCACGGCGAATGTCAGATAGGG-3') of the 1kb regions of homologous recombination, resulting in a 4.5kb amplicon from the BCGΔ*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. BCGΔ*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 $OVA_{323–339}$ 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 [110]. Bone marrow cells were isolated from C57BL/6J mice and differentiated for 7

days at 37°C with 5% CO_2 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 $3x10^5$ 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 [513]. 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-stimulatign 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 $3x10^5$ 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 $1x10^6$/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' instrucutions: 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 BCGΔ*hip1* at an MOI of 30 or left unstimulated in media for 24 hours. BMDCs were then washed, resuspended in PBS and intratracheally instilled ($1x10^6$ per mouse in 50 ul 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 $1x10^6$ cells were plated for phenotypic analysis or antigen restimulation. Cells were exposed to media (unstimulated), PMA/ionomycin (80ng/ml and 500ng/ml, respectively), or 10ug/ml whole cell lysate (WCL). Cells were then incubated at 37°C for 1.5 hours before addition of BFA (5ug/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 γδ 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).

M. tuberculosis aerosol challenge and enumeration of bacteria

Groups of five mice were intratracheally instilled with $1x10^6$ BMDCs, rested for 14 days, and then challenged with a low dose (~100 CFU) of *M. tuberculosis* 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.

Acknowledgements

We would like to thank Dr. Miriam Braunstein (University of North Carolina, Chapel Hill), and her current and former lab members, Dr. Ellen Perkowski and Kate Zulauf, for providing the pYUB657 plasmid, and for their helpful advice for generating the BCGΔ*hip1* strain. We would also like to thank past and present members of the Rengarajan lab for helpful discussions.

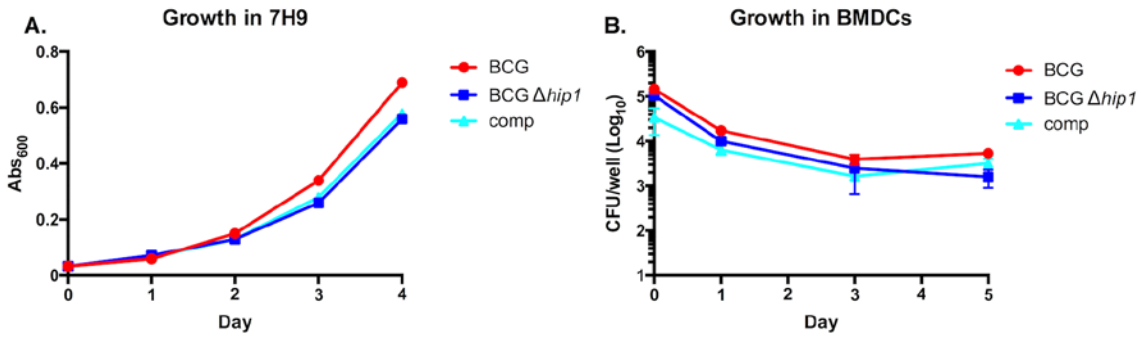


Figure 1. Deletion of *hip1* from BCG does not affect growth of BCG in broth or in dendritic cells.

(A) BCG Danish, BCGΔ*hip1*, or complemented BCGΔ*hip1* (comp) were inoculated into 7H9 liquid medium supplemented with OADC and glycerol at a starting OD_{600} of 0.05, were incubated at 37°C shaking, and the absorbance of the cultures at OD_{600}, was recorded daily. (B) BMDCs from C57BL/6J mice were infected with BCG, BCGΔ*hip1*, or complemented BCGΔ*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 ±SD.

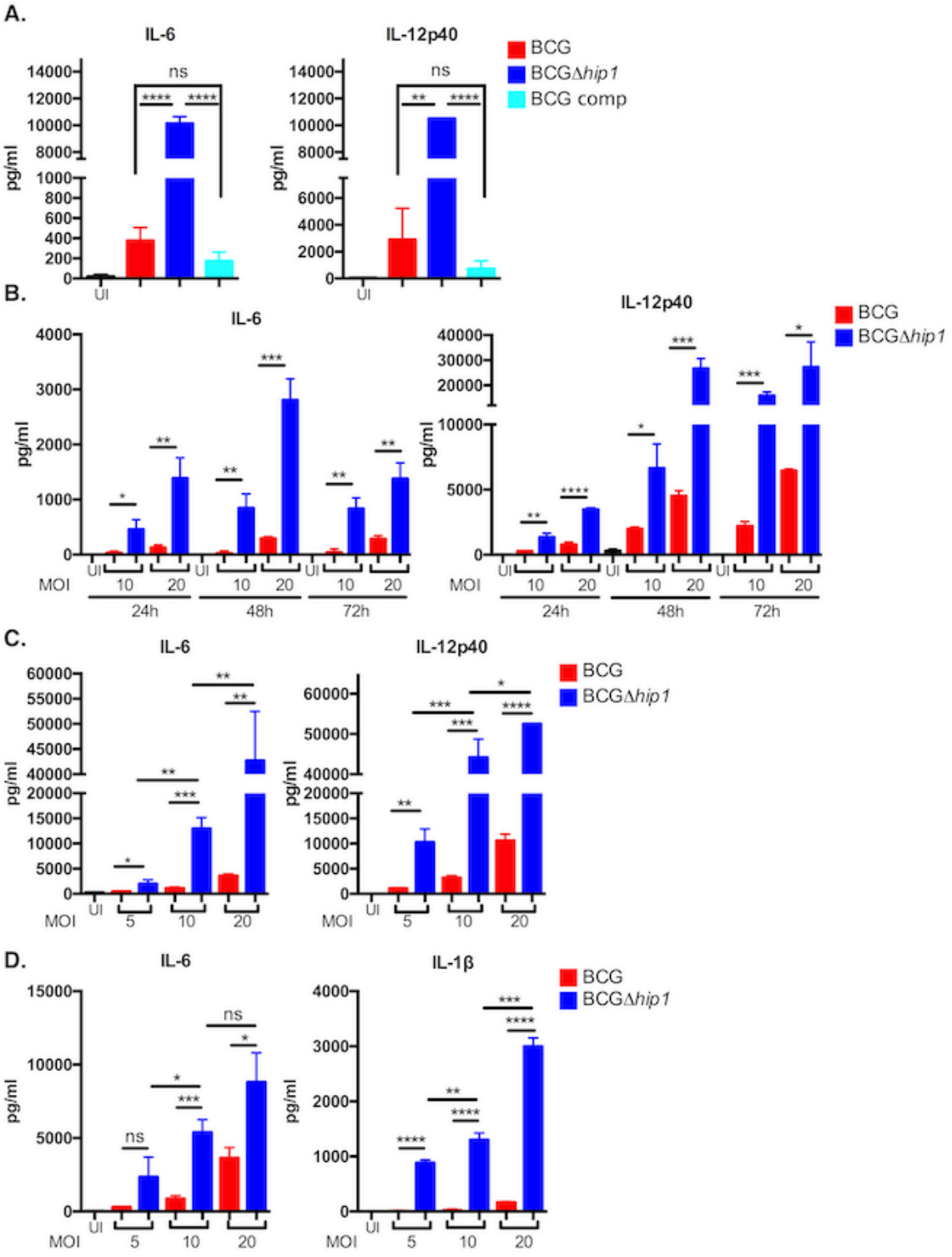

A.
IL-6
IL-12p40
BCG
BCGΔhip1
BCG comp
pg/ml
ns
UI
B.
IL-6
IL-12p40
BCG
BCGΔhip1
pg/ml
MOI
UI
10
20
24h
48h
72h
C.
IL-6
IL-12p40
BCG
BCGΔhip1
pg/ml
MOI
UI
5
10
20
D.
IL-6
IL-1β
BCG
BCGΔhip1
pg/ml
MOI
UI
5
10
20
ns

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

(A) BMDCs from C57BL/6J mice were infected with BCG, BCGΔ*hip1*, or BCGΔ*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 BCGΔ*hip1*, and culture supernatants were assayed for IL-6 and IL-12p40 at 24, 48, and 72 hours post-infection. Heat-killed BCG or BCGΔ*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-1β (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 ±SD. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001, ns= no significance, ui= uninfected

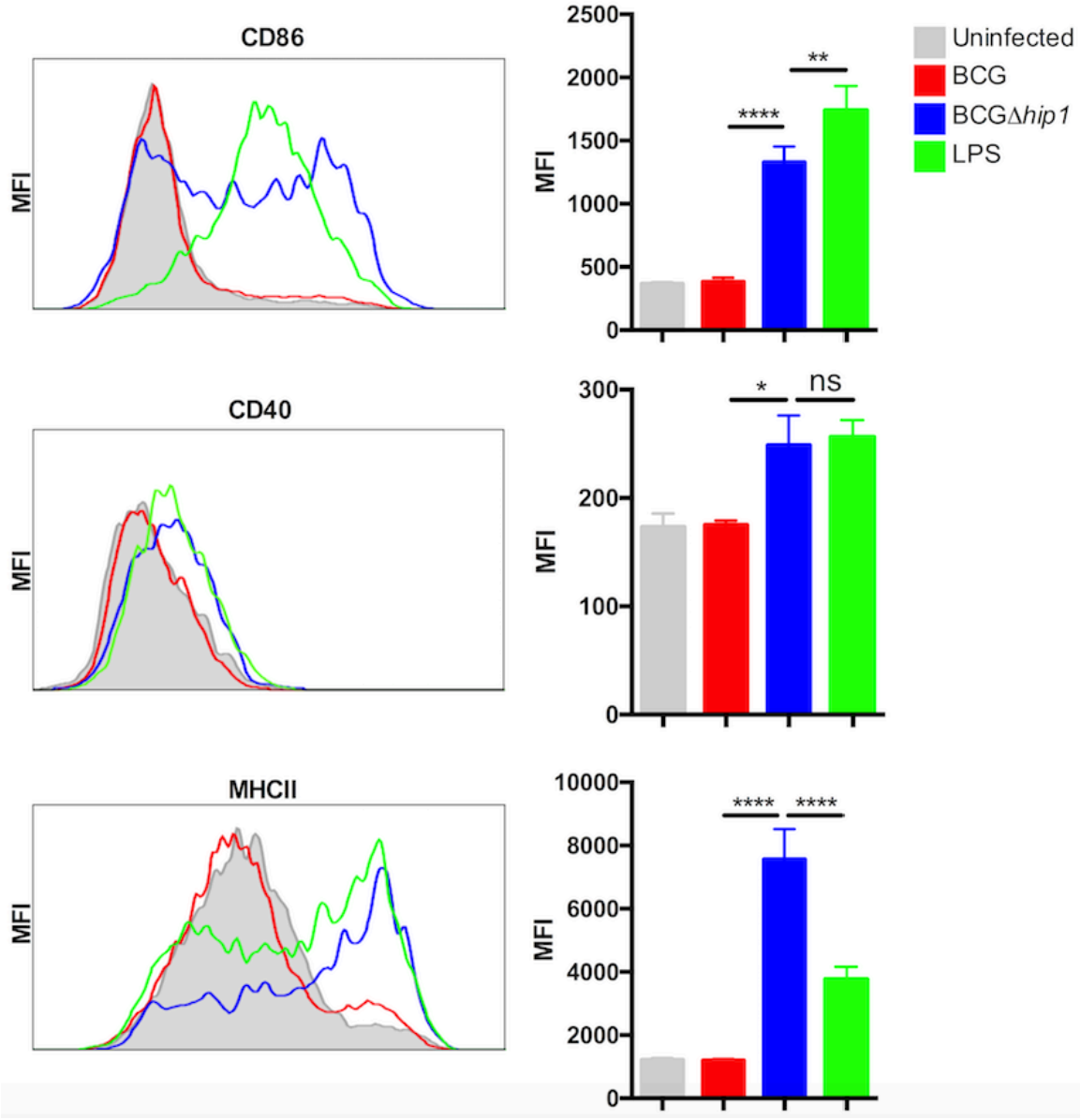

CD86
MFI
2500
2000
1500
1000
500
0
****
**
Uninfected
BCG
BCGΔhip1
LPS
CD40
MFI
300
200
100
0
*
ns
MHCII
MFI
10000
8000
6000
4000
2000
0
****
****

Figure 3. BCGΔ*hip1* enhances expression of costimulatory molecules on infected DCs

BMDCs from C57BL/6J mice were either uninfected, infected with BCG or BCGΔ*hip1* (MOI 10), or stimulated with LPS (1 μg/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 ±SD. *$p<0.05$, **$p<0.01$, ****$p<0.0001$, ns= no significance.

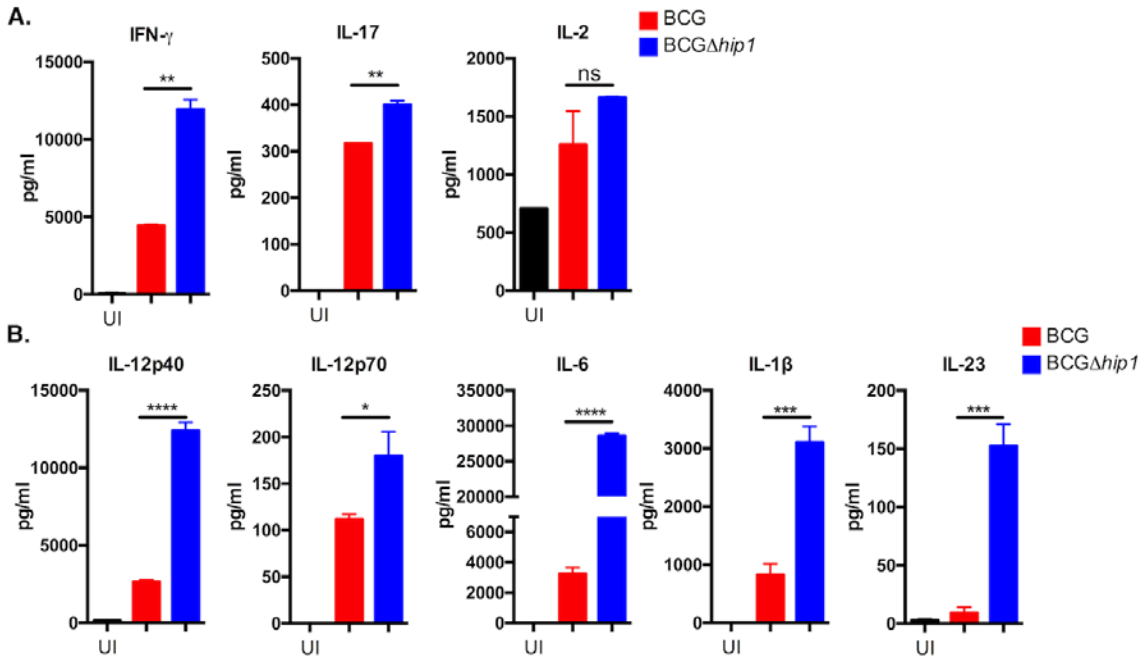


Figure 4. DCs infected with BCGΔ*hip1* induce higher levels of IFN-γ and IL-17 production from CD4 T cells compared to BCG.

BMDCs from C57BL/6J mice were pulsed with $OVA_{323-339}$ peptide, and 6 hrs later, infected with BCG or BCGΔ*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-1β, 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 ±SD. *$p<0.05$, **$p<0.01$, ns= no significance.

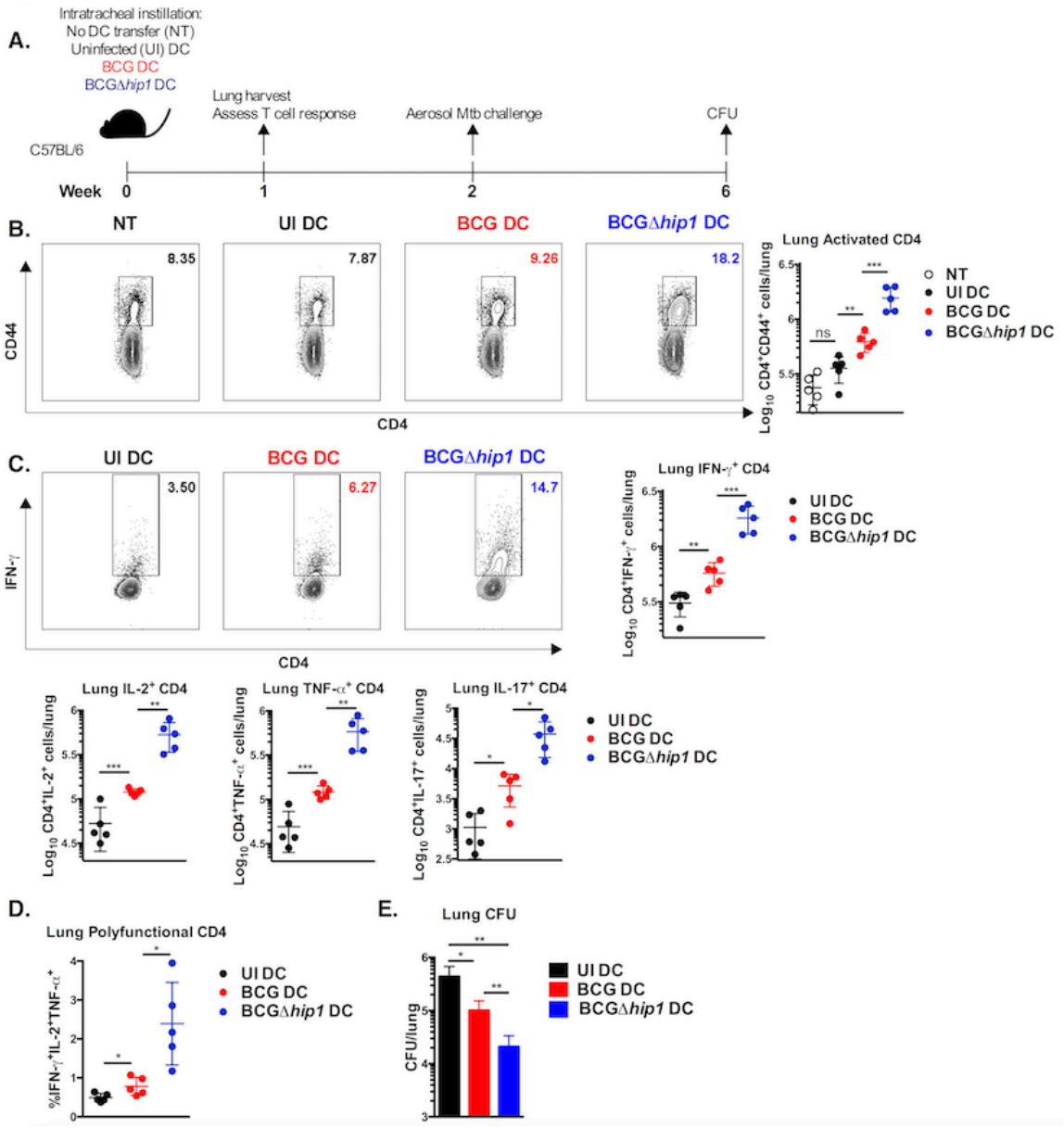
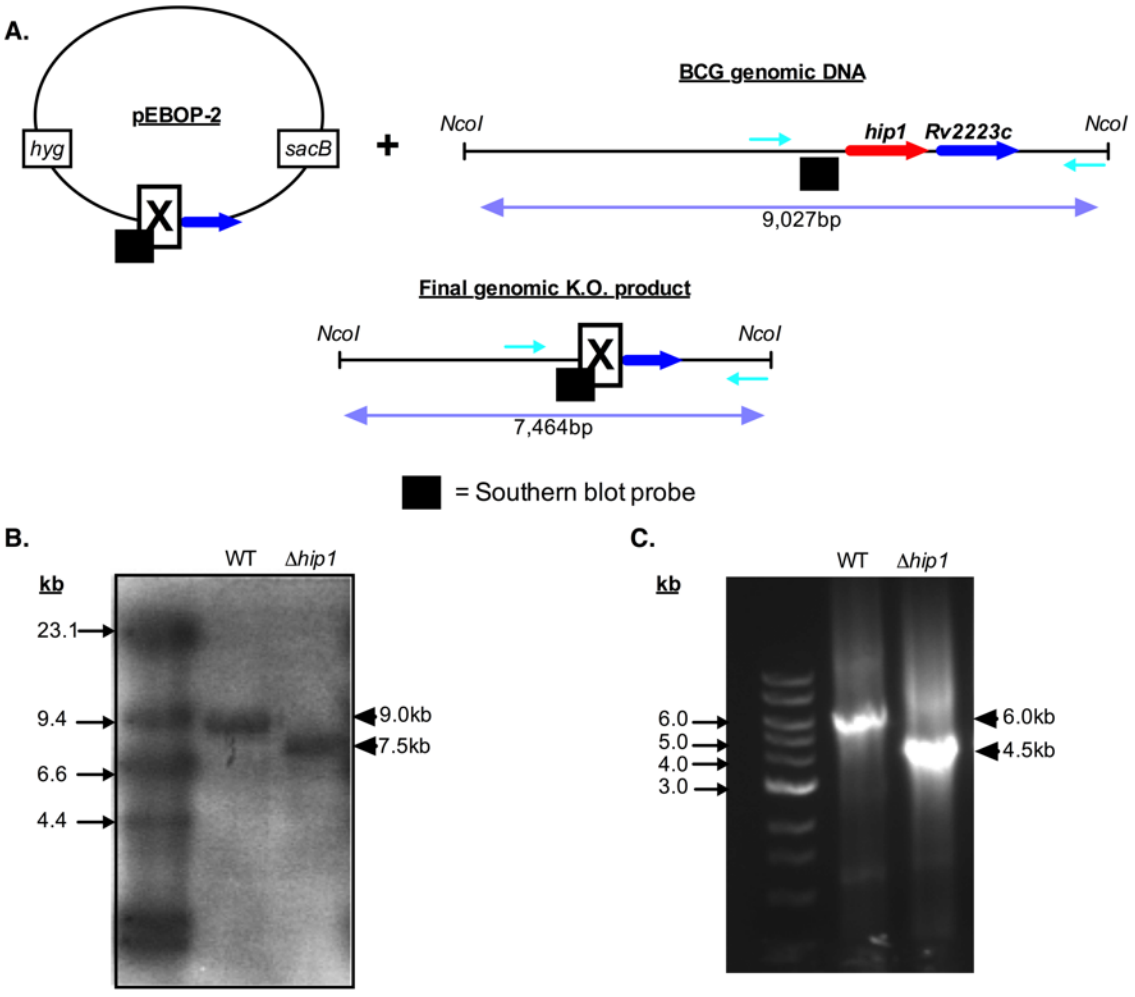

A.
Intratracheal instillation:
No DC transfer (NT)
Uninfected (UI) DC
BCG DC
BCGΔhip1 DC
C57BL/6
Lung harvest
Assess T cell response
Aerosol Mtb challenge
CFU
Week 0 1 2 6
B.
NT
UI DC
BCG DC
BCGΔhip1 DC
8.35
7.87
9.26
18.2
CD44
CD4
Lung Activated CD4
Log10 CD4+CD44+ cells/lung
ns
NT
UI DC
BCG DC
BCGΔhip1 DC
C.
UI DC
BCG DC
BCGΔhip1 DC
3.50
6.27
14.7
IFN-γ
CD4
Lung IFN-γ+ CD4
Log10 CD4+IFN-γ+ cells/lung
Lung IL-2+ CD4
Log10 CD4+IL-2+ cells/lung
Lung TNF-α+ CD4
Log10 CD4+TNF-α+ cells/lung
Lung IL-17+ CD4
Log10 CD4+IL-17+ cells/lung
D.
Lung Polyfunctional CD4
%IFN-γ+IL-2+TNF-α+
E.
Lung CFU
CFU/lung

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

(A) Diagram of experimental design. BMDCs were exposed to BCG or BCGΔ*hip1* or were left uninfected for 24 hours prior to being intratracheally instilled ($1x10^6$ 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 *M. tuberculosis* (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 1week 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 BCGΔ*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 ±SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns= no significance.

Supplemental Figure 1



Supplemental Figure 1. Southern blot and PCR analysis of BCGΔ*hip1*.

(A) pEBOP-2 suicide vector schematic, chromosomal locus for *hip1*, and schematic of Δ*hip1* allele. *NcoI* restriction endonuclease sites are denoted. The Southern probe used is represented by a black box, while cyan arrows indicate PCR amplification primers. (B) Southern blot of *NcoI* digested genomic DNA purified from WT BCG or BCGΔ*hip1* probed with digoxigenin (DIG)-labeled amplicon of the 1kb region upstream of *hip1*. Black arrows denote molecular weight marker values, while black arrowheads indicate DNA detected by probe. (C) PCR amplification of the genomic region surrounding *hip1* using primers 1.6kb upstream and 2.8kb downstream of *hip1*. Black arrows denote molecular weight marker values, while black arrowheads indicate amplified DNA. The contrast and brightness of the Southern blot and DNA gel were adjusted for clarity.

Chapter V

Discussion and future directions

M. tuberculosis is an ancient scourge inexorably intertwined with human history and culture by evolving with and causing disease in our ancestors. Our relationship with *M. tuberculosis* can be inferred from historical and molecular epidemiologic evidence: the ancestor of modern strains of *M. tuberculosis* is estimated to have emerged roughly 15,000 years ago[545-547]; *M. tuberculosis* DNA, as well as characteristic lesions, have been identified in anthropological findings from the New Kingdom of Egypt to pre-Columbian Peru and Chile [548-550]; and works of art and the annals of historical texts, medical or otherwise, are rife with references to TB[551, 552]. The 20th century witnessed the development and implementation of medical advances that have vastly improved human health. It is remarkable, then, that *M. tuberculosis* continues to be a global public health challenge today. Understanding the immunological response to *M. tuberculosis* is paramount in developing effective strategies to curtail the global TB burden. I outlined some of the ways in which *M. tuberculosis* subverts innate and adaptive immune responses in the introduction. In the following section, I will summarize the published and unpublished findings that encompass the work contained within this dissertation, address concepts and hypotheses that have emerged from these findings, and discuss the significance of these findings to the broader context of TB vaccines.

Summary of findings

This dissertation contains two main themes:

1. DCs are readily subverted by *M. tuberculosis* infection, which leads to suboptimal development of antigen-specific CD4 T cell immunity.

2. Improving DC responses to infection or vaccination can improve antigen-specific CD4 T cell immunity and host control of *M. tuberculosis*.

M. tuberculosis readily infects and subverts DCs, but the consequences of this subversion are relatively poorly understood. In Chapter II, we demonstrated that DC cytokine production, co-stimulation, and antigen-presentation are attenuated by *M. tuberculosis* Hip1. From these studies, we also provided an important and novel insight: DC subversion by *M. tuberculosis* substantially affects the development of the IL-17 producing CD4 T cell subset. Whereas DCs infected with wild-type *M. tuberculosis* primarily elicited an IFN-γ response from CD4 T cells, we found that DCs infected with the *hip1* mutant *M. tuberculosis* promoted more robust antigen-specific production of both IFN-γ and IL-17. Unpublished data from a kinetic study of *in vivo* lung responses to ESAT-$6_{1\text{-}20}$ showed that mice infected with the *hip1* mutant *M. tuberculosis* developed an early, robust, and durable IL-17 response relative to mice infected with wild-type *M. tuberculosis* (Appendix, Fig. 1). Interestingly, both sets of animals developed an IFN-γ response of similar magnitude. Collectively, these data focused our attention towards the relatively poorly understood development of Th17 cells in *M. tuberculosis* infection.

Although Th17 cells are known to be important in the context of subunit vaccination[293, 482], their role in natural immunity to *M. tuberculosis* was unknown because H37Rv does not elicit significant levels of antigen-specific Th17 cells and little was understood regarding their development beyond the cytokines necessary for their polarization. In Chapter III, we demonstrated that the co-stimulatory molecule, CD40, on DCs is required

for the development of an antigen-specific Th17 response during infection with *M. tuberculosis*. We also established that Th17 development requires CD40L signaling in antigen-specific CD4 T cells. Further, we show that ligation of CD40 on DCs infected with *M. tuberculosis* enhances DC cytokine production and polarization of CD4 T cells to the Th17 subset *in vitro* and *in vivo*. Interestingly, ligation of CD40 on infected DCs lowered the antigenic requirements for Th17 development, which identifies this pathway as an attractive target to augment levels of antigen-specific Th17 cells early during infection when antigen load is low. Finally, we demonstrated that mucosal transfer of antigen-loaded, CD40-ligated DCs enhanced lung CD4 T cell immunity and host control of lung bacterial burden following *M. tuberculosis* challenge. Unpublished data suggested that CD40 ligation requires TLR2 signaling to maximally promote Th17 polarization (Appendix, Fig. 2), though the molecular mechanisms underlying this synergy requires further investigation. Notably, this study suggests that DC subversion by *M. tuberculosis* hinders the development of a Th17 response and that targeting the CD40 pathway can overcome *M. tuberculosis* subversion. We also clarify the role of Th17 cells during infection with *M. tuberculosis* and show that early induction of antigen-specific Th17 cells enhances host immunity against infection.

In Chapter IV, we extended our findings to understand the limited immunogenicity of BCG. There has been a spate of efforts in TB vaccinology to improve BCG immunogenicity by providing *M. tuberculosis* antigens, but we considered the hypothesis that BCG immunogenicity may be limited by shared mycobacterial immune subversion strategies between BCG and *M. tuberculosis*. Notably, BCG shares an identical Hip1

protein with *M. tuberculosis*. E. Bizzell generated a strain of BCG lacking Hip1 (BCGΔ*hip1*) and *in vitro* studies supported the hypothesis that BCG Hip1 also serves as an immunomodulatory protease that attenuates DC and macrophage responses. We found that BCGΔ*hip1* infected DCs displayed a more mature phenotype that included robust expression of co-stimulatory molecules relative to BCG infected DCs. Consequently, BCGΔ*hip1* infected DCs better induced Th1 and Th17 responses upon co-culture with antigen-specific CD4 T cells. We demonstrated that mucosal transfer of DCs infected with BCGΔ*hip1* conferred enhanced antigen-specific CD4 T cell responses in the lungs that ultimately augmented host control of *M. tuberculosis* bacterial burden following aerosol challenge relative to transfer of BCG infected DCs.

Future considerations

Seq'ing answers in the infected DCs

Global approaches to determine the transcriptional response of *M. tuberculosis* infected cells are becomingly increasingly utilized. In order to gain a more comprehensive understanding of *M. tuberculosis* subversion of DCs, we propose to leverage transcriptional profiling using RNA-seq. The transcriptional responses of *M. tuberculosis* infected myeloid cells have been compared to infection with phylogenetically distinct pathogens[553]. Other groups have investigated cell-type specific or whole blood transcriptional profiles of clinically distinct patient cohorts[170, 554-557]. While there are advantages to these approaches, including the identification of *M. tuberculosis* specific transcriptional signatures that can be leveraged for biomarker discovery, the comparisons utilized in these studies make it difficult to distinguish pathogenic immunity

derived from infection and inflammation from beneficial immunity that leads to enhanced host control of infection.

Based on the findings from Chapter II and III, we propose to transcriptionally profile *M. tuberculosis* infected DCs compared to *hip1* mutant infected DCs, with and without CD40 ligation on both conditions. This can be a prelude to or in conjunction with similar experiments assessing transcriptional signatures of infected lung DCs sorted on the basis of infection with fluorescently labeled *M. tuberculosis* or *hip1* mutant. Further studies to compare the transcriptional signatures of DCs infected with other *M. tuberculosis* mutants (e.g. *nuoG* mutant, *RD1* mutant) or with a variety of clinical isolates (e.g. CDC1551, HN878) can reveal shared and distinct pathways that can be linked to the outcome of infection and *in vivo* T cell functionality. Finally, there is a dearth of knowledge on the transcriptional signature of DCs in the context of vaccination. Our own studies in Chapter III and IV and data from others[239] demonstrated that DCs can enhance vaccine-mediated immunity, but much of our knowledge regarding vaccination involves the phenotypic, functional, and transcriptional characterization of lymphocyte populations. DCs from vaccinated or unvaccinated hosts encounter phenotypically and functionally distinct T cells, but the impact of these unique encounters on the DC is unknown. Understanding the interactions between memory T cells and *M. tuberculosis* infected DCs can shed light on vaccine-mediated immunity. We can leverage well-established subunit, live-attenuated, or inactivated whole cell vaccinations and challenge with fluorescently labeled *M. tuberculosis*. This design allows us to transcriptionally profile sorted *M. tuberculosis* infected DCs from the lungs and mediastinal lymph nodes

at different timepoints after challenge of vaccinated or unvaccinated hosts. Transcriptional signatures can be validated *in vitro* and *in vivo* to inform us about how manipulation of DC functions impacts the efficacy of vaccination and the outcome of infection. Taken together, these experiments can provide a set of data revealing novel mechanisms of DC subversion by *M. tuberculosis*. This knowledge can be strategically employed for the creation of better adjuvants, vaccines, or other host-directed therapies for TB.

Impact of ligating other DC co-stimulatory molecules during infection and vaccination

This dissertation work uncovered a novel role for CD40 co-stimulation in the development of antigen-specific Th17 responses during *M. tuberculosis* infection and demonstrated that strategically targeting the CD40 pathway via ligation on DCs enhances host immunity. DCs also express a wide range of other co-stimulatory molecules whose roles during T cell priming and *M. tuberculosis* infection have not yet been fully explored. This dissertation work raises the possibility that ligation of other DC co-stimulatory molecules following infection with *M. tuberculosis* can regulate DC-CD4 T cell interactions and affect infection outcome. Using flow cytometry, we want to define the spectrum of DC co-stimulatory molecules differentially expressed following infection with *M. tuberculosis* or *hip1* mutant, with and without ligation of CD40. Subsequently, we can study the effects of ligating selected DC co-stimulatory molecules, singly or in combination, on DC and antigen-specific T cell responses to infection. In preliminary studies taking this approach, we identified the OX40-OX40L pathway in the modulation of *M. tuberculosis* infected DC cytokine production and CD4 T cell responses.

Identification of other candidate co-stimulatory molecules of interest can also be derived from aforementioned transcriptional profiling experiments. Ligation of DC co-stimulatory molecules may be viewed from the lens of host-directed therapy. However, there is precedence in using co-stimulatory pathways to enhance vaccine-mediated innate and adaptive immunity to viral pathogens[558, 559], but not to TB. Therefore, substantial opportunity and rationale exists for the identification of DC co-stimulatory pathways that can be targeted during infection and vaccination in order to improve host immunity against *M. tuberculosis*.

Considering alternative consequences of engaging DC co-stimulatory molecules

Although the most well-known consequence of CD40 ligation on DCs is the production of pro-inflammatory cytokines, early studies also implicated CD40 ligation with extended DC survival[560-562]. Pro-survival signals relayed by CD40 ligation are dependent on Bcl-x(L)[563] and components of the canonical NF-κB pathway[564], though other pathways are likely involved. Little is known regarding the duration of antigen-presentation following infection with *M. tuberculosis*. It is possible that CD40 ligation of *M. tuberculosis* infected DCs extends their survival and antigen-presentation to CD4 T cells in addition to enhancing DC cytokine production. Based on studies described in Chapter III, we have also demonstrated that CD40 ligation of *M. tuberculosis* infected DCs can modulate the antigen dose required to elicit an antigen-specific IL-17 response, which may be due to enhanced cytokine production from the ligated DC. However, the possibility that CD40 ligation is also altering the infected DC to promote more efficient physical DC-CD4 T cell interactions is intriguing. There is evidence that CD40 ligation

promotes accumulation of MHCII and other co-stimulatory molecules in lipid rafts on the surface of B-cells, which is associated with enhanced antigen-presentation[565]. We propose that CD40 ligation enhances formation of immunological synapses leading to stable interactions between infected DCs and CD4 T cells and more efficient T cell polarization. Interactions between APCs and antigen-specific lymphocytes is fleeting in the chronic mycobacterial granuloma[314], which negatively affects T cell functionality. Future studies can address whether ligation of DC co-stimulatory molecules improves physical interactions between infected DCs and antigen-specific lymphocytes.

Integration and challenges in context of TB vaccines

The "End TB" strategy proposed by the World Health Organization envisions dramatic reductions in TB incidence, morbidity, and mortality in 2030 compared to 2015[1]. I frame the global TB challenge with the following statistics:

1. The average annual decline in the global TB incidence rate between 2000 and 2016 is 1.4% but must accelerate to a historic 4-5% decline per year in order to meet "End TB" milestones in 2020[1].
2. Globally, there is an 83% treatment success rate, on average, for new and relapse TB cases in 2015[1].

While the treatment success rate for TB cases is relatively high, the stagnant rate of decline for TB incidence suggests that breaking the transmission cycle is key to significantly reducing the global TB burden. Introduction of a more efficacious TB vaccine would be crucial in this regard, but absence of validated correlates of protection

is a considerable obstacle. Other significant challenges also remain. We still do not understand the spectrum of antigen-specific responses during the course of infection with *M. tuberculosis* and whether responses to certain antigens are preferable to others. The early events leading to the development of antigen-specific immunity following infection with *M. tuberculosis* remains incompletely understood. Furthermore, translation of fundamental insights on infection immunity to the development of novel vaccines and therapeutics remains a significant challenge in the TB field.

Much of the work in Chapter III is based on the use of intratracheally instilled, *ex vivo* manipulated DCs. This approach was taken in order to specifically investigate the role of DCs during infection as well as to avoid non-specific and potentially pathological effects of systemic CD40 ligation. Although there are multiple, ongoing clinical trials assessing the use of autologous DC therapy for the treatment of various cancers, this approach does not have traction in the context of a TB vaccine. Therefore, in addition to identifying candidate DC pathways to activate, it will be important to identify adjuvants that augment expression of DC co-stimulatory molecules, suitable antigens to utilize, and delivery methods that specifically target DCs *in vivo*. Finally, our studies described in Chapter IV demonstrated that BCGΔ*hip1* improves host immunity against *M. tuberculosis* challenge in the context of DC transfer. In order to assess the vaccine potential of BCGΔ*hip1* compared to BCG, future studies using traditional vaccination approaches will investigate different routes of delivery, strategies to further enhance immunogenicity, and efficacy as measured by reductions in *M. tuberculosis* burden and survival after challenge.

Overall, this dissertation contributes to a growing body of evidence that *M. tuberculosis* is able to evade adaptive immune responses by subverting the DC. The long and dark history between us and *M. tuberculosis* is becoming progressively illuminated by a deeper understanding of the immune response to infection. Further understanding the role of the DC during infection and vaccination can provide insights for the development of more effective TB vaccines and immunotherapies.

Chapter VI

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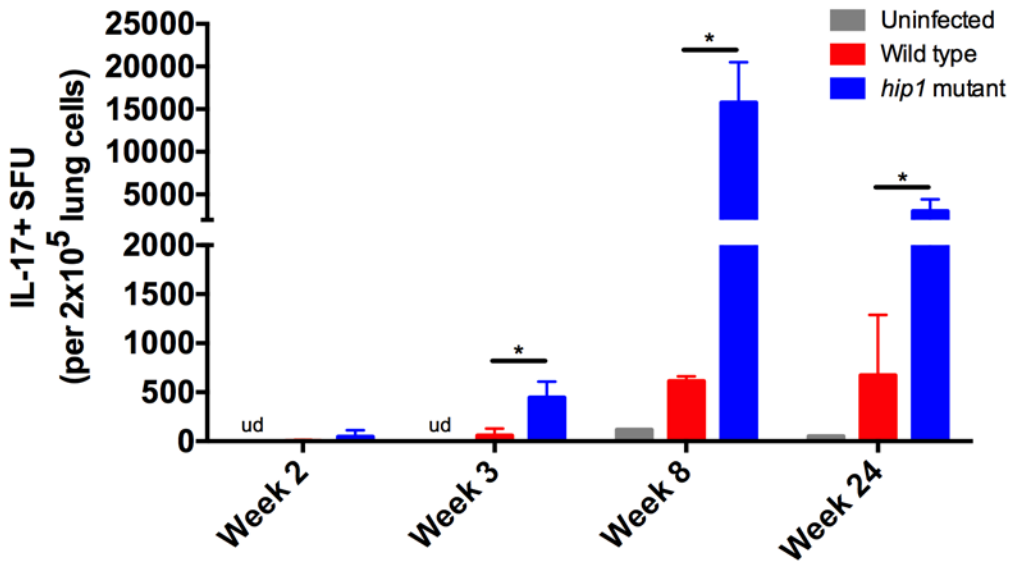
Appendix


IL-17+ SFU
(per 2x10^5 lung cells)
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Uninfected
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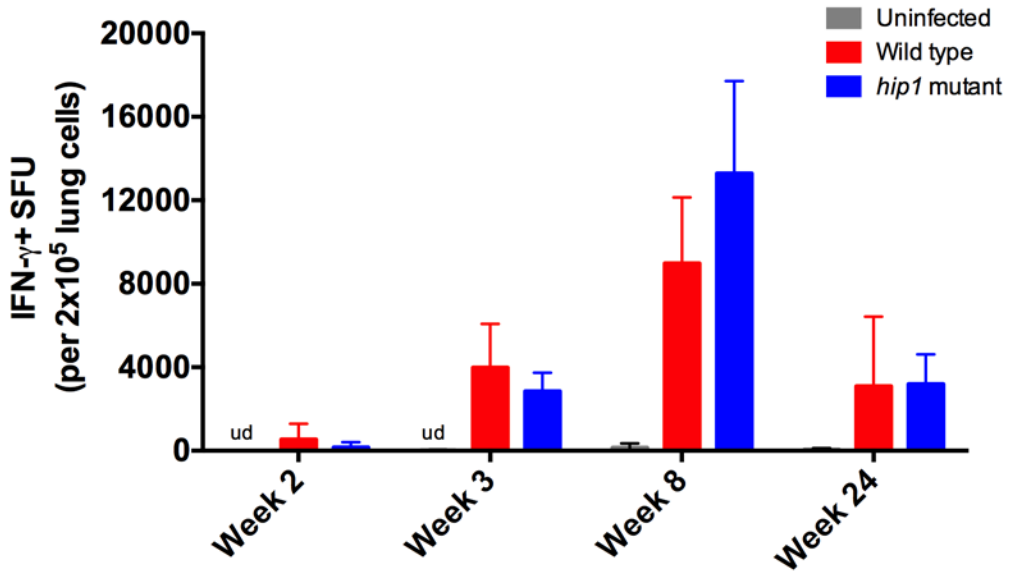
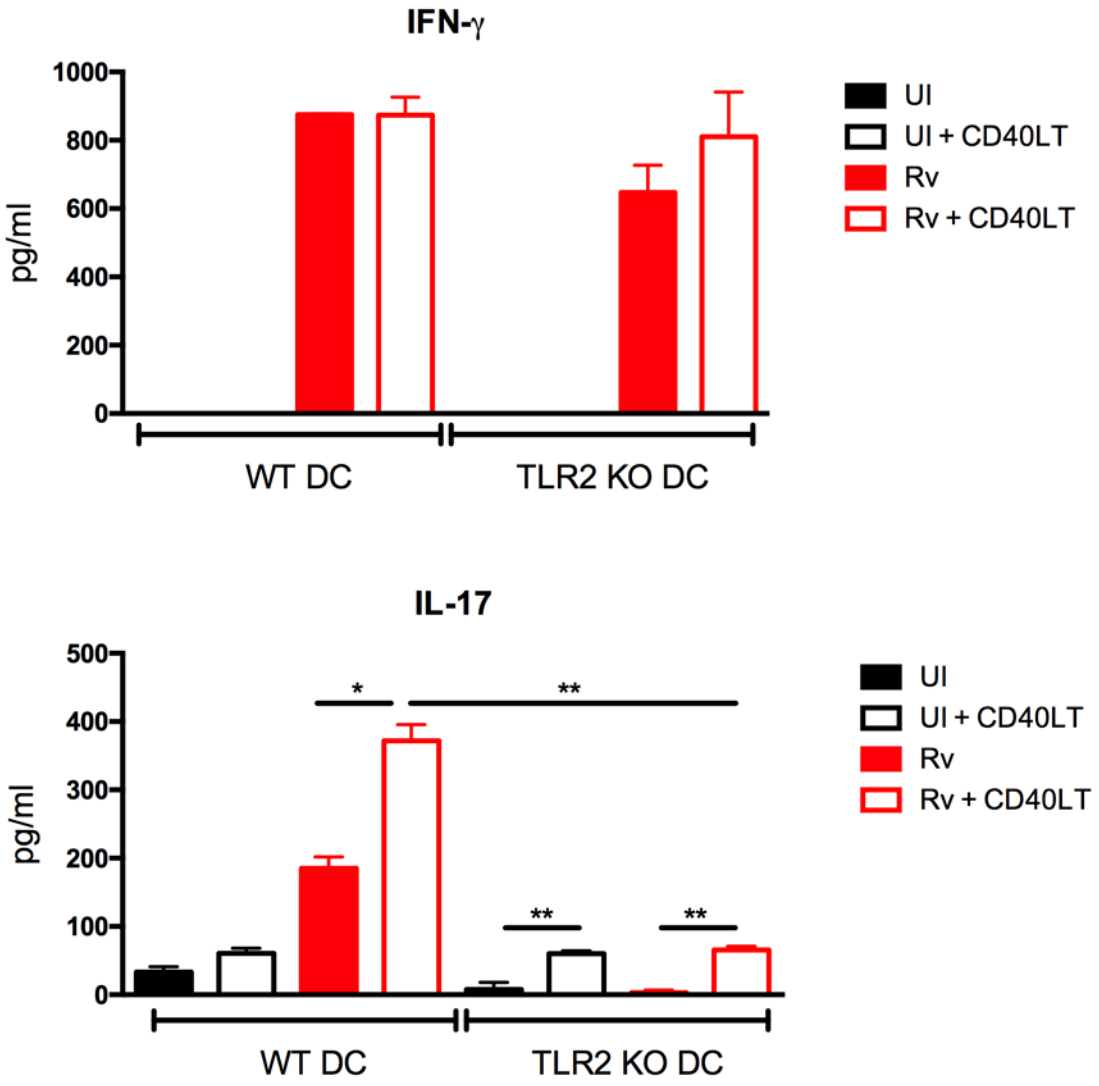

IFN-γ+ SFU
(per 2x10^5 lung cells)
20000
16000
12000
8000
4000
0
Uninfected
Wild type
hip1 mutant
ud
ud
Week 2
Week 3
Week 8
Week 24

Figure 1. Time-course of ESAT-6_{1-20} specific lung responses following aerosol *M. tuberculosis* or *hip1* mutant infection.

C57BL/6J mice (n=5 per group) were infected with 100-150 CFU of *M. tuberculosis* H37Rv or *hip1* mutant *M. tuberculosis* by aerosol. Uninfected mice were utilized as controls. At indicated timepoints post-infection, lungs were harvested for CFU enumeration (upper right lobe) and immune assays (remaining lung tissue). Lung cells were processed to single-cell suspensions and $2x10^5$ cells were stimulated with 10 μg/ml ESAT-6_{1-20} for 48 hours. Antigen-specific responses were measured as spot-forming units (SFU) by ELISPOT. Statistical significance was determined using one-way analysis of variance (ANOVA) correcting for multiple comparisons. * $p<0.05$


IFN-γ
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UI
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Rv
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IL-17
500
400
300
200
100
0
pg/ml
*
**
**
**
WT DC
TLR2 KO DC
UI
UI + CD40LT
Rv
Rv + CD40LT

Figure 2. Comparing WT and TLR2 KO DC induction of antigen-specific IFN-γ and IL-17 responses.

Wild-type or TLR2 KO BMDCs were generated as previously described. $3x10^5$ DCs were infected with heat-killed *M. tuberculosis* (MOI 10) or left uninfected (UI) in conjunction with CD40LT treatment (1 μg/ml) as indicated. After 24 hours, CD4-purified OTII T cells were co-cultured at a ratio of 1:4 DC:T cell alongside cognate peptide. Cell-free supernatants were harvested after 72 hours and IFN-γ and IL-17 levels were measured by ELISA. Statistical significance was determined by unpaired two-tailed student's t-test. Values are presented as mean ±SD. *$p<0.05$, **$p<0.01$

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Review Article

Innate Immune Defenses in Human Tuberculosis: An Overview of the Interactions between *Mycobacterium tuberculosis* and Innate Immune Cells

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Tuberculosis (TB) remains a serious global public health problem that results in up to 2 million deaths each year. TB is caused by the human pathogen, *Mycobacterium tuberculosis* (Mtb), which infects primarily innate immune cells patrolling the lung. Innate immune cells serve as barometers of the immune response against Mtb infection by determining the inflammatory milieu in the lungs and promoting the generation of adaptive immune responses. However, innate immune cells are also potential niches for bacterial replication and are readily manipulated by Mtb. Our understanding of the early interactions between Mtb and innate immune cells is limited, especially in the context of human infection. This review will focus on Mtb interactions with human macrophages, dendritic cells, neutrophils, and NK cells and detail evidence that Mtb modulation of these cells negatively impacts Mtb-specific immune responses. Furthermore, this review will emphasize important innate immune pathways uncovered through human immunogenetic studies. Insights into the human innate immune response to Mtb infection are necessary for providing a rational basis for the augmentation of immune responses against Mtb infection, especially with respect to the generation of effective anti-TB immunotherapeutics and vaccines.

1. Introduction

Infection with *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), was responsible for 1.5 million deaths and 9 million cases of TB in 2013, according to the World Health Organization [1]. While only 5–10% of individuals infected with Mtb progress to active TB disease, approximately one-third of the world population, or over 2 billion people, are estimated to have latent Mtb infection (LTBI) [2]. Latently infected individuals control Mtb infection and are clinically asymptomatic but retain a significant risk of progressing to TB by reactivation of latent Mtb when immune compromised [3]. This is due to the ability of Mtb to persist within granulomatous lesions in the lungs of individuals and the inability of host immunity to completely eradicate mycobacteria from host tissues [4]. Granuloma formation is initiated by Mtb-infected macrophages and continues with the development of multinucleated giant cells (MGCs) and lipid-filled foamy macrophages surrounded by a ring of lymphocytes encapsulated in a fibrotic cuff [5, 6]. Although macrophages and T cells play a central role in the formation of the granuloma, the complete cellular composition of the human granuloma throughout Mtb infection remains unclarified and other cell types, including dendritic cells (DCs), neutrophils, and B lymphocytes, are present and have been shown to contribute to cellular recruitment and the maturation of the granuloma [7]. Thus, granulomas are a testament to the involvement of both innate and adaptive immune cells in the human immune response to TB. Huge strides have been made towards understanding the acute and chronic T cell response to Mtb infection from studies in animal models, but the earliest encounters between Mtb and

the human innate immune system are incompletely understood.

Investigations in animal models of TB, human clinical and epidemiological studies on the genetics of mycobacterial susceptibility, and *in vitro* work with primary human cells strongly indicate that innate immune responses play a major role in determining the outcome of TB by helping control bacterial load and through shaping the nature and magnitude of adaptive immune responses [8–10]. While innate antimicrobial pathways that are activated early and throughout infection play a role in limiting disease, myeloid cells also serve as the primary niches for Mtb replication. Moreover, Mtb has evolved multiple strategies to modulate innate immune responses and prevent optimal activation of adaptive immunity. Thus, understanding the crosstalk between innate and adaptive immune cells in human TB is critical for identifying novel targets of immunomodulatory therapies and for elucidating mechanisms of protective immunity. However, innate immune responses to Mtb infection in humans remain relatively poorly understood, largely because of the inherent difficulties in studying lung-specific immunity in humans. This review will focus on the key innate immune cell types implicated in the human response to Mtb, the interaction of innate immune cells with Mtb, and their influence on adaptive immune responses and the course of disease. We will also review specific human immunogenetics studies that link perturbations in innate immunity to mycobacterial susceptibility.

2. Friendly Guardians: Innate Immune Cells in TB

The major innate cell types that have been studied in humans are macrophages, neutrophils, DCs, and natural killer (NK) cells. Recently, other cell types not classically defined as immune cells, such as airway epithelial cells, have been shown to contribute to the immune response against Mtb in animal models of TB [11] and *in vitro* studies with human cell lines [12]. However, this review will focus on the roles of the classically defined innate immune cells in human TB as these cells serve as both the primary cellular niches for Mtb replication as well as the initial sources of immune pressure to contain infection.

2.1. Macrophages. Alveolar macrophages are one of the first host cell types to encounter Mtb in the lungs following aerosol transmission. While macrophages function as the first line of defense against Mtb infection, early interactions between macrophages and Mtb favor the bacteria. Thus, macrophages are a major cellular niche for bacterial replication during early infection and serve as reservoirs for persistent bacteria within the lung granulomas during chronic infection. In human TB, several aspects of macrophage functions have been investigated, including phagocytosis of bacteria, induction of antimicrobial pathways, and responsiveness to interferon gamma (IFN-γ) (Figure 1).

A number of receptors recognizing a wide variety of mycobacterial ligands play a role in human macrophage phagocytosis of Mtb. Collectins (e.g., surfactant proteins A and D and mannose-binding lectin), C-type lectins (e.g., mannose receptor, DC-SIGN, and Dectin-1), toll-like receptors (TLRs; e.g., TLR-2, TLR-4, and TLR-9), and many others have been implicated in the recognition and uptake of mycobacterial glycolipids, lipoproteins, and carbohydrates [10]. Of these, the best characterized are mannose-binding lectin (MBL) and mannose receptor (MR). MBL belongs to a family of soluble C-type lectins, called collectins, which are involved in the recognition and clearance of apoptotic cells via calreticulin and CD91 mediated phagocytosis [13]. During Mtb infection, MBL recognizes mannosylated lipoarabinomannan (ManLAM) and phosphatidylinositol mannosides (PIMs) [10]. In addition to recognition of Mtb ligands and phagocytosis, macrophage receptors are also involved in the activation of specific downstream pathways. As an example, MR, a transmembrane C-type lectin, ligates Mtb lipoarabinomannan and activates macrophage peroxisome proliferator activated receptor gamma (PPARγ) expression in a phospholipase A2 and TLR-2 dependent manner [14, 15]. In contrast to the avirulent vaccine strain *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), virulent Mtb activates macrophage PPARγ and induces the production of cyclooxygenase 2 and IL-8, which regulate inflammatory responses via arachidonic acid metabolites and the recruitment of neutrophils, respectively [14]. These studies suggest that the earliest Mtb interactions with macrophages at the level of receptor-mediated phagocytosis can influence the ensuing inflammatory response. Moreover, it is likely that Mtb manipulates these responses in order to promote its survival and dissemination. No single receptor has been demonstrated to be essential for macrophage phagocytosis of Mtb during human infection and it is clear that Mtb is recognized by numerous receptors which induces a network of coordinated receptor-mediated signaling pathways that lead to distinct gene expression profiles of infected macrophages at different stages of disease. Studies centered on the gene expression profiles of Mtb-infected macrophages have largely been explored in murine cells, though a few studies have examined proinflammatory cytokine profiles in Mtb-infected human macrophages [16, 17] as well as global gene expression after infection of *in vitro* blood monocyte-derived macrophages or human monocytic cell lines with Mtb [18–22]. These early gene profiling studies in infected human macrophages provided evidence for the importance of IFN-γ transcription in suppressing Mtb gene expression [17], highlighted a prominent role for IL-1β and other proinflammatory cytokines at early and late timepoints after infection, and showed that macrophage responses to pathogenic mycobacteria differed from responses to infection with nonpathogenic mycobacteria [18–22]. Additionally, transcriptional profiling studies of blood monocyte-derived human macrophages after Mtb infection *in vitro* have provided corollary evidence for the importance of known factors such as IL-12 in combating Mtb as well as new insights into other factors not known to be important previously, including macrophage-derived chemokine CCL22 (MDC) and macrophage inflammatory protein-1α (MIP-1α/CCL3) [17]. Importantly, a study that examined gene expression of *ex vivo* stimulated macrophages from TB patients resulted in

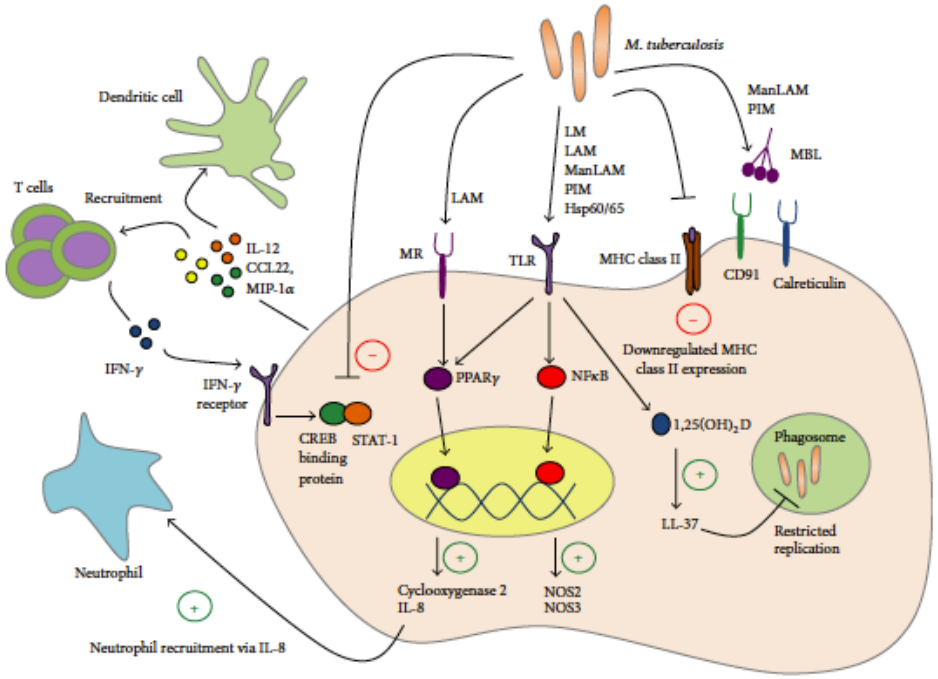

FIGURE 1: Human alveolar macrophages possess an array of receptors to recognize *M. tuberculosis* (Mtb). In response to Mtb infection, macrophages upregulate effector and signalling pathways to both prevent bacterial replication and recruit other immune cells into the site of infection. *M. tuberculosis* components, including lipoarabinomannans (LAMs), lipomannans (LMs), phosphatidylinositol mannosides (PIMs), and heat shock proteins (HSPs), are recognized by a variety of pattern recognition receptors. Following recognition of Mtb, host effectors, such as NF-κB and PPARγ, are activated to upregulate antimicrobial factors. These antimicrobial peptides (e.g., LL-37) possess both effector and signalling functions to actively interfere with bacterial replication as well as recruit and activate neutrophils, dendritic cells, and T cells. However, Mtb interferes with macrophage effector and signalling pathways. Most importantly, Mtb downregulates MHCII expression on macrophages to prevent optimal interaction with antigen specific T-cells. Furthermore, Mtb interferes with IFNγ signaling, a T cell cytokine mediator critical for upregulating the inherent antimicrobial capacity of macrophages during infection.

the association of CCL1 with TB susceptibility [23]. Studies that investigate gene expression profiles of primary macrophage cells from patients with TB are limited but are required to yield the most relevant insights into how Mtb interacts with human macrophages *in vivo*. With a few exceptions, the gene expression studies in Mtb-infected human macrophages and macrophage cell lines highlighted here have validated and supplemented data derived from other mechanistic studies in human monocytic cell lines and mouse cells. Much remains unknown about the gene expression profile of Mtb-infected primary macrophages from TB patients and studies utilizing primary samples from TB cohorts will provide the best insight into the human macrophage response to Mtb infection *in vivo*.

Insights into the survival and replication of Mtb within macrophage phagosomes have largely been derived from studying murine macrophages, and studies on Mtb manipulation of phagosomal function in human macrophages have been relatively limited. Interestingly, recent studies have examined alveolar macrophages from the bronchoalveolar lavage (BAL) of patients coinfected with Mtb and HIV [24, 25] and demonstrate that Mtb resides within relatively nonacidified compartments in otherwise functionally capable macrophages. This suggests that the phagosome in human macrophages is specifically modulated by Mtb to make it a preferential niche and further studies will be needed to clarify mechanisms of immune evasion that specifically target the human macrophage phagosome. Tools developed for the assessment of innate immune functions, including vacuole acidification and superoxide burst, will be important in answering questions as to why bacteria are able to replicate within otherwise hostile environments [26, 27]. The IFN-γ pathway remains a critical pathway for resistance against

mycobacterial infection, as highlighted by increased susceptibility to mycobacterial infections in humans with genetic impairments in the IL-12/STAT-1 pathway [8, 9, 28, 29], though *in vitro* evidence indicates that IFN-γ alone does not fully limit Mtb replication in human macrophages and that Vitamin D signaling pathways augment macrophage IFN-γ responsiveness [30–37]. Vitamin D was shown to act synergistically with IFN-γ to augment antimycobacterial activity in human monocytes [37]. Vitamin D treatment enhances a variety of important downstream pathways in macrophages, including autophagy, phagosomal maturation, and the production of antimicrobial peptides [30, 35]. Additional studies have demonstrated that the bioactive 1,25-dihydroxyvitamin D3 can restrict Mtb replication within infected human macrophages [36] in a phosphatidylinositol 3-kinase-dependent [34] and TLR-dependent [33] manner. Mechanistically, Vitamin D upregulates gene expression of macrophage *hCAP-18*, which encodes for the antimicrobial peptide LL-37 (cathelicidin), and LL-37 trafficking to Mtb-containing phagosomes is purported to mediate the antimycobacterial effects of Vitamin D [31, 32].

Mtb contains numerous pathogen associated molecular patterns (PAMPs) that are recognized by a variety of cell surface and intracellular pattern recognition receptors (PRRs) on macrophages. Engagement of PRRs leads to activation of antimicrobial effector functions within the macrophage. For example, TLR2 recognizes mycobacterial mannosylated lipoarabinomannans and engagement of this receptor-ligand pair leads to downstream NF-κB activation and inducible nitric oxide synthase (*iNOS*) gene transcription [38]. NOS2 and NOS3 expression has been implicated in the production of nitric oxide (NO) in human macrophages [39] and clear induction of macrophage NOS2 mRNA can be seen in the BAL of TB patients compared to healthy controls [35, 40]. In contrast to murine studies, NO seems to have limited bactericidal or bacteriostatic effects against Mtb during *in vitro* infection of human alveolar macrophages and primary monocytes post-IFN-γ treatment [39, 41], suggesting that the critical immune responses to Mtb garnered from studies in animal models may not be as important during human infection. Alternatively, antimycobacterial effects of NO may in fact occur *in vivo* within the lung microenvironment.

The IFN-γ/IL-12 axis is critical in host resistance to Mtb in mice and in humans [42–46]. Clinical observations of increased levels of IFN-γ in the pleural fluid and BAL of patients with confirmed pulmonary TB compared to healthy controls suggest that IFN-γ plays a prominent role in human TB infection [47–50]. While murine macrophages activated by IFN-γ alone show distinctly augmented capacity for antimycobacterial functions compared to untreated macrophages, human macrophages require additional factors such as Vitamin D, in addition to IFN-γ, to maximize antimycobacterial functions [37]. This is perhaps due to Mtb-mediated inhibition of critical STAT1 protein-protein interactions with cAMP response element binding (CREB) binding protein [51–53], leading to hyporesponsiveness to IFN-γ stimulation. Additionally, MHC class II expression, normally upregulated after IFN-γ activation of macrophages, is downregulated after Mtb infection of human macrophages via decreased expression of class II transactivator (CIITA) [52, 54, 55]. This may play a role in dampening adaptive immune responses by attenuating T cell recognition of infected macrophages and could explain the reported defects in antigen recognition by Mtb-specific lymphocytes in the granuloma [56].

Overall, macrophages are clearly at least capable of restricting Mtb bacilli given appropriate activation signals from antigen specific T cells and the local lung microenvironment. However, questions remain regarding whether effective juxtaposition of infected macrophages and activated T cells occurs within the confines of the lung. Studies aimed at answering basic questions about infected macrophages in human TB, including signaling pathways subverted during infection, the activation status of Mtb-infected human alveolar macrophages, and the crosstalk between macrophages and T cells within infected lungs, are critical for developing immunomodulatory therapies for TB.

2.2. Neutrophils. During infection, neutrophils phagocytose bacteria discharge antimicrobial effectors from their granules and constitute a potent population of effector cells that can mediate both antimycobacterial activity and immunopathology in human TB (Figure 2). This is because release of factors such as elastase, collagenase, and myeloperoxidase by neutrophils during their respiratory burst indiscriminately damages bacterial and host cells alike. Neutrophils are the most abundant cell type found in the BAL and sputum of active pulmonary TB patients and are second only to lymphocytes within lungs [57]. One study found an inverse correlation between the development of pulmonary TB and the number of neutrophils in peripheral blood of contacts of active TB patients and *in vitro* depletion of neutrophils from whole blood led to poor induction of antimicrobial peptides (AMPs) and failure to restrict BCG and Mtb growth [58]. Apoptotic neutrophils and purified neutrophil granules, both of which still contain active antimicrobial peptides, have been demonstrated to be taken up by infected macrophages and can lead to impairment of bacterial replication *in vitro* [59].

Apart from their degranulation capacity, neutrophils have recently been implicated in a more immunoregulatory role during Mtb infection. Interaction between programmed death ligand 1 (PD-L1) on myeloid cells and programmed death receptor (PD-1) on lymphocytes is thought to promote the development of dysfunctional, or exhausted, lymphocyte responses during chronic infections [60–62]. Recent transcriptional profiling studies determined that cell surface expression of programmed death ligand 1 (PD-L1) by neutrophils was primarily responsible for high levels of PD-L1 expression in whole blood of active TB patients [63]. Another study described a 393 blood-based transcript signature that differentiated active TB infection from healthy individuals with LTBI. From this, the authors derived an 86-gene signature that corresponded to neutrophil expression of type I and type II interferon inducible genes that distin-guished active TB infection from other inflammatory conditions [64]. It will be important to extend such global transcriptional analyses to the lung, which is the primary site

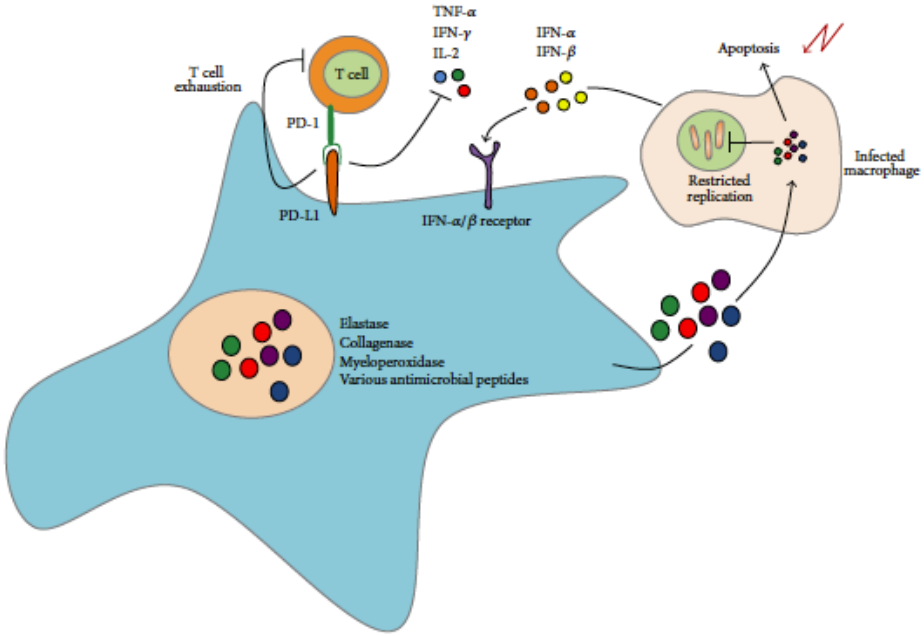

FIGURE 2: Neutrophils constitute a major subset of innate immune cells in the BAL and sputum of patients with active pulmonary TB. During infection with *M. tuberculosis*, neutrophils produce and secrete a variety of antimicrobial enzymes to restrict bacterial growth within infected macrophages. These neutrophil effectors promote apoptosis of infected macrophages, thereby limiting Mtb survival within infected host cells. However, these enzymes also mediate lung tissue damage and sustained, hyperactivated inflammatory response. Furthermore, transcriptional profiling studies have demonstrated the importance of PD-L1, a cell-surface associated molecule, in modulating T cell responses during infection with Mtb. Additional transcriptional studies have identified a blood based IFN-inducible gene signature in neutrophils that is unique to tuberculosis-specific immune responses.

of Mtb infection in pulmonary TB. Indeed, a recent study utilizing biopsy samples from a variety of human tissues to investigate the steady-state T cell compartment in different places throughout the body demonstrated that different tissue compartments, including the lung, contained distinct T cell populations [65] and it is likely that the same principles apply to innate immune populations during states of infection. Since neutrophils comprise a significant percentage of cells that infiltrate the lung during human TB, it will be important to determine their roles in lung tissue and their contribution to uncontrolled inflammation and immunopathology.

2.3. Dendritic Cells. DCs are critical cell types involved in bridging innate and adaptive immunity. DCs are the primary antigen presenting cells that initiate adaptive immune responses through their capacity to present antigen, their costimulatory capacity, and secretion of T-helper polarizing cytokines (Figure 3). In mouse models of TB, it has been shown that DCs constitute a significant population of cells harboring Mtb *in vitro* and *in vivo* [66, 67]. However, whether or not human DCs serve as a major cellular niche for Mtb replication *in vivo* remains unclear. *In vitro* studies in monocyte-derived DCs suggest low levels of bacterial replication within these DCs [68], but further studies are needed to substantiate these observations. Monocyte-derived human DCs express mannose receptors, CD11b, CD11c, and DC-SIGN, all of which are capable of recognizing Mtb ligands. Indeed, DC-SIGN has been shown to serve as a major receptor for Mtb entry into DCs via recognition of ManLAM [69]. Under homeostatic conditions, DC-SIGN functions by binding ICAM-2 on endothelial surfaces to allow for efficient DC migration. During Mtb infection, ligation of DC-SIGN by Mtb ManLAM leads to the induction of the anti-inflammatory cytokine IL-10, which has been implicated in the impairment of DC maturation and expression of costimulatory molecules [70]. Other studies suggest that ManLAMs are capable of inducing a negative signal that inhibits IL-12 production through both mannose receptor and DC-SIGN [71]. These data suggest that Mtb may be modulating

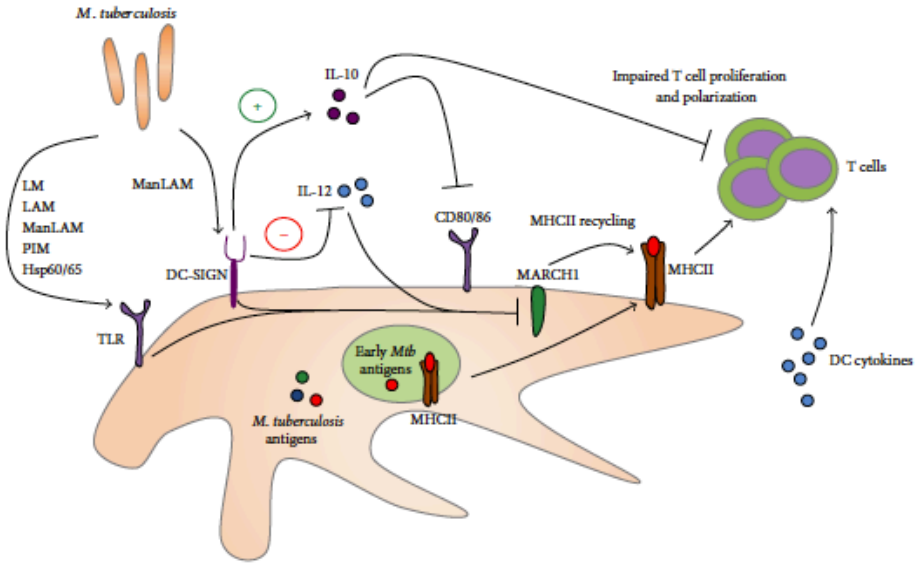

FIGURE 3: DCs are the primary antigen presenting cells (APCs) in the immune system and play a central role in activation and differentiation of T cells by presenting antigenic peptides. DCs recognize a variety of *M. tuberculosis* components directly primarily through TLRs and DC-SIGN. Mtb impacts DC maturation and CD80/CD86 expression via induction of the immunosuppressive IL-10 mediator. Furthermore, engagement of TLRs and DC-SIGN during Mtb infection downregulates MARCH1, a ubiquitin ligase critical for recycling of MHCII on the cell surface. Downregulation of MARCH1 early during infection may play a role in limiting the repertoire of presented Mtb antigens and narrows the adaptive immune response in human TB.

DC functions in order to prevent optimal induction of host adaptive immunity.

Subversion of DC functions by Mtb represents an ideal strategy for slow growing Mtb to evade adaptive immunity. Manipulation of DC maturation, cytokine production, and antigen presentation will affect the kinetics, nature, and magnitude of the T cell response and can provide Mtb with time to establish a foothold within the lungs. Studies that show impaired ability of Mtb-infected monocyte-derived DCs to stimulate lymphoproliferation of naïve and memory CD4s and CD8s provide strong evidence suggesting that Mtb infection of DCs can impair T cell responses during human infection [72]. Since Mtb is a slow growing organism, antigen availability, especially at the early stages of infection, may be an additional reason for poor T cell responses. Under homeostatic conditions, DCs are able to retain peptide-MHC complexes at the cell surface much more efficiently than macrophages, primarily due to the downregulation of membrane associated RING-CH-1 protein (MARCH1), a ubiquitin E3 ligase that helps recycle cell surface MHC complexes [73, 74]. MHC class II cycling from the phagosome to the plasma membrane is induced by DC maturation when TLRs first engage Mtb ligands but may occur before the availability of loadable Mtb antigens, thereby leading to Mtb immune evasion from CD4 T cells [75]. These data might suggest that Mtb antigens are not properly represented during the initiation of adaptive immune responses and may lead to an overabundance of antigen specific T cells that are specific for antigens that may not be relevant at different stages of infection. It has been previously shown that BCG vaccination fails to elicit human T cell responses to latency associated Mtb antigens [76] and vaccination strategies implementing latency associated antigens have shown some promise in the mouse model [77]. The DC plays a central role in the presentation of any Mtb antigen throughout infection and future studies must look to the DC in order to understand why certain antigens are under or nonrepresented at the T cell level. Many questions remain regarding the mechanisms that Mtb employs to manipulate DCs and the subsequent consequences of that manipulation on the nature, kinetics, and magnitude of the adaptive immune response. Studies in humans will remain limited as DCs are poorly represented in BAL, and peripheral blood derived DCs may not be representative of DCs found in the lungs. Nevertheless, it will be important to pursue mechanistic studies on lung DC biology during Mtb infection in humans as well as in animal models of TB, including in mice and nonhuman primates where immunological reagents are readily available.

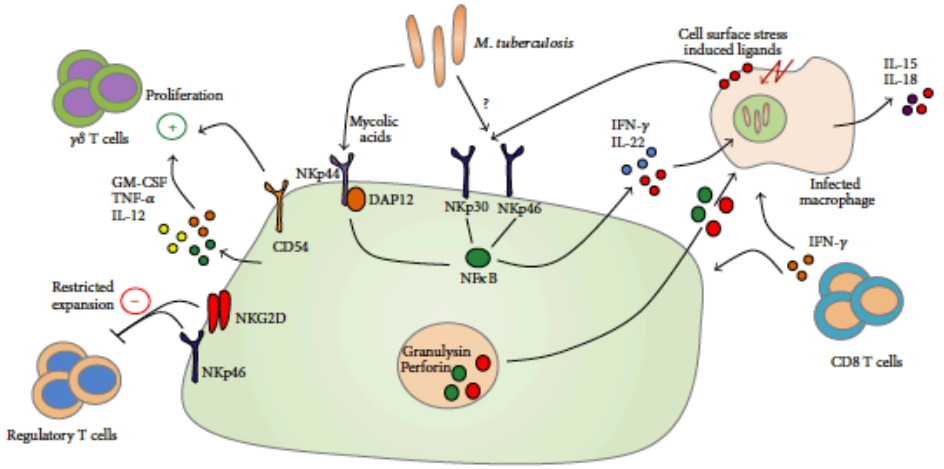

FIGURE 4: Natural killer (NK) cells have the capacity to restrict *M. tuberculosis* replication through the production of soluble mediators such as GM-CSF, IL-12, TNF-α, IL-22, and IFN-γ. These upregulate the antimicrobial function of infected macrophages and activate antigen-specific T cell responses during *M. tuberculosis* infection. NK cell-derived antimicrobial factors such as granulysin and perforin indirectly restrict Mtb growth via the lysis of infected host cells. Several studies suggest that NK cells directly recognize Mtb-derived mycolic acids via NKp44. Aside from direct recognition of Mtb ligands, NKp30 and NKp46 recognize a variety of stress molecules upregulated on the surface of infected host cells.

2.4. Natural Killer Cells. Natural killer (NK) cells are granular innate lymphocytes possessing potent cytolytic capacity. NK cells act early during infection, are not MHC-restricted, and depend upon licensing based on engagement of various activating receptors found on their cell surface by ligands upregulated by stressed or infected target cells (Figure 4). Various Mtb cell wall components, such as mycolic acids, are direct ligands for the natural cytotoxicity receptor (NCR) NKp44 on NK cells [78], and human NK cells exhibit the capacity to lyse Mtb-infected macrophages *in vitro* [79, 80]. Additionally, NK cells can also produce IFN-γ and IL-22, which can inhibit intracellular growth of Mtb *in vitro* by enhancing phagolysosomal fusion [81], or can promote the production of IFN-γ from CD8 T cells by stimulating IL-15 and IL-18 production from Mtb-infected monocytes *in vitro* [82].

Studies on the functionality of NK cells in human TB are limited, but there are indications that NK cells may be functionally impaired during TB. Patients newly diagnosed with pulmonary TB display decreased frequencies of NK cell subsets, coinciding with lowered expression of NKp30, NKp46, and IFN-γ [83]. Anti-TB treatment regimens leading to reductions in mycobacterial load have been shown to partially restore cytolytic capabilities of NK cells [84]. Furthermore, NK cells in patients with tuberculous pleurisy express high levels of ICAM-1 [85], important for the establishment of the immunological synapse, chemokine receptors, and TLR expression [86], and are able to activate autologous lymphocytes under *ex vivo* conditions [85].

In addition to direct killing, NK cells can also promote γδ T cell proliferation via CD54, TNFα, GM-CSF, and IL-12 [87] and, upon recognition of an NK ligand, ULBP1 can restrict the expansion of regulatory T cells in an NKG2D/NKp46 dependent manner [88]. These cells are sensitive to the local microenvironment and monocyte produced IL-10 has been shown to impair NK cell lytic capacity and decrease expression of activating NK cell receptors [89]. Very little is known about NK cells in human TB, but evidence suggests that they play a role in restricting bacterial growth indirectly, via promotion of CD8 [82] and γδ T cell responses [87], and directly, via killing of Mtb-infected monocytes and macrophages [79, 80].

The success of Mtb infection likely hinges upon its early interactions with cells of the innate immune system. Macrophages and neutrophils can take up and kill bacteria but can be subverted by Mtb to promote chronic inflammatory conditions harmful to the lung. Additionally, DCs are central to the generation of Mtb-specific T cells that can bolster immunity but are manipulated to establish poor or misdirected T cell responses. NK cells are capable of directly and indirectly promoting killing of Mtb, but their functional capacity is diminished and little is known about how well they are activated during infection. Each cell type has distinct roles to play in defending the host against Mtb, but they are also readily coopted into helping Mtb establish a long term infection. The difficulties of *in vivo* and lung *in situ* human studies are major roadblocks towards the understanding of innate immune responses during Mtb infection, but population

based immunogenetics studies can offer important insights into innate immune pathways critical for antimycobacterial immunity.

3. Human Innate Immunogenetics and Mycobacterial Susceptibility

Historically, our knowledge regarding human innate and adaptive immune pathways involved during Mtb infection stems from clinical observations in patients suffering from Mendelian Susceptibility to Mycobacterial Diseases (MSMD) and then validated in murine models of TB. Patients suffering from MSMD have genetic polymorphisms that predispose them to infections with various environmental mycobacteria as well as infection with classically avirulent mycobacteria such as BCG, though a significant portion of MSMD patients also suffer from disseminated TB [44]. The importance of innate immunity in combating mycobacterial infections is highlighted in patients with mutations in two innate immune autosomal genes (*IL12B* and *IL12Rβ1*), who suffer widespread and recurrent mycobacterial infections early in life [8, 28, 29]. Fortunately, individuals with polymorphisms in the IL-12 locus can receive treatment with exogenous IL-12 and are less likely to suffer from fatal infections, highlighting a role for macrophage and/or DC-derived IL-12 in the generation of IFN-γ responses that control infection [9]. Mutations in *IL12Rβ1* are among the most common genetic factors associated with MSMD resulting in susceptibility to primary mycobacterial infections [8, 28]. However, BCG vaccination of these individuals can confer resistance, which indicates that IL-12 signaling, and IFN-γ responses dependent on IL-12, may not be completely required for secondary immunity [8, 28]. Additionally, mutations in the leucine zipper domain of NF-κB essential modulator (*NEMO*, also known as inhibitor of NF-κB kinase subunit gamma or *IKK-γ*), encoding an intracellular protein involved in the activation of the NF-κB pathway, has been demonstrated to predispose individuals to recurrent mycobacterial infections due to a lack of IL-12 production from monocytes and DCs [90].

Immunogenetics studies have also implicated other innate pathways, especially those related to pathogen sensing or cytokine and chemokine production, in immunity to mycobacterial infection. Polymorphisms in TLR2, TLR9 [91], TLR1 [92], TLR8 [93], and the intracellular signaling molecule TIRAP [94] have all been associated with susceptibility to mycobacterial infection. The mechanisms for the association between TLR and mycobacterial infection are still unclear and studies in the murine model of TB seem to indicate redundant roles for TLRs and TLR-associated molecules such as MyD88 in the generation of adaptive immune responses to Mtb [95–100]. Aside from PRRs, individuals with mutations in the inflammasome pathway have provided insight into the regulatory role of the inflammasome during mycobacterial infection. A gain of function gene variant in caspase-1 coupled with a loss of function for inhibitory caspase recruitment domain family member 8 (CARD8) promotes inflammatory diseases such as rheumatoid arthritis, but macrophages isolated from these individuals are more efficient at restricting Mtb growth *in vitro* [101]. Polymorphisms in the *Il1* gene cluster and macrophage chemoattractant protein 1 (MCP-1) also predispose individuals to TB, presumably due to an inadequate inflammatory response against infection [102–104]. Indeed, IL-1 responses in humans seem to be linked to higher eicosanoid induction that curtails excessive inflammation promoted by type I IFNs [105]. Collectively, these data suggest that the inflammasome pathway, and IL-1 in particular, may be critical in promoting enhanced immunity against Mtb in humans. In another example, a population with low serum levels of Vitamin D3 metabolites displayed increased susceptibility to active TB [106], validating *in vitro* results from human primary cells. These examples highlight the idea that immunogenetic studies on a population level are important parallel approaches that strengthen *in vitro* derived results in elucidating genes important in immunity against Mtb.

Human immunogenetic studies provide an attractive avenue for the validation of several mechanisms of resistance against Mtb observed in animal models of TB and can complement observations from *in vitro* human cells, but the innate immune response against pathogens, especially one as complex as Mtb, is multigenic and very complex. It will be interesting, and potentially very rewarding, to examine innate immune genes in studies examining individuals who are highly exposed to Mtb but who do not progress to active TB disease. This relatively resistant population, for example, health care workers in high-burden TB settings, should provide important clues to how the innate immune response may successfully handle Mtb infection.

4. Perspectives and Conclusions

Innate immunity is a crucial component of the immune response against Mtb but has received relatively little attention in studies of human TB. While myeloid cells serve as niches for bacterial replication, innate antimicrobial pathways activated early and throughout infection play a role in limiting disease and serve as potent regulators of antigen-specific adaptive immunity. TB disease results when pathological responses that promote chronic inflammation and lung damage dominate over protective responses that limit disease and eliminate bacteria. There is growing evidence indicating that innate immune cells are uniquely positioned to determine that balance between protective and pathogenic immune responses in human TB. However, Mtb employs myriad potent mechanisms for evading antimicrobial responses and subverting the innate immune crosstalk with adaptive immunity, thereby tilting the balance towards pathological rather than protective immune responses. Further study of human innate immune pathways during Mtb infection will be important for developing host-directed immunomodulatory therapies for TB. Further, a greater understanding of how innate immune responses impact adaptive immunity is critical for designing efficacious TB vaccines.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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