Identification of Bhlhe40 and Irg1 as essential regulators of the inflammatory response to Mycobacterium tuberculosis

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Identification of Bhlhe40 and Irg1 as Essential Regulators of the Inflammatory Response to *Mycobacterium tuberculosis*

by

Jeremy P. Huynh

A dissertation presented to
The Graduate School
of Washington University in Saint Louis in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2018
Saint Louis, Missouri
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List of Abbreviations

10BiT  IL-10 BAC-in transgene
BAC  Bacterial artificial chromosome
BCG  *Mycobacterium bovis* Bacillus Calmette-Guérin
Bhlhe40  Basic helix-loop-helix family, member e40
BMDM  Bone marrow-derived, M-CSF-cultured macrophage
CD  Cluster of differentiation
CFU  Colony-forming units
DC  Dendritic cell
dpi  days post-infection
EAE  Experimental autoimmune encephalomyelitis
fl/fl  Denotes that the preceding gene has an exon flanked by LoxP sites
GFP  Green fluorescent protein
GM-CSF  Granulocyte-macrophage colony stimulating factor
HDAC  Histone deacetylase
H&E  Haematoxylin and eosin
ICL  isocitrate lyase
IFN  Interferon
IGRA  Interferon-γ release assay
IL  Interleukin
Irg1  Immune-regulated Gene 1
M-CSF  Macrophage colony stimulating factor
MFI  (Geometric) Mean fluorescence intensity

*Mtb*  *Mycobacterium tuberculosis*

NOS2  nitric oxide synthase 2 (also known as iNOS)

ROS  reactive oxygen species

TB-IRIS  tuberculosis-associated immune reconstitution inflammatory syndrome

TCA  Tricarboxylic acid

TNF-α  Tumor necrosis factor α

Tg  Transgenic

T_H  T helper cell

WT  Wild type
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Jeremy Huynh

Washington University in Saint Louis

August 2018
Dedicated to my parents,

Phuon and Deborah Huynh

for encouraging me to challenge myself and for their unconditional love.
Abstract of the Dissertation

Identification of Bhlhe40 and Irg1 as Essential Regulators of the Inflammatory Response to
*Mycobacterium tuberculosis*

by

Jeremy P. Huynh

Doctor of Philosophy in Biology and Biomedical Sciences
Molecular Microbiology and Microbial Pathogenesis
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Professor Christina L. Stallings, Chair

Protective immune responses to *Mycobacterium tuberculosis* (*Mtb*) must induce bactericidal functions while minimizing damage to the lung. Such responses require precise control of pro- and anti-inflammatory factors to regulate the recruitment and function of protective immune cells but the mechanisms by which this control is exerted remain incompletely defined. Basic helix-loop-helix family, member e40 (Bhlhe40) is a transcription factor known to regulate production of pro- and anti-inflammatory cytokines that affect protective immunity to *Mtb*. Immune-responsive Gene 1 (Irg1) is an enzyme that generates itaconate, a metabolite with potential anti-inflammatory and antibacterial roles during *Mtb* infection. The impact of Bhlhe40 and Irg1 on protective immunity to *Mtb* was unknown prior to the studies detailed in this dissertation.

We utilized genetically deficient mice to assess the roles of Bhlhe40 and Irg1 expression on control of *in vivo* *Mtb* infection. We found that Bhlhe40 enables protective immune responses to *Mtb* by restricting expression of the anti-inflammatory cytokine IL-10 and is required in T
cells and CD11c+ cells. Bhlhe40 does so by repressing Il10 transcription, likely by direct binding to the Il10 locus. Additionally, we discovered that Irg1 functions in LysM+ and CD11c+ cells to prevent neutrophil-mediated immunopathology. Irg1 expression restricts inflammatory responses in myeloid cells at a transcriptional level, likely through production of the metabolite itaconate. Thus, Bhlhe40 and Irg1 directly and indirectly tune the inflammatory response to Mtb at a transcriptional level. These findings advance our understanding of protective immunity to Mtb by revealing novel mechanisms used by specific immune cell types to promote bacterial killing and restrict pathologic inflammation.
Chapter 1

Introduction
Mycobacterium tuberculosis (Mtb) is an acid-fast bacterium of the phylum Actinobacteria and the etiological agent of the disease tuberculosis. Mtb is an obligate human pathogen first isolated by Robert Koch in 1882 that has infected and co-evolved with humans for an estimated 70,000 years.\textsuperscript{1,2} The burden that tuberculosis imposes on global public health is illustrated by a 2017 report by the World Health Organization estimating that 1.7 billion people are currently infected with Mtb and that tuberculosis has become the leading cause of death by a single infectious agent.\textsuperscript{3} Infection is initiated when aerosolized Mtb bacilli are expelled from an infected host and inhaled by a new, susceptible host. Pulmonary tuberculosis is the most common form of the disease, but dissemination to extrapulmonary sites can occur.\textsuperscript{4} Tuberculosis encompasses a dynamic spectrum of disease states that vary by the presence of disease symptoms and transmissibility. Despite this heterogeneity, patients are broadly classified as having either latent or active tuberculosis.\textsuperscript{4} Approximately 90% of Mtb infections result in latent tuberculosis, a disease state in which the host immune system limits bacterial replication and minimizes lung pathology. Latent tuberculosis patients are generally asymptomatic and unable to transmit infection but maintain a life-long risk of progression to active tuberculosis. In active tuberculosis, which accounts for approximately 10% of cases, the immune response is unable to control bacterial replication and patients are able to transmit infection.\textsuperscript{5,6} Active tuberculosis patients exhibit symptoms such as fever, fatigue, weight loss, persistent coughing, and coughing up blood.\textsuperscript{6}

Most Mtb infections can be treated by a six month course of antibiotic therapy consisting of isoniazid, rifampicin, pyrazinamide, and ethambutol for two months, followed by isoniazid and rifampicin for four months. The long duration and potential toxicity of current antibiotic
treatment regimens often leads to problems with patient adherence. Incomplete patient adherence promotes the development of multiple drug resistant-tuberculosis (MDR-TB) strains resistant to rifampicin or isoniazid and extremely drug resistant-tuberculosis (XDR-TB), defined as MDR-TB with resistance to a second-line antibiotic. MDR-TB cases currently comprise 4% of new cases and 19% of previously treated cases. The emergence and spread of difficult to treat MDR-TB and potentially incurable XDR-TB strains highlights the need for therapeutics with novel mechanisms of action and shorter treatment durations. Therapies that target the host immune system to enhance bacterial control and minimize lung pathology have gained interest as an approach for treating drug resistant \textit{Mtb} but their development requires a detailed understanding of how the immune system succeeds or fails in controlling \textit{Mtb} infection.

Vaccines are another attractive approach for combating \textit{Mtb} and also rely on knowledge of basic host-pathogen interaction. \textit{Mycobacterium bovis} Bacillus Calmette-Guérin (BCG) is currently the only approved vaccine for prevention of tuberculosis. BCG was developed in the early 1900s by attenuation of a \textit{Mycobacterium bovis} isolate through serial passage \textit{in vitro} for 13 years. Over 3 billion doses of BCG have been administered and it has proven effective at preventing severe forms of disseminated tuberculosis such as tuberculous meningitis and military tuberculosis in infants. However, its efficacy in preventing pulmonary tuberculosis in adolescents and adults varies wildly and reports of protective efficacy range from 0-80%. BCG vaccination has successfully reduced infant mortality, but a vaccine that prevents establishment of pulmonary infection in adults will be necessary to halt transmission and ultimately eradicate tuberculosis.
The immune response to *Mycobacterium tuberculosis*

Our current understanding of how the immune system controls *Mtb* infection is largely based on study of humans that develop severe forms of tuberculosis and animal disease models. These approaches enable identification of gene signatures and specific genes that are associated with or contribute to different disease outcomes. Once identified, protective genes can be mechanistically investigated in animal models to determine their function and the quality of the immune responses they promote, then further assessed for relevance in humans. Such studies have advanced understanding of the pathogenesis of tuberculosis and will inform the development of novel diagnostics, therapeutics, and vaccines to treat and prevent tuberculosis.

The genetically tractable mouse model of *Mtb* infection has proven to be an invaluable tool for dissecting how host genes and immunological processes affect disease outcome. The Cre-Flox conditional gene deletion system has proven especially useful in this regard. In this system, LoxP DNA recognition sites flanking an exon of a gene of interest are inserted into the genome to generate a “flanked by LoxP” or “floxed” allele, denoted by “Gene*fl/fl*.” When floxed mice are crossed to mice that express Cre recombinase under the control of cell-type specific promoters, Cre mediates deletion of the DNA sequence between the LoxP sites. This generates a mouse with conditional deletion of the gene of interest only in specified cell types and is a highly useful tool for identification of the key cell types that must express the gene as well as study of gene function in those cell types.

Experimental tuberculosis infection in mice is initiated by delivering 100-200 colony-forming units (CFUs) of aerosolized *Mtb* to the lungs of each mouse. After deposition in the lung bacilli are phagocytosed by lung resident alveolar macrophages. Alveolar macrophages fail to clear infection and produce inflammatory signals that recruit other innate immune cell types such
as neutrophils, monocytes, macrophages, and dendritic cells (DCs) to the site of infection. These recruited innate immune cell types also fail to clear infection and \textit{Mtb} replicates logarithmically in them until the onset of adaptive immunity. Around 8-9 days post-infection (dpi) DCs migrating from the lung arrive in the mediastinal lymph node. Once in the lymph node DCs present \textit{Mtb} antigens to naïve T cells and secrete IL-12, causing the naïve T cells to expand and differentiate into T helper (T$_{H1}$) 1-polarized effector cells. T cells are an essential component of protective immunity to \textit{Mtb}, as demonstrated by the propensity of HIV/AIDS patients with latent tuberculosis to progress to active tuberculosis as their T cell counts decline.$^{15,16}$ Antigen-specific T cells reach the lung 18-20 dpi where they release cytokines, such as interferon (IFN)-\(\gamma\), that activate bactericidal effector mechanisms and limit pathologic inflammatory responses. \textit{Mtb} burden in the lung peaks around 21 dpi, decreases slightly, then remains constant for the remainder of the lifetime of the mouse. The arrival of effector T cells in the lung enables the formation of structured innate and adaptive immune cell aggregates, referred to as “granulomas” in humans and “lesions” in mice that confine \textit{Mtb} bacilli.$^{17}$ Human immune responses to \textit{Mtb} are thought to progress in a similar fashion as those in mice, although differences in the amount of time before an antigen-specific immune response can be detected suggest that the timing of each event differs by species.$^{18}$ These immune responses are sufficient to limit infection to a latent, non-transmissible state in 90\% of infected humans. Thus, a primary function of the immune system is to induce a durable, protective response that activates bactericidal mechanisms and limits the spread of \textit{Mtb} while avoiding pathologic responses.
**IFN-γ-mediated protective responses to Mtb**

One important component of protective immunity to *Mtb* is IFN-γ. IFN-γ is a cytokine that activates cell-autonomous and non-autonomous mechanisms enabling control of *Mtb*. The importance of IFN-γ for control of *Mtb* infection is demonstrated by the predisposition to severe tuberculosis in humans with in-born errors in genes essential for IFN-γ-producing Th1 responses and genes in the IFN-γ signaling pathway.19–22 Protective cell-intrinsic effects of IFN-γ signaling include increased fusion of *Mtb*-containing phagosomes with bactericidal low pH lysosomes via induction of LRG47 (also known as IRGM1),23 restriction of neutrophil survival,24 prevention of Th1 cell apoptosis by LRG47-mediated autophagy,25 expression of antimicrobial peptides such as cathelicidin and β-defensin in human cells26 or ubiquicidin-like peptides in mouse cells,27 induction of reactive oxygen species (ROS),28 divalent cation restriction or hyperaccumulation,29,30 and tryptophan limitation via indoleamine-2,3-dioxygenase (IDO).31 Although many studies have posited a critical role for IFN-γ-driven canonical autophagy, this process is dispensable for *in vivo* control of *Mtb* infection.32 IFN-γ is typically considered a pro-inflammatory cytokine but it also prevents pathologic neutrophil-dominated inflammation by restricting neutrophil recruitment24 and inducing nitric oxide production via nitric oxide synthase 2 (NOS2).33 Mice deficient in IFN-γ production or signaling rapidly succumb to *Mtb* infection.23,34,35 Th1-polarized CD4+ T cells are the primary source of IFN-γ and production of IFN-γ is necessary but not sufficient for the full extent of their protective capacity.24,36,37 Natural killer cell production of IFN-γ also contributes to resistance in the context of T cell deficiency.38 Collectively, these studies demonstrate that IFN-γ is necessary, but not sufficient, for host resistance to *Mtb* and mechanisms that support its production and function play important roles in protective immunity to *Mtb*.
IL-10 is an immunoregulatory cytokine that suppresses the production and effects of IFN-γ. Nearly all innate and adaptive immune cell types, with the exception of plasmacytoid DCs, can produce IL-10 during *Mtb* infection and it is detrimental for control of *Mtb* infection. IL-10 antagonizes IFN-γ-associated pathways by suppressing macrophage responsiveness to IFN-γ, modulating T-helper (T_H) 1 cell IFN-γ production, and restricting production of the hallmark T_H1-inducing cytokine IL-12. IL-10 can also inhibit DC migration to the lung-draining lymph node and limit secretion of myeloid cell-derived proinflammatory cytokines. Many observations have suggested an important role for IL-10 in human tuberculosis pathogenesis. Human single nucleotide polymorphisms in the *IL10* gene promoter have been linked to tuberculosis susceptibility in particular ethnic groups. Patients with active tuberculosis express higher amounts of IL-10 in their lung pleural fluid, bronchoalveolar lavage fluid, sputum, and serum as compared to healthy controls or controls with non-mycobacterial lung disease. IL-10 has also been shown to decrease the amount of IFN-γ produced by *Mtb*-stimulated peripheral blood mononuclear cells from active tuberculosis patients or tuberculin skin test-positive individuals and can block phagosome maturation in *Mtb* infected human monocyte-derived macrophages. Surprisingly, studies on mice globally deficient in IL-10 production have either reported no role for IL-10 or that it causes a mild impairment of control during chronic *Mtb* infection. This impairment has been attributed to IL-10 production by T cells and CD11c+ cells. *In vivo* over-expression of IL-10 by T cells or macrophages has also supported a negative role for IL-10 during chronic *Mtb* infection, although differences in mouse genetic background and the transgenic promoters used have resulted in an unclear picture.
Given that IL-10 has the potential to negatively impact key protective immune responses to *Mtb* but global loss of IL-10 function does not result in excessive reduction of *Mtb* burden in mice, it is likely that cell-autonomous mechanisms that limit IL-10 induction pathways are important for control of *Mtb*. Mycobacteria directly stimulate IL-10 production from monocytes, macrophages, DCs, and neutrophils via signaling through pattern-recognition receptors such as toll-like receptor 2 (TLR2)\textsuperscript{72,73} and the C-type lectin receptor DC-specific ICAM3-grabbing non-integrin (DC-SIGN).\textsuperscript{74} In addition, different T\textsubscript{H} cell subsets and innate immune cell types produce IL-10 in response to distinct combinations of cytokines.\textsuperscript{75} These signals lead to the binding of diverse transcription factors at promoter and enhancer elements within the *Il10* locus to activate transcription within myeloid and lymphoid cells.\textsuperscript{75–77} Many transcription factors restricting IL-10 production have been identified, but none have been assessed for IL-10-dependent roles during *in vivo* *Mtb* infection. The transcription factors known to repress *Il10* in innate immune cells include B cell CLL/lymphoma 3 (Bcl3),\textsuperscript{78} histone deacetylase 11 (HDAC11),\textsuperscript{79} poly(ADP-ribose) polymerase 1 (PARP-1),\textsuperscript{80} nuclear receptor subfamily 1 group D member 1 (NR1D1, Rev-erb\textalpha{}),\textsuperscript{81} programmed cell death protein 4 (PCDP4),\textsuperscript{82} and interferon regulatory factor 5 (IRF5)\textsuperscript{83} in macrophages as well as class II MHC transactivator (CIITA) in DCs.\textsuperscript{84} NR1D1 is notable for its role in promoting *Mtb* control by human macrophages through repression of IL-10.\textsuperscript{81} Repressors of *Il10* in adaptive immune cells include E26 transformation specific 1 (ETS-1)\textsuperscript{85} and PU.1\textsuperscript{86} in T cells as well as forkhead box D3 (Foxd3) in B cells.\textsuperscript{87} The ability of IL-10 to suppress the actions of IFN-\gamma suggests that it must be carefully regulated in order to allow the induction and maintenance of protective responses to *Mtb* but the mechanisms of its regulation during *Mtb* infection are poorly characterized.
Pathologic responses to Mtb

In addition to establishing protective responses the immune system must also avoid responses that fail to restrict Mtb replication or cause lung pathology. The importance of strictly controlling the immune response to Mtb is illustrated by tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS), a condition that occurs in 18% of HIV/Mtb co-infected patients as anti-retroviral therapy allows the immune system to recover from the effects of HIV infection. During TB-IRIS the recovering immune system mounts an excessive pro-inflammatory cytokine and cellular response to Mtb that causes immunopathology and worsens tuberculosis-associated symptoms. Murine studies have generated complementary data showing that even cellular and cytokine responses normally associated with resistance to Mtb can be pathological if produced in excess.

Studies in humans and mice have suggested that neutrophils are key mediators of inflammatory responses that poorly control Mtb and cause lung pathology. In active tuberculosis patients neutrophils are the predominantly infected cell type and active tuberculosis patients can be identified by a neutrophil-driven transcriptional signature. Tuberculosis patients with higher blood neutrophil counts also have increased disease severity and higher case fatality rates. Acutely susceptible mouse strains often display neutrophilic inflammation that can cause or result from failed immunity to Mtb. Neutrophils are thought to mediate susceptibility to Mtb by releasing pro-inflammatory cytokines that can exacerbate non-protective inflammation as well as proteases and reactive oxygen species (ROS) that damage the lung. The resulting pathology may be an important cause of granuloma breakdown and lung cavitation, processes that allow Mtb to re-enter the respiratory tract and transmit to a new host. In addition to causing lung pathology, neutrophils also provide a nutrient-rich replicative niche for Mtb.
Restraint of pro-inflammatory cytokine production and neutrophilic inflammation are important components of protective immunity to *Mtb*, but the mechanisms by which the immune system controls these processes remain poorly defined.

**Bhlhe40**

Homologs of the gene now officially named Bhlhe40 (basic helix-loop-helix family member e40) were identified by several independent groups and have thus been referred to by multiple names (Bhlhb2, Clast5, Dec1, Sharp2, Stra13) over the course of its study. For simplicity, this gene will henceforth be referred to as “Bhlhe40,” regardless of the homolog name used in the original study. Bhlhe40 is a 411 amino acid protein of the basic helix-loop-helix (BHLH)-Orange subfamily of transcription factors which are characterized by a basic patch that enables DNA binding, a helix-loop-helix domain that enables protein dimerization, and an Orange domain conferring functional specificities that vary by family member. Bhlhe40 is expressed in a wide range of organs and cell types and can be induced by many different stimuli. Cell types that express Bhlhe40 include neurons, chondrocytes, epithelial cells, innate immune cells, and adaptive immune cells. Stimuli inducing Bhlhe40 transcription include growth factors, light, starvation, hypoxia, anti-CD3 stimulation, anti-CD28 stimulation, hormones, retinoic acid, cytokines, and B cell receptor cross-linking. Roles for Bhlhe40 have been reported in a wide variety of cellular processes including cell cycle arrest, apoptosis, epithelial to mesenchymal transition, cellular differentiation, circadian rhythm, stress response, and immune system function. Bhlhe40 typically mediates these effects by transcriptional repression and several mechanisms of action have been reported. Direct binding to the class B E-box DNA motif
CACGTG allows Bhlhe40 to repress transcription, likely by occluding other transcription factors.\textsuperscript{112,124} Bhlhe40 can also associate with other transcription factors and inhibit their ability to bind DNA and activate transcription\textsuperscript{102,112,125,126} or recruit histone deacetylases to modify chromatin accessibility.\textsuperscript{102,127} Many of the stimuli that induce Bhlhe40 expression are present in the \textit{Mtb} infected lung, suggesting that it may be operative in lung resident and recruited cells during infection.

\textbf{Bhlhe40 in the immune system}

Study of Bhlhe40 in the context of the immune system has been limited to lymphoid cell types and multiple studies have identified it as an important regulator of cytokine production, particularly in T cells and in the context of autoimmunity. Naïve \textit{Bhlhe40}\textsuperscript{-/-} mice appear phenotypically similar to wild-type (WT) mice.\textsuperscript{109} Bhlhe40 expression has no effect on \(\alpha\beta\) T cell polarization but promotes expression of many pro-inflammatory cytokines such as IL-2, IL-4, IL-17, IFN-\(\gamma\), and GM-CSF while suppressing anti-inflammatory IL-10.\textsuperscript{109,114,128,129} Bhlhe40 expression is also required for production of IFN-\(\gamma\) by invariant natural killer T cells\textsuperscript{111} and GM-CSF by \(\gamma\delta\) T cells.\textsuperscript{128} Bhlhe40 may also promote systemic immune regulatory functions by associating with the transcription factor Runx1 to sustain IL-2R\(\alpha\) (CD25) expression, thus enabling long-term maintenance of regulatory T cells.\textsuperscript{130} Aged \textit{Bhlhe40}\textsuperscript{-/-} mice exhibit an accumulation of activated B cells that may be due to a role for Bhlhe40 in inhibiting B cell activation and proliferation by blocking cell cycle progression.\textsuperscript{110} Notably, Bhlhe40-deficient mice are highly resistant to the induction of experimental autoimmune encephalomyelitis (EAE), a T cell-driven model of multiple sclerosis-like neuroinflammation.\textsuperscript{108,114,128,129} Resistance to EAE in \textit{Bhlhe40}\textsuperscript{-/-} mice is attributed to their decreased \textit{Csf2} (GM-CSF) and increased \textit{Il10} (IL-
10) transcription in T cells and is dependent on signaling through the IL-10 receptor. Collectively, these studies demonstrate that Bhlhe40 regulates multiple T cell functions important for control of *Mtb* infection, such as production of IL-10 and IFN-γ. Despite its potential impact on *Mtb* infection, no prior studies had examined the role of Bhlhe40 in the context of an infectious disease.

**Irg1**

*Immune-responsive Gene 1* (Irg1, also known as *cis*-aconitate decarboxylase 1 [Acod1]) is an enzyme that links cellular metabolism to immune system functionality. It is distinct from the similarly named Immunity-Related GTPase (IRG) proteins that play a prominent role in *Toxoplasma gondii* infection. Innate immune cells such as macrophages, microglia, and neutrophils strongly express Irg1 upon activation, whereupon it localizes to the mitochondria. Irg1 is also inducible in non-hematopoietic cell types such as uterine epithelial cells in response to progesterone signaling and neurons. Many types of pro-inflammatory stimuli such as lipopolysaccharide (LPS) and CpG methylated DNA, as well as viral, parasitic, and bacterial infection are able to induce Irg1 expression. Notably, such bacterial species include both pathogenic and non-pathogenic mycobacteria. Induction by these stimuli is likely mediated by inflammatory cytokines such as IFN-α, IFN-β, IFN-γ, TNF-α, IL-1β, and IL-17. Upon its expression, Irg1 catalyzes the decarboxylation of *cis*-aconitate, a metabolic intermediate of the tricarboxylic acid (TCA) cycle, thus producing the metabolite itaconic acid. This enzymatic activity is mediated by its five *cis*-aconitate decarboxylase domains (CADs), all of which are fully conserved in mouse and human Irg1. Itaconic acid is often referred to as itaconate, its neutral salt. Dimethyl itaconate (DI), an
itaconate derivative with increased membrane permeability, has been used to study the biological functions of itaconate.\textsuperscript{138} Thus far, Irg1 is only known source of itaconate in mammalian cells and no itaconate-independent functions for Irg1 have been described. Reports of Irg1 induction during mycobacterial infection and the presence of Irg1-inducing cytokines in \textit{Mtb} infected lungs strongly suggest that Irg1 is expressed during \textit{Mtb} infection.

\textit{Antibacterial roles for Irg1}

Irg1 has gained attention for the potential of its metabolite product, itaconate, to function as an endogenous antibacterial effector molecule by inhibiting isocitrate lyase (ICL).\textsuperscript{144} ICLs function in two important metabolic pathways, the glyoxylate shunt and the methylcitrate cycle.\textsuperscript{145,146} The glyoxylate shunt enables certain bacteria and fungi to utilize fatty acids as alternative carbon sources and persist under what would otherwise be nutrient-limiting conditions by modifying their tricarboxylic acid (TCA) cycle.\textsuperscript{147} The TCA cycle generates the essential cellular energy source adenosine triphosphate (ATP), but its intermediates can also be diverted into biosynthetic pathways. The glyoxylate shunt is an anaplerotic pathway that bypasses two decarboxylation reactions of the TCA cycle, allowing \textit{Mtb} to use even-chain fatty acid substrates, such as host-derived lipids, to replenish TCA cycle intermediates that are diverted to biomass production.\textsuperscript{147} ICLs in \textit{Mtb} also possess methylisocitrate lyase activity that allows them to function in the methylcitrate cycle.\textsuperscript{148} The methylcitrate cycle enables regeneration of the TCA cycle intermediates oxaloacetate and malonate while also preventing build-up of toxic propionyl-CoA and methylmalonyl-CoA that result from β-oxidation of odd-chain fatty acids such as cholesterol.\textsuperscript{146} Loss of methylcitrate cycle function can lead to defects in
Mtb TCA cycle activity, pH homeostasis, and membrane potential homeostasis but it may not be essential for in vivo survival of Mtb.\textsuperscript{146,148}

The glyoxylate shunt is an important metabolic pathway for persistence in pathogenic Mycobacteria, Salmonella, and Yersinia species.\textsuperscript{145,149} The commonly studied Mtb Erdman strain has two isocitrate lyases, ICL1 and ICL2, encoded by the genes icl1 and icl2 (also known as aceA). ICL1-deficient (Δicl1) Mtb exhibits a persistence defect during chronic in vivo infection of mice but ICL2-deficient (Δicl2) Mtb exhibits no defects, suggesting that while ICL1 and ICL2 have some level of functional redundancy ICL1 plays a slightly more important role.\textsuperscript{145,150} In contrast, Δicl1/Δicl2 Mtb is dramatically attenuated in vivo and is cleared from the lung by two weeks post-infection.\textsuperscript{145} An antibacterial role for itaconate was supported by the findings that it inhibits purified Mtb ICL proteins in vitro\textsuperscript{151} and that itaconate concentrations two- to three-fold higher than those observed in activated macrophages had bacteriostatic effects on Mtb growth in liquid culture under conditions requiring the glyoxylate shunt.\textsuperscript{143} Irg1 has also been reported to promote the generation of potentially bactericidal mitochondrial reactive oxygen species (ROS) during Salmonella infection of zebrafish.\textsuperscript{152} However, Irg1 had no effect on ROS production in murine Legionella infection,\textsuperscript{135} suggesting that this capability may be pathogen- or species-dependent. These reports suggested that Irg1 might restrict Mtb growth by promoting ROS production or inhibiting the glyoxylate shunt through itaconate production.

\textit{Irg1 as a modulator of inflammation}

Recent studies have linked Irg1-mediated itaconate production to modulation of immune cell metabolism and inflammatory responses, with the majority of studies finding that it has a net anti-inflammatory effect. In bone marrow-derived macrophages (BMDMs), itaconate suppressed
a subset of pro-inflammatory cytokines including IFN-β, IL-1β, IL-6, IL-12, and IL-18 through several different mechanisms. Inhibition of succinate dehydrogenase by itaconate reduces production of IL-1β and IL-18, while suppression of IL-6 and IL-12 is mediated by Atf3-induction that results in a translation-level block of IκBζ, a transcription factor promoting Il6 and Il12b expression. Itaconate and its derivatives were also found to limit IL-1β and IFN-β production by promoting post-translational modification-mediated degradation of KEAP1, a repressor of the anti-inflammatory transcription factor Nrf2. Irg1 has also been proposed to suppress IL-1β and IL-6 production through induction of the anti-inflammatory transcription factor TNFAIP3 (also known as A20). These anti-inflammatory effects on cytokine production are associated with improved outcomes in sepsis and psoriasis models in which pathology is mediated by excessive inflammation.

Irg1 has been linked to the generation of ROS but reports vary as to whether it promotes or inhibits ROS production and the resulting effect on inflammation. ROS can mediate pro- or anti-inflammatory effects and the amount produced may be the determining factor. Decreased ROS levels have been associated with itaconate-mediated inhibition of succinate dehydrogenase activity and protection from ischemic reperfusion injury. During respiratory syncytial virus infection Irg1 is reported to increase ROS levels, leading to increased inflammation and lung injury.

Macrophage-produced itaconate can also be secreted into the extracellular environment, suggesting that it may mediate immunomodulatory effects in trans on other cell types in the inflammatory environment. The capacity of Irg1 and itaconate to modulate cell-autonomous and nonautonomous effects on inflammation suggested that it could have an
important role in the context of *Mtb* infection wherein immune responses must be precisely calibrated to balance bacterial clearance and prevention of tissue pathology.\textsuperscript{24,32}
Chapter 2

The role of Bhlhe40 in control of

*Mycobacterium tuberculosis* infection

Portions of this chapter are excerpted from

Abstract

The cytokine IL-10 antagonizes pathways that control *Mycobacterium tuberculosis* (*Mtb*) infection. Nevertheless, the impact of IL-10 during *Mtb* infection has been difficult to decipher because loss-of-function studies in animal models have yielded only mild phenotypes. We have discovered that the transcription factor basic helix-loop-helix family member e40 (Bhlhe40) is required to repress *Il10* expression during *Mtb* infection. Loss of Bhlhe40 in mice results in higher *Il10* expression, higher bacterial burden, and early susceptibility similar to that observed in mice lacking IFN-γ. Deletion of *Il10* in *Bhlhe40*+/− mice reverses these phenotypes. Bhlhe40 deletion in T cells or CD11c+ cells is sufficient to cause susceptibility to *Mtb*. Bhlhe40 represents the first transcription factor found to be essential during *Mtb* infection to specifically regulate *Il10* expression, revealing the importance of strict control of IL-10 production by innate and adaptive immune cells during infection. Our findings uncover a previously elusive but significant role for IL-10 in *Mtb* pathogenesis.

Introduction

Host immune responses mediate both bacterial control and pathology during *Mycobacterium tuberculosis* (*Mtb*) infection. IFN-γ signaling through the transcription factor STAT1 is essential for the control of mycobacterial infections in humans and mice. IL-10 is an immunoregulatory cytokine produced by a wide variety of innate and adaptive immune cell types that antagonizes IFN-γ-associated pathways by suppressing macrophage responsiveness to IFN-γ, modulating T-helper (T_H) 1 cell IFN-γ production, and restricting production of the hallmark T_H1-inducing cytokine IL-12. IL-10 can also inhibit
dendritic cell (DC) migration and limit secretion of myeloid cell-derived proinflammatory cytokines. Global loss-of-function studies have demonstrated a detrimental role for IL10 expression in the control of chronic Mtb infection in mice, although the magnitude of this effect appears dependent on genetic background and is generally mild. More recently, conditional deletion of Il10 in T cells or CD11c+ cells showed that IL-10 production by these two cell types exacerbates Mtb infection. Overexpression studies in mice have also supported a negative role for IL-10 in controlling mycobacterial infection, although differences in genetic background, transgenic (Tg) promoters, and mycobacterial species used have resulted in an unclear picture.

Given the potential for IL-10 to negatively impact protective immune responses, cell-autonomous mechanisms likely exist to regulate IL-10 expression. However, the factors required for this regulation remain poorly understood. Mtb directly stimulates IL-10 production from monocytes, macrophages, DCs, and neutrophils via pattern-recognition receptor signaling. In addition, different Th cell subsets produce IL-10 in response to distinct combinations of cytokines. These signals lead to the binding of diverse transcription factors at various promoter and enhancer elements within the Il10 locus to activate transcription within myeloid and lymphoid cells. Less is known about transcriptional pathways that limit production of IL-10.

The transcription factor basic helix-loop-helix family member e40 (Bhlhe40) has been shown to regulate production of cytokines, including IL-10, by T cells in the experimental autoimmune encephalomyelitis model, a T cell-driven model of neuroinflammation. Prior to this study Bhlhe40 was uncharacterized in an infectious disease context. We utilized mice with genetic deletions of Bhlhe40 to characterize its importance and function during in vivo
*Mtb* infection. Here we report that Bhlhe40 serves an essential role in resistance to *Mtb* infection by repressing *Il10* expression in both T cells and myeloid cells.

**Results**

*Lower BHLHE40 expression is associated with active tuberculosis*

When we analyzed publicly available whole-blood gene expression datasets, we found that *BHLHE40* transcripts were present at a significantly lower abundance in patients with active tuberculosis as compared with healthy controls, those with latent tuberculosis infection, or those with lung cancer, pneumonia, or sarcoidosis (*Fig. 1A*). This expression pattern contrasted with that of STAT1, whose expression was significantly increased in active tuberculosis patients (*Fig. 1B*) as previously noted.

*Bhlhe40 is required to control mycobacterial infection*

Our finding that *BHLHE40* expression is decreased in patients with active tuberculosis infection led us to investigate a role for this transcription factor during *Mtb* infection in mice. We infected WT, *Bhlhe40*+/−, and *Bhlhe40*−/− mice on the C57BL/6J background with the *Mtb* Erdman strain and monitored morbidity and mortality. WT and *Bhlhe40*+/− mice displayed no signs of morbidity and survived beyond 100 days post-infection (dpi). *Bhlhe40*−/− mice began losing weight around 21 dpi and succumbed to infection between 32 and 40 dpi with a median survival time of 33 dpi (*Fig. 2A, B*). This severe susceptibility phenotype is similar to that of mice lacking STAT123 and is more severe than that of mice lacking NF-κB p50,169 both of which are central transcriptional regulators of the immune system. By 21 dpi, *Mtb* colony-forming units (CFUs) in *Bhlhe40*−/− mice were 23-fold higher in the lung and 5-fold higher in the spleen as
Figure 1. Lower BHLHE40 expression is specifically associated with active tuberculosis.

Expression of (A) BHLHE40 and (B) STAT1 in human whole blood from healthy controls or patients with latent tuberculosis (TB), active TB, or other lung diseases. Data are derived from the analysis of the indicated publicly available GEO microarray datasets using the indicated probe sets. Each point represents data from one human. The mean ± SEM is graphed. Statistical differences were determined by two-tailed unpaired Student’s t test or unpaired one-way ANOVA for normally distributed groups and unpaired Kruskal-Wallis test or two-tailed unpaired Mann-Whitney test for non-normally distributed groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Previously published in Huynh and Lin et al., 2018.

compared with WT mice (Fig. 2C, D). The differences in Mtb CFUs in both organs became even more pronounced at 28 dpi, demonstrating an ongoing defect in the ability of Bhlhe40−/− mice to control Mtb replication. We next tested the requirement for two important Mtb virulence factors, the ESX-1 secretion system and the membrane lipid phthiocerol dimycoserosate (PDIM), to cause susceptibility in Bhlhe40−/− mice. Bhlhe40-deficient mice infected with isogenic Mtb
strains lacking ESX-1 (ΔESX-1) or PDIM (ΔPDIM) exhibited no susceptibility over 80 days of infection (Fig. 2E). At 81 dpi, lung CFU counts for PDIM-deficient Mtb were below the limit of detection in Bhlhe40−/− mice while ESX-1 deficient Mtb counts trended higher in Bhlhe40−/− lungs compared to WT Mtb (Fig. 2F). These experiments lack the statistical power to draw strong conclusions but suggest that expression of ESX-1 and PDIM by Mtb is required for the acute susceptibility of Bhlhe40−/− mice. Bhlhe40−/− mice may also have a defect in control of ΔESX-1 Mtb, though not to the same extent as WT Mtb.

We next tested whether Bhlhe40−/− mice were susceptible to a related mycobacterial species, Mycobacterium bovis Bacillus Calmette-Guérin (BCG) (Fig. 2G). No survival defect was observed prior to nine weeks post-infection but BCG lung titers were significantly higher in the lungs of Bhlhe40−/− mice. This demonstrated that Bhlhe40−/− mice have a reduced capacity to control lung infection by multiple mycobacterial species, although their susceptibility is species- and virulence-factor dependent. Upon infection with the intracellular bacterial pathogen Listeria monocytogenes, Bhlhe40−/− mice showed no increased susceptibility (Fig. 2H) and mounted a robust response to secondary L. monocytogenes challenge (Fig. 2I), indicating that their impaired immune response may be specific to mycobacterial infection.

**Bhlhe40 deficiency leads to severe lung inflammation**

Shortly before succumbing to infection, it was evident by gross examination that Bhlhe40−/− lungs had developed larger lesions than WT lungs (Fig. 3A). Histological analysis confirmed that although there were no differences in pulmonary inflammation before infection, the lungs of Bhlhe40−/− mice had developed larger neutrophil- and acid-fast bacilli-rich lesions with more widespread inflammation than WT lungs by 21 dpi (Fig. 3B, C).
Figure 2. Bhlhe40 is required to control mycobacterial infection.

(A-D) Mice were infected with WT Mtb and monitored for changes in (A) body weight, (B) survival, (C) lung Mtb burden, and (D) spleen Mtb burden. (E-F) Mice were infected with WT, ΔESX-1, and ΔPDIM Mtb then monitored for (E) survival and (F) lung Mtb burden at 81 dpi. Bhlhe40−/− mice infected with WT Mtb did not survive to 81 dpi and thus could not be assessed at this time point. (G) Mice were infected intranasally with M. bovis BCG and monitored for
lung BCG burden at 62 and 65 dpi. Data from 62 and 65 dpi is graphed and analyzed together. (H) Mice were intravenously infected with $10^6$ or $10^7$ CFU of *L. monocytogenes* and monitored for survival. (I) Mice were intravenously infected with $10^6$ CFU and *L. monocytogenes* burden in the spleen and liver was assessed at 3 days after primary or secondary infection. Dotted lines indicate limits of detection ([B] 500, [B] 50 and 500, and [I] 100 CFU). The mean ± SEM is graphed. Statistical differences were determined by log-rank Mantel-Cox test (B, E, H), two-tailed unpaired Student’s t test for normally distributed groups, and two-tailed unpaired Mann-Whitney test for non-normally distributed groups (C, D, G, I). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from one (E, F, G, I) or two independent experiments (A-D, G). Panels (A-D, H-I) were previously published in Huynh and Lin et al., 2018.

**Figure 3. Bhlhe40 deficiency leads to severe lung inflammation.**

(A-C) Mice were left uninfected or were infected with *Mtb*. (A) Gross lung pathology was assessed at 33 dpi. Lung histopathology in (B) naïve mice or (B, C) at 21 dpi was assessed by H&E staining. Images shown are at 4X magnification. (C) Acid-fast bacteria in lung samples were visualized by Ziehl-Neelsen stain at 21 dpi. Scale bars indicate 5 mm (A), 500 µm (B), 100 µm (C, H&E), or 50 µm (C, Ziehl-Neelsen). Images are representative of two independent experiments with three biological replicates per experiment. Previously published in Huynh and Lin et al., 2018.
We analyzed these histological differences further by performing flow cytometry on the immune cell populations present in the lungs of mice before and after *Mtb* infection. By 21 dpi, neutrophils were the predominant CD45+ cell type in the lungs of *Bhlhe40<sup>−−</sup>* mice, and the absolute number of neutrophils in *Bhlhe40<sup>−−</sup>* lungs was three times greater than in WT lungs (Fig. 4A, D). The number and frequency of neutrophils in the lungs of *Bhlhe40<sup>−−</sup>* mice further increased as the infection progressed (Fig. 4C, D). The number of CD11b<sup>+</sup>Ly6<sup>C<sub>high</sub></sup> monocyte-derived DCs (moDCs) was 12-fold lower and CD8<sup>+</sup> T cell counts trended downward in *Bhlhe40<sup>−−</sup>* mice at 21 dpi, suggesting that Bhlhe40 may regulate the development, recruitment, or survival of these populations (Fig. 4A). There were no significant differences in the populations sizes of other myeloid or lymphoid cell types analyzed at 21 dpi (Fig. 4A), nor were there any differences in the frequency of *Mtb* antigen-specific T cells in lungs or mediastinal lymph nodes (Fig. 4B).

The timing of the increased inflammation correlated with our initial observation of higher *Mtb* burden in the lungs of *Bhlhe40<sup>−−</sup>* mice, leading us to investigate whether the accumulating myeloid cells were infected with *Mtb*. We infected WT and *Bhlhe40<sup>−−</sup>* mice with a strain of *Mtb* Erdman that stably expresses GFP and monitored the number and frequency of *Mtb*-infected cells at 21 dpi. Neutrophils, CD11b<sup>+</sup> macrophages, inflammatory monocytes, moDCs, and CD11b<sup>+</sup> DCs were all infected at a higher frequency in *Bhlhe40<sup>−−</sup>* lungs compared with WT lungs (Fig. 4E). Absolute numbers of infected neutrophils, inflammatory monocytes, and CD11b<sup>+</sup> DCs were also significantly higher in *Bhlhe40<sup>−−</sup>* lungs (Fig. 4F), suggesting that *Mtb* residing within these cell types accounts for the difference in pulmonary *Mtb* burden in *Bhlhe40<sup>−−</sup>* lungs at 21 dpi. Infected *Bhlhe40<sup>−−</sup>* neutrophils, inflammatory monocytes, moDCs, and CD11b<sup>+</sup> DCs also exhibited an increase in mean fluorescence intensity (MFI) for GFP.
compared with infected WT neutrophils, suggesting that they harbored more *Mtb* on a per-cell basis (Fig. 4H).

**Neutrophil and monocyte inflammation do not drive susceptibility in Bhlhe40<sup>−/−</sup> mice.**

To test whether the influx of neutrophils contributed to the susceptibility of *Bhlhe40<sup>−/−</sup>* mice, we used an anti-Ly6G monoclonal antibody to specifically deplete neutrophils between 10 and 30 dpi. Neutrophil-depleted *Bhlhe40<sup>−/−</sup>* mice did not exhibit any improvements in morbidity, survival time, or control of *Mtb* replication (Fig. 5A-C). We further tested a role for neutrophils in susceptibility by infecting *Bhlhe40<sup>−/−</sup>* mice lacking granulocyte colony stimulating factor receptor (G-CSFR, *Csf3r*) (Fig. 5D). *Csf3r<sup>−/−</sup>* mice are reported to have basal neutropenia<sup>170</sup> although we did not confirm neutropenia in this setting. *Csf3r<sup>−/−</sup>*/*Bhlhe40<sup>−/−</sup>* mice exhibited a statistically significant extension of survival time compared to *Csf3r<sup>+/−</sup>*/*Bhlhe40<sup>−/−</sup>* mice, and the difference in survival time between *Bhlhe40<sup>+/−</sup>* and *Csf3r<sup>−/−</sup>*/*Bhlhe40<sup>−/−</sup>* mice approached significance (*p* = 0.052), suggesting that loss of G-CSFR signaling is partially protective in the context of Bhlhe40-deficiency. *Csf3r<sup>−/−</sup>* mice were susceptible in chronic *Mtb* infection, demonstrating that G-CSFR-signaling is host-protective in WT mice (Fig. 5D). We next tested a role for monocytes in susceptibility by infecting *Bhlhe40<sup>−/−</sup>* mice lacking C-C motif chemokine receptor 2 (CCR2, *Ccr2*) (Fig. 5E). CCR2 is essential for survival of high dose *Mtb* infection and *Ccr2<sup>−/−</sup>* mice exhibit defects in inflammatory monocyte recruitment,<sup>171</sup> although we did not confirm reduced monocyte recruitment in this setting. CCR2-deficiency had no impact on the susceptibility of *Bhlhe40<sup>−/−</sup>* mice. These results show that although neutrophils and monocytes are prominent replicative niches for *Mtb* and neutrophils may have a detrimental role, these cell types are not the sole cause of susceptibility to *Mtb* infection in *Bhlhe40<sup>−/−</sup>* mice.
Figure 4. Bhlhe40 deficiency leads to neutrophil-dominated inflammation.

(A-G) Mice were infected with *Mtb*. (A) Absolute cell counts for pulmonary immune cells before infection and at 21 dpi. MoDC counts in four naive WT and three naive Bhlhe40−/−
samples were below the limit of detection (∼100 cells). (B) Frequency of Mtb antigen-specific CD4+ T cells at 21 dpi. (C) Representative flow cytometry plots for neutrophils as a percentage of the total CD45+ population. (D) Absolute count and percentage of the total CD45+ population for lung neutrophils. (E) Representative flow cytometry plots for GFP+ (Mtb+) neutrophils at 21 dpi. (F) Frequency of GFP+ (Mtb+) cells in myeloid lung populations at 21 dpi. (G) Absolute count for GFP+ (Mtb+) lung myeloid cells at 21 dpi. Four WT alveolar macrophage, four WT and five Bhlhe40−/− CD103+ DC, and two WT and one Bhlhe40−/− eosinophil samples were below the limit of detection (∼850 cells). (H) MFI for FITC in GFP+ (Mtb+) lung cell populations at 21 dpi. Mac., macrophage; Inf. Mono., inflammatory monocyte. The mean ± SEM is graphed. Statistical differences were determined by two-tailed unpaired Student’s t test for normally distributed groups or two-tailed unpaired Mann-Whitney test for nonnormally distributed groups as appropriate. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from or representative of two (A-D) or five (E-H) independent experiments. Previously published in Huynh and Lin et al., 2018.

**Bhlhe40 is required within hematopoietic cells**

Bhlhe40 is expressed in specific immune cell populations and in nonhematopoietic cell types. To dissect which cells required Bhlhe40 expression during Mtb infection, we infected reciprocal bone marrow chimeric mice with Mtb and monitored survival (Fig. 6A). As expected, all but one WT→WT chimeric mouse survived past 100 dpi, and Bhlhe40−/−→Bhlhe40−/− chimeras succumbed 27–35 dpi (median survival time of 33 days). WT→Bhlhe40−/− chimeras also survived past 100 dpi, whereas Bhlhe40−/−→WT chimeras died between 29-37 dpi (median survival time of 32 days), similar to Bhlhe40−/−→Bhlhe40−/− chimeras. These data demonstrate a specific role for Bhlhe40 in radiosensitive hematopoietic cells during Mtb infection.

To determine which hematopoietic cells express Bhlhe40 during Mtb infection, we used Bhlhe40-GFP reporter mice (Bhlhe40GFP). We monitored GFP expression as a proxy for Bhlhe40 expression in the lungs of naive and Mtb infected Bhlhe40GFP mice. Sizeable fractions of neutrophils, alveolar macrophages, CD11b+ DCs, CD103+ DCs, and eosinophils expressed GFP both before infection and at 21 dpi (Fig. 6B-D), with smaller fractions of CD11b+ macrophages, moDCs, B cells, and CD8+ T cells also showing this pattern of expression. Similar
Figure 5. Neutrophils and monocytes do not drive susceptibility in Bhlhe40−/− mice.

(A-C) Mice were infected with Mtb and treated with rat IgG or anti-Ly6G mAb from 10-30 dpi. Mice were monitored for changes in (A) body weight, (B) survival, and (C) lung Mtb burden at 21 dpi. (D, E) Mice were infected with Mtb and monitored for survival. The mean ± SEM is graphed. Statistical differences were determined by log-rank Mantel-Cox test (B, D, E) or unpaired Kruskal-Wallis test with Dunn’s multiple comparison test (B), *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from one (E) or two (A-D) independent experiments. Panels (A-C) were previously published in Huynh and Lin et al., 2018.

Trends were observed at 14 dpi (data not shown). Therefore, these cell types constitutively express Bhlhe40, and this expression pattern does not change during Mtb infection. In contrast, CD4+ T cells in naïve mice expressed very little Bhlhe40 but at 21 dpi, Bhlhe40 levels increased in this cell type (Fig. 6B-D). These data show that Bhlhe40 is expressed in both myeloid and lymphoid cells during Mtb infection.
Figure 6. Bhlhe40 is expressed and required in hematopoietic cells.
(A) WT or Bhlhe40<sup>−/−</sup> mice were lethally irradiated, reconstituted with WT or Bhlhe40<sup>−/−</sup> bone marrow, and monitored for survival after Mtb infection. (B) Frequency of GFP<sup>+</sup> (Bhlhe40<sup>+</sup>) cells
for lung cell populations in non-transgenic (Tg) and Bhlhe40GFP mice before infection and at 21 dpi. (C, D) Representative flow cytometry plots for GFP (Bhlhe40) in immune cells from the lungs of non-Tg WT and Tg Bhlhe40GFP mice before infection (C) or at 21 dpi (D). Bold numbers are the percentages of cells that fall within the gate. Mac., macrophage; Inf. Mono., inflammatory monocyte; Alv. Mac., alveolar macrophage. Data are from or representative of two (A) or three (B-D) independent experiments. The mean ± SEM is graphed. Statistical differences were determined by log-rank Mantel-Cox test (A) and one-way unpaired ANOVA with Tukey’s post-test for normally distributed groups or unpaired Kruskal-Wallis test with Dunn’s multiple comparison test for non-normally distributed groups (B). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Previously published in Huynh and Lin et al., 2018.

**Bhlhe40 enables control of Mtb in the context of T cell deficiency**

Bhlhe40 expression is required in CD4+ T cells to regulate cytokine production in the experimental autoimmune encephalomyelitis (EAE) model of autoimmunity. However, Bhlhe40−/− mice succumbed to Mtb infection between 32 and 40 dpi, which is earlier than Rag1−/− mice that lack mature B and T cell and succumb to Mtb infection between 46 and 50 dpi. The timing of the death of Mtb infected Bhlhe40−/− mice suggests that there is a defect in the innate immune response to Mtb, as it preceded the point at which adaptive immunity is required, or the presence of pathological T cells is leading to earlier death than would occur in the absence of T cells, as previously observed in Pdcd1−/− mice.

To measure the contribution of Bhlhe40 to host resistance in the absence of B and T cells, we used anti-CD4 and anti-CD8 monoclonal antibodies to deplete CD4+ and CD8+ T cells in WT and Bhlhe40−/− mice, infected with Mtb, and measured Mtb burden at 22 dpi (Fig. 7A). CFU counts in T cell-depleted WT and Bhlhe40−/− mice were not significantly different from undepleted mice of the same genotype. However, when we assessed the numbers of lung CD4+ and CD8+ T cells we found that although depletion led to significant reductions, most mice retained a sizable number of T cells at 22 dpi (Fig. 7B). This suggested that the absence of observable differences in Mtb burden after depletion might be due to effects from residual T
cells. To avoid the potentially cofounding effects of residual T cells we utilized \( \text{Rag}1^{-/-} \) mice that lack mature adaptive immune cells. We compared the survival of \( \text{Mtb} \) infected WT, \( \text{Bhlhe40}^{-/-} \), \( \text{Rag}1^{-/-} \), and \( \text{Rag}1^{-/-}\text{Bhlhe40}^{-/-} \) mice (Fig. 7C). \( \text{Rag}1^{-/-}\text{Bhlhe40}^{-/-} \) mice (median survival time of 33 days) were significantly more susceptible than \( \text{Rag}1^{-/-} \) mice (median survival time of 43 days) to \( \text{Mtb} \) infection and succumbed to infection at the same time as \( \text{Bhlhe40}^{-/-} \) mice. These data demonstrate that Bhlhe40 is required during the innate immune response in the absence of T cells to prolong survival during \( \text{Mtb} \) infection and suggest that our T cell depletion experiments were confounded by the presence of residual T cells. \( \text{Mtb} \) CFUs were 4-fold higher in \( \text{Rag}1^{-/-}\text{Bhlhe40}^{-/-} \) mice compared with \( \text{Rag}1^{-/-} \) mice at 21 dpi (Fig. 7D), and the frequency of neutrophils in the lungs of \( \text{Rag}1^{-/-}\text{Bhlhe40}^{-/-} \) mice was also significantly elevated (Fig. 7E). The frequency of infected neutrophils, CD11b+ macrophages, and moDCs was significantly higher in \( \text{Rag}1^{-/-}\text{Bhlhe40}^{-/-} \) mice at 21 dpi (Fig. 7F). We also observed significantly higher MFI s for GFP in \( \text{Rag}1^{-/-}\text{Bhlhe40}^{-/-} \) neutrophils, CD11b+ macrophages, inflammatory monocytes, and CD11b+ DCs, suggesting that these cell types harbored more \( \text{Mtb} \) on a per-cell basis compared with \( \text{Rag}1^{-/-} \) cells (Fig. 7G). Therefore, loss of Bhlhe40 in innate immune cells compromises their ability to control inflammation and \( \text{Mtb} \) replication independent of adaptive immunity.

**Bhlhe40 is required in CD11c\(^+\), Zbtb46\(^+\), and T cells**

To determine more precisely which cells require Bhlhe40 expression to control \( \text{Mtb} \) infection, we infected mice that conditionally delete Bhlhe40 in specific cell types. After \( \text{Mtb} \) infection, \( \text{Bhlhe40}^{fl/fl}\text{-Lysm-Cre} \) and \( \text{Bhlhe40}^{fl/fl}\text{-Mrp8-Cre} \) mice survived past 100 dpi (Fig. 8A) and showed no signs of morbidity over the first 45 days of infection (Fig. 8B), indicating that loss of Bhlhe40 in LysM\(^+\) or Mrp8\(^+\) cells was not sufficient to generate susceptibility to
Figure 7. Bhlhe40 enables control of Mtb in the context of T cell deficiency.

(A, B) Mice were treated with anti-CD4/anti-CD8 monoclonal antibodies, infected with Mtb, and assessed for (A) lung CFUs or (B) lung CD4$^+$ and CD8$^+$ T cells at 22 dpi. (C-G) Mice were infected with Mtb and monitored for (C) survival or analyzed at 21 dpi for (D) lung CFUs, (E) neutrophilic inflammation, (F) frequency of GFP$^+$ (Mtb$^+$) cells, and (G) MFI for GFP in GFP$^+$ (Mtb$^+$) cells. Mac., macrophage; Inf. Mono., inflammatory monocyte; Alv. Mac., alveolar macrophage. Data are from two (A, B) or five (C-G) independent experiments. The mean ± SEM is graphed. Statistical differences were determined by log-rank Mantel-Cox test (C), two-tailed unpaired Student’s t test or one-way unpaired ANOVA with Tukey’s post-test for normally
distributed groups and two-tailed unpaired Mann-Whitney test or unpaired Kruskal-Wallis test with Dunn’s multiple comparison test for non-normally distributed groups, as appropriate (A,B, D-G). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Panels (C-G) were previously published in Huynh and Lin et al., 2018.

infection. In contrast, Bhlhe40^{fl/fl}-Cd11c-Cre and Bhlhe40^{fl/fl}-Cd4-Cre mice succumbed to infection at 34-62 dpi (median survival time of 56 days) and 31-73 dpi (median survival time of 52 days), respectively (Fig. 8A). Therefore, Bhlhe40 expression in both T cells and CD11c^{+} cells is required to control Mtb infection. Given that the Lysm (also known as Lyz2) promoter can drive conditional deletion in alveolar macrophages but deletes poorly in myeloid DCs, whereas the Cd11c (also known as Itgax) promoter deletes equally well in both alveolar macrophages and DCs,\textsuperscript{174} one interpretation is that loss of Bhlhe40 in alveolar macrophages is not sufficient to cause susceptibility and that loss of Bhlhe40 in DCs contributes to the susceptibility observed in Bhlhe40^{fl/fl}-Cd11c-Cre mice. The Cd11c promoter has also been reported to drive conditional deletion in a subpopulation of T cells\textsuperscript{174} that could be contributing to susceptibility. In order to examine a role for Bhlhe40 specifically in conventional DCs we infected a Bhlhe40^{fl/fl}-Zbtb46-Cre mouse and found that it succumbed at 116 dpi (Fig. 8A). Although only one mouse was tested, its susceptibility demonstrates that loss of Bhlhe40 in conventional DCs is sufficient for susceptibility.

The longer survival times of Bhlhe40^{fl/fl}-Cd11c-Cre and Bhlhe40^{fl/fl}-Cd4-Cre mice compared to Bhlhe40^{−/−} mice indicated that loss of Bhlhe40 in CD11c^{+} and T cells separately did not account for the entirety of the defect observed in mice with global Bhlhe40-deficiency. To test whether simultaneous loss of Bhlhe40 in CD11c^{+} and T cells accounted for the severe defect in Bhlhe40^{−/−} mice we infected Bhlhe40^{fl/fl}-Cd4-Cre/Cd11c-Cre mice and tracked survival (Fig. 8A). Bhlhe40^{fl/fl}-Cd4-Cre/Cd11c-Cre were significantly more susceptible than Bhlhe40^{fl/fl}-
Figure 8. Bhlhe40 is required in CD11c⁺, Zbtb46⁺, and T cells.

(A-E) Mice were infected with Mtb and monitored for (A) survival and (B) weight loss or (C) lung Mtb burden, (D) lung neutrophil and moDC absolute counts and frequency in the total...
CD45+ population, and (E) frequency of GFP+ (Mtbb+) lung cells at 21 dpi. Mac., macrophage; Inf. Mono., inflammatory monocyte; Alv. Mac., alveolar macrophage. (F) Mice were vaccinated with M. bovis BCG, rested for 30 days, then infected with Mtbb and monitored for survival. The mean ± SEM is shown. Statistical differences were determined by log-rank Mantel-Cox test (A, F) or one-way unpaired ANOVA with Tukey’s post-test for normally distributed groups and unpaired Kruskal-Wallis test with Dunn’s multiple comparison test for non-normally distributed groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from one to four (A), two (B), seven (C-E), or one (F) independent experiments. Parts of panel (A) and all of panels (B-D) were previously published in Huynh and Lin et al., 2018.

Cd11c-Cre and Bhlhe40fl/fl-Cd4-Cre mice, but not as susceptible as Bhlhe40−/− mice. These results confirm that loss of Bhlhe40 in CD11c+ and T cells accounts for the majority of susceptibility observed in Bhlhe40−/− mice but other cell types make minor contributions to resistance.

To assess the role of myeloid cell expression of Bhlhe40 in a T cell-sufficient context we monitored the survival of Bhlhe40fl/fl-Lysm-Cre/Cd11c-Cre mice (Fig. 8A). Only two mice were tested, but their survival times displayed a trend towards increased susceptibility as compared to Bhlhe40fl/fl-Cd11c-Cre mice, suggesting that Bhlhe40 expression in LysM+ cells plays a protective but minor role in resistance to Mtbb.

We next analyzed susceptible Bhlhe40fl/fl-Cd11c-Cre and Bhlhe40fl/fl-Cd4-Cre mice to determine how loss of Bhlhe40 in CD11c+ or T cells contributed to phenotypes observed in Bhlhe40−/− mice. By 21 dpi, pulmonary Mtbb burden in Bhlhe40fl/fl-Cd11c-Cre and Bhlhe40fl/fl-Cd4-Cre mice was 3- and 4-fold higher than Bhlhe40fl/fl controls, respectively (Fig. 8C).

Bhlhe40fl/fl-Cd11c-Cre and Bhlhe40fl/fl-Cd4-Cre mice also exhibited an increase in the frequency and absolute number of lung neutrophils (Fig. 8D). The frequency of moDCs was significantly lower in Bhlhe40fl/fl-Cd4-Cre and Bhlhe40fl/fl-Cd11c-Cre lungs, but the absolute number of moDCs was decreased in Bhlhe40fl/fl-Cd4-Cre lungs only, suggesting that loss of Bhlhe40 in T cells leads to the decreased number of moDCs observed in Bhlhe40−/− mice (Fig. 8D).
Neutrophils, inflammatory monocytes, moDCs, and CD11b+ DCs were infected at a higher frequency in both Bhlhe40^fl/fl-Cd4-Cre and Bhlhe40^fl/fl-Cd11c-Cre mice (Fig. 8E). Collectively, the survival, Mtb burden, neutrophilic inflammation, and frequency of cellular infection phenotypes evident in mice with conditional deletions of Bhlhe40 indicate that expression of Bhlhe40 in CD11c^+ and T cells provides essential and synergistic functions that enable resistance to Mtb infection.

Our identification of an essential role for Bhlhe40 in T cells led us to assess the functionality of vaccination-elicited adaptive immune responses in Bhlhe40^−/− mice. Mice were vaccinated with M. bovis BCG and then monitored for survival after Mtb infection (Fig. 8F). Vaccination significantly extended the survival time of Bhlhe40^−/− mice, although they remained susceptible to Mtb. These data suggested that a pre-existing adaptive immune response in Bhlhe40^−/− mice can confer a degree of resistance, although it is ultimately insufficient to overcome defects that arise during Mtb infection.

Bhlhe40 deficiency alters cytokine transcription after exposure to Mtb

Bhlhe40 has previously been found to regulate cytokine production by CD4^+ T cells in the EAE model.\(^{114,128}\) If Bhlhe40 functions analogously during Mtb infection, Bhlhe40^−/− cells could exert a dominant effect on WT cells through the production of secreted cytokines. We tested this by generating mixed bone marrow chimeric mice. Congenically marked WT (CD45.1/.2) recipients were lethally irradiated and reconstituted with a 1:1 mixture of WT (CD45.1/.1) and Bhlhe40^−/− (CD45.2/.2) bone marrow cells. Control mice included mixed chimeras generated with WT (CD45.1/.1) and WT (CD45.2/.2) bone marrow cells as well as nonchimeric WT (CD45.2/.2) and Bhlhe40^−/− (CD45.2/.2) mice. In mixed chimeras, Bhlhe40^−/−
Figure 9. Bhlhe40 deficiency alters cytokine transcription after exposure to Mtb.
(A) Mixed bone marrow chimera mice were generated and the frequency of CD45.1⁺, CD45.2⁺, and CD45.1⁺CD45.2⁺ lung cells was assessed before infection. (B-D) Mixed bone marrow chimera mice were infected with Mtb. (B, C) The frequency of infected lung neutrophils and (D) lung Mtb burden was assessed in nonchimeric and mixed bone marrow chimeric mice infected with GFP-Mtb at 21 dpi. (E-G) Cytokine transcript levels in total lung samples were assessed by
bone marrow cells were capable of normal reconstitution of the hematopoietic compartment within the lung (Fig. 9A). Chimeric mice were infected with GFP-expressing Mtb and analyzed at 21 dpi. WT + WT chimeras controlled Mtb infection with a low frequency of neutrophil infection (Fig. 9B, C). Neutrophils of both genotypes displayed a high frequency of Mtb infection (Fig. 9B, C), which correlated with a trend toward higher CFUs in the lungs of mixed WT + Bhlhe40−/− chimeras (Fig. 9D). These results demonstrate that Bhlhe40−/− cells, even when present as only half of all hematopoietic cells, exert a dominant influence in trans on the ability of WT cells to control neutrophil accumulation and Mtb infection. These data indicate that Bhlhe40 likely regulates secreted factors such as cytokines or chemokines, which can impact other cells. Therefore, we analyzed cytokine and chemokine levels in total lung samples from WT and Bhlhe40−/− mice by quantitative RT-PCR. In the lungs of naive WT and Bhlhe40−/− mice, there were no differences in the levels of Csf3 (G-CSF), Il10 (IL-10), Il12b (IL-12/23p40), or Ifng (IFN-γ) transcript (Fig. 9E), indicating that loss of Bhlhe40 does not impact expression of these genes in naive mice. At 14 dpi, transcripts for Il12b and Ifng were significantly decreased in the lungs of Bhlhe40−/− mice (Fig. 9F). At 21 dpi, transcripts for the neutrophil-associated cytokines Csf3, Ccl3 (MIP-1α), and Ccl4 (MIP-1β) were up-regulated in Bhlhe40−/− lungs (Fig. 9G) as expected given the neutrophil-dominated inflammation observed. We also found that at 21 dpi, Il10 transcript levels were 3-fold higher in Bhlhe40−/− lungs, while Il12b and Ifng transcript levels were 3- and 2-fold lower in Bhlhe40−/− lungs, respectively, compared with WT

qRT-PCR (E) prior to infection, (F) at 14 dpi, and (G) at 21 dpi. The mean ± SEM is graphed. Statistical differences were determined by two-tailed unpaired Student’s t test for normally distributed groups or two-tailed unpaired Mann-Whitney test for nonnormally distributed groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from one (A) or two (B-G) independent experiments. Panels (A-D, G) were previously published in Huynh and Lin et al., 2018.
lungs (Fig. 9G). This finding was of particular interest because Bhlhe40 represses \textit{Il10}
transcription in CD4\(^+\) T cells in the EAE model\(^{128}\) and IL-10 has been shown to inhibit IL-12/23p40 and IFN-\(\gamma\) expression during \textit{Mtb} infection.\(^{40,43,48,50}\)

\textit{Bhlhe40 suppresses IL-10 production during in vivo Mtb infection}

The increased \textit{Il10} transcript levels in conjunction with decreased \textit{Il12b} and \textit{Ifng}
transcript levels in \textit{Bhlhe40}\(^{-/-}\) lungs at 21 dpi indicated that Bhlhe40 could be regulating \textit{Il10}
expression. To identify the cell types responsible for the increased \textit{Il10} expression in \textit{Bhlhe40}\(^{-/-}\)
lungs, we used \textit{Il10} bacterial artificial chromosome (BAC)-in transgene (10BiT) IL-10 reporter
mice,\(^{175}\) where \textit{Il10}-expressing cells dis- play Thy1.1 on their surface. We observed a similar
distribution of \textit{Il10}-expressing cells in 10BiT\(^+\) WT mice as reported in a recent study that used
these reporter mice to characterize sources of IL-10 before and after \textit{Mtb} infection.\(^{44}\) We crossed
10BiT and \textit{Bhlhe40}\(^{-/-}\) mice to generate a \textit{Bhlhe40}\(^{-/-}\) 10BiT\(^+\) strain, and then we analyzed Thy1.1
expression as a proxy for \textit{Il10} expression. We observed low levels of Thy1.1 expression in naive
10BiT\(^+\) WT and 10BiT\(^+\) \textit{Bhlhe40}\(^{-/-}\) mice (Fig. 10). At 21 dpi, the frequency of Thy1.1\(^+\)
neutrophils, inflammatory monocytes, moDCs, CD11b\(^+\) DCs, B cells, CD4\(^+\) T cells, and CD8\(^+\) T
cells was significantly higher in \textit{Bhlhe40}\(^{-/-}\) mice (Fig. 11A, C), but no differences were observed
when comparing the absolute number of Thy1.1\(^+\) cells (Fig. 11B). In addition, when we
compared MFIs for Thy1.1 on Thy1.1\(^+\) cells, we found that not only were a higher percentage of
\textit{Bhlhe40}\(^{-/-}\) moDCs and B cells expressing Thy1.1, but these cell types also expressed more
Thy1.1 on a per-cell basis during \textit{Mtb} infection (Fig. 11D). Significantly higher Thy1.1 MFI was
also observed on \textit{Bhlhe40}\(^{-/-}\) Thy1.1\(^+\) alveolar macrophages and CD11b\(^+\) macrophages (Fig.
11D). The increased frequency of Thy1.1 positivity observed in \textit{Bhlhe40}\(^{-/-}\) CD4\(^+\) T cells was
Figure 10. Bhlhe40 deficiency does not cause IL-10 overexpression in the absence of infection.
(A-B) Uninfected 10BiT+ mice were analyzed for the frequency of Thy1.1+ (IL-10+) lung immune cells was assessed in uninfected mice. Mac., macrophage; Inf. Mono., inflammatory monocyte; Alv. Mac., alveolar macrophage. The mean ± SEM is graphed. Statistical differences were determined by two-tailed unpaired Student’s t test for normally distributed groups or two-tailed unpaired Mann-Whitney test for non-normally distributed groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from one experiment (A, B).
Figure 11. Bhlhe40 deficiency causes overexpression of IL-10 during Mtb infection.

(A-E) Mice were infected with Mtb and assessed for (A, B) frequency and (C) absolute number of Thy1.1⁺ (IL-10⁺) lung immune cells at 21 dpi. (D) MFI for FITC (Thy1.1 [IL-10]) on Thy1.1⁺ lung cells at 21 dpi. Mac., macrophage; Inf. Mono., inflammatory monocyte; Alv. Mac., alveolar macrophage. Thy1.1⁺ (E) Ag85B- or ESAT6-specific CD4⁺ T cells as a percentage of the total Ag85B- or ESAT6-specific CD4⁺ T cell population in the lung or mediastinal lymph node at 21 dpi. The mean ± SEM is graphed. Statistical differences were determined by two-tailed unpaired Student’s t test for normally distributed groups or two-tailed unpaired Mann-
Whitney test for non-normally distributed groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from two independent experiments (A-E). Panels (A-E) were previously published in Huynh and Lin et al., 2018.

even more pronounced in \textit{Bhlhe40}^{−/−} \textit{Mtb}-specific CD4\(^{+}\) T cells in the mediastinal lymph node and lung, indicating that \textit{Mtb}-specific CD4\(^{+}\) T cells take on an immunosuppressive phenotype in the absence of Bhlhe40 (Fig. 11E). These data demonstrated that the frequency and per-cell expression of \textit{Il10} was increased in multiple Bhlhe40\(^{−/−}\) myeloid and lymphoid populations.

\textbf{Bhlhe40 suppresses IL-10 expression in myeloid cells in vitro}

We next sought to identify an \textit{in vitro} myeloid cell culture system that recapitulates the effects of Bhlhe40 deficiency on IL-10 production in response to \textit{Mtb}. We cultured WT bone marrow cells with GM-CSF or macrophage colony stimulating factor (M-CSF) and determined by Western blot analysis that only cells cultured with GM-CSF, which comprise a mixture of granulocytes, macrophage-like cells, and DC-like cells,\textsuperscript{176,177} express Bhlhe40 (Fig. 12A).

Analysis of GM-CSF- and M-CSF-cultured cells from \textit{Bhlhe40}\textsuperscript{GFP} reporter mouse bone marrow confirmed that only GM-CSF-cultured bone marrow-derived cells expressed GFP (Fig. 12B).

This expression of Bhlhe40 in granulocytes, macrophage-like cells, and DC-like cells cultured with GM-CSF may relate to the expression of Bhlhe40 by these cell types in the lung (Fig. 6),\textsuperscript{108,178} where GM-CSF is abundant and plays an important part in the development and function of lung myeloid cells.\textsuperscript{179}

We next assessed IL-10 expression from WT and \textit{Bhlhe40}^{−/−} cells cultured with GM-CSF. In the absence of stimulation with heat-killed \textit{Mtb}, minimal IL-10 protein was detected in culture supernatants (Fig. 12C). In contrast, \textit{Bhlhe40}^{−/−} GM-CSF-cultured bone marrow-derived
Figure 12. Bhlhe40 represses IL-10 in myeloid cells after Mtb stimulation.

(A-B) Bone marrow cells were differentiated in the presence of M-CSF or GM-CSF and analyzed for expression of Bhlhe40 by (A) immunoblot (IB) with HDAC1 as a loading control or (B) GFP as a reporter for Bhlhe40 expression. MW, molecular weight. (C) GM-CSF-cultured bone marrow-derived cells were left unstimulated or were stimulated with heat-killed (HK) Mtb for 24 hrs, and culture supernatants were analyzed for IL-10 production by ELISA. IL-10 expression in six unstimulated WT and 12 unstimulated Bhlhe40−/− samples was below the limit of detection (~0.1 ng/ml). (D-F) 10BiT+ WT and Bhlhe40−/− bone marrow cells were differentiated in the presence of GM-CSF and stimulated with heat-killed Mtb antigen for 24 hrs. Thy1.1 was assessed on three subpopulations of cells within the GM-CSF cultures. These included Ly6G+ cells, GM-macrophages (GM-Macs; identified as CD11c+CD11bhighMHC-IImid), and GM-DCs (identified as CD11c+CD11bmidMHC-IIhigh). (D) Frequency of Thy1.1+ (IL-10+) cells after 24 hrs of stimulation. (E) MFI for Thy1.1 on Thy1.1+ cells after 24 hrs of stimulation.
(F) Culture supernatants were analyzed for IL-10 production by ELISA. IL-10 expression in four unstimulated 10BiT+ WT and five unstimulated 10BiT+ Bhlhe40−/− samples was below the limit of detection (~0.1 ng/ml). (G) GM-CSF-cultured bone marrow-derived cells were infected with Mtb, in some cases treated with IFN-γ, and CFUs were measured. The mean ± SEM is graphed. Statistical differences were determined by two-tailed unpaired Student’s t test for normally distributed groups or two-tailed unpaired Mann-Whitney test for non-normally distributed groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from or representative of two (A, B, D-F), five (C), or one (G) independent experiments. Panels (A-G) were previously published in Huynh and Lin et al., 2018.

cells stimulated with heat-killed Mtb for 24 hours produced significantly more IL-10 than WT cells (Fig. 12C). We also stimulated 10BiT+ WT and Bhlhe40−/− GM-CSF-cultured cells with heat-killed Mtb, assessed Thy1.1 expression by flow cytometry, and measured IL-10 in culture supernatants by ELISA. Mtb stimulation increased the frequency of Thy1.1+ cells in both WT and Bhlhe40−/− cultures relative to unstimulated cultures, and this increase was greater in Bhlhe40−/− cells compared with WT cells (Fig. 12D). Mtb stimulation also increased the amount of Thy1.1 expression on a per-cell basis, where Bhlhe40−/− CD11c+CD11b+MHC-IIhigh (GM-DC) cells exhibited a 1.7-fold higher MFI than WT GM-DCs (Fig. 12E). ELISAs confirmed that 10BiT+ Bhlhe40−/− cells secreted more IL-10 than 10BiT+ WT cells after stimulation with heat-killed Mtb (Fig. 12F). These experiments confirm that loss of Bhlhe40 in GM-CSF-cultured cells results in higher levels of IL-10 production. Loss of Bhlhe40 did not significantly affect the ability of GM-CSF-cultured cells to control Mtb replication in the presence or absence of IFN-γ (Fig. 12G), suggesting that Bhlhe40 is dispensable for the ability of these cell types to control Mtb replication in vitro.
Figure 13. Bhlhe40 directly binds the \textit{Il10} locus in myeloid and lymphoid cells.  
(A-D) Bone marrow cells were differentiated in the presence of GM-CSF and stimulated with heat-killed \textit{Mtb} for 4 hrs. CD4\(^+\) T cells were isolated from the spleen of naïve mice and T\(_{H1}\)
polarized in vitro for 4 days. DNA was immunoprecipitated using anti-Bhlhe40 antibody and sequenced. (A) Sequence motifs present within DNA bound by Bhlhe40 were analyzed by MEME-ChIP. (B) Bhlhe40 binding sites were annotated using ChIP-seeker v3.5. (C) Functions of cis-regulatory regions were predicted for Bhlhe40 binding site data using GREAT. The top five most highly enriched gene sets with minimum region-based fold enrichment of 2 and binomial and hypergeometric false discovery rates of ≤0.05 in the Gene Ontology (GO) Biological Process and MSigDB Pathway gene sets are displayed for each dataset (in GM-CSF–cultured cells, only three MSigDB Pathway gene sets met these criteria). NFAT, nuclear factor of activated T cells. (D) ChIP-seq binding tracks for Bhlhe40 at the Bhlhe40, Il10, and Ifng loci in WT and Bhlhe40−/− myeloid and lymphoid cells. Vertebrate conservation of each genomic region is displayed in blue, and peaks identified by MACS are indicated by arrows. Bracketed numbers indicate the trace height range. Data are from a single experiment. Panels (A-D) were previously published in Huynh and Lin et al., 2018.

**Bhlhe40 binds directly to the Il10 locus in T$_{H1}$ cells and myeloid cells**

We performed chromatin immunoprecipitation sequencing (ChIP-seq) experiments in in vitro-polarized T$_{H1}$ cells and GM-CSF-cultured bone marrow-derived cells to interrogate whether Bhlhe40 directly binds to the Il10 locus in these cells as well as to identify other genes that may be directly regulated by Bhlhe40. We performed these studies with WT and Bhlhe40−/− cells, where Bhlhe40−/− cells served as controls for nonspecific binding by the anti-Bhlhe40 antibody. Bhlhe40 bound 379 sites in WT GM-CSF-cultured cells and 5,532 sites in WT T$_{H1}$-polarized T cells. Of these sites, 273 were found in both datasets. Bhlhe40 can directly bind to regulatory DNA elements as a homodimer through recognition of E-box sites, with a preference for the sequence CACGTG. Motif finding identified the expected E-box (CACGTG) as the most frequent motif present within the peaks identified in both cell types (Fig. 13A). Analysis of binding sites revealed that Bhlhe40 predominantly bound promoters within 1 kb of the transcriptional start site, introns, and distal intergenic regions (Fig. 13B). Pathway analysis of predicted Bhlhe40-regulated genes revealed an enrichment in immune activation and cytokine response pathways in both datasets (Fig. 13C). Bhlhe40 is known to bind a conserved
autoregulatory site in the Bhlhe40 promoter, and this binding site was identified as a peak in both datasets (Fig. 13D). Importantly, our ChIP-seq experiment identified a Bhlhe40 binding site at +6 kb relative to the transcriptional start site of Il10 in both datasets, coinciding with an evolutionarily conserved region that is close to a +6.45 kb site previously identified as an enhancer element in T\textsubscript{H}2 cells (Fig. 13D). These data suggest that Bhlhe40 directly represses Il10 transcription in myeloid and lymphoid cells through direct binding of a downstream cis-regulatory element. ChIP-seq analysis did not reveal binding of Bhlhe40 to the Il12b locus in either dataset. Bhlhe40 did not bind the Ifng locus in GM-CSF-cultured cells but bound two sites distal (−33.5 kb and +53.2 kb) to the Ifng transcriptional start site in T\textsubscript{H}1 cells (Fig. 13D). These findings suggest that the transcriptional down-regulation of Il12b in Bhlhe40\textsuperscript{−/−} total lung samples (Fig. 9F, G) is indirect and likely a result of increased IL-10 signaling, whereas the decreased levels of Ifng may result from either increased IL-10 signaling or T cell-intrinsic loss of direct regulation by Bhlhe40.

**IL-10 deficiency protects Bhlhe40\textsuperscript{−/−} mice from Mtb infection**

To investigate the role of IL-10 production in the susceptibility of Bhlhe40\textsuperscript{−/−} mice, we generated II10\textsuperscript{−/−}/Bhlhe40\textsuperscript{−/−} mice and compared their survival to WT, Bhlhe40\textsuperscript{−/−}, and II10\textsuperscript{−/−} mice after Mtb infection. The absence of IL-10 signaling resulted in near-complete rescue of the acute susceptibility phenotype caused by Bhlhe40 deficiency as shown by the significant increase in the median survival time of II10\textsuperscript{−/−}/Bhlhe40\textsuperscript{−/−} mice (122 days) compared with Bhlhe40\textsuperscript{−/−} mice (31 days) (Fig. 14A). The increased survival of II10\textsuperscript{−/−}/Bhlhe40\textsuperscript{−/−} mice was accompanied by decreased Mtb titer in II10\textsuperscript{−/−}/Bhlhe40\textsuperscript{−/−} lungs (8-fold) and spleens (5-fold).
Figure 14. IL-10 deficiency protects Bhlhe40−/− mice from Mtb infection.

(A-F) Mice were infected with Mtb and monitored for (A) survival and (B) lung and spleen CFU, (C, D) frequency and absolute number of lung neutrophils, and (E) cytokine transcript levels in total lung samples at 21 dpi. (F, G) Mice were infected with Mtb and treated with IL-12 three times per week for the first 4 weeks of infection. (F) Weight loss and (G) survival were monitored. The mean ± SEM is graphed. Statistical differences were determined by log-rank Mantel-Cox test (A, B) and one-way unpaired ANOVA with Tukey’s post-test for normally
distributed groups or unpaired Kruskal-Wallis test with Dunn’s multiple comparison test for non-normally distributed groups (B, D, E). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from or representative of two (A, E), four (B, C, D), or one (F, G) independent experiments. Parts of panels (A, B) and all of panels (C-E) were previously published in Huynh and Lin et al., 2018.

compared with Bhlhe40−/− mice at 21 dpi (Fig. 14B). These data demonstrate that the inability of Bhlhe40−/− mice to control acute phase Mtb replication is likely caused in large part by higher IL-10 levels. When compared with Bhlhe40−/− lungs, Il10+/−Bhlhe40+/− lungs contained a significantly lower frequency and total number of neutrophils (Fig. 14C, D). However, the frequency of neutrophils in Il10+/−Bhlhe40+/− lungs remained higher than in WT or Il10−/− lungs (Fig. 14C, D). Il10−/−Bhlhe40−/− lungs also contained 2-fold more Il12b and Ifng transcripts than Bhlhe40−/− lungs at 21 dpi, demonstrating that IL-10 signaling was at least partially responsible for the decreased expression of these genes in Bhlhe40−/− lungs (Fig. 14E). The association of higher Il12b transcript levels and increased resistance in Il10−/−Bhlhe40−/− mice suggested that an early defect in IL-12 production might contribute to the susceptibility of Bhlhe40−/− mice. We tested this with an IL-12 administration regimen previously shown to extend the susceptibility of Il12b−/− mice.182 Bhlhe40−/− mice were treated with IL-12 during Mtb infection and weight loss (Fig. 14F) and survival (Fig. 14G) were monitored. IL-12 administration had no effect on weight loss or survival, suggesting that lower Il12b levels are not limiting protective immunity or that there are additional obstacles to protective immunity. These data establish Bhlhe40 as an essential regulator of Il10 expression in myeloid cells and lymphocytes during Mtb infection and reveal the importance of IL-10 regulation for innate and adaptive immune responses that control Mtb infection.
Discussion

We have identified Bhlhe40 as a transcription factor that is essential for coordinating immune responses that protect the host from *Mtb* infection. Based on our research, we propose the following model (Fig. 15). During the acute phase of infection, CD11c^+^ cells encounter *Mtb* antigens that trigger a putative transcriptional activator to induce *Il10* expression, the activity of which is restricted by the binding of Bhlhe40 to a cis-acting regulatory region +6 kb downstream of the *Il10* transcriptional start site. The identity of the activator that induces *Il10* transcription in the absence of Bhlhe40 during *Mtb* infection is currently unknown but may be one of the activators previously described.\(^75\) In addition, Bhlhe40 is induced in T cells during *Mtb* infection, where it also represses *Il10* transcription. The repression of *Il10* expression by Bhlhe40 in these cell types allows for higher expression of *Il12b* and *Ifng*, both of which are essential for control of *Mtb* replication.\(^34,35,183,184\)

These findings represent the first study of roles for Bhlhe40 in the immune response to an infection and within myeloid cells, although a report that T cell expression of Bhlhe40 is essential for control of *Toxoplasma gondii* infection was published concurrently.\(^129\) Additionally, although other negative regulators of *Il10* transcription have been described,\(^78–81,83,84\) Bhlhe40 is the first transcription factor that has been shown to be essential during *Mtb* infection specifically to regulate *Il10* expression. Our findings reveal the importance of controlling IL-10 generation by both innate and adaptive immune cells and shed light on how different levels of IL-10 could impact tuberculosis disease in humans. Survival experiments showed that about one quarter of *Bhlhe40^fl/fl-Cd11c-Cre* and *Bhlhe40^fl/fl-Cd4-Cre* mice recapitulated the very early susceptibility of *Bhlhe40^−/−* mice (Fig. 8A), with the remainder of these Bhlhe40 conditionally deleted mice dying between 50 and 75 dpi. In the case of the *Bhlhe40^fl/fl-Cd4-Cre* strain, the finding that 4 out
of 12 of these mice succumbed at 31-38 dpi (Fig. 8A), whereas Rag1−/− mice succumbed at ~45 dpi (Fig. 7C), indicates that Bhlhe40−/− T cells can be actively pathological and generate a worse outcome than the absence of T cells, likely through the production of a factor such as IL-10. However, the extension of survival observed in BCG-vaccinated Bhlhe40−/− mice (Fig. 8F) suggests that Bhlhe40−/− T cells may have some protective capacity in specific contexts. The delay in susceptibility of most of the Bhlhe40fl/fl-Cd11c-Cre and Bhlhe40fl/fl-Cd4-Cre mice as compared with Bhlhe40−/− mice could indicate that the susceptibility of Bhlhe40−/− mice is a result of the combination of loss of Bhlhe40 in both CD11c+ and T cells, insufficient ability of the Cd11c and Cd4 promoters to drive Bhlhe40 exon deletion in all Cre-expressing cells, or the ability of Bhlhe40 deficiency in a non-CD11c+ or -CD4+ cell type to enhance susceptibility.

Although only one Bhlhe40fl/fl-Zbtb46-Cre mouse was tested, it proved to be susceptible during chronic infection, demonstrating the requirement for Bhlhe40 expression in DCs. The delayed susceptibility of Bhlhe40fl/fl-Zbtb46-Cre mice compared to Bhlhe40fl/fl-Cd11c-Cre mice may be due to incomplete deletion in all DCs, Cd11c promoter-driven deletion of Bhlhe40 in non-DCs, or a protective role for Bhlhe40 in alveolar macrophages when other CD11c+ cell lack Bhlhe40. IL-10 production by T cells and CD11c+ cells during Mtb infection in mice was recently shown to contribute to host susceptibility, further supporting the notion that regulation of Il10 expression in these cell populations would be important during Mtb infection.

Our analyses of Il10−/− Bhlhe40−/− mice revealed that IL-10 deficiency conferred a near-complete rescue of multiple phenotypes associated with the acute susceptibility of Bhlhe40−/− mice (Fig. 14), indicating that the up-regulation of Il10 transcription in the absence of Bhlhe40 is a major contributor to the acute susceptibility of these mice. Nonetheless, some phenotypes partially remained in the absence of Il10. For example, nearly all Il10−/− Bhlhe40−/− mice
succumbed by 200 dpi (Fig. 14A) and Il10−/−/Bhlhe40−/− mice had a significantly higher frequency of lung neutrophils at 21 dpi compared with WT mice (Fig. 14C, D). The incomplete rescue of these phenotypes indicates that Bhlhe40 likely regulates the expression of other genes during Mtb infection that impact pathogenesis. Future studies will explore the other Bhlhe40-bound loci identified by ChIP-seq to identify additional Bhlhe40 targets that contribute to the control of Mtb infection.

Patients with active tuberculosis have increased levels of IL-10 in their serum and lungs, suggesting a link between increased IL-10 levels and tuberculosis disease. Although it is unclear whether the reduced expression of BHLHE40 in the blood of patients with active tuberculosis (Fig. 1A) is a cause or effect of their disease or related to the higher IL-10 levels found in this form of tuberculosis, this correlation agrees with the increased susceptibility of mice lacking Bhlhe40 and suggests that our experiments could be relevant to human Mtb infection.
Figure 15. Model of Bhlhe40 function during Mtb infection.

During Mtb infection of WT cells, Il10 transcription is restricted by Bhlhe40 in CD11c+ DCs and T cells through direct binding to an enhancer site +6 kb downstream of the Il10 transcriptional start site (TSS). The resulting amount of IL-10 expression is insufficient to compromise host resistance, leading to an immunological stalemate in which Mtb replication is controlled for the remainder of the lifetime of the host. In Bhlhe40−/− mice, the absence of Bhlhe40 in CD11c+ DCs and T cells allows for high levels of Il10 expression. Excessive IL-10 signaling then acts on lung immune cells to suppress the production and protective effects of IL-12 and IFN-γ, both of which are essential for control of Mtb pathogenesis. As a result, Mtb lung burdens are higher and neutrophil-dominated inflammation is uncontrolled in Bhlhe40−/− mice, ultimately resulting in susceptibility. Previously published in Huynh and Lin et al., 2018.
Experimental Procedures

Bacterial strains and cultures

*Mtb* strain Erdman and its derivatives were cultured at 37°C in 7H9 broth (Sigma-Aldrich) or on 7H11 agar (BD) medium supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC), 0.5% glycerol, and 0.05% Tween-80 (broth only). *Mtb*-GFP was generated by transforming *Mtb* strain Erdman with a plasmid (pMV261-kan-GFP) that drives constitutive expression of GFP under the control of the *hsp60* promoter. Cultures were grown in the presence of kanamycin to ensure plasmid retention. ΔPDIM *Mtb* was generated by transducing *Mtb* strain Erdman with a phage containing homology to nucleotides 3243062-3243567 and 3272196-3272718. ΔESX-1 *Mtb* was generated by transducing *Mtb* strain Erdman with a phage containing homology to nucleotides 4349945-4350677 and 4356534-4357206. Phage transduction was used to replace the endogenous *fadD26* and *ppsABCDE* (ΔPDIM strain) and *pe35*, *ppe68*, *esxAB*, *espI*, and *eccD1* (ΔESX-1 strain) genes with a hygromycin resistance cassette. Mutants were selected by culture on hygromycin-containing 7H11 agar. Individual hygromycin-resistant colonies were expanded by inoculation into hygromycin-supplemented 7H9 media. Replacement of target genes with the hygromycin resistance cassette was confirmed by Southern blotting of genomic DNA from expanded cultures.

*M. bovis* BCG was a gift from S. Khader (Washington University in Saint Louis, Saint Louis, MO). Bacteria were not cultured and were used directly after thawing.

*L. monocytogenes* expressing chicken ovalbumin (LM-Ova) on the 10403S genetic background was a gift from H. Shen (University of Pennsylvania, Philadelphia, PA). Bacteria were grown to mid-logarithmic phase with shaking at 37°C in brain-heart infusion broth (HiMedia Laboratories) before washing and storage as glycerol stocks at −80°C.
Mouse strains

All mice used were on a C57BL/6 background. C57BL/6 (Taconic), B6.SJL (CD45.1; Taconic), Bhlhe40\(^{GFP}\) BAC Tg (N10 to C57BL/6),\(^{108}\) Bhlhe40\(^{-/-}\) (N10 to C57BL/6),\(^{109}\) Rag1\(^{-/-}\), I\(l10^{-/-}\), and 10BiT IL-10 reporter mice\(^{175}\) were maintained in a specific pathogen-free facility. Bhlhe40\(^{-/-}\) mice were crossed to Ccr2\(^{-/-}\), Csf3r\(^{-/-}\), I\(l10^{-/-}\), Rag1\(^{-/-}\), or 10BiT mice for some experiments. Bhlhe40\(^{fl/fl}\) mice were generated with two loxP sites flanking exon 4 of Bhlhe40. These mice originated as a KO-first promoter-driven mouse line from the Knockout Mouse Project (KOMP) and were purchased from The Jackson Laboratory (Bhlhe40\(^{tm1a(KOMP)Wtsi}\); 024395). These initial mice contained a Bhlhe40 allele with a splice acceptor-LacZ reporter and a Neo cassette flanked by two Frt sites, which were removed by crossing them to B6N.129S4-Gt(Rosa)26Sor\(^{tm1(FLP1)Dym/J}\) mice (016226; The Jackson Laboratory), leaving behind an allele of Bhlhe40 with a loxP-flanked exon 4. Subsequent crosses yielded Bhlhe40\(^{fl/fl}\) mice with no residual Rosa26-Flp transgene. Bhlhe40\(^{fl/fl}\) mice were crossed with one of four mouse strains, Mrp8-Cre (B6.Cg-Tg(S100A8-cre,-EGFP)1Ilw/J; 021614), Lysm-Cre (B6N.129P2(B6)-Ly2\(^{tm1(cre)If0/J}; 018956), Cd11c-Cre (B6. Cg-Tg(Itgax-cre)1-1Reiz/J; 008068), and Cd4-Cre (B6.Cg-Tg(Cd4- cre)1Cwi/Bflu/J; 022071), all from The Jackson Laboratory.

Age-matched littermate adult mice (9-23 weeks of age) of both sexes were used, and mouse experiments were randomized. No blinding was performed during animal experiments. All procedures involving animals were conducted following the National Institutes of Health guidelines for housing and care of laboratory animals, and they were performed in accordance with institutional regulations after protocol review and approval by the Institutional Animal Care and Use Committee of The Washington University in St. Louis School of Medicine (protocol 20160094, Immune System Development and Function, and protocol 20160118, Analysis of
Mycobacterial Pathogenesis). Washington University is registered as a research facility with the United States Department of Agriculture and is fully accredited by the American Association of Accreditation of Laboratory Animal Care. The Animal Welfare Assurance is on file with Office for Protection from Research Risks–National Institutes of Health. All animals used in these experiments were subjected to no or minimal discomfort. All mice were euthanized by CO\textsubscript{2} asphyxiation, which is approved by the American Veterinary Association Panel on Euthanasia.

**Generation of bone marrow chimeric mice**

Bone marrow chimeric mice were generated by lethal irradiation (1,000 rads) of WT or *Bhlhe40\textsuperscript{−/−}* recipients and reconstitution with 1-2 x 10\textsuperscript{7} bone marrow cells from WT or *Bhlhe40\textsuperscript{−/−}* donors. Mixed bone marrow chimeric mice were generated by lethal irradiation (1,000 rads) of WT (CD45.1/2) recipients and reconstitution with 1-2 x 10\textsuperscript{7} bone marrow cells from WT (CD45.1/1) and either WT (CD45.2/2) or *Bhlhe40\textsuperscript{−/−}* (CD45.2/2) donors mixed at a 1:1 ratio before transfer. Mice received drinking water containing 1.3 mg sulfamethoxazole and 0.26 mg trimethoprim per ml for 2 weeks after reconstitution and were allowed to reconstitute for at least 8 weeks before infection with *Mtb*.

**Cell culture**

Bone marrow cells were isolated from femurs and tibias of mice and treated with ACK lysis buffer (0.15 M NH\textsubscript{4}Cl, 10 mM KHCO\textsubscript{3}, and 0.1 mM EDTA) to lyse red blood cells. To generate M-CSF bone marrow-derived macrophages, bone marrow cells were cultured in complete IMDM (10% heat-inactivated FBS + 2 mM l-glutamine + 1X penicillin/streptomycin + 55 µM β-mercaptoethanol + 1X MEM nonessential amino acids + 1 mM sodium pyruvate) in
Petri dishes with the addition of 20 ng/ml M-CSF (PeproTech). To generate GM-CSF-cultured bone marrow-derived DCs and macrophages, bone marrow cells were cultured in complete RPMI (10% heat-inactivated FBS + 2 mM l-glutamine + 1X penicillin/streptomycin + 55 µM β-mercaptoethanol) in six-well plates with the addition of 20 ng/ml GM-CSF (PeproTech). Cells were incubated at 37°C in 8% CO₂ for 8-9 days. At the end of the cultures, cells were harvested, counted, and adjusted to the desired cell concentrations. For flow cytometry analysis and ELISA assays, cells were seeded at a concentration of 5 x 10⁵ cells/well in 96-well plates and were stimulated with or without 50 µg/ml heat-killed *Mtb* (H37Ra strain; Difco) for 18 (FACS) or 24 (ELISA) hrs. In some experiments, suspension cells were stimulated in the presence of 10 µg/ml heat-killed *Mtb* (H37Ra strain; Difco) for 4 hrs for ChIP assays.

To generate *in vitro*-polarized Th1 cells, naive splenic CD4⁺ T cells (Easysep mouse naive CD4⁺ T cell isolation kit, typical purity ~90-96%; StemCell Technologies, Inc.) were cultured in IMDM with plate-bound anti-CD3 (2 µg/ml; clone 145-2C11; BioLegend) and anti-CD28 antibodies (2 µg/ml; clone 37.51; BioLegend) in the presence of IL-12 (10 ng/ml; BioLegend) and anti-IL-4 (20 µg/ml; Leinco). Cultures were split on day 3 and used for ChIP-seq on day 4.

**Bone marrow-derived GM-CSF-cultured cell infections**

For *Mtb* infection, cells were seeded at a concentration of 2.5 x 10⁵ cells/well in 96-well plates. *Mtb* was washed with PBS + 0.05% Tween-80, sonicated to disperse clumps, diluted in antibiotic-free cell culture media, and added to cells at a multiplicity of infection of 1. After 4 hrs of incubation, cells were pelleted and washed twice with PBS, fresh culture media was added, and cells were incubated at 37°C in 5% CO₂. In some cases, cells were pre-treated with 50 ng
IFN-γ (BioLegend)/ml for 12 hrs before infection. CFUs were enumerated by pelleting cells, removing supernatant, lysing with PBS + 0.5% Triton X-100, and plating serial dilutions on 7H11 agar. Colonies were counted after 3 weeks of incubation at 37°C in 5% CO₂.

Mouse infections

*Mtb* cultures in logarithmic growth phase (OD₆₀₀ nm = 0.5-0.8) were washed with PBS + 0.05% Tween-80, sonicated to disperse clumps, and diluted in sterile water. Mice were exposed to 1.6 x 10⁸ *Mtb* CFUs, a dose chosen to deliver 100–200 CFUs of aerosolized *Mtb* per lung using an Inhalation Exposure System (Glas-Col). Within the first 24 hrs of each infection, lungs were harvested from at least two control mice, homogenized, and plated on 7H11 agar to determine the input CFU dose. The mean dose determined at this time point was assumed to be representative of the dose received by all other mice infected simultaneously. At each time point after infection, *Mtb* titers were determined by homogenizing the superior, middle, and inferior lobes of the lung or the entire spleen and plating serial dilutions on 7H11 agar. Colonies were counted after 3 weeks of incubation at 37°C in 5% CO₂.

*M. bovis* BCG was thawed and diluted in PBS. Mice were sedated with ketamine (Henry Schein) and xylazine (Akorn) and intranasally infected with 2 x 10⁵ CFU. BCG titers were determined by homogenizing the superior, middle, and inferior lobes of the lung and plating serial dilutions on 7H11 agar. Colonies were counted after 3 weeks of incubation at 37°C in 5% CO₂.

LM-Ova was diluted into pyrogen-free saline for i.v. injection into mice. For experiments involving secondary infection, some mice were initially infected with a sublethal dose of 10⁴ LM-Ova i.v. and rested for 30 d before secondary challenge with 10⁶ LM-Ova i.v. After 3 days,
spleens and livers were homogenized separately in PBS + 0.05% Triton X-100. Homogenates were plated on brain-heart infusion agar, and *L. monocytogenes* CFUs were determined after growth at 37°C overnight.

**Flow cytometry**

Lungs were perfused with sterile PBS before harvest. Lungs and lymph nodes were digested at 37°C with 630 µg/ml collagenase D (Roche) and 75 U/ml DNase I (Sigma-Aldrich). All antibodies were used at a dilution of 1:200. Single-cell suspensions were preincubated with anti-CD16/CD32 Fc Block antibody (BD) in PBS + 2% heat-inactivated FBS for 10 min at RT before surface staining. The following anti–mouse antibodies were obtained from BioLegend: PE-Cy7 anti-CD4 (RM4-5), APC-Cy7 anti-CD8α (53–6.7), APC anti-CD11b (M1/70), BV605 anti-CD11c (N418), BV605 anti-CD19 (6D5), APC anti-CD45.2 (104), FITC anti-CD90.1 (Thy-1.1; OX-7), PerCP-Cy5.5 anti-CD103 (2E7), Pacific blue (PB) or PerCP-Cy5.5 anti-Ly6C (HK1.4), PE anti-Ly6G (1A8), PB anti-TCRβ (H57-597), and APC anti-TCRγδ (GL3). The following anti–mouse antibodies were purchased from BD: PB anti-CD3ε (145-2C11), BV510 anti-CD45 (30F11), and PE anti–Siglec F (E50-2440). The following anti–mouse antibodies were obtained from Tonbo Biosciences: redFluor710 anti-CD44 (IM7), V450 or PerCP-Cy5.5 anti-CD45.1 (A20), PE-Cy7 anti-Ly6G (1A8), and redFluor710 anti-I-A/I-E (M5/114.15.2).

Cells were stained for 20 min at 4°C, washed, and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 20 min at 4°C. Cell counts were determined by hemocytometer.
Figure 16. Flow cytometry gating strategy used in Bhlhe40 experiments.
(A) Gating strategy for innate immune cell types in the lung. (B) Gating strategy for adaptive immune cell types in the lung. Previously published in Huynh and Lin et al., 2018.

For identification of antigen-specific T cells, APC-conjugated tetramers of Ag85B<sub>280–294</sub> peptide (FQDAYNAAGHNAVF) or ESAT6<sub>4–17</sub> peptide (QQWNFAGIEAAASA) bound to MHC-II<sup>1-A<sub>b</sub></sup> (National Institutes of Health Tetramer Core) were added to digested cells at final dilutions of 1:25 or 1:100, depending on the age of the tetramer stock, and incubated at RT for 75
min. Cells were then surface stained as above. Antigen-specific cells were defined as CD45⁺/CD3ε⁺/CD4⁺/CD44⁺/tetramer⁺.

Flow cytometry data were acquired on an LSR Fortessa cytometer (BD) and analyzed using FlowJo software (TreeStar). Gating strategies are depicted in Figure 16. Gates for transgenic (Bhlhe40GFP and 10BiT) mice were set using non-transgenic mice to control for background staining.

In vivo cell depletions

For neutrophil depletion, mice were i.p. injected with 200 µg monoclonal anti-Ly6G antibody (clone 1A8; BioXCell or Leinco) or 200 µg polyclonal rat serum IgG (Sigma-Aldrich) diluted in sterile PBS (HyClone) every 48 hrs beginning at 10 dpi and ending at 30 dpi.

For T cell depletion, mice were i.p. injected with 250 µg anti-CD4 (clone GK1.5; Leinco) and 250 µg anti-CD8 (clone YTS-169; Leinco) monoclonal antibody diluted in sterile PBS beginning 2 days before infection then at 4, 11, and 18 dpi.

IL-12 administration

Mice were treated using the protocol published by Feng et al 2005. Mice were i.p. injected with 0.25 µg recombinant IL-12p70 (Leinco) diluted in PBS. Treatment was initiated at 0 dpi and mice were injected three times per week (Monday, Wednesday, Friday) for 4 weeks.
**BCG vaccination**

Mice were sedated with ketamine (Henry Schein) and xylazine (Akorn) then subcutaneously injected with $2 \times 10^5$ CFUs of *M. bovis* BCG. Mice were infected with *Mtb* 30 days after vaccination.

**Quantitative RT-PCR**

Lung samples were lysed by bead-beating in TRIzol reagent (Invitrogen), pelleted to remove beads, and stored at $-80^\circ$C until RNA extraction. RNA was purified from TRIzol using the Direct-zol RNA miniprep kit (Zymo Research) and immediately reverse transcribed with SuperScript III reverse transcription using Oligo-dT primers (Thermo Fisher Scientific). Quantitative RT-PCR was performed using iTAQ SYBR green (Bio-Rad Laboratories) on a C1000 thermal cycler with the CFX96 real-time system (Bio-Rad Laboratories). Transcript levels were analyzed using the $2^{\Delta\Delta C_t}$ method normalized to Actb ($\beta$-actin) as the reference gene. Primer sequences are listed in **Table 1**.
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Table 1. Primers used for qRT-PCR in Bhlhe40 experiments.
Western blots

Bone marrow-derived cells were counted and lysed at $10^6/40 \mu l$ in Laemmli sample buffer (Bio-Rad Laboratories) containing 2.5% β-mercaptoethanol. Cell lysates were loaded and separated by 12% SDS-PAGE (Bio-Rad Laboratories) and transferred to Bio-Blot–polyvinylidene difluoride membranes (Costar). Blots were incubated with anti-Bhlhe40 (1:1,000; NB100-1800, Lot A; Novus Biologicals) or anti-HDAC1 (1:2,000; Abcam) primary antibodies at 4°C overnight with shaking. Blots were washed four to five times before incubation with anti–rabbit IgG-HRP (clone 5A6- 1D10 [light chain specific]; Jackson ImmunoResearch Laboratories, Inc.) at RT for 60 min with shaking. After five washes, Clarity Western ECL substrate (Bio-Rad Laboratories) was applied, and blots were placed on blue basic autoradiography film (Gene-Mate). Film was developed with a medical film processor (model SRX-101A; Konica Minolta).

Cytokine ELISAs

IL-10 ELISA assays were performed on Nunc Maxisorp plates using IL-10 ELISPOT antibody pairs (BD). The enzyme reaction was developed with streptavidin-HRP (BioLegend) and 3,3′,5,5′-tetramethylbenzidine substrate (BioLegend). Recombinant mouse IL-10 (BioLegend) was used to generate the standard curve.

Histology

Lung samples were fixed in 10% buffered formalin (Thermo Fisher Scientific), embedded in paraffin, sectioned, and stained with H&E or Ziehl-Neelsen stain to identify acid-fast bacilli.
**Chromatin immunoprecipitation Sequencing (ChIP-seq)**

Anti-Bhlhe40 ChIP was modified from the protocol published by Chou et al. 2016.\(^\text{186}\) GM-CSF-cultured cells were harvested after 9 days and stimulated with 10 µg/ml heat-killed *Mtb* (strain H37Ra; Difco) for 4 hrs. *In vitro*–polarized T\(_\text{H1}\) cells were stimulated with PMA (50 ng/ml; Enzo Life Sciences) and ionomycin (1 µM; Enzo Life Sciences) for 1.5 hrs. Stimulated cells were fixed for 10 min at RT with 1% paraformaldehyde with shaking. Cross-linked chromatin was fragmented by sonication and then immunoprecipitated with polyclonal rabbit anti-Bhlhe40 antibody (NB100-1800, Lot A or Lot C1; Novus Biologicals). After immunoprecipitation, DNA was purified by the GenElute PCR cleanup kit (Sigma-Aldrich). Purified DNA was used for library construction followed by single-read sequencing on a HiSeq3000 system (Illumina) at the Genome Technology Access Center at Washington University in St. Louis. Read lengths were 101 bp and 50 bp in the case of GM-CSF-cultured bone marrow cells and T\(_\text{H1}\) cells, respectively. Fastq files were provided by the Genome Technology Access Center sequencing facility. Quality control of fastq files was performed using FastQC (0.11.3). Raw reads were mapped on the mm10 mouse reference genome using Bowtie (1.1.1). *Bhlhe40*\(^{+/−}\) samples were used as input samples for peak calling on WT samples. Peak calling was performed using MACS v1.4\(^\text{187}\) with the following flags: macs14-t WT-cKO-f BAM-g mm-n WTvsKO-p 0.00001. BEDtools (2.6; bedClip and bedGraphToBigWig) were used to visualize raw alignments. Normalized tracks were built using Deeptools (2.5.3). The UCSC Genome Browser was used for visualization. R package ChIPseeker (1.14.1) was used for peak annotation. Multiple EM for Motif Elicitation (MEME)-ChIP (4.12.0)\(^\text{188}\) was used for motif enrichment analysis using all acquired peaks. Genomic Regions Enrichment of Annotations Tool
(GREAT; 3.0.0) was used to predict the function of \textit{cis}-regulatory regions for Gene Ontology Biological Process and MSigDB Pathway gene sets.\textsuperscript{189} Links for raw sequencing data are available at GSE113054. All primary processed data (including mapped reads) for ChIP-seq experiments are also available there.

\textit{Analysis of human expression microarrays}

We used the GEO2R web tool (www.ncbi.nlm.nih.gov/geo/geo2r) to query the expression of genes in three publicly available GEO datasets (GSE19491\textsuperscript{92}, GSE28623\textsuperscript{167}, and GSE42834\textsuperscript{168}) that compared the whole-blood transcriptomes of humans with active tuberculosis to other humans with either no disease, latent tuberculosis, or in some cases, lung cancer, pneumonia, or sarcoidosis. The following probesets were used to examine the expression of \textit{BHLHE40} (previously called \textit{BHLHB2}): for GSE19491 and GSE42834, ILMN_1768534; and for GSE28623, Agilent Technologies feature number 37383. The following probesets were used to examine the expression of \textit{STAT1}: for GSE19491 and GSE42834, ILMN_1690105, ILMN_1691364, and ILMN_1777325; and for GSE28623, Agilent Technologies feature numbers 1928, 4610, 4763, 15819, 24587, 29771, 37967, and 42344. For analysis of GSE19491, the training and test sets, both encompassing samples from the United Kingdom, were combined, and the validation set containing samples from South Africa was analyzed separately. GSE28623 contained samples from The Gambia. For analysis of GSE42834, the training, test, and validation sets encompassing samples from the United Kingdom and France were combined.
Data and statistics

Samples represent biological (not technical) replicates of mice randomly sorted into each experimental group. No blinding was performed during animal experiments. Mice were excluded only when pathology unrelated to Mtb infection was present (i.e., weight loss caused by malocclusion or cage flooding). Statistical differences were calculated using Prism (7.0; GraphPad Software) using log-rank Mantel-Cox tests (survival), unpaired two-tailed Student’s t tests (to compare two groups with normal distributions), unpaired two-tailed Mann-Whitney tests (to compare two groups with non-normal distributions), one-way ANOVA with Tukey’s multiple comparisons tests (to compare more than two groups with normal distributions), or unpaired Kruskal-Wallis tests with Dunn’s multiple comparisons tests (to compare more than two groups with non-normal distributions). Normality was determined using a D’Agostino-Pearson omnibus normality test. Sample sizes were sufficient to detect differences as small as 10% using the statistical methods described. When used, center values and error bars represent means ± SEM.
Chapter 3

The role of Irg1 in control of

*Mycobacterium tuberculosis* infection

Portions of this chapter are excerpted from

Abstract

Immune-Responsive Gene 1 (Irg1) is a mitochondrial enzyme that produces itaconate under inflammatory conditions principally in cells of myeloid lineage. Cell culture studies suggest that itaconate regulates inflammation through its inhibitory effects on cytokine and reactive oxygen species production. To evaluate the functions of Irg1 in vivo, we challenged wild-type (WT) and Irg1<sup>−/−</sup> mice with Mycobacterium tuberculosis (Mtb) and monitored disease progression. Irg1<sup>−/−</sup> but not WT mice succumbed rapidly to Mtb, and mortality was associated with increased infection, inflammation, and pathology. Infection of Irg1<sup>fl/fl-Lysm-Cre</sup>, Irg1<sup>fl/fl-Mrp8-Cre</sup>, and Irg1<sup>fl/fl-Cd11c-Cre</sup> conditional knockout mice along with neutrophil depletion experiments revealed a role for Irg1 in LysM<sup>+</sup> myeloid cells in preventing neutrophil-mediated immunopathology and disease. RNA-sequencing analyses suggest that Irg1 and its production of itaconate temper Mtb-induced inflammatory responses in myeloid cells at the transcriptional level. Thus, an Irg1 regulatory axis modulates inflammation to curtail Mtb-induced lung disease.

Introduction

Mycobacterium tuberculosis (Mtb), the etiologic agent of tuberculosis (TB), causes up to 10.4 million new clinical infections and 1.3 million deaths annually. Following inhalation of Mtb, the interplay between the immune response and the bacilli determines whether infection leads to active disease or latency. Although innate immune pathways that are triggered early can limit disease, myeloid cells also serve as a niche for Mtb replication. Indeed, Mtb has adapted to its human host to persist in myeloid cells despite their ability to induce an antibacterial inflammatory immune response.
In the mouse model of tuberculosis, pulmonary infection is established after exposure to aerosolized bacteria. The acute phase of infection is marked by exponential growth of the bacteria in the lungs. In its persistence phase, \textit{Mtb} undergoes a metabolic shift triggered by changes in nutrient availability, as substrates for glycolysis become limited. The bacterial glyoxylate shunt pathway, which is absent in mammals, is an alternative anaplerotic pathway that facilitates the use of fatty acids derived from host lipids and cholesterol as a carbon source for biosynthetic pathways and generation of ATP. \textit{Mtb} strains encode at least one and sometimes two isocitrate lyases, ICL1 (\textit{icl1}) and ICL2 (\textit{icl2} or \textit{aceA}) that function within the glyoxylate shunt. The \textit{in vivo} attenuation exhibited by \textit{Mtb} mutants lacking ICL expression has provided evidence for a role of the glyoxylate shunt in \textit{Mtb} pathogenesis.

The host Immune-Responsive Gene 1 (Irg1, also called Acod1) is a mitochondrial enzyme induced under inflammatory conditions that produces the metabolite itaconate by decarboxylating \textit{cis}-aconitate, a tricarboxylic acid (TCA) cycle intermediate. Since itaconate production in activated myeloid cells reportedly inhibits the activity of bacterial ICLs, it has gained interest as an endogenous antibacterial effector molecule. This role was supported by a study showing that non-physiologically relevant (25 to 50 mM) concentrations of exogenously administered itaconate had bacteriostatic effects on \textit{Mtb} growth during liquid culture under conditions requiring the glyoxylate shunt pathway. Some bacteria such as \textit{Yersinia pestis} and \textit{Pseudomonas aeruginosa} have genes that can metabolize itaconate, thereby avoiding its antimicrobial activity and achieving persistence in the host. Thus, one hypothesis is that Irg1 produced itaconate would act as a direct antimicrobial molecule during \textit{Mtb} infection.

Beyond its possible antibacterial functions, itaconate also links immune cell metabolism and inflammatory responses. \textit{Irg1} mRNA is highly expressed in macrophages in response to pro-
inflammatory stimuli that induce type I and type II interferon (IFN) signaling. Experiments in bone marrow-derived macrophages (BMDM) showed that itaconate suppressed the production of mitochondrial reactive oxygen species (ROS) as well as pro-inflammatory cytokines, including IL-1β, IL-6 and IL-12p70. This capacity to modulate inflammation could be of particular importance during *Mtb* infection because excessive immune responses can prevent bacterial clearance and cause pathology. Thus, Irg1 and its production of itaconate could modulate *Mtb* infection and pathogenesis indirectly through their signaling and regulatory functions.

We addressed the *in vivo* function of Irg1 in regulating immune responses and *Mtb* pathogenesis using mice with global and conditional deletions of *Irg1*. A complete absence of Irg1 during *Mtb* infection resulted in severe pulmonary disease and ultimately death, with greater numbers of infected myeloid cells and higher production of inflammatory cytokines and chemokines. Bone marrow chimera studies suggested that Irg1 expression in hematopoietic cells was necessary to control neutrophil recruitment, *Mtb* infection, and immune-mediated tissue injury. This data was supported by experiments in mice with conditional deletion of Irg1 in myeloid cells. Transcriptional profiling of *Mtb* infected WT and Irg1−/− BMDM and neutrophils *ex vivo* and *in vivo* revealed a principal role for Irg1 and itaconate in regulating the expression of genes associated with inflammation. Collectively, our studies define a key role for Irg1 in regulating immune cell metabolism in subsets of myeloid cells, which minimizes the pathological neutrophil-mediated immune response that contributes to pulmonary disease caused by *Mtb*.
Results

*Irg1 enables resistance to a subset of pathogenic mycobacteria.*

To determine the role of Irg1 during *Mtbc* infection, *Irg1*<sup>−/−</sup> C57BL/6N mice<sup>138</sup> were monitored for clinical and bacterial outcomes following aerosol inoculation with the *Mtbc* Erdman strain. Whereas all WT and *Irg1*<sup>+/−</sup> mice survived past 80 days post-infection (dpi), approximately 75% of *Irg1*<sup>−/−</sup> mice succumbed to *Mtbc* 30-40 dpi, a phenotype similar to animals lacking IFN-γ signaling (Fig. 17A).<sup>24,34,35</sup> The timing of the susceptibility of *Irg1*<sup>−/−</sup> mice to *Mtbc* infection is earlier than *Rag1*<sup>−/−</sup> mice that lack mature B and T cells and succumb between 46 and 50 dpi,<sup>24</sup> suggesting there is a defect in the innate immune response to *Mtbc* that precedes the time point at which adaptive immunity is required. At 21 dpi, the lungs of *Irg1*<sup>−/−</sup> mice infected with *Mtbc* had larger numbers and size of macroscopic lesions compared to infected WT mice (Fig. 17B). To determine whether Irg1 controls *Mtbc* infection, we monitored bacterial burdens in *Mtbc* infected WT and *Irg1*<sup>−/−</sup> mice. Although bacterial levels were similar in the lung and spleen of both mouse strains at 10 dpi, by 14 and 21 dpi *Mtbc* burden was 3- and 25-fold higher, respectively, in the lungs of *Irg1*<sup>−/−</sup> compared to WT mice (Fig. 17C). At 21 dpi, *Mtbc* burden also was 8-fold higher in the spleens of *Irg1*<sup>−/−</sup> compared to WT mice (Fig. 17C).

Having found that Irg1 played an essential role in restricting *Mtbc* infection, we next asked whether it was important for control of two other pathogenic mycobacteria, *Mycobacterium abscessus* (strain L948) and *Mycobacterium avium* (strain TMC724). *Irg1*<sup>−/−</sup> mice infected intranasally with *M. abscessus* showed no signs of morbidity (Fig. 17D) but bacterial burden was nearly 100-fold higher in the lung as compared to WT at 10 dpi (Fig. 17E). After plating *M. abscessus* from the lungs of WT and *Irg1*<sup>−/−</sup> mice at 10 dpi we observed a significantly lower frequency of colonies with a rough morphotype in *Irg1*<sup>−/−</sup> mice (Fig. 17F). The *M. abscessus*
Figure 17. Irg1 enables resistance to a subset of pathogenic mycobacteria. (A-C) Mice were infected with aerosolized Mtb and monitored for (A) survival, (B) gross lung pathology at 21 dpi, and (C) Mtb burden in lung and spleen at 10, 14, and 21 dpi. (D-F) Mice were intranasally infected with M. abscessus and monitored for (D) weight loss and (E) lung CFUs at 10 dpi. (F) The frequency of M. abscessus rough morphotype colonies in WT and Irg1<sup>−/−</sup> lungs at 10 dpi was assessed. (G-H) Mice were intranasally infected with M. avium and monitored for (G) weight loss and (H) lung CFUs at 10 dpi. (I) Mice were intravenously infected with L. monocytogenes (LM) and monitored for survival. (J-K) Mice were intranasally infected with IAV and monitored for (J) survival and weight loss, as well as (K) lung viral burden at 5 dpi. The median ± SEM is graphed. Statistical differences were determined by log-rank Mantel-Cox test (A, I, J) and two-tailed unpaired Student’s t test for normally distributed groups or two-tailed unpaired Mann-Whitney test for non-normally distributed groups (C, E, F, H, J, K). n.s., not significant, *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from or representative of two (A-C, I-K) or one (D-H) independent experiments. Panels (A-C, I-K) were previously published in Nair and Huynh et al., 2018.
rough morphotype is associated with increased virulence and ability to persist in vivo\textsuperscript{193,194} and its decreased frequency suggests that \textit{M. abscessus} may be encountering a less restrictive host environment in the \textit{Irg1\textsuperscript{+}} lung that allows improved persistence of the less virulent smooth morphotype. Intranasal \textit{M. avium} infection did not induce morbidity in \textit{Irg1\textsuperscript{−}} mice (Fig. 17G) and they were able to restrict infection to the same extent as WT mice (Fig. 17H). These results demonstrate that Irg1 is important for control of a subset of pathogenic mycobacteria.

We also tested the effects of Irg1 on two other pathogens, the intracellular bacterium, \textit{Listeria monocytogenes} (strain EGD) and Influenza A virus (IAV strain A/California/04/2009 H1N1), a respiratory pathogen (Fig. 17D-F). Loss of Irg1 expression in mice did not result in altered susceptibility to \textit{L. monocytogenes} (Fig. 17I) or IAV infection (Fig. 17E-F). These results suggest that Irg1 is not broadly protective in bacterial and viral infections but rather has a more specific role in restricting a subset of mycobacterial infections.

**Figure 18. GFP-\textit{Mtb} is as virulent as WT \textit{Mtb}.**
Mice were infected with WT or GFP-\textit{Mtb} and bacterial burden was analyzed in the lung and spleen at 21 dpi. The mean ± SEM is graphed. Statistical differences were determined by one-way ANOVA with Tukey’s post-test. n.s., not significant. Data are from three independent experiments. Previously published in Nair and Huynh et al., 2018.
Figure 19. Irg1 modulates cellular inflammation after Mtb infection.

(A-H) Mice were infected with Mtb. (A) Histopathology was visualized by H&E staining of lungs at 21 dpi. Bars: 2.5 mm (1.25x); 50 µm (40x). (B) Representative flow cytometry plots for neutrophils as a percentage of total lung CD45+ cells in lungs before Mtb infection and at 10, 14, and 21 dpi. Absolute number of innate immune cell populations in lungs at (C) 10 dpi, (D) 14 dpi, and (E) 21 dpi. (F) Absolute number of adaptive immune cell populations in lungs at 21 dpi. (G) Acid-fast bacilli in Mtb-infected lungs at 21 dpi. Bars, 10 µm. (H) Representative flow cytometry plots for GFP+ (Mtb+) neutrophils in lungs at 10, 14, and 21 dpi. SSC, side scatter. (I) Frequency of GFP+ (Mtb+) cells in indicated cell types at 10, 14, and 21 dpi. The median ± SEM is graphed. Statistical differences were determined by Mann–Whitney test. Ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001). Mφ, macrophages. Data are from or representative of two (A, C, D, G, I) or three (B, E, F, H) independent experiments. Previously published in Nair and Huynh et al., 2018.
**Irg1 modulates lung inflammatory responses after Mtb infection.**

Since Irg1 has been reported to regulate inflammatory responses\(^{138}\) we next evaluated cellular inflammation in the lungs of WT and *Irg1*\(^{−/−}\) mice infected with a strain of *Mtb* Erdman that stably expresses GFP and has the same virulence properties as WT *Mtb* (Fig. 18). Histological analysis at 21 dpi showed that lung lesions in *Irg1*\(^{−/−}\) mice were larger and contained denser cellular infiltrates than those in WT mice (Fig. 19A). To define the composition of these infiltrates, we performed a kinetic analysis of immune cells in infected lungs by flow cytometry (gating strategy defined in Fig. 29). At 10 dpi, no differences in the number of neutrophils, inflammatory monocytes, infiltrating (CD11b\(^{+}\)) macrophages, alveolar macrophages, CD11b\(^{+}\) and CD103\(^{+}\) DCs, NK and NKT cells, or eosinophils were detected in the lungs of WT and *Irg1*\(^{−/−}\) mice (Fig. 19B, C). However, by 14 dpi infected lungs from *Irg1*\(^{−/−}\) mice had 4-fold more neutrophils and 3-fold more eosinophils compared to WT mice, and this difference remained at 21 dpi for neutrophils (Fig. 19B, D, E). At 21 dpi, lungs from *Irg1*\(^{−/−}\) mice also had 1.3-fold fewer alveolar macrophages and 3-fold fewer NK1.1\(^{+}\) cells. Analysis of other innate immune cell populations in the lungs at 14 and 21 dpi revealed no significant differences in numbers of inflammatory monocytes, infiltrating macrophages, and DCs between WT and *Irg1*\(^{−/−}\) mice. In addition, no differences in the numbers of CD4\(^{+}\) T cells, CD8\(^{+}\) T cells, and B cells were found between WT and *Irg1*\(^{−/−}\) mice at 21 dpi (Fig. 19F).

As the timing of the increased myeloid cell inflammation correlated with the higher *Mtb* burden in the lungs of Irg1\(^{−/−}\) mice, we evaluated whether the accumulating myeloid cells were infected with *Mtb*. Acid-fast staining of tissue sections at 21 dpi revealed that bacilli were predominantly found in lung lesions, with greater numbers of bacilli present in *Irg1*\(^{−/−}\) mice (Fig. 19G). To analyze the extent of infection in each myeloid cell type, we monitored the presence of
*Mtb*-GFP in the various cell populations by flow cytometry. At 10 and 14 dpi myeloid cell types in WT and *Irg1*−/− lungs were infected at similar frequencies ([Fig. 19H, I]). However, by 21 dpi lungs from *Irg1*−/− mice had higher percentages of infected neutrophils (8-fold), inflammatory monocytes (19-fold), infiltrating macrophages (19-fold), and CD11b+ DCs (3-fold) compared to WT mice. Collectively, this data suggests that Irg1 regulates inflammatory responses during *Mtb* infection and controls the recruitment of multiple types of myeloid cells, many of which become targets for subsequent rounds of infection.

**Figure 20.** Irg1 modulates inflammatory cytokine production after *Mtb* infection. (A-C) Cytokine and chemokine levels in *Mtb* infected lungs at (A) 10 dpi, (B) 14 dpi, and (C) 21 dpi. (D) Lung TNF-α levels. The median ± SEM is graphed. Statistical differences were determined by Mann-Whitney test. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001). Mφ, macrophages. Data are from or representative of two (A, B, D) or three (C) independent experiments. Previously published in Nair and Huynh et al., 2018.
Given the increased inflammatory cell recruitment in Irg1\(^{-/-}\) mice, we measured the effect of Irg1 on the accumulation of inflammatory mediators in the lung during Mtb infection. Equivalent levels of chemokines and pro-inflammatory cytokines were detected in lung homogenates of WT and Irg1\(^{-/-}\) mice at 10 dpi (Fig. 20A, D), a time point at which we observed no difference in the numbers of lung immune cells. By 14 dpi, higher levels of several pro-inflammatory cytokines (IL-1\(\beta\), IL-6, IL-17, G-CSF) and chemokines (CXCL1, CCL3, CCL4) were detected in the lungs of Irg1\(^{-/-}\) mice compared to WT mice (Fig. 20B), although TNF-\(\alpha\) levels remained unchanged (Fig. 20D). By 21 dpi, all eleven pro-inflammatory cytokines (IL-1\(\beta\), IL-6, IL-12p70, IL-17, IFN-\(\gamma\), TNF-\(\alpha\), G-CSF) and chemokines (CCL2, CXCL1, CCL3, CCL4) measured were higher in the lungs of Irg1\(^{-/-}\) mice compared to WT mice (Fig. 20C, D). Thus, enhanced accumulation of immune cells in the lungs of infected Irg1\(^{-/-}\) mice at 14 and 21 dpi correlated with increased expression of pro-inflammatory cytokines and chemokines.

**Neutrophil depletion extends the survival of Irg1\(^{-/-}\) mice during Mtb infection.**

Excessive neutrophil recruitment is associated with tissue damage and progression of Mtb disease.\(^{24,32}\) We speculated that the susceptibility of Irg1\(^{-/-}\) mice to Mtb infection was related to the increased accumulation of neutrophils in their lungs. To test this hypothesis, we administered a neutrophil depleting anti-Ly6G (\(\alpha\)Ly6G) or isotype control monoclonal antibody to WT and Irg1\(^{-/-}\) mice every other day, beginning at 10 dpi and ending at 34 dpi. Flow cytometry analysis demonstrated that anti-Ly6G treatment of Irg1\(^{-/-}\) mice led to a 7-fold reduction in the number of lung neutrophils compared to isotype control-treated mice at 21 dpi (Fig. 21A, F). Neutrophil depleted Irg1\(^{-/-}\) mice exhibited less weight loss (Fig. 21B) and extended survival time (Fig. 21C).
Figure 21. Neutrophil depletion enhances the survival of Irg1−/− mice.

(A-G) Mice were infected with Mtb and treated with anti-Ly6G (αLy6G) or isotype control antibodies beginning at 10 dpi. (A) Representative flow cytometry for lung neutrophils at 21 dpi. (B) Weight loss kinetics. (C) Survival analysis. (D) Lung and spleen Mtb burden at 21 dpi. (E) Lung histopathology and acid-fast bacilli at 21 dpi were visualized with H&E (top) and acid-fast (bottom) stains. Bars: 1 mm (top); 100 µm (bottom). (F) Innate immune cell populations in the lung at 21 dpi. (G) Lung cytokine levels at 21 dpi. Statistical differences were determined by one-way ANOVA with Tukey’s post-test (D, F) and Mann-Whitney test (G). ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from or representative of two (A-G) independent experiments. Panels (A, C-G) were previously published in Nair and Huynh et al., 2018.
after *Mtb* infection. Once neutrophil depletion was halted and neutrophils were allowed to accumulate in the lung, disease ensued and all *Irg1*−/− mice succumbed to infection (mean time to death for isotype control and anti-Ly6G-treated *Irg1*−/− mice was 30 and 62 days, respectively). This demonstrated that the excessive accumulation of neutrophils contributed to the early susceptibility of *Irg1*−/− mice and that Irg1 is required to restrict neutrophilic inflammation during acute and chronic *Mtb* infection. Neutrophil depletion also reduced lung *Mtb* burden in *Irg1*−/− mice by 19-fold at 21 dpi, but had no effect on splenic *Mtb* burden (Fig. 21D). However, lung *Mtb* levels in neutrophil-depleted *Irg1*−/− mice remained higher (9-fold) than in anti-Ly6G-treated WT mice (Fig. 21D), indicating incomplete neutrophil depletion or that recruitment and infection of neutrophils only partially contributed to the higher bacterial burden in *Mtb* infected *Irg1*−/− mice. Correspondingly, diminished numbers of acid-fast bacilli were present in lung sections of neutrophil depleted *Irg1*−/− mice compared to controls (Fig. 21E). Histological analysis showed that lung lesions from infected, neutrophil depleted *Irg1*−/− mice were smaller than their isotype treated counterparts (Fig. 21E). Other than a 1.5-fold decrease in inflammatory monocytes, the recruitment of which could be altered by decreases in lung cytokine and chemokine expression caused by neutrophil depletion, all other innate immune cell subsets (infiltrating and alveolar macrophages, NK1.1+ cells, DCs, eosinophils) were unaffected by neutrophil depletion in *Irg1*−/− mice (Fig. 21F). Cytokine (IL-1β, IL-6, IL-12p70, G-CSF, IFN-γ) and chemokine (CCL2, CCL3, CCL4, CXCL1) levels in the lung also were decreased in neutrophil depleted *Irg1*−/− mice at 21 dpi, demonstrating that neutrophils contributed to the heightened inflammatory response at this time point (Fig. 21G). Collectively, these experiments show that depletion of neutrophils mitigates the bacterial and pro-inflammatory pathological phenotypes observed at 21 dpi in
Figure 22. Expression of Irg1 in myeloid cell subsets enables survival of Mtb infection.

(A, B, D-J) Mice were infected with Mtb. (A) Survival analysis of bone marrow chimeric mice. (B) Lung Mtb burden in one-way and mixed bone marrow chimeric mice at 21 dpi. (C) Cells
were isolated from naive \(Irg1^{+/+}\) and \(Irg1^{+/+}\)-Cre-expressing mice. mRNA was then extracted and reverse transcribed, and a section of the \(Irg1\) gene spanning exon 4 was amplified by PCR. Non-recombined transcript yields a 490 bp amplicon, whereas Cre recombinase-mediated excision of exon 4 results in a 290 bp amplicon. Recombined \(Irg1\) exon 4 in cells from Cre-negative \(Irg1^{+/+}\), \(Irg1^{+/+}\)-Mrp8-Cre (bone marrow neutrophils), \(Irg1^{+/+}\)-Cd11-Cre (alveolar macrophages from BAL and total splenic CD11c\(^+\) cells), and \(Irg1^{+/+}\)-Lysm-Cre mice (bone marrow neutrophils, alveolar macrophages from BAL, splenic monocytes, and total splenic CD11c\(^+\) cells). (D) Survival analysis of conditional deletion mice. (E) Lung and spleen \(Mtb\) burden at 21 dpi. (F) Neutrophils as a percentage of the total lung CD45\(^+\) population. (G-I) Quantitation of lung myeloid cells at 21 dpi in (G) \(Irg1^{+/+}\)-Mrp8-Cre, (H) \(Irg1^{+/+}\)-Cd11c-Cre, and (I) \(Irg1^{+/+}\)-Lysm-Cre. Mφ, macrophages. (J) Lung cytokine levels at 21 dpi. The median ± SEM is graphed. Statistical differences were determined by log-rank Mantel-Cox test (A, D), one-way ANOVA with Tukey’s post-test (B, E, F, J), or Mann-Whitney test (G-I). n.s., not significant, *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\); ****, \(P < 0.0001\). Data are from or representative of two (A-C, I-K) or one (D-H) independent experiments. Panels (A, C-E, G-J) and parts of panel (B) were previously published in Nair and Huynh et al., 2018.

\(Irg1^{+/+}\) mice and suggest that a dysfunctional neutrophil response contributes to the susceptibility of these mice to \(Mtb\) infection.

**Expression of Irg1 in LysM\(^+\) and CD11c\(^+\) cells enables survival of Mtb infection.**

In order to define the cell types that require Irg1 expression for control of \(Mtb\) infection, we generated reciprocal bone marrow chimeric mice, infected them with \(Mtb\), and monitored survival (Fig. 22A). Whereas WT→WT and WT→\(Irg1^{+/+}\) mice survived past 80 dpi, \(Irg1^{+/+}\)→WT mice succumbed by 32 dpi, similar to the phenotype observed with \(Irg1^{+/+}\) mice. This result demonstrated that the protective effects of Irg1 were derived from radio-sensitive cells, likely in the hematopoietic compartment. Consistent with the survival data, \(Mtb\) titers in the lungs and the spleen of \(Irg1^{+/+}\)→WT mice were higher than WT→WT mice at 21 dpi (lung, 10-fold; spleen, 3-fold) (Fig. 22B). Intriguingly, lung and spleen CFU counts in mixed WT + \(Irg1^{+/+}\)→WT chimeras were similar to those of WT→WT chimeras and significantly lower than in \(Irg1^{+/+}\)→WT
chimeras. This result suggested that the presence of Irg1-expressing cells could compensate for the defective ability of Irg1<sup>-/-</sup> cells to control Mtb infection. However, the proportions of WT and Irg1<sup>-/-</sup> cells in the lungs of mixed bone marrow chimera mice prior to infection were not assessed and phenotypes observed in these mice could be due to differences in the engraftment ability of WT and Irg1<sup>-/-</sup> cells.

Since bone marrow chimera experiments revealed a role for Irg1 in hematopoietic cells, we sought to identify the Irg1-expressing cell types that modulated neutrophil accumulation and Mtb disease pathogenesis. Because Irg1 is expressed principally in cells of myeloid lineage,<sup>134,138,143,152</sup> we used conditional deletion mice to test its role in myeloid cells during Mtb infection. Mice with a loxP site-flanked exon of Irg1 (Irg1<sup>fl/fl</sup>) were crossed to mice that expressed Cre recombinase under the control of various myeloid cell-specific promoters and targeted cells were isolated and assessed for Irg1 exon deletion (Fig. 22C). As expected, we did not detect Irg1 exon deletion in Cre-negative Irg1<sup>fl/fl</sup> mice. The expected patterns of deletion were detected in Irg1<sup>fl/fl</sup>-Mrp8-Cre (deletion in neutrophils) and Irg1<sup>fl/fl</sup>-Lysm-Cre mice (deletion in monocytes, alveolar macrophages, neutrophils, and some DCs). However, we found that Irg1<sup>fl/fl</sup>-Cd11c-Cre mice exhibited strong deletion in alveolar macrophages and poor deletion in splenic DCs, suggesting that lung DCs might have a similarly low level of deletion. Conditional deletion mice were infected with Mtb and monitored for survival (Fig. 22D). All Cre-negative Irg1<sup>fl/fl</sup> and Irg1<sup>fl/fl</sup>-Mrp8-Cre mice survived past 150 dpi, but Irg1<sup>fl/fl</sup>-Lysm-Cre mice succumbed within 40 dpi, a phenotype similar to that of Irg1<sup>-/-</sup> mice (Fig. 17A). In comparison, all Irg1<sup>fl/fl</sup>-Cd11c-Cre mice succumbed by 135 dpi. Consistent with these survival results, Mtb levels in the lungs of Irg1<sup>fl/fl</sup>-Mrp8-Cre mice were similar to littermate Cre-negative Irg1<sup>fl/fl</sup> controls, whereas Irg1<sup>fl/fl</sup>-Lysm-Cre and Irg1<sup>fl/fl</sup>-Cd11c-Cre mice both had greater higher lung Mtb burden at 21 dpi.
(14-fold and 4-fold respectively) (Fig. 22E). Splenic Mtb burdens were also higher in \( IrgI^{fl/fl} - Lysm-Cre \) and \( IrgI^{fl/fl} - Cd11c-Cre \) mice at 21 dpi compared to controls (Fig. 22E).

We next assessed cellular inflammation in the lung at 21 dpi and found that \( IrgI^{fl/fl} - Lysm-Cre \) had a higher frequency (Fig. 22F) and absolute number (Fig. 22I) of neutrophils in the lung as compared to Cre-negative \( IrgI^{fl/fl} \) controls, although their neutrophil frequency remained lower than that of \( IrgI^{+/+} \) mice. \( IrgI^{fl/fl} - Cd11c-Cre \) mice had greater numbers of lung neutrophils compared to Cre-negative \( IrgI^{fl/fl} \) mice, but \( IrgI^{fl/fl} - Mrp8-Cre \) mice did not (Fig. 22G, H). Loss of Irg1 expression in myeloid cells (\( IrgI^{fl/fl} - Lysm-Cre \) mice) resulted in higher levels of cytokines and chemokines in the lungs at 21 dpi (Fig. 22J). Loss of Irg1 expression in alveolar macrophages and some DCs (\( IrgI^{fl/fl} - Cd11c-Cre \) mice) also resulted in greater amounts of inflammatory cytokines and chemokines. In contrast, deletion of Irg1 in neutrophils (\( IrgI^{fl/fl} - Mrp8-Cre \) mice) had little effect on cytokine responses. These data show that Irg1 expression in CD11c\(^+\) and LysM\(^+\) cell subsets regulates neutrophil recruitment and inflammation. Although neutrophils mediate Mtb-induced immunopathology in \( IrgI^{+/+} \) mice, their cell-intrinsic expression of Irg1 is dispensable for this process.

**Susceptibility of \( IrgI^{+/+} \) mice is dependent on Mtb expression of PDIM and ESX-1, but not ICL1**

We next investigated the requirement for key Mtb virulence factors in the susceptibility of \( IrgI^{+/+} \) mice. The ESX-1 secretion system and the Mtb membrane lipid phthiocerol dimycoserosate (PDIM) enable Mtb to modulate host immune responses\(^{195-197}\) and thus may have important effects on the inflammatory response observed in \( IrgI^{+/+} \) mice. \( IrgI^{+/+} \) mice infected with isogenic Mtb strains lacking ESX-1 (\( \Delta ESX-1 \)) or PDIM (\( \Delta PDIM \)) exhibited no
Figure 23. Susceptibility of Irg1<sup>−/−</sup> mice is dependent on Mtb expression of PDIM and ESX-1, but not ICL1.

(A-B) Mice were infected with WT, ΔPDIM or ΔESX-1 Mtb and monitored for (A) survival or (B) lung Mtb burden at 81 dpi. Dotted lines in (B) indicate limit of detection (WT, 50 CFU; Irg1<sup>−/−</sup>, 500 CFU). (C-G) Mice were infected with WT or Δicl1 Mtb and monitored for (C) weight loss kinetics, (D) survival, (E) lung and spleen burden at 21 dpi, (F) myeloid lung cell populations at 21 dpi, and (G) lung cytokines at 21 dpi. The median ± SEM is graphed. Statistical differences were determined by log-rank Mantel-Cox test (A, D), one-way unpaired Student’s t test (B), or Mann-Whitney test (C, E-G). n.s., not significant, *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from or representative of one (A, B) or two (C-G) independent experiments.

Susceptibility over 80 days of infection (Fig. 23A). At 81 dpi lung CFU counts for ΔPDIM Mtb were below the limit of detection in Irg1<sup>−/−</sup> mice while ΔESX-1 Mtb counts were 25-fold higher in Irg1<sup>−/−</sup> lungs compared to WT mice (Fig. 23B). These experiments show that expression of
ESX-1 and PDIM by *Mtb* is required for the acute susceptibility of *Irg1*−/− mice, but ESX-1 is not required for defective control of *Mtb* by *Irg1*−/− mice.

Since Irg1 catalyzes the production of itaconate, a metabolite that can inhibit the glyoxylate shunt enzymes ICL1 and ICL2, it has been suggested that Irg1 restricts *Mtb* infection by blocking the glyoxylate shunt. The strain of *Mtb* used in this study, Erdman, encodes two ICL genes, ICL1 and ICL2, although ICL1 is considered functionally dominant. In *vivo*, Δicl1 *Mtb* replicates normally during the acute phase but is attenuated during the chronic phase of infection, whereas Δicl2 *Mtb* shows no defects during *in vivo* infection. In comparison, Δicl1/Δicl2 strains grow poorly in liquid culture and are completely avirulent *in vivo*. To test the impact of Irg1 on restriction of ICL1 activity *in vivo*, we infected WT and *Irg1*−/− mice with WT and isogenic Δicl1 *Mtb* strains. As anticipated, WT mice infected with WT or Δicl1 *Mtb* showed no signs of morbidity (Fig. 23C) and survived past 80 dpi (Fig. 23D). In contrast, all *Irg1*−/− mice infected with Δicl1 *Mtb* succumbed to infection with weight loss (Fig. 23C) and survival (Fig. 23D) patterns highly similar to that of *Irg1*−/− mice infected with an isogenic WT *Mtb* strain. Moreover, Δicl1 *Mtb* titers were higher in the lung (83-fold) and spleen (12-fold) of *Irg1*−/− mice compared to WT mice (Fig. 23E). Our findings that Δicl1 *Mtb* is still virulent in *Irg1*−/− mice suggest that the susceptibility of *Irg1*−/− mice is independent of ICL1 activity and that Irg1 likely restricts *in vivo* *Mtb* infection independently of its activity on ICL1.

Since *Irg1*−/− mice infected with WT or Δicl1 *Mtb* succumbed to infection at equivalent rates (Fig. 23D), we asked whether the pattern of inflammatory responses in these mice also was similar. Indeed, *Irg1*−/− mice infected with WT or Δicl1 *Mtb* showed similarly increased accumulation of neutrophils in their lungs at 21 dpi (Fig. 23F). Correspondingly, pro-inflammatory cytokines (IL-1β, IL-6, IL-12p70, IL-17, G-CSF, IFN-γ) and chemokines (CCL2,
CCL3, CCL4, CXCL1) were higher in $\Delta icl1 \ Mtb$-infected $Irg1^{-/-}$ mice compared to WT mice (Fig. 23G). The similar inflammatory responses to WT and $\Delta icl1 \ Mtb$ in $Irg1^{-/-}$ mice further supports the hypothesis that the defective control of $Mtb$ infection in these mice is independent of ICL1 activity.

**Figure 24.** $Irg1$ is not required for induction of ROS, NOS2, or control of $Mtb$ in BMDMs. (A-B) Mice were infected with $Mtb$ and at 21 dpi lung neutrophils were assessed for (A, B) reactive oxygen species by MitoSOX Red dye or intracellular NOS2 expression. (C) BMDMs were IFN-$\gamma$ pretreated or not, infected with $Mtb$ (multiplicity of infection = 1), and CFUs were assessed at 0, 24, and 96 hpi. The median ± SEM is graphed. Statistical differences were determined by Mann-Whitney test (B) or one-way ANOVA with Tukey’s post-test (C). n.s., not significant, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data are from or representative of three (A, B) or two (C) independent experiments.

**Irg1 is not required for induction of ROS and NOS2 or control of Mtb in macrophages**

Reactive oxygen species (ROS) have been proposed as a cell autonomous antibacterial mechanism during $Mtb$ infection and Irg1 has been reported to promote production of antibacterial reactive oxygen species during Salmonella enterica infection. However, overproduction of ROS has been linked to inflammatory pathology and exogenous itaconate suppresses ROS production by macrophages in vitro. We asked whether neutrophils might be contributing to susceptibility through altered ROS production and addressed this question by
quantifying ROS-producing lung neutrophils in WT and Irg1−/− mice at 21 dpi using a dye that reports ROS production, MitoSOX Red. We observed an increase in intracellular ROS (Fig. 24A) and a higher number of ROS+ (MitoSOX+) neutrophils in Irg1−/− mice, but no difference in the frequency of ROS+ neutrophils (Fig. 24B). The increased number of ROS+ neutrophils was expected, given the higher number of neutrophils present in the lung at this time point (Fig. 19E). No differences in ROS expression were detected in Ly6G-negative immune cells (data not shown). These data suggest Irg1−/− neutrophils and macrophages are not failing to produce potentially bactericidal ROS and neutrophils may be contributing to lung pathology through overproduction of ROS.

Inducible nitric oxide synthase (NOS2) restricts pathologic, granulocyte-dominated inflammatory responses33,96 that appear grossly similar to the neutrophil-dominated inflammation we observe in Irg1−/− mice. However, overexpression of NOS2 by macrophages has been associated with a hyperinflammatory state and tissue pathology.138 We asked whether the susceptibility of Irg1−/− mice might be associated with alterations in NOS2 expression and addressed this by measuring expression of NOS2 in lung neutrophils at 21 dpi. The frequency and absolute number of NOS2+ neutrophils were significantly higher in Irg1−/− mice (Fig. 24A, B). No differences in NOS2 expression were detected in Ly6G-negative immune cells (data not shown). These data suggest that Irg1−/− neutrophils may be in a pathologic, hyper-inflamed state.

We next investigated whether Irg1 deficiency impacted control of Mtb by macrophages, a cell type identified as a key expresser of Irg1 by the susceptibility of Irg1fl/fl-Lysm-Cre mice (Fig. 22D-J). Irg1 had no effect on IFN-γ-independent or -dependent control of Mtb in vitro, as no differences in Mtb titer were detected in WT and Irg1−/− bone marrow-derived macrophages (BMDMs) (Fig. 24C). These data suggest that Irg1 is dispensable for control of Mtb in
Figure 25. Irg1 alters transcription of inflammatory and metabolic genes in neutrophils during *Mtb* infection.

Neutrophils were infected *ex vivo* and analyzed at 0 (naïve), 2, and 6 hpi. Experiments were performed in triplicate for each cohort. (A) PCA plot: PCA component 1 indicates difference between naive and infected samples. PCA component 2 shows a time-dependent change in
infected samples. (B) Heat map shows the relative expression intensity of the indicated inflammatory genes in WT and Irg1−/− neutrophils. (C) Diagram shows the numbers of differentially expressed genes between WT and Irg1−/− neutrophils over the time course of ex vivo infection. (D) Venn diagrams show comparison of differentially expressed genes between WT and Irg1−/− neutrophils at 2 hpi versus those at 6 hpi. Tables show the top enriched pathways as indicated for each diagram. (E-G) Transcriptome data from WT and Irg1−/− neutrophils purified from lungs at 16 dpi or from the bone marrow of naïve mice (as baseline comparison) (n = 3 per cohort). (E) PCA plot. PCA component 1 indicates the difference between samples derived from infected lungs and naïve bone marrow. The second component indicates difference between WT and Irg1−/− neutrophils after infection. (F) Data sets from WT and Irg1−/− neutrophils in vivo were compared to data sets from WT and Irg1−/− neutrophils ex vivo (2 and 6 hpi combined). Genes that are upregulated in ex vivo infected Irg1−/− neutrophils also are enriched in Irg1−/− neutrophils isolated from lung at 16 dpi. Left plot shows gene expression table of WT and Irg1−/− neutrophils from infected mice. Columns and rows show conditions and genes, respectively. Genes are ranked according to significance of differential expression and direction of change. Orange bars (right side) represent genes that become upregulated in the combined transcriptome of Irg1−/− neutrophils at 2 + 6 hpi. Right plot shows the running score for the gene set as the analysis moves down the ranked list. The score at the peak of the plot (the score furthest from 0.0) is the score for the gene set. (G) Gene set enrichment analysis comparison between WT and Irg1−/− neutrophils from infected lungs described above. Each dot on volcano plot (left) represents a pathway and is characterized by two values: NES (normalized enrichment score) shows direction and strength of change; minus logarithm of adjusted P value represents the significance of the pathway. Significantly enriched pathways are highlighted in red and listed on the table along with adjusted P values. (H) Heat map of selected inflammatory genes in neutrophils isolated from WT and Irg1−/− lungs at 16 dpi.

macrophages. Collectively, these findings indicate that Irg1−/− cells are in a hyper-inflamed state, rather than being defective in cell autonomous control of Mtb.

Irg1 regulates inflammatory responses in myeloid cells during Mtb infection.

To gain insight into why a lack of Irg1 expression in myeloid cells resulted in excessive accumulation of neutrophils and hyper-inflammation in Irg1−/− mice during Mtb infection we examined the expression profiles of Mtb-infected WT and Irg1−/− neutrophils and BMDMs.

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Ex vivo analysis of naïve neutrophils isolated from WT and Irg1−/− bone marrow showed a highly overlapping gene expression profile (Fig. 25A), indicating that the basal gene expression pattern of both genotypes was similar. Within 2 hrs of Mtb infection ex vivo, Irg1−/− neutrophils had higher expression of Nos2, Il6, Il1b, and Nlrp3 but not Tnfa and Cxcl2 transcripts (Fig. 25B), indicating that Irg1 expression in neutrophils controls expression of a subset of pro-inflammatory genes. Indeed, Irg1−/− neutrophils exposed to Mtb induced the differential expression of twice as many transcripts as WT cells (Fig. 25C). Pathway enrichment analysis of samples at 2 hours post-infection (hpi) revealed that infected Irg1−/− neutrophils had prominent inflammatory response transcriptional signatures compared to WT cells (Fig. 25D). At 6 hpi, Irg1−/− neutrophils had down-regulated transcripts of several genes involved in the electron transport chain, TCA cycle, and oxidative phosphorylation (Fig. 25D), which is consistent with the reported metabolic functions of Irg1.138,155 We then analyzed neutrophils isolated directly from lungs of mice at 16 dpi (Fig. 25E-G). WT and Irg1−/− neutrophils were distinguished by global gene expression patterns (Fig. 25E), confirming a marked effect of Irg1 on neutrophil responses during Mtb infection in vivo. Genes that were upregulated in Irg1−/− neutrophils ex vivo after Mtb infection were also upregulated in Irg1−/− neutrophils isolated from the lung at 16 dpi (Fig. 25F).

Neutrophils in Irg1−/− mice at 16 dpi upregulated transcriptional signatures associated with inflammation, including NF-κB and pro-inflammatory signaling pathways downstream of cytokine receptors and interferons (Fig. 25G, H). This pronounced pro-inflammatory phenotype of Irg1−/− neutrophils isolated in vivo was accompanied by altered expression of genes involved in oxidative phosphorylation, which links the anti-inflammatory action of Irg1 with its control of cellular metabolic activity.
Figure 26. Irg1 alters the transcriptional signature of Mtb infected BMDMs.

(A-C) Transcriptomic data from WT, Irg1<sup>−/−</sup>, and Irg1<sup>−/−</sup> + itaconate (ITA)-treated BMDMs infected with Mtb and analyzed at 4 hrs post-infection (hpi). (A, left) Heat map comparing the transcriptional changes that occur at 4 hpi in Irg1<sup>−/−</sup> BMDMs ± ITA and WT BMDMs. Genes that are up-regulated in WT BMDMs also are enriched in Irg1<sup>−/−</sup> + ITA BMDMs at 4 hpi. Columns and rows show conditions and genes, respectively. Genes are ranked according to significance of differential expression and direction of change. (A, right) Plot shows the running score for NF-κB gene set as the analysis moves down the ranked list. (B) Gene set enrichment analysis comparison for genes in NF-κB signaling and (C) inflammatory chemokines Cxcl11 and Il1b between WT versus Irg1<sup>−/−</sup>, WT versus Irg1<sup>−/−</sup> + ITA, and Irg1<sup>−/−</sup> versus Irg1<sup>−/−</sup> + ITA BMDMs. The median ± SEM is graphed. Data is from one experiment.

We next compared the expression profiles of Mtb infected WT and Irg1<sup>−/−</sup> BMDMs.

Within just 4 hours of Mtb infection, the transcriptional signature in Irg1<sup>−/−</sup> BMDMs differed markedly from WT BMDMs. Many of the genes that were upregulated in Irg1<sup>−/−</sup> cells after Mtb
infection were reciprocally downregulated in WT BMDMs (**Fig. 26A, B**). Exogenous addition of itaconate (ITA) to *Mtb* infected *Irg1*−/− BMDMs switched their transcriptional signature to one similar to *Mtb* infected WT BMDMs (**Fig. 26A, B**), suggesting that the effect of *Irg1* deletion on transcriptional profiles was due principally to the loss of itaconate production. Pathway enrichment analysis indicated that a lack of *Irg1* expression in *Mtb* infected BMDMs resulted in induction of inflammatory and chemoattractant genes downstream of NF-κB signaling, and correspondingly, addition of itaconate to *Irg1*−/− BMDMs reverses this effect (**Fig. 26B, C**). Nonetheless, and consistent with our *in vivo* experiments (**Fig. 20D**), *Tnfa* expression levels were similar in *Mtb* infected WT and *Irg1*−/− BMDMs in the presence or absence of itaconate treatment. Collectively, this data shows that *Irg1* and its product itaconate function to limit inflammation during *Mtb* infection at the transcriptional level.

**Figure 27. Irg1 is the sole source of lung itaconate during *Mtb* infection and i.p. administration of dimethyl itaconate fails to rescue *Irg1*−/− susceptibility.**

**(A-C)** Mice were infected with *Mtb*. (A) Relative abundance of itaconate in total lung samples from 21 dpi. (B-C) Mice were administered dimethyl itaconate (DI) i.p. daily from 10-20 dpi and monitored for (B) weight loss kinetics and (C) *Mtb* burden in lung and spleen. The mean ± SEM is graphed. Statistical differences were determined by two-tailed unpaired Student’s t test (A) or unpaired Kruskal-Wallis test (C). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from two independent experiments.
**Irg1 is the sole source of itaconate in the lung during Mtb infection**

The ability of exogenous itaconate to restore the transcriptional profile of Irg1−/− BMDMs to a profile resembling that of WT BMDMs raised the questions of whether there were Irg1-independent sources of itaconate during *Mtb* infection and if administration of an exogenous itaconate analog could rescue the susceptibility of Irg1−/− mice to *Mtb*. We performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) on methanol-soluble extracts of total lung samples taken from *Mtb* infected mice to quantify relative levels of itaconate. Itaconate was not detected in WT mice at 7 dpi (data not shown) but was detected at 21 dpi. In contrast to WT mice, no itaconate was detected in any Irg1−/− lung samples, demonstrating that Irg1 is the sole source of itaconate in the lung during *Mtb* infection (Fig. 27A).

We next attempted to complement the susceptibility associated phenotypes in Irg1−/− mice through exogenous delivery of dimethyl itaconate (DI), a more cell permeable analog of itaconate. Daily intraperitoneal administration of DI had no effect on weight loss kinetics (Fig. 27B) or *Mtb* burden in the lung or spleen (Fig. 27C). Additionally, DI administration was poorly tolerated by mice and induced hunching and temporary hind limb paralysis. Presence of DI in the lung was not evaluated and thus it is unclear whether it failed to rescue Irg1−/− susceptibility because DI did not reach the lung, was not consistently present at an effective concentration, did not have the same effect as itaconate in this context, or because Irg1 is required for itaconate-independent functions. Experiments that utilize alternative routes of delivery (for example, intranasal) and monitor bioavailability of itaconate in the lung will be necessary to draw conclusions about the ability of exogenously administered itaconate to rescue Irg1−/− mouse susceptibility.
Discussion

Active *Mtb* infection in humans induces a potent inflammatory response characterized by neutrophilia in the blood. Studies in mice have shown that regulation of neutrophil recruitment to the lung during early stages of *Mtb* infection is a determinant of *Mtb* pathogenesis, as these cells can exert protective or deleterious effects. Our results demonstrate that Irg1 has an important role in hematopoietic cells in regulating inflammation to prevent neutrophil-mediated immunopathology during *Mtb* infection in mice. Whereas infection of WT mice with *Mtb* induced an inflammatory response that resulted in long-term survival, infection of mice lacking Irg1 led to heightened inflammation characterized by increased production of pro-inflammatory cytokines and chemokines and recruitment of granulocytes (neutrophils and eosinophils) by 14 dpi. By 21 dpi uncontrolled accumulation of neutrophils provided an expanded niche for *Mtb* replication that extended to infection of other myeloid cells subsets recruited to the lung. Loss of Irg1 expression in CD11c+ and LysM+ cells led to increased neutrophil accumulation in the lung by 21 dpi. Together, these bacterial and immune consequences contributed to the severe lung pathology and subsequent death of *Irg1*−/− mice within 40 days. As depletion of neutrophils in *Irg1*−/− mice reduced bacterial burden, prolonged survival, and dampened lung inflammation, we suggest that neutrophils are a principal cell type contributing to the immunopathology of *Irg1*−/− mice after *Mtb* infection.

Several groups have reported Irg1 as a highly induced protein in activated macrophages. We observed that Irg1 regulates expression of pro-inflammatory and chemoattractant genes in macrophages within 4 hours of *Mtb* infection. Irg1 may be regulating inflammation during *Mtb* infection in several ways. Irg1 has been reported to suppress production of Toll-like receptor (TLR)-triggered NF-κB-dependent cytokines by promoting the
activity of anti-inflammatory factors such as TNFAIP3 (also known as A20), Nrf2, and Atf3.\textsuperscript{153,154,156} Although an increased inflammatory response was detected in the lungs of Irg1\textsuperscript{-/-} mice at 14 dpi, we failed to detect differences in TNF-α, a cytokine regulated by TNFAIP3,\textsuperscript{154,157} compared to WT lung homogenates. Thus, in the context of Mtb infection Irg1 appears to modulate inflammatory responses by dampening expression of a selected subset of NF-κB regulated genes in both macrophage and neutrophils, perhaps through Nrf2- or Atf3-dependent mechanisms. Irg1 also has been implicated in inducing the production of reactive oxygen species, which can have antibacterial or immunomodulatory effects \textit{in vivo}.\textsuperscript{152,160} The neutrophil-dependent pathology in Irg1\textsuperscript{-/-} mice infected with Mtb could be due to overproduction of NOS2 production in neutrophils or the increased number of ROS-producing neutrophils.

An established function of Irg1 is its \textit{cis}-aconitate decarboxylase activity, which produces itaconate through the decarboxylation of the TCA intermediate, \textit{cis}-aconitate.\textsuperscript{143} Irg1 has been proposed to have an important protective role in Mtb infection through production of itaconate, which blocks the glyoxylate shunt by inhibiting ICL1 and ICL2.\textsuperscript{143,144,202} Our experiments with Δicl1 Mtb suggest that \textit{in vivo}, Irg1 exerts its action against Mtb by regulating inflammatory responses, rather than by inhibiting ICL1. The Δicl1 Mtb strain was as virulent as WT Mtb in Irg1\textsuperscript{-/-} mice even though its glyoxylate shunt capacity is suboptimal.\textsuperscript{150} However, we cannot rule out an effect of Irg1 on the glyoxylate shunt because Mtb Erdman encodes a second ICL enzyme, ICL2, which could compensate for loss of ICL1. Nonetheless, our immunologic and transcriptional profiling data are more consistent with a model where the dominant role for Irg1 is to restrict production of pro-inflammatory chemokines and accumulation of myeloid cells that provide a niche for Mtb replication. Consistent with this hypothesis, we observed no difference in the ability of WT and Irg1\textsuperscript{-/-} BMDMs to restrict Mtb growth \textit{in vitro}. Intriguingly, \textit{Mtb}, \textit{M}.
abscessus, and M. avium all possess ICL homologs but the susceptibility and degree of bacterial control observed in Irg1−/− mice varied dramatically by species. This suggests that the protective function of Irg1 may vary by mycobacterial species. Future studies can address this topic by comparing host inflammatory responses and species-specific requirements for the glyoxylate shunt.

Our data support a model (Fig. 28) where CD11c+ and LysM+ cells require Irg1 to dampen pro-inflammatory cytokine production and subsequent neutrophil inflammation. Our transcriptomic analysis after Mtb infection of Irg1−/− BMDCs revealed a pro-inflammatory signature that was reversed by the addition of exogenous itaconate. Thus, the distinct gene expression profiles in Irg1-sufficient and Irg1-deficient cells likely are due to differences in the production of itaconate, a metabolite that is absent in the lung homogenates of Mtb infected Irg1−/− mice. Collectively, our data suggest a key role for Irg1 and itaconate in regulating inflammation in the context of Mtb infection. Infection studies in gene-edited mice expressing forms of Irg1 that lack the ability to generate itaconate will be the focus of future work to confirm this hypothesis.

In summary, our experiments establish an essential role for Irg1 in regulating neutrophil-dependent inflammation during Mtb infection of the lung. We show that Irg1, likely through its ability to convert the TCA cycle intermediate cis-aconitate to itaconate, shapes the host immune responses through an immunometabolism axis to curtail Mtb-induced lung disease. As murine Irg1 is ~80% identical in amino acid sequence to human IRG1 with all five predicted cis-aconitate decarboxylase domains fully conserved, the development of pharmacological agents that enhance Irg1 function or promote itaconate production might minimize pathological
inflammatory responses that cause severe lung injury associated with tuberculosis disease progression.

Figure 28. Model of Irg1 function during Mtb infection.
During *Mtb* infection of WT mice, Irg1 is induced in LysM⁺ and CD11c⁺ cells such as macrophages, monocytes, and DCs. Irg1 acts to suppress transcriptional upregulation of pro-inflammatory cytokines and NK-κB-regulated genes, likely through production of itaconate. This prevents recruitment of pathologic neutrophils and enables survival. In *Irg1⁻/⁻* mice, *Mtb* infection strongly induces pro-inflammatory cytokine production by LysM⁺ and CD11c⁺ cells, resulting in excessive neutrophil recruitment that promotes *Mtb* growth and causes lung pathology, ultimately resulting in susceptibility.

Experimental Procedures

*Ethics statement*

All procedures involving animals were conducted following the National Institutes of Health (NIH) guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after protocol review and approval by the Institutional Animal Care and Use Committee of the Washington University in St. Louis School of Medicine (protocol no. 20160118). Washington University is registered as a research facility with the United States Department of Agriculture and is fully accredited by the American Association of Accreditation of Laboratory Animal Care. The Animal Welfare Assurance is on file with Office for Protection
from Research Risks–NIH. All animals used in these experiments were subjected to no or minimal discomfort. All mice were euthanized by CO₂ asphyxiation, which is approved by the American Veterinary Association Panel on Euthanasia.

**Mouse strains**

C57BL/6N (WT) mice were either purchased from Charles River or bred in-house. No differences in survival or disease progression during Mtb infection were observed in *Irg1*<sup>+/+</sup> littermate controls or *Irg1*<sup>+/+</sup> C57BL/6N mice obtained from Charles River in three independent experiments. B6.SJL (CD45.1) mice were obtained from Jackson Laboratories. *Irg1*<sup>−/−</sup> mice (embryonic stem cells obtained from KOMP [C57BL/6N background reporter-tagged insertion with conditional potential, MGI: 103206]) were generated at Washington University and have been described previously. Adult mice (6–13 weeks of age) of both sexes were used, and sex was randomized between experiments. Cre-negative *Irg1*<sup>fl/fl</sup> mice were generated as described previously<sup>204</sup> with two loxP sites flanking exon 4 of *Irg1*. *Irg1*<sup>−/−</sup> mice were bred with FLPe “deleter” mice<sup>205</sup> to facilitate deletion of *lacZ* and neomycin resistance cassettes between FRT sites and create *Irg1*<sup>fl/+</sup> founder mice. The founder mice were backcrossed to C57BL6/J background using speed congenic approaches (>99% purity) and then interbred to generate *Irg1*<sup>fl/fl</sup> mice. These animals were crossed with *Mrp8-Cre*<sup>+</sup>, *Lysm-Cre*<sup>+</sup>, and *Cd11c-Cre*<sup>+</sup> (Jackson Laboratory) mice to delete exon 4 of *Irg1* from specific subsets of myeloid cells.

**Generation of bone marrow chimeric mice**

Bone marrow chimeric mice were generated by irradiation (900 Gy) of WT or *Irg1*<sup>−/−</sup> recipients and reconstitution with 10⁷ bone marrow cells from WT or *Irg1*<sup>−/−</sup> donors. 6-8 weeks
after bone marrow transplantation, \textit{Irg1}−/− (CD45.2) → WT (CD45.1), WT (CD45.1) → \textit{Irg1}−/− and WT (CD45.1) → WT (CD45.2), and WT (CD45.1) + \textit{Irg1}−/− (CD45.2) → WT (CD45.1) mice were bled to confirm chimerism by flow cytometry before \textit{Mtb} infection.

\textit{Mouse infections}

\textit{Mtb} cultures in logarithmic growth phase (OD$_{600\text{ nm}}$ = 0.5-0.8) were washed with PBS + 0.05% Tween 80, sonicated to disperse clumps, and diluted in sterile water. Mice were exposed to 1.6 × 10$^8$ CFUs of \textit{Mtb}, a dose chosen to deliver 100-200 CFUs of aerosolized \textit{Mtb} per lung using an inhalation exposure system (Glas-Col). For each infection, lungs were harvested from at least two control mice, homogenized, and plated on 7H11 agar to confirm the input CFU dose. The mean dose determined at this time point was assumed to be representative of the dose received by all other mice infected concurrently. \textit{Mtb} burden was determined after homogenizing the superior, middle, and inferior lobes of the lung or the entire spleen and plating serial dilutions on 7H11 agar. Colonies were counted after 3 weeks of incubation at 37°C in 5% CO$_2$.

\textit{M. abscessus} (strain L948) and \textit{M. avium} (strain TMC724) cultures in logarithmic growth phase (OD$_{600}$ = 0.5-0.8) were washed with PBS + 0.05% Tween 80, passaged through a 27 G needle three times to disperse clumps, and diluted in sterile water. Mice were sedated with ketamine (Henry Schein) and xylazine (Akorn), then infected by intranasal inoculation of 1.5 × 10$^7$ CFU. For each infection, lungs were harvested from at least two control mice, homogenized, and plated on 7H11 agar to confirm the input CFU dose. The mean dose determined at this time point was assumed to be representative of the dose received by all other mice infected concurrently. Bacterial burden was determined after homogenizing the superior, middle, and
inferior lobes of the lung and plating serial dilutions on 7H11 agar. Colonies were counted after 5 days (*M. abscessus*) or 3 weeks (*M. avium*) of incubation at 37°C in 5% CO₂.

*Listeria monocytogenes* (strain EGD) was stored at mid-logarithmic growth as frozen glycerol stocks. Thawed bacteria were diluted into pyrogen-free saline for intravenous injection into mice at a dose of 10⁵ bacteria/mouse in 200 µl.

Mice were infected with IAV strain A/California/04/2009 H1N1 by intranasal inoculation of 10⁵ or 10⁶ tissue culture dose. At 5 dpi viral titer was assessed from bronchoalveolar (BAL) fluid by tissue culture dose.

**Neutrophil depletions**

Mice were administered 200 µg of anti-Ly6G monoclonal antibody (clone 1A8; BioXCell) or rat IgG2a isotype control antibody (clone 2A3; BioXCell) diluted in sterile PBS (Hyclone) by intraperitoneal injection every 48 hrs, beginning at 10 dpi and ending at 20 or 34 dpi, depending on the experiment. For confirmation of depletion during survival experiments, lungs from one anti-Ly6G treated *Irg1*⁻/⁻ control mouse and one isotype treated *Irg1*⁻/⁻ control mouse were harvested from each independent experiment and analyzed by flow cytometry for reduction in CD45⁺/CD11b⁺/Ly6Cmid cells.

**In vivo itaconate administration**

Mice were intraperitoneally administered 500 µl of 250 mM dimethyl itaconate (Alfa Aesar) daily, beginning at 10 dpi and ending at 20 dpi.
Metabolomics profiling of Mtb infected lung cells

The superior, middle, and inferior lobes of the lung were left unperfused, minced with a razor blade, and placed into 800 μl 80% HPLC-grade methanol/20% deionized water. Samples were bead-beaten three times and kept on ice between cycles. Cell debris were pelleted by centrifugation at 17,000 x g for 5 min at room temp. Supernatants were sterilized by filtration through two 0.22 μm Spin-X cellulose acetate filters (Costar) and stored at -80°C until analysis by liquid chromatography-tandem mass spectrometry.

Infection of neutrophils

Neutrophils were purified from bone marrow of naïve mice by negative selection using magnetic-activated cell sorting beads (Miltenyi). Neutrophil purity (>95%) was assessed by flow cytometry as the percentage of Ly6G+CD11b+ cells. Neutrophils were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids at 37°C, 5% CO₂. *Mtb* was grown to mid-log phase, washed with PBS, sonicated to disperse clumps, and resuspended in neutrophil culture media. *Mtb* was then was opsonized prior to infection by mixing with an equal volume of normal mouse sera (Sigma) and incubation at room temperature for 30 min. Neutrophils were mock-infected or infected with opsonized *Mtb* at MOI 1 and incubated at 37°C, 5% CO₂.

Infection of BMDMs

Macrophages were obtained by culturing bone marrow cells in RPMI-1640 (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% nonessential amino acids, 100 U penicillin per ml, 100 μg streptomycin per ml, and 22 ng M-CSF (Peprotech)/ml for 6
days at 37°C, 5% CO₂. Fresh media was added on day 3 of culture. After 6 days of culture, nonadherent cells were discarded. Adherent macrophages were switched into antibiotic-free media and seeded at 10⁵ cells per well and 9 x 10⁵ cells per well in tissue culture-treated 96- and 6-well plates, respectively. In some cases, macrophages were treated with physiologically relevant concentrations of 0.25 mM itaconic acid (Sigma) or 50 ng IFN-γ (Peprotech)/ml for 12 hrs before infection with *Mtb*.¹⁴³ *Mtb* was grown to OD₆₀₀=0.6-0.8, washed with PBS, sonicated to disperse clumps, and resuspended in antibiotic-free macrophage culture media. Macrophage cultures were infected by adding *Mtb*-containing media at a multiplicity of infection of 1 and centrifuging for 10 min at 200 x g. Cells were washed twice with PBS to remove unbound *Mtb*, fresh culture media was added, and cells were incubated at 37°C, 5% CO₂. In some cases, culture media was supplemented with 0.25 mM itaconic acid.

*Bacterial cultures*

*Mtb* strain Erdman, mutant *Mtb* strains, *M. abscessus*, and *M. avium* were cultured at 37°C in 7H9 broth (Sigma) or on 7H11 agar (BD Difco) medium supplemented with 10% oleic acid/albumin/dextrose/catalase, 0.5% glycerol, and 0.05% Tween 80 (broth only).

*Listeria monocytogenes* was grown in a shaking culture in brain-heart infusion broth at 37°C to mid-logarithmic growth phase, washed in PBS, and frozen as glycerol stocks at −80°C.

*Generation of Mtb mutants*

*Mtb*-GFP was generated by transforming *Mtb* strain Erdman with a plasmid (pMV261-kan-GFP) that drives constitutive expression of GFP under the control of the hsp60 promoter. Cultures were grown in the presence of kanamycin to ensure plasmid retention. Δicl1 *Mtb* was
generated by transducing *Mtb* strain Erdman with a phage containing homology to nucleotides 556805–557527 and 558797–559447. ΔPDIM *Mtb* was generated by transducing *Mtb* strain Erdman with a phage containing homology to nucleotides 3243062-3243567 and 3272196-3272718. ΔESX-1 *Mtb* was generated by transducing *Mtb* strain Erdman with a phage containing homology to nucleotides 4349945-4350677 and 4356534-4357206. Phage transduction was used to replace the endogenous *icl1* (Δ*icl1* strain); *fadD26* and *ppsABCDE* (ΔPDIM strain); and *pe35*, *ppe68*, *esxAB*, *espI*, and *eccD1* (ΔESX-1 strain) genes with a hygromycin resistance cassette. Mutants were selected by culture on hygromycin-containing 7H11 agar. Individual hygromycin-resistant colonies were expanded by inoculation into hygromycin-supplemented 7H9 media. Replacement of the target genes with the hygromycin resistance cassette was confirmed by Southern blotting of genomic DNA from expanded cultures.

**Viral cultures**

A/California/04/2009 H1N1 influenza viral stocks were prepared as previously described.206

**Bronchoalveolar lavage (BAL) of IAV infected mice**

For analysis of BAL fluid, mice were sacrificed by Avertin overdose, followed by anterior neck dissection and cannulation of the trachea with a 22-G catheter. BAL was performed with three washes of 0.8 ml of sterile Hanks’ balanced salt solution. BAL fluid was centrifuged and the cell-free supernatant was collected and stored for viral titer analysis.
Flow cytometry

Lungs were perfused with sterile PBS and left lobes of lungs were digested at 37°C with 630 µg/ml collagenase D (Roche) and 75 U/ml DNase I (Sigma). All antibodies were used at a dilution of 1:200. Single cell suspensions were preincubated with Fc Block antibody (BD Pharmingen) in PBS + 2% heat-inactivated FBS for 10 min at room before staining. Cells were incubated with antibodies against the following markers: V500 anti-CD45.1 (clone A20), AF700 anti-CD45.2 (clone 104; eBioscience), AF700 anti-CD45 (clone 30 F-11), APC-Cy7 anti-CD11c (clone N418), PE anti-SiglecF (clone E50-2440; BD), PE-Cy7 anti-Ly6G (clone 1A8), PB or Qdot605 anti-Ly6C (clone AL-21/HK1.4; Biolegend/ BD), PerCP-Cy5.5 anti-CD11b (clone M1/70), APC anti-CD103 (clone 2E7; eBioscience), PB anti-CD3 (clone 17A2), PE-Cy7, APC anti-CD4 (clone RM4-5), PE-Cy7 anti-CD8 (clone53-6.7), anti-NK1.1 (clone PK136), and APC anti-Nos2 (clone CXN FT; all from eBioscience). Intracellular ROS were stained with MitoSOX red dye (Thermo Fisher). Absolute cell counts were determined using TruCount beads (BD). Cells were stained for 20 min at 4°C, washed, and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 20 min at 4°C. Flow cytometry data were acquired on a cytometer (LSR Fortessa; BD Biosciences) and analyzed using FlowJo software (Tree Star). Gating strategies are depicted in Figure 29.

Cytokine/chemokine quantification

Lungs were homogenized in 5 ml of PBS supplemented with 0.05% Tween 80. Homogenates were filtered (0.22 µm) twice and analyzed by BioPlex-Pro Mouse Cytokine 23-Plex Immunoassay (Bio-Rad).
**Histology and imaging**

Lung samples were fixed in 10% buffered formalin (Thermo Fisher). Gross pathology images were acquired using a Power Shot G9 camera (Canon). For detailed histological analysis, lung lobes were embedded in paraffin, sectioned, and stained with H&E (Pulmonary Morphology Core, Washington University). Acid-fast bacilli were stained with Ziehl-Neelsen

Figure 29. Flow cytometry gating strategy used in Irg1 experiments.
stain (Pulmonary Morphology Core). Images were acquired using NanoZoomer 2.0-HT System (Hamamatsu) or an Eclipse E400 camera (Nikon).

**RNA-seq**

Neutrophils were purified by magnetic-activated cell sorting from the bone marrow of naïve mice (negative selection) or whole lungs of *Mtb* infected mice at 16 dpi (selection for Ly6G+ cells) (Miltenyi). Neutrophil purity was defined by flow cytometry as the percentage of Ly6G+CD11b+ cells (negatively selected samples) or Gr-1highCD11b+ cells (positively selected samples). BMDMs were harvested at 4 h after *Mtb* infection, and RNA was extracted using TriZol reagent (Invitrogen).

Total RNA was isolated from TriZol by chloroform extraction, precipitated with isopropanol and high salt buffer (0.8 M sodium citrate, 1.2 M NaCl in RNase-free water), washed with 80% ethanol/20% RNase-free water, dried for 10 min at room temp, and resuspended in 20 μl RNase-free water.

mRNA was extracted with oligo-dT beads (Invitrogen) and cDNA synthesized using custom oligo-dT primer with a barcode and adaptor-linker sequence (CCT ACA CGA CGC TCT TCC GAT CT-XXX XXX XX-T15). Samples were pooled based on Actb qPCR values, RNA–DNA hybrids degraded with acid-alkali treatment and a second sequencing linker (5′-AGA TCG GAA GAG CAC ACG TCTG- 3′) was ligated with T4 ligase (NEB). After SPRI bead cleanup (Agencourt AMPure XP; Beckman Coulter), the mixture was PCR-enriched (12 cycles) and SPRI-purified, yielding final strand-specific RNA-seq libraries. Libraries were sequenced on a HiSeq 2500 (Illumina) using 40 x 10 bp paired-end sequencing. Second read (read-mate) was used for sample demultiplexing. Files obtained from the sequencing center were demultiplexed
with fastq-multx tool. Fastq files for each sample were aligned to mm10 genome (Release M8 Gencode; GRCm38.p4) using STAR. Each sample was evaluated according to a variety of both pre- and post-alignment quality control measures with Picard tools. Aligned reads were quantified using a quant3p script (Computer Technologies Laboratory, 2016) to account for specifics of 3’ sequencing: higher dependency on good 3’ annotation and lower level of sequence specificity close to 3’ end. Actual read counts were performed by HTSeq with enriched genome annotation and alignment with fixed multimapper flags. DESeq2 was used for analysis of differential gene expression. Preranked GSEA analysis was used to identify pathway enrichments in hallmark and canonical pathways.

Quantification and statistical analysis

All data are from at least two independent biological experiments with multiple mice in each group. No blinding was performed during animal experiments. Statistical differences were calculated using Prism 7 (GraphPad) using log-rank Mantel-Cox tests (survival), unpaired two-tailed Mann-Whitney tests (to compare two groups with non-normal data distribution), and one-way ANOVA with Tukey’s multiple comparisons tests (to compare more than two groups with normal data distribution). Differences with a p-value of <0.05 were defined as statistically significant.

Data and software availability

RNA-seq data are available in the Gene Expression Omnibus repository under reference series number GSE98458.
Chapter 4

Conclusions
Introduction

Protective immune responses to *Mycobacterium tuberculosis* (*Mtb*) must induce bactericidal functions while minimizing damage to the lung. Such responses require precise control of pro- and anti-inflammatory factors to regulate the recruitment and function of protective immune cells but the mechanisms by which this control is exerted remain incompletely defined. *Bhlhe40* is a transcription factor known to regulate production of pro- and anti-inflammatory cytokines that affect protective immunity to *Mtb*. *Irg1* is an enzyme that generates itaconate, a metabolite with potential anti-inflammatory and antibacterial roles during *Mtb* infection.

We found that *Bhlhe40* enables protective immune responses to *Mtb* in T cells and CD11c+ cells and enables resistance to acute infection by restricting expression of the anti-inflammatory cytokine IL-10. *Bhlhe40* does so by repressing *Il10* transcription, likely by direct binding to the *Il10* locus. Additionally, we discovered that *Irg1* functions in LysM+ and CD11c+ cells to prevent neutrophil-mediated immunopathology. *Irg1* expression restricts inflammatory responses in myeloid cells at a transcriptional level, likely through production of the metabolite itaconate. The impact of *Bhlhe40* and *Irg1* on protective immunity to *Mtb* was unknown prior to the studies detailed in this dissertation.

Discussion and Future Directions

*Bhlhe40 expression patterns*

Using *Bhlhe40*\textsuperscript{GFP} transgenic reporter mice that express green fluorescent protein (GFP) under the control of the genomic regions flanking the *Bhlhe40* gene we demonstrated that *Bhlhe40* is expressed in multiple innate and adaptive lung immune cell types before infection.
and during acute infection. Mice with conditional deletions of *Bhlhe40* in T cells and CD11c+ cells were susceptible to *Mtb*, indicating that these are the key cell types that require *Bhlhe40* expression. Studies in other model systems have demonstrated that *Bhlhe40* expression is dynamic and regulated by multiple stimuli. Given the importance of *Bhlhe40* for control of *Mtb* infection, it is possible that these dynamics impact control of *Mtb* infection. *Bhlhe40*GFP mice could be highly useful in future studies that seek to answer questions about where *Bhlhe40*-expressing cells localize within the *Mtb* infected lung, the infection status of *Bhlhe40*-expressing cells, and patterns of *Bhlhe40* expression in specific cell types over a prolonged course of *Mtb* infection. These questions could be answered by flow cytometry and immunohistochemistry studies on lung lesions in *Bhlhe40*GFP mice infected with a *Mtb* strain that expresses a non-GFP fluorescent marker and flow cytometric analysis of *Bhlhe40*GFP mice at later points in infection. Additionally, we did not address the importance of *Bhlhe40* in chronic *Mtb* infection but could do so using *Bhlhe40*fl/fl mice that express Cre recombinase under the control of a tamoxifen-inducible promoter, such as *ERT2-Cre* mice.

*Bhlhe40* expression patterns could also be modulated by diet, with potentially important effects on control of *Mtb* infection. Epidemiological207,208 and *in vitro*209 studies have suggested that vitamin A levels are associated with resistance to *Mtb* and all-trans retinoic acid, the biologically available form of vitamin A, is known to induce *Bhlhe40* expression.106,123 Together with our findings on the importance of *Bhlhe40* in control of murine tuberculosis and lower *BHLHE40* expression in patients with active tuberculosis, these reports raise the possibility of a *Bhlhe40*-mediated link between micronutrient availability, disease severity, and variation in long-term control of disease. Experiments characterizing how vitamin A availability modulates *Bhlhe40* expression and associated control of *Mtb* in mice in conjunction with prospective
studies in humans tracking serum metabolites, Bhlhe40 expression, and progression to active tuberculosis could address this relationship.

**Bhlhe40 and IL-10-dependent control of Mtb infection**

We assessed the role of Bhlhe40 expression on control of *in vivo* Mtb infection using genetically deficient mice. Bhlhe40 was required in T cells and CD11c+ cells and enabled protective immune responses to acute *Mtb* infection by restricting expression of IL-10. Bhlhe40 does so by repressing *Il10* transcription, likely by direct binding to the *Il10* locus. Multiple groups had previously demonstrated that IL-10 has detrimental effects on control of *Mtb* infection but these phenotypes were mild or occurred late in infection. Our findings show for the first time that IL-10 can have major impacts on control of *Mtb* and that Bhlhe40 is an essential regulator of IL-10. However, it remains unclear exactly how increased IL-10 production causes susceptibility in *Bhlhe40−/−* mice. Our observations of decreased *Il12b* and *Ifng* transcript aligns with a large body of literature demonstrating that IL-10 interferes with mechanisms involved in inducing and responding to IFN-γ, but studies comparing the transcriptional and functional state of WT and *Bhlhe40−/−* cells *in vivo* will be necessary to fully prove that this is the case. *Bhlhe40−/−* cells would be expected to exhibit a transcriptional profile characterized by reduced expression of IFN-γ-stimulated genes after the induction of *Mtb*-specific adaptive immune responses.

We have identified multiple immune cell populations that overexpress IL-10 as well as the key cell types that must express Bhlhe40 during *Mtb* infection. We also observed increased IL-10 expression in non-Bhlhe40-expressing cell types in the context of global Bhlhe40 deficiency which raises the possibility that non-Bhlhe40-expressing cells may be important sources of IL-10. It remains to be determined which of these cell types mediate IL-10 dependent
susceptibility. Cell types that cause susceptibility by overproducing IL-10 in a globally Bhlhe40-deficient setting can be identified by generating Bhlhe40^{+/}Il10^{fl/fl} mice that express Cre recombinase in the cell types we found to be over-producers of IL-10 in our analysis of Bhlhe40^{+/}10BiT^{+} mice. Cell types in which Bhlhe40 is essential to regulate IL-10 production can be identified by generating Bhlhe40^{fl/fl}Il10^{fl/fl} mice that express Cre recombinase in the cell types we identified as expressing Bhlhe40 in our analysis of Bhlhe40^{GFP} mice. Susceptibility-associated phenotypes such as survival, Mtb burden, and inflammation can then be compared in Cre-positive and Cre-negative Bhlhe40^{+/}Il10^{fl/fl} mice or Bhlhe40^{fl/fl}Il10^{WT/WT} mice with the same Cre expression pattern. Cre-positive Bhlhe40^{+/}Il10^{fl/fl} and Bhlhe40^{fl/fl}Il10^{fl/fl} mice that show improvement in susceptibility-associated phenotypes will reveal key cell types that mediate IL-10-dependent susceptibility.

Additionally, we have yet to identify the cell types that mediate susceptibility by responding to the increased IL-10. Such cell types can be identified by comparing susceptibility-associated phenotypes in Cre-positive and Cre-negative Bhlhe40^{+/}Il10ra^{fl/fl} mice. Key cell types will be identified by Cre-positive mice that show improvements in susceptibility-associated phenotypes as compared to Cre-negative mice.

While we have established that Bhlhe40 is a direct regulator of IL-10, the pathway of IL-10 induction in which Bhlhe40 functions remains to be determined. IL-10 can be induced by a wide variety of extracellular signals during Mtb infection\textsuperscript{75,165} and the ability of Bhlhe40 to repress IL-10 may vary according to the induction pathway. We found that Bhlhe40^{+} cells and mice do not overexpress Il10 prior to infection which strongly implies that a transcriptional activator is triggered by Mtb infection. The identity of this putative activator is currently unknown and there are many possibilities, but c-Maf, AP-1 family members, and IRF4 stand out.
by virtue of their reported ability to bind the \textit{Il10} locus close to the Bhlhe40 binding site we identified at \textit{Il10} +6 kb. c-Maf (encoded by the \textit{Maf} gene) is induced in T cells by cytokine signals\textsuperscript{210,211} and promotes T cell production of IL-10 in infectious disease, allergy, and autoimmune contexts.\textsuperscript{212} Bhlhe40 expression was found to be upregulated in c-Maf-deficient T cells\textsuperscript{212} and \textit{Maf} expression is increased in Bhlhe40-deficient T cells,\textsuperscript{129} suggesting that these transcription factors regulate each other. In support of this idea, our ChIP-seq data identified \textit{Maf} as a gene predicted to be regulated by Bhlhe40 in T\textsubscript{H}1 cells. Binding sites for c-Maf have been reported in the \textit{Il10} promoter and near the \textit{Il10} +6 kb site bound by Bhlhe40.\textsuperscript{212} Interferon regulatory factor 4 (IRF4) promotes IL-10 production after T cell activation and has been shown to bind a \textit{Il10} +6.45 kb enhancer site in T\textsubscript{H}2 cells.\textsuperscript{213} Several members of the activator protein (AP)-1 family of transcription factors have also been reported to bind the \textit{Il10} +6.45 kb site in T cells after T cell receptor stimulation, including JunB and c-Jun.\textsuperscript{181,214} The AP-1 family member c-fos promotes IL-10 production in myeloid DCs after stimulation with toll-like receptor (TLR) agonists and could potentially bind to the same AP-1 site as JunB or c-Jun in DCs, but direct binding has yet to be demonstrated.\textsuperscript{215} Bhlhe40 has also been reported to interact with histone deacetylases (HDACs)\textsuperscript{102} and could be preventing binding of an activator elsewhere in the \textit{Il10} locus by controlling chromatin accessibility. Thus, these transcription factors represent likely candidates for the activator of \textit{Il10} transcription that is restricted by Bhlhe40.

CRISPR or small-interfering RNA knock-down approaches could be used to target the activator protein candidates mentioned above in \textit{Bhlhe40}\textsuperscript{-/-} T cells and GM-CSF cultured cells. \textit{Il10} transcript levels could then be measured after treatment with IL-10-inducing stimuli such as anti-CD3/anti-CD28 and PMA/Ionomycin or heat-killed \textit{Mtb} antigen. Knock-down of the activator protein will result in reduced \textit{Il10} transcription in \textit{Bhlhe40}\textsuperscript{-/-} cells. Activator protein
redundancy is also a possibility and multiple activator candidates may need to be targeted simultaneously. HDAC-dependent mechanisms could be identified through treatment with trichostatin A, a pan-HDAC inhibitor, profiling of histone acetylation status, and co-immunoprecipitation mass spectrometry to identify HDAC interactions or potential activators that Bhlhe40 represses through direct binding. The importance of activator candidates in *Mtb* infection can then be tested by generation of *Bhlhe40*<sup>−/−</sup> mice that also lack the protein of interest and comparison of susceptibility-associated phenotypes with *Bhlhe40*<sup>−/−</sup> mice. Once an activator protein is identified, the key upstream signaling pathways that trigger it can be identified by genetic knock-out or antibody blockade and treatment with IL-10-inducing stimuli.

Another IL-10-dependent role for Bhlhe40 concerns clinical tuberculosis diagnostics. IFN-γ release assays (IGRAs) are a commonly used method of diagnosing *Mtb* exposure, but more than 10% of culture-positive tuberculosis patients test negative by IGRA. These patients tend to have worse disease outcomes, likely due to delayed treatment. IGRAs work by stimulating a sample of peripheral blood with *Mtb* antigens and measuring IFN-γ production. We found that Bhlhe40-deficiency leads to increased production of IL-10 and that patients with active tuberculosis had lower *BHLHE40* expression in their peripheral blood. Since IL-10 is well established as a suppressor of IFN-γ, our findings suggest that patients with lower expression of Bhlhe40 in peripheral blood could produce increased IL-10 with a suppressive effect on IFN-γ production. Thus, lower Bhlhe40 expression levels could have an adverse effect on IGRA specificity and patient outcomes. Future studies could address this question by obtaining peripheral blood samples from discordant active tuberculosis patients who tested negative for tuberculosis by IGRA, but positive by bacterial culture and concordant patients who were positive by IGRA and culture. *Bhlhe40* expression levels in unstimulated and stimulated
peripheral blood samples could then be compared in concordant and discordant active tuberculosis patients to determine if lower BHLHE40 expression before stimulation is correlated with a false negative IGRA result. Stimuli that induce Bhlhe40 expression, such as retinoic acid, could be used to treat blood samples from discordant patients and demonstrate an active role for Bhlhe40. IL-10-neutralizing antibodies could be used to test the role of IL-10 in any observed effects.

**IL-10-independent roles for Bhlhe40 in Mtb infection**

We have demonstrated that the acute susceptibility of Bhlhe40−/− mice is largely IL-10-dependent, but loss of IL-10 does not confer complete restoration of WT phenotypes. Il10−/−Bhlhe40−/− mice have more neutrophilic inflammation at 21 dpi and significantly shorter survival time compared to WT mice. These findings demonstrate that Bhlhe40 has IL-10-independent functions that are important during Mtb infection. The key cell types mediating IL-10-independent functions of Bhlhe40 can be identified by comparison of Cre-positive Bhlhe40fl/fl and Cre-positive Bhlhe40fl/fl Il10fl/fl. Susceptibility associated phenotypes found in both strains with the same Cre expression pattern would be due to IL-10-independent functions of Bhlhe40 in Cre-expressing cells. Identification of IL-10-independent functions for Bhlhe40 could be identified in an unbiased manner by transcriptomic comparison of WT, Bhlhe40−/−, and Il10−/−Bhlhe40−/− lung hematopoietic cells at 21 dpi, a time point at which Il10−/−Bhlhe40−/− mice already have a higher frequency of neutrophils compared to WT mice.

We have also identified genes that Bhlhe40 is predicted to regulate based on DNA binding sites and this data could also be used to address IL-10-independent roles. Bhlhe40 has been reported to function in a wide variety of biological processes including cell death and
regulation of non-IL-10 cytokine production. Our studies of Irg1 and Bhlhe40 have demonstrated the importance of inflammatory control for infection and cell death modalities are known to impact control of Mtb. Perturbation of these processes in Bhlhe40+ cells could adversely affect control of inflammation and Mtb growth in the absence of IL-10. Additionally, we have observed that Il10−/−Bhlhe40−/− mice have a stronger neutrophil response than WT mice as early as 21 dpi. Based on literature reports and our data, investigation of Bhlhe40-regulated genes that are likely to regulate cytokine production, cell death, or have previously been identified as important for control of Mtb should be prioritized. Three candidate genes, all of which were identified as potentially regulated by Bhlhe40 in both myeloid and lymphoid cells, are discussed below.

Tristetraprolin (TTP, encoded by Zfp36) is an RNA-binding protein that regulates mRNA stability. TTP has been reported to repress production of IL-12 and IFN-γ, and transcript levels for both of these cytokines trend downward in Il10−/−Bhlhe40−/− compared to WT mice. TTP can also regulate other proinflammatory cytokines of potential importance in Il10−/−Bhlhe40−/− mouse susceptibility during chronic infection. TTP also regulates RIP1 kinase activity, and decreased RIP1 activity could lead to detrimental cell death pathways. Forkhead box protein O3 (Foxo3) is a transcription factor that exerts predominantly anti-inflammatory effects on immune cells. Foxo3 has been reported to promote T cell apoptosis and to repress pro-inflammatory responses by DCs and T cells. Dysregulated Foxo3 activity could lead to over or underproduction of inflammatory cytokines or be detrimental for survival of effector T cells during Mtb infection. Hypoxia inducible factor-1α (Hif-1α) is a transcription factor that mediates a wide variety of IFN-γ-stimulated responses and is essential for control of Mtb infection. However, over-expression of Hif-1α can cause proteinase-mediated tissue pathology. Dysregulated Hif-1α activity could compromise protective functions of IFN-γ during Mtb
infection or promote immunopathology. Thus TTP, Foxo3, and Hif-1α all modulate key immune cell processes involved in control of Mtb and could promote IL-10-independent susceptibility if dysregulated in Bhlhe40<sup>−/−</sup> mice.

**Irg1 and restriction of neutrophil-mediated immunopathology**

We have discovered that Irg1 expression restricts neutrophil-dominated inflammatory responses in myeloid cells at a transcriptional level, likely through production of the metabolite itaconate. In support of itaconate as an anti-inflammatory mediator, we found that exogenous itaconate treatment was sufficient to revert Irg1<sup>−/−</sup> macrophages to a less inflammatory, WT-like transcriptional profile and suppressed transcription of the neutrophil recruitment signal CXCL2 and the broadly inflammatory gene Il1b. Intriguingly, we found that levels of IL-17, a cytokine that triggers and sustains neutrophilic inflammation, were also highly upregulated in the lungs of Irg1<sup>−/−</sup> mice. This suggests that Irg1 restricts neutrophilic inflammatory responses by limiting production of cytokines such as CXCL2, IL-1β, and IL-17.

However, the mechanism by which Irg1 exerts its anti-inflammatory effects during Mtb infection remains unclear. Studies in other models of immunopathology have reported that Irg1 or itaconate promotes the activity of anti-inflammatory factors such as TNFAIP3 (also known as A20), Nrf2, and Atf3, leading to the repression of a subset of pro-inflammatory cytokines that includes IFN-β, IL-1β, IL-6, and IL-12. Unpublished data from our co-authors suggests that itaconate may also regulate IL-17 production by interfering with expression of the transcription factor BATF (Lampropolou & Artyomov, in preparation). Our findings that IL-1β, IL-6, and IL-17 are more highly expressed in Mtb infected Irg1<sup>−/−</sup> lungs a 14 dpi suggest that they might lead to neutrophilic inflammation. Future studies could test this by blockade or genetic
deletion of IL-6R and IL-17R and treatment with the IL-1R antagonist anakinra, followed by assessment of neutrophil accumulation and other susceptibility-associated phenotypes in Irg1+/− mice.

Our studies implicate Irg1-derived itaconate as a key suppressor of neutrophilic inflammation. Since neutrophil-dominated responses to Mtb are associated with active tuberculosis in humans, itaconate supplementation is a potential therapeutic approach for limiting neutrophil-mediated immunopathology in humans. Our efforts to modulate the pulmonary response to Mtb through intraperitoneal delivery of dimethyl itaconate were unsuccessful, but this may be due to insufficient bioavailability in the lung. Itaconate or an analog could be delivered intravenously or intranasally and Irg1+/− mice could be assessed for reduction neutrophil recruitment or inflammatory mediators such as IL-6 and IL-17. It is also possible that Irg1 has itaconate-independent effects in vivo. Future studies utilizing comparing Irg1+/− mice with mice that express a catalytically-inactivated version of Irg1 during Mtb infection will be required to identify such effects.

Myeloid cell-specific requirements for Irg1

We found that although the susceptibility of Irg1+/− mice is mediated by neutrophils, neutrophil-specific loss of Irg1 expression was not sufficient for susceptibility to Mtb infection. In contrast, loss of Irg1 expression in LysM+ and CD11c+ cells was sufficient to cause susceptibility. These susceptibility patterns point to a requirement for Irg1 in macrophages, monocytes, and DCs during Mtb infection. Irg1 deficiency in LysM+ cells led to an earlier susceptibility than deficiency in CD11c+ cells, demonstrating that expression in macrophages and monocytes enables survival of acute infection. Neutrophils also express LysM and Irg1-
deficient neutrophils are likely an important contributor to the acute susceptibility of \(Irg1^{fl/fl}\)-Lysm-Cre mice, despite being insufficient for susceptibility when other myeloid cells express Irg1. We do not yet know why loss of Irg1 in neutrophils alone has no impact on susceptibility but it may be due to the ability of Irg1-expressing cell types to secrete itaconate and thereby provide \textit{in trans} complementation to Irg1-deficient neutrophils.\textsuperscript{138} Transcriptomic comparison of neutrophils isolated from Mtb-infected Cre-negative \(Irg1^{fl/fl}\), \(Irg1^{fl/fl}\)-Mrp8-Cre, and \(Irg1^{+/+}\) lungs would reveal whether Irg1-deficient neutrophils take on a less inflammatory profile when other Irg1-sufficient cells are present.

\textit{Impact of Irg1 on adaptive immunity}

Irg1 and itaconate exert host-protective effects on innate immune cell function during Mtb infection, but no studies have yet examined their impact on adaptive immune cells. The lungs of Mtb infected \(Irg1^{+/+}\) mice contained increased amounts of IFN-\(\gamma\) and IL-17 at 21 dpi, cytokines produced in large quantities by T helper (T\(H\)) 1 and T\(H\)17-polarized CD4\(^+\) T cells, respectively. IFN-\(\gamma\) and IL-17 have important protective functions in primary- and vaccine-elicited responses that control Mtb infection\textsuperscript{34,35,227} but both can cause pathology and susceptibility if over-produced.\textsuperscript{37,228} The ability of IL-17-producing CD4\(^+\) T cells to drive pathologic granulocyte-dominated inflammation during Mtb infection\textsuperscript{228} is of particular interest given that the susceptibility of \(Irg1^{+/+}\) mice is mediated by neutrophils, a type of granulocyte strongly associated with failure to control Mtb infection.\textsuperscript{24,32,33} Our observations of increased IFN-\(\gamma\) and IL-17 and neutrophil-mediated susceptibility in Mtb infected \(Irg1^{+/+}\) mice suggest that Irg1 may restrict the generation of pathologic T cell responses that contribute to susceptibility.
Irg1 could be regulating T cell survival, recruitment, polarization, or effector function. Our studies focused on the role of Irg1 in myeloid cells and thus we did not test any of these possibilities. Still, some of our observations are suggestive of potential effects. Our finding that \( Irg1^{-/-} \) mice had no differences in the absolute number of lung CD4\(^+\) T cells at 21 dpi suggests that T cell survival and recruitment are unaffected. The fold increase in IL-17 levels in infected \( Irg1^{-/-} \) lungs compared to WT was much greater than the fold increase in IFN-\(\gamma\) levels, implicating over-production of IL-17 as the dominant effector cytokine and a cause of neutrophil-mediated immunopathology. Recently generated data from our co-authors shows that secreted itaconate from myeloid cells inhibits T\(\text{H}17\) cell effector function (Lampropoulou & Artyomov, in preparation). This raises the possibility that loss of itaconate in \( Irg1^{-/-} \) mice leads to unrestrained T\(\text{H}17\) cell production of IL-17 and recruitment of pathologic neutrophils. Future studies should assess the role of T cells in \( Irg1^{-/-} \) mouse susceptibility by characterizing \textit{in vivo} expression of lineage-defining transcription factors, ability to generate antigen-specific responses, antigen-specific cytokine production, and T cell transcriptional profiles.

If Irg1 is found to be important for regulating T cell responses, follow-up studies could utilize \( Irg1^{flo/flo}-Cd4-Cre \) mice examine a requirement for T cell-intrinsic Irg1 expression and the ability of secreted itaconate to modulate T cell metabolism and effector function during \textit{Mtb} infection.

\textit{Direct antibacterial roles for Irg1 in Mtb infection}

A direct antibacterial role for Irg1 has been hypothesized based on the ability of itaconate to inhibit \textit{Mtb} ICLs \textit{in vitro}. We have demonstrated that neutrophil depletion reduces WT \textit{Mtb} burden in \( Irg1^{-/-} \) mice but does not restore it to levels observed in WT mice. This partial rescue
could be due to incomplete neutrophil depletion, cytokine-mediated reductions in bactericidal capacity, or a direct antibacterial role for Irg1. We have also shown that the susceptibility of $Irg1^{+/}$ mice is ICL1-independent and that $Irg1^{+/}$ BMDMs have no defect in control of WT $Mtb$. These results do not support a direct antibacterial role for Irg1 during $Mtb$ infection but are not definitive. Further studies are required and must account for the redundancy of ICL1 and ICL2 as well as confounding factors such as host-mediated pathology, the potential for $Mtb$ to degrade itaconate, and the involvement of ICLs in other metabolic pathways.

The compensatory effects of ICL2 in $\Delta icl1$ $Mtb$ limit our ability to identify an inhibitory role for Irg1. Comparison of bacterial burden and susceptibility in WT and $Irg1^{+/}$ mice infected with $\Delta icl1/\Delta icl2$ $Mtb$ will be necessary to draw more comprehensive conclusions on a role for Irg1 in restricting the glyoxylate shunt. However, it is likely that interpreting the results of such an experiment will be complicated by the severe $in$ $vivo$ attenuation of $\Delta icl1/\Delta icl2$ $Mtb$.$^{145}$ This attenuation has attributed to the dual functionality of ICL1 and ICL2 in the glyoxylate shunt for carbon assimilation and the methylcitrate cycle for detoxification of propionate.$^{146}$ The finding that $Irg1^{+/}$ mice are equally susceptible to WT and $\Delta icl1/\Delta icl2$ $Mtb$ would demonstrate that susceptibility is independent of the glyoxylate shunt and support restraint of inflammation as the crucial function for Irg1 in acute infection but would not rule out a simultaneous defect in antibacterial activity. An antibacterial role for Irg1 would be supported if $\Delta icl1/\Delta icl2$ $Mtb$ in an $Irg1^{+/}$ mouse is cleared at rates equivalent to a WT mouse, but only if confounding inflammatory effects are avoided by limiting neutrophilic inflammation in the $Irg1^{+/}$ mice to levels comparable to a WT mouse. This could be accomplished by neutrophil-specific depletion and antibody-mediated reduction of over-produced pro-inflammatory cytokines such as IL-17 in the $Irg1^{+/}$ mice. Measurement of glyoxylate levels during $in$ $vivo$ or $in$ $vivo$ infection of WT and $Irg1^{+/}$
macrophages and mice by mass spectrometry would also provide a direct assessment of whether Irg1 restricts glyoxylate shunt activity since the glyoxylate cycle is absent from mammals.\textsuperscript{147}

It is also unclear whether the potentially antibacterial activity of itaconate is being blocked by itaconate-degrading enzymes in \textit{Mtb}. Mammals and several \textit{Yersinia}, \textit{Pseudomonas}, and \textit{Salmonella} species encode three enzymes that enable detoxification of itaconate: succinyl-CoA:itaconyl-CoA transferase (ICT), itaconyl-CoA hydratase (ICH), and citramalyl-CoA lyase (CCL).\textsuperscript{149} Genes that may provide the same functionality in \textit{Mtb} include \textit{scoAB} and \textit{Rv3551/Rv3552} for ICT; \textit{echA1-echA21}, \textit{Rv1136}, and \textit{Rv3538} for ICH; and \textit{citE} for CCL. Future studies could test the ability of \textit{Mtb} to degrade itaconate by radiolabeling itaconate and performing mass spectrometry to look for its breakdown products. The necessity of the genes listed above for itaconate degradation could be determined by genetic knock-out. However, these genes are likely to have roles in multiple processes and the likelihood of pleiotropic effects is high. For example, CitE is predicted to generate acetyl-CoA for fatty acid biosynthesis based on its genomic location,\textsuperscript{229} and fatty acid biosynthesis is essential for survival of \textit{Mtb} within macrophages.\textsuperscript{230}

\textbf{Explanations for virulence of \textit{Δicl1 Mtb} in Irg1\textsuperscript{−/−} mice}

The persistence defect of \textit{Δicl1 Mtb} during chronic infection demonstrates that glyoxylate shunt function is suboptimal in this strain.\textsuperscript{145} Since Irg1 is hypothesized to inhibit the glyoxylate shunt through itaconate, we asked whether \textit{Δicl1 Mtb}, a strain with suboptimal glyoxylate shunt function, might phenocopy the inhibitory effects of itaconate on the glyoxylate shunt and exhibit reduced virulence in \textit{Irg1\textsuperscript{−/−}} mice and if the susceptibility of \textit{Irg1\textsuperscript{−/−}} mice was independent of ICL1 function. ICL1-deficient \textit{Mtb} proved not to be attenuated in \textit{Irg1\textsuperscript{−/−}} mice and we found that \textit{Irg1\textsuperscript{−/−}}
mice were equally susceptible to WT and Δicl1 Mtb. There are several possible explanations for this lack of a difference. First, it is possible that the glyoxylate shunt is not active during acute infection and thus inhibition by itaconate would have no effect on virulence. The essentiality of malate synthase (glcB) to detoxify ICL-generated glyoxylate during acute infection suggests that ICLs and the glyoxylate shunt are active during acute infection and argues against this possibility. Second, it is possible that ICL2 is compensating for loss of ICL1 during acute infection. This is very likely because Δicl1/Δicl2 Mtb is severely attenuated during acute infection, while Δicl1 Mtb is not. Third, Irg1−/− mice may succumb to inflammatory defects at a time point before effects of glyoxylate shunt inhibition by itaconate can be observed. This is suggested by the observations that Irg1−/− mice succumb 5-6 weeks post-infection but the persistence defect of Δicl1 Mtb is first seen at 8 weeks post-infection. Fourth, the neutrophil-dominated inflammation observed in Irg1−/− mice may generate a nutrient rich, growth-permissive environment in which the glyoxylate shunt is not needed. Such an environment could compensate for any attenuation caused by loss of ICL1. A fifth and final possibility is that during acute infection itaconate does not inhibit ICL1 or ICL2 enough to affect Mtb growth or survival. This idea is supported by the report that Δicl1 Mtb in WT mice is attenuated in chronic infection, but we and others find that WT and Δicl1 Mtb reach similar titers by 21 dpi in WT and Irg1−/− mice. This lack of an inhibitory effect could be due to the presence of a degradative pathway for itaconate in Mtb, as mentioned previously.

**Itaconate catabolism and vitamin B12 deficiency**

An intermediate in the metabolic pathway responsible for itaconate catabolism has been shown to cause vitamin B12 depletion in human cells. Since mammals and bacteria utilize a
similar pathway for itaconate break down, vitamin B12 deficiency is likely to occur in bacteria as well. As stated previously, it is unclear whether or not Mtb can catabolize itaconate. If so, this process could lead to vitamin B12 deficiency that inhibits the methylmalonyl-CoA cycle, one of several processes used by Mtb to detoxify propionate generated during metabolism of host fatty acids. If allowed to accumulate, propionate causes defects in intrabacterial pH and membrane potential. Thus, itaconate could limit vitamin B12 availability, metabolic flexibility, and homeostasis in Mtb through its breakdown products.

Itaconate catabolism-driven consumption of vitamin B12 may also cause localized vitamin B12 depletion in host cells. Vitamin B12 is used as a coenzyme by two human enzymes, MUT, which recycles carbon from the methylmalonyl cycle back into the TCA cycle and MTR, which synthesizes methionine. Lower circulating vitamin B12 levels are associated with increased expression of inflammatory biomarkers. The effect of vitamin B12 deficiency on inflammatory responses is currently unclear, but may be important for understanding the progression of itaconate-mediated effects. Future studies could address this by treating mice with itaconyl-CoA, the vitamin B12-depleting itaconate metabolite, to deplete vitamin B12 in the absence of other itaconate-derived effects and analyzing its impact on Mtb-elicited inflammation.

Possible cross-talk between Bhlhe40 and Irg1

Irg1-sufficient (Irg1+/+) bone marrow-derived macrophages generated by culture of bone marrow cells with M-CSF (BMDMs) do not express Bhlhe40 before or after Mtb infection. However, we found that Bhlhe40 transcript is highly induced in Irg1+/+ BMDMs as early as four hours after Mtb infection and that itaconate treatment blocks Bhlhe40 induction in Irg1+/+ BMDMs. This demonstrates that expression of Bhlhe40 in certain macrophage subsets is
repressed by Irg1 and itaconate. In conjunction with previous reports that Irg1 and itaconate are highly expressed by activated macrophages, these observations may explain why minimal Bhlhe40 expression is detected in CD11b+ macrophages during \textit{in vivo} Mtb infection. By extension, Irg1 expression in LysM+ cells, such as macrophages, during \textit{in vivo} infection may repress Bhlhe40 and explain why loss of Bhlhe40 expression in LysM+ cells had no effect on \textit{in vivo} control of Mtb. Irg1 is not predicted to be regulated by Bhlhe40 based on binding site data, although it could still be regulated through DNA binding-independent functions of Bhlhe40. Future studies could determine whether itaconate suppresses transcription of Bhlhe40 \textit{in vivo} by comparing Mtb infected Bhlhe40\textsuperscript{GFP} and Irg1\textsuperscript{-/-}Bhlhe40\textsuperscript{GFP} mice. \textit{In vitro} studies of GM-CSF-cultured cells treated with itaconate could use Western blotting and mass spectrometry to determine whether itaconate might directly repress Bhlhe40 through post-translational modification leading to degradation, as has been shown for KEAP1.\textsuperscript{156} Comparison of the resulting transcriptomic changes in WT, Bhlhe40\textsuperscript{+/+}, and Irg1\textsuperscript{-/-} cells of the same type would enable large scale conclusions on how their effects synergize to promote protective responses to Mtb. DCs may be an appropriate starting cell type for such a transcriptional comparison since we have demonstrated that expression of both Bhlhe40 and Irg1 in CD11c+ cells is essential for resistance to Mtb.

\textbf{Closing remarks}

The work presented in this dissertation has relied heavily on insights gained from studies on the functions of Bhlhe40 in neuroinflammation and Irg1 in macrophage activation. The unexpected roles that these genes had in regulating inflammatory responses provided a rationale for investigation of their function during Mtb infection. Using mice with global and conditional
deletions of Bhlhe40 and Irg1 in conjunction with in vitro models we have discovered novel and essential components of aspects of protective immunity to Mtb.

Prior to these studies it was unclear why the broadly anti-inflammatory cytokine IL-10 had such mild effects on control of Mtb infection. A multitude of reports in other disease models had demonstrated the potential of IL-10 to suppress protective immune responses but its effects on Mtb infection appeared to be limited to chronic infection and varied by genetic background. The work presented here has revealed that IL-10 can compromise protective immunity to Mtb if overexpressed during acute infection and has identified Bhlhe40 as an essential transcriptional regulator that prevents over-expression of IL-10. This regulatory function of Bhlhe40 is one mechanism by which the immune system promotes host-protective responses by suppressing non-protective responses.

Irg1 had gained a great deal of interest for its ability to generate a potentially antibacterial effector but little attention was given to its immunomodulatory capabilities. We demonstrate here that Irg1 has an unexpected and essential role in preventing pathologic inflammation by limiting neutrophil recruitment. This selective immunosuppression allows the immune system to avoid responses that fail to limit Mtb replication and severely damage the lung.

These studies establish the ability of Bhlhe40 and Irg1 to directly and indirectly tune the inflammatory response to Mtb at a transcriptional level. In summation, my dissertation work advances understanding of protective immunity to Mtb by revealing novel mechanisms used by specific innate and adaptive immune cell types to promote bactericidal activity and suppress pathologic inflammation.
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