ARTICLE

Bhlhe40 is an essential repressor of IL-10 during *Mycobacterium tuberculosis* infection

Jeremy P. Huynh1*, Chih-Chung Lin2*, Jacqueline M. Kimmey1, Nicholas N. Jarjour2, Elizabeth A. Schwarzkopf2, Tara R. Bradstreet2, Irina Shchukina3, Oleg Shpynov3,4, Casey T. Weaver5, Reshma Taneja1, Maxim N. Artyomov2, Brian T. Edelson2, and Christina L. Stallings1

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 the disease outcome and the pathology of tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) infection. IFN-γ signaling through the transcription factor STAT1 is essential for the control of mycobacterial infections in humans and mice (Cooper et al., 1993; Flynn et al., 1993; MacMicking et al., 2003; Bustamante et al., 2014). IL-10 is an immunoregulatory cytokine produced by innate and adaptive immune cell types (Gabryšová et al., 2014; Moreira-Teixeira et al., 2017) that antagonizes IFN-γ–associated pathways by suppressing macrophage responsiveness to IFN-γ (Gazzinelli et al., 1992), modulating T-helper (Th1) 1 cell IFN-γ production (Turner et al., 2002; Beamer et al., 2008; Redford et al., 2010), and restricting production of the hallmark Th1-inducing cytokine IL-12 (Roach et al., 2001; Demangel et al., 2002; Schreiber et al., 2009). IL-10 can also inhibit dendritic cell (DC) migration (Demangel et al., 2002) and limit secretion of myeloid cell–derived proinflammatory cytokines (de Waal Malefyt et al., 1991). 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 the genetic background and is generally mild (Roach et al., 2001; Beamer et al., 2008; Redford et al., 2010). 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 (Moreira-Teixeira et al., 2017). Overexpression of IL-10 in mice has 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 (Murray et al., 1997; Feng et al., 2002; Turner et al., 2002; Schreiber et al., 2009).

Given the potential for IL-10 to negatively impact protective immune responses, cell-intrinsic 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 (Redford et al., 2011). In addition, different Th1 cell subsets produce IL-10 in response to distinct combinations of cytokines (Gabryšová et al., 2014). 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 (Saraiva and O’Garra, 2010; Gabryšová et al., 2014; Hörber et al., 2016). Much less is known about transcriptional pathways that limit the production of IL-10 (Iyer and Cheng, 2012). In this study, we report that the transcription factor basic helix-loop-helix family member e40 (Bhlhe40) serves an essential role in resistance to *Mtb* infection by repressing *Il10* expression in both T cells and myeloid cells.

1Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO; 2Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO; 3JetBrains Research, Saint Petersburg, Russia; 4Department of Pathology, University of Alabama at Birmingham, Birmingham, AL; 5Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore.

*J.P. Huynh and C.-C. Lin contributed equally to this paper; Correspondence to Christina L. Stallings: stallings@wustl.edu; Brian T. Edelson: bedelson@path.wustl.edu.*
Results
Bhlhe40 is required to control Mtb infection
We have previously shown that the transcription factor Bhlhe40 regulates cytokine production by T cells in a mouse model of multiple sclerosis (Lin et al., 2014, 2016). When we analyzed publicly available whole-blood gene expression datasets (Berry et al., 2010; Maertzdorf et al., 2011; Bloom et al., 2013), we found that BHLHE40 transcripts were present at a significantly lower abundance in patients with active TB as compared with healthy controls, those with latent TB 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 patients with active TB (Fig. 1A) as previously noted (Berry et al., 2010; Bloom et al., 2013). This finding of decreased BHLHE40 expression in patients with active TB led us to investigate a role for this transcription factor during Mtb infection in mice. We infected Bhlhe40+/+, Bhlhe40−/−, and Bhlhe40−/+ mice on the C57BL/6 background with the Mtb Erdman strain and monitored morbidity and mortality. Bhlhe40−/− and Bhlhe40−/+ mice displayed no signs of morbidity and survived beyond 100 d postinfection (dpi). Bhlhe40−/+ mice began losing weight at ~21 dpi and succumbed to infection between 32 and 40 dpi with a median survival time of 33 d (Fig. 1, B and C). This severe susceptibility phenotype is similar to that of mice lacking STAT1 (MacMicking et al., 2003) and is more severe than that of mice lacking NF-kB p50 (Yamada et al., 2001), both of which are central transcriptional regulators of the immune system. By 21 dpi, Mtb CFUs in Bhlhe40−/− mice were 23-fold higher in the lung and fivefold higher in the spleen as compared with Bhlhe40+/+ mice (Fig. 1, D and E). 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. Upon infection with the intracellular bacterium Listeria monocytogenes, Bhlhe40−/− mice showed no increased susceptibility (Fig. 1, F and G) and mounted a robust response to secondary L. monocytogenes challenge (Fig. 1G), indicating that their susceptibility to Mtb was caused by an impaired response specific to this pathogen.

Bhlhe40 deficiency leads to neutrophil-dominated inflammation
Shortly before succumbing to infection, it was evident by gross examination that Bhlhe40−/− lungs had developed larger lesions than Bhlhe40+/+ lungs (Fig. 2A). 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 Bhlhe40+/+ lungs by 21 dpi (Figs. 2B and S1A). We analyzed these 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−/− mice, and the absolute number of neutrophils in Bhlhe40−/− lungs was three times greater than in Bhlhe40+/+ lungs (Fig. 2, C and D). The number and frequency of neutrophils in the lungs of Bhlhe40−/− mice further increased as the infection progressed (Fig. 2, C and D). The number of CD11b+Ly6C(high) monocyte-derived DCs (moDCs) was 12-fold lower in Bhlhe40−/− mice at 21 dpi, suggesting that Bhlhe40 may regulate the development, recruitment, or survival of this population (Fig. S1B). There were no significant differences in the populations sizes of other myeloid or lymphoid cell types analyzed at 21 dpi (Fig. S1B), nor were there any differences in the frequency of Mtb antigen-specific T cells in the lungs or mediastinal lymph nodes (Fig. S1C).

The timing of the increased inflammation correlated with our initial observation of higher Mtb burden in the lungs of Bhlhe40−/− mice, leading us to investigate whether the accumulating myeloid cells were infected with Mtb. We infected Bhlhe40−/+ and Bhlhe40−/− 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+ macrophages, inflammatory monocytes, moDCs, and CD11b+ DCs were all infected at a higher frequency in Bhlhe40−/− lungs compared with Bhlhe40−/+ lungs (Fig. 2, E and F). Absolute numbers of infected neutrophils, inflammatory monocytes, and CD11b+ DCs were also significantly higher in Bhlhe40−/− lungs (Fig. S1D), suggesting that Mtb residing within these cell types accounts for the difference in pulmonary Mtb burden in Bhlhe40−/− lungs at 21 dpi. Infected Bhlhe40−/− neutrophils, inflammatory monocytes, moDCs, and CD11b+ DCs also exhibited an increase in mean fluorescence intensity (MFI) for GFP compared with infected Bhlhe40−/+ neutrophils, suggesting that they harbored more Mtb on a per-cell basis (Fig. S1E).

To test whether the influx of neutrophils contributed to the susceptibility of Bhlhe40−/− mice, we used an anti-Ly6G monoclonal antibody to specifically deplete neutrophils between 10 and 30 dpi (Kimmey et al., 2015). Neutrophil-depleted Bhlhe40−/− mice did not exhibit any improvements in morbidity, survival time, or control of Mtb replication (Fig. S1, F–H). Therefore, although neutrophils serve as a prominent replicative niche for Mtb, they are not the sole cause of susceptibility to Mtb infection in Bhlhe40−/− mice.

Bhlhe40 functions in innate and adaptive immune cells
Bhlhe40 is expressed in specific immune cell populations and in nonhematopoietic tissues (Ow et al., 2014). To dissect which cells required Bhlhe40 expression during Mtb infection, we first generated reciprocal and control bone marrow chimeraic mice, infected them with Mtb, and monitored for survival (Fig. 3A). As expected, all but one Bhlhe40−/−→Bhlhe40−/+ chimeric mouse survived beyond 100 dpi, and Bhlhe40−/+→Bhlhe40−/− chimeras succumbed between 27 and 35 dpi (median survival time of 33 d). Bhlhe40−/+→Bhlhe40−/− chimeras also survived past 100 dpi, whereas Bhlhe40−/−→Bhlhe40−/+ chimeras died between 29 and 37 dpi (median survival time of 32 d), similar to the 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−/−→Bhlhe40−/+ GFP reporter mice (Bhlhe40GFP; Schmidt et al., 2013; Lin et al., 2016). We monitored GFP expression as a proxy for Bhlhe40 expression in the lungs of naive and Mtb-infected Bhlhe40−/−GFP mice. Sizeable fractions of neutrophils, alveolar macrophages, CD11b+ DCs, CD103+ DCs, and eosinophils expressed GFP both before infection and at 21 dpi.
Huynh et al.
Bhlhe40 represses IL-10 during M. tuberculosis infection

3

(Fig. S2), with smaller fractions of CD11b+ macrophages, moDCs, B cells, and CD8+ T cells also showing this pattern of expression. Therefore, these cell types constitutively express Bhlhe40, and this expression pattern does not change during Mtb infection. In contrast, CD4+ T cells in naive mice expressed very little Bhlhe40, but at 21 dpi, Bhlhe40 levels increased in this cell type (Fig. S2). These data show that Bhlhe40 is expressed in both myeloid and lymphoid cells during Mtb infection.

Bhlhe40 expression is required in CD4+ T cells to regulate cytokine production in the experimental autoimmune encephalomyelitis (EAE) model of autoimmunity (Martínez-Llordella et al., 2013; Lin et al., 2014). Bhlhe40−/− mice were infected with 100–200 CFU aerosolized Mtb and monitored for body weight (B, n = 6 per group), survival (C, n = 9–10 per group), pulmonary Mtb burden (D, n = 8 per group), and splenic Mtb burden (E, n = 8 per group). (F) Mice were infected with 10^6 or 10^7 CFU of L. monocytogenes i.v. and monitored for survival (n = 5 per group). The dotted lines in E and G indicate limits of detection (500 [E] and 100 [G] CFU). Each point represents data from one human or mouse. The mean ± SEM is graphed. Statistical differences were determined by two-tailed unpaired Student’s t test, unpaired one-way ANOVA, or unpaired Kruskal-Wallis test (A), log-rank Mantel-Cox test (C and F), and two-tailed unpaired Student’s t test for normally distributed groups or two-tailed unpaired Mann-Whitney test for nonnormally distributed groups (D, E, and G). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from one (F and G) or two independent experiments (B–E).

To measure the contribution of Bhlhe40 to host resistance in the absence of mature B and T cells, we compared the survival of Mtb-infected Bhlhe40+/+, Bhlhe40−/−, Rag1−/−, and Rag1−/− Bhlhe40−/− mice. Rag1−/− Bhlhe40−/− mice (median survival time of 33 d) were significantly more susceptible than Rag1−/− mice (median survival time of 43 d) to Mtb infection and succumbed to infection at the same time as Bhlhe40−/− mice (Fig. 3B). These data demonstrate that Bhlhe40 is required during the innate immune response in the absence of T cells to prolong survival during Mtb infection. Mtb CFUs were fourfold higher in Rag1−/− Bhlhe40−/− mice compared with Rag1−/− mice at 21
dpi (Fig. 3 C), and the frequency of neutrophils in the lungs of 
\textit{Rag1}^{−/−} \textit{Bhlhe40}^{−/−} mice was also significantly elevated (Fig. S3 A). The frequency of infected neutrophils, CD11b+ macrophages, and moDCs was significantly higher in \textit{Rag1}^{−/−} \textit{Bhlhe40}^{−/−} mice at 21 dpi (Fig. S3 B). We also observed significantly higher MFIs for GFP in \textit{Rag1}^{−/−} \textit{Bhlhe40}^{−/−} neutrophils, CD11b+ macrophages, inflammatory monocytes, and CD11b+ DCs, suggesting that these cell types harbored more \textit{Mtb} on a per-cell basis compared with \textit{Rag1}^{−/−} cells (Fig. S3 C). Therefore, loss of \textit{Bhlhe40} in innate immune cells compromises their ability to control inflammation and \textit{Mtb} replication independent of adaptive immunity.

To determine more precisely which cells require \textit{Bhlhe40} expression to control \textit{Mtb} infection, we infected mice that conditionally delete \textit{Bhlhe40} in specific cell types. After \textit{Mtb} infection, \textit{Bhlhe40}^{−/−}\textit{Lysm-Cre} and \textit{Bhlhe40}^{−/−}\textit{Cd11c-Cre} mice showed no signs of morbidity (Fig. S3 D) and survived past 100 dpi (Fig. 3 D), indicating that loss of \textit{Bhlhe40} in LysM+ or Mrp8+ cells was not sufficient to generate susceptibility to infection. In contrast, \textit{Bhlhe40}^{−/−}\textit{Cd11c-Cre} and \textit{Bhlhe40}^{−/−}\textit{Cd4-Cre} mice succumbed to infection at 34–62 d (median survival time of 56 d) and 31–73 d (median survival time of 52 d), respectively (Fig. 3 D). Therefore, \textit{Bhlhe40} expression in both T cells and CD11c+ cells is required to control \textit{Mtb} infection. Given that the \textit{Lysm} promoter can drive conditional deletion in alveolar macrophages but deletes poorly in myeloid DCs, whereas the \textit{Cd11c} promoter deletes equally well in both alveolar macrophages and DCs (Abram et al., 2014), one interpretation is that loss of \textit{Bhlhe40} in alveolar macrophages is not sufficient to cause susceptibility and that loss of \textit{Bhlhe40} in DCs contributes to the susceptibility observed in \textit{Bhlhe40}^{−/−}\textit{Cd11c-Cre} mice.

We next analyzed susceptible \textit{Bhlhe40}^{−/−}\textit{Cd4-Cre} and \textit{Bhlhe40}^{−/−}\textit{Cd11c-Cre} mice to determine how loss of \textit{Bhlhe40} in CD11c+ or T cells contributed to phenotypes observed in \textit{Bhlhe40}^{−/−} mice. By 21 dpi, pulmonary \textit{Mtb} burden in \textit{Bhlhe40}^{−/−}.
Cd4-Cre and Bhlhe40fl/fl-Cd11c-Cre mice was four- and threefold higher than Bhlhe40fl/fl controls, respectively (Fig. 3 E). Bhlhe40fl/fl-Cd4-Cre and Bhlhe40fl/fl-Cd11c-Cre mice also exhibited an increase in the frequency and absolute number of pulmonary neutrophils (Fig. 3 F). 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 (Figs. 3 F and S1 B). Neutrophils, inflammatory monocytes, moDCs, and CD11b+ DCs were infected at a higher frequency in both Bhlhe40fl/fl-Cd4-Cre and Bhlhe40fl/fl-Cd11c-Cre mice (Fig. 3 G). Although survival, Mtb burden, neutrophilic inflammation, and frequency of cellular infection phenotypes were evident in Bhlhe40−/−, Cd4-Cre and Bhlhe40−/−, Cd11c-Cre mice, they were less severe than in Bhlhe40−/− mice, suggesting that defects caused by Bhlhe40 deficiency in Cd11c+ cells, T cells, and potentially other cell types combine to undermine protective responses to Mtb.

Bhlhe40 represses IL-10 production after exposure to Mtb

Bhlhe40 has previously been found to regulate cytokine production by CD4+ T cells in the EAE model (Martínez-Llordella et al., 2013; Lin et al., 2014). If Bhlhe40 functions analogously during Mtb infection, Bhlhe40−/− cells could exert a dominant effect on Bhlhe40+/+ cells through the production of secreted cytokines. We tested this by generating mixed bone marrow chimeric mice. Congenically marked Bhlhe40+/+ (CD45.1/.2) recipients were lethally irradiated and reconstituted with a 1:1 mixture of Bhlhe40+/+ (CD45.1/1) and Bhlhe40−/− (CD45.2/2) bone marrow cells. Control mice included mixed chimeras generated with Bhlhe40+/+ (CD45.1/1) and Bhlhe40−/− (CD45.2/2) bone marrow cells as well as nonchimeric Bhlhe40+/+ (CD45.2/2) and Bhlhe40−/− (CD45.2/2) mice. In mixed chimeras, Bhlhe40−/− bone marrow cells were capable of normal reconstitution of the hematopoietic compartment within the lung (Fig. 4 A). Chimeric mice were infected with GFP-expressing Mtb and analyzed at 21 dpi. Bhlhe40+/+ + Bhlhe40−/− chimeras controlled Mtb infection with little neutrophilic infiltrate (unpublished data) and a low
frequency of neutrophil infection (Fig. 4, B and C). Lungs of mixed \( \text{Bhlhe40}^{+/+} + \text{Bhlhe40}^{-/-} \) chimeras contained large populations of infiltrating neutrophils of both genotypes (unpublished data). Neutrophils of both genotypes displayed a high frequency of \( \text{Mtb} \) infection (Fig. 4, B and C), which correlated with a trend toward higher CFUs in the lungs of mixed \( \text{Bhlhe40}^{+/+} + \text{Bhlhe40}^{-/-} \) chimeras (Fig. S3 E). These results demonstrate that \( \text{Bhlhe40}^{-/-} \) cells, even when present as only half of all hematopoietic cells, exert a dominant influence in trans on the ability of \( \text{Bhlhe40}^{+/+} \) cells to control neutrophil accumulation and \( \text{Mtb} \) infection.

These data indicate that \( \text{Bhlhe40} \) 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 \( \text{Bhlhe40}^{+/+} \) and \( \text{Bhlhe40}^{-/-} \) mice by quantitative RT-PCR. At 21 dpi, transcripts for the neutrophil-associated cytokines \( \text{Csf3} \) (G-CSF), \( \text{Ccl3} \) (MIP-1α), and \( \text{Ccl4} \) (MIP-1β) were up-regulated in \( \text{Bhlhe40}^{-/-} \) lungs (Fig. 4 D) as expected given the neutrophil-dominated inflammation observed. We also found that at 21 dpi, \( \text{Il10} \) (IL-10) transcript levels were three-fold higher in \( \text{Bhlhe40}^{-/-} \) lungs, and \( \text{Il12b} \) (IL-12/23p40) and \( \text{Ifng} \) (IFN-γ) transcript levels were three- and twofold lower in \( \text{Bhlhe40}^{-/-} \) lungs, respectively, compared with \( \text{Bhlhe40}^{+/+} \) lungs (Fig. 4 D). This finding was of particular interest because \( \text{Bhlhe40} \) represses \( \text{Il10} \) transcription in CD4+ T cells in the EAE model (Lin et al., 2014) and IL-10 has been shown to inhibit \( \text{Il12b} \) and IFN-γ expression during \( \text{Mtb} \) infection (Roach et al., 2001; Turner et al., 2002; Schreiber et al., 2009; Redford et al., 2010). In the lungs of naive \( \text{Bhlhe40}^{+/+} \) and \( \text{Bhlhe40}^{-/-} \) mice, there were no differences in the levels of \( \text{Ifng} \) and \( \text{Csf3} \) transcript, and \( \text{Il10} \) and \( \text{Il12b} \) transcript levels were below the level of detection (unpublished data), indicating that loss of \( \text{Bhlhe40} \) impacts expression of these genes during \( \text{Mtb} \) infection but not in naive mice.

The increased \( \text{Il10} \) transcript levels in conjunction with decreased \( \text{Il12b} \) and \( \text{Ifng} \) transcript levels in \( \text{Bhlhe40}^{-/-} \) lungs at 21 dpi indicated that \( \text{Bhlhe40} \) could be regulating \( \text{Il10} \) expression. To identify the cell types responsible for the increased \( \text{Il10} \) expression in \( \text{Bhlhe40}^{-/-} \) lungs, we used \( \text{Tg} \, \text{Il10} \) bacterial artificial chromosome (BAC)-transgene (10BiT) IL-10 reporter mice (Maynard et al., 2007), where \( \text{Il10} \)-expressing cells display Thy1.1 on their surface. We observed a similar distribution of \( \text{Il10} \)-expressing cells in 10BiT + \( \text{Bhlhe40}^{+/+} \) and \( \text{Bhlhe40}^{-/-} \) lungs at 21 dpi (n = 5 per group).
sources of IL-10 before and after Mtb infection (Moreira-Teixeira et al., 2017). We crossed 10BiT and Bhlhe40−/− mice to generate a Bhlhe40−/− 10BiT-positive strain, and then we analyzed Thy1.1 expression as a proxy for IL10 expression. We observed low levels of Thy1.1 expression in naive 10BiT− Bhlhe40+/− and 10BiT− Bhlhe40−/− mice (Fig. S4 A). At 21 dpi, no differences were observed when comparing the absolute number of Thy1.1+ cells, but the frequency of Thy1.1+ neutrophils, inflammatory monocyes, moDCs, CD11b+ DCs, B cells, CD4+ T cells, and CD8+ T cells was significantly higher in Bhlhe40−/− mice (Figs. 4 E and S4, B and C). In addition, when we compared the MFIs for Thy1.1 on Thy1.1− cells, we found that not only were there a higher percentage of Bhlhe40−/− moDCs and B cells expressing Thy1.1, but these cell types also expressed more Thy1.1 on a per-cell basis during Mtb infection (Fig. 4 F). Significantly higher Thy1.1 MFI was also observed on Bhlhe40−/− Thy1.1+ alveolar macrophages and CD11b+ macrophages (Fig. 4 F). The increased frequency of Thy1.1 positivity observed in Bhlhe40−/− CD4+ T cells was even more pronounced in Bhlhe40−/− Mtb−specific CD4+ T cells in the mediastinal lymph node and lung, indicating that Mtb-specific CD4+ T cells take on an immunosuppressive phenotype in the absence of Bhlhe40 (Fig. 4 G). These data demonstrated that the frequency and per-cell expression of IL10 was increased in multiple Bhlhe40−/− myeloid and lymphoid populations.

Bhlhe40 suppresses IL-10 expression in myeloid cells in vitro
We sought to identify an in vitro myeloid cell culture system that recapitulates the effects of Bhlhe40 deficiency on IL-10 production in response to Mtb. We cultured Bhlhe40−/− bone marrow cells with GM-CSF or macrophage CSF (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 (Helft et al., 2015; Poon et al., 2015), express Bhlhe40 (Fig. 5 A). Analysis of GM-CSF− and M-CSF−cultured cells from Bhlhe40ΔGFP reporter mouse bone marrow confirmed that only GM-CSF−cultured bone marrow−derived cells expressed GFP (Fig. 5 B). 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. S2; Heng et al., 2008; Lin et al., 2016), where GM-CSF is abundant and plays an important part in the development and function of lung myeloid cells (Kopf et al., 2015). 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. S5 A), suggesting that Bhlhe40 is dispensable for the ability of these cell types to control Mtb replication.

We next assessed IL-10 expression from Bhlhe40+/+ and Bhlhe40−/− cells cultured with GM-CSF. In the absence of stimulation with heat-killed Mtb, minimal IL-10 protein was detected in culture supernatants (Fig. 5 C). In contrast, Bhlhe40+/+ GM-CSF−cultured bone marrow−derived cells stimulated with heat-killed Mtb for 24 h produced significantly more IL-10 than Bhlhe40−/− cells (Fig. 5 C). We also stimulated 10BiT+ Bhlhe40+/+ 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 Bhlhe40+/+ and Bhlhe40−/− cultures relative to unstimulated cultures, and this increase was greater in Bhlhe40+/+ cells compared with Bhlhe40−/− cells (Fig. 5 D). 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 Bhlhe40+/+ GM-DCs (Fig. 5 E). ELISAs confirmed that 10BiT Bhlhe40−/− cells secreted more IL-10 than 10BiT Bhlhe40+/+ cells after stimulation with heat-killed Mtb (Fig. 5 F). These experiments confirm that loss of Bhlhe40 in GM-CSF−cultured cells results in higher levels of IL-10 production.

Bhlhe40 binds directly to the IL10 locus in T1,1 cells and myeloid cells
We performed chromatin immunoprecipitation sequencing (ChIP-seq) experiments in in vitro−polarized T1,1 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 this research with Bhlhe40+/+ and Bhlhe40−/− cells, where Bhlhe40−/− cells served as controls for nonspecific binding by the anti-Bhlhe40 antibody. Bhlhe40 bound 379 sites in Bhlhe40+/+ GM-CSF−cultured cells and 5,532 sites in Bhlhe40−/− T1,1−polarized T cells (Tables S1 and S2). 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 CACCGTG (St-Pierre et al., 2002). Motif finding identified the expected E-box (CACGGTG) as the most frequent motif present within the peaks identified in both cell types (Fig. 6 A). Analysis of binding sites revealed that Bhlhe40 predominantly bound promoters within 1 kb of the transcriptional start site, introns, and distal intergenic regions (Fig. 6 B). Pathway analysis of predicted Bhlhe40−regulated genes revealed an enrichment in immune activation and cytokine response pathways in both datasets (Fig. 6 C). Bhlhe40 is known to bind a conserved autoregulatory site in the Bhlhe40 promoter (Sun and Taneja, 2000), and this binding site was identified as a peak in both datasets (Fig. 6 D). 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 T1,2 cells (Fig. 6 D; Jones and Flavell, 2005). 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 (Tables S1 and S2). 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 T1,1 cells (Fig. S5 B). These findings suggest that the transcriptional down-regulation of Il12b in Bhlhe40+/+ total lung samples (Fig. 4 D) 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−/− mice from Mtb infection
To investigate the role of IL-10 production in the susceptibility of Bhlhe40−/− mice, we generated Il10−/− Bhlhe40−/− mice and...
compared their survival to *Bhlhe40*+/+, *Bhlhe40*−/−, and *Il10*−/− mice after *Mtb* infection. The absence of IL-10 signaling resulted in near-complete rescue of the susceptibility phenotype caused by *Bhlhe40* deficiency as shown by the significant increase in the median survival time of *Il10*−/− *Bhlhe40*+/+ mice (31 d; Fig. 7 A). The increased survival of *Il10*−/− *Bhlhe40*+/+ mice was accompanied by an eightfold decrease in pulmonary *Mtb* titer in *Il10*−/− *Bhlhe40*+/+ lungs compared with *Bhlhe40*−/− lungs at 21 dpi (Fig. 7 B). These data demonstrate that the inability of *Bhlhe40*−/− mice to control *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. 7 C and D). However, the frequency of neutrophils in *Il10*−/− *Bhlhe40*−/− lungs remained higher than in *Bhlhe40*−/− or *Il10*−/− lungs (Fig. 7 C and D). *Il10*−/− *Bhlhe40*−/− lungs also contained twofold 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. 7 E). 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. 8). 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 (Gabryšová et al., 2014). 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 (Cooper et al., 1993; Flynn et al., 1993; Hölscher et al., 2001). These findings represent the first study of roles for *Bhlhe40* in the immune response to an infection and within myeloid cells. Additionally, although other negative regulators of *Il10* transcription have been described (Riemann et al., 2005; Yee et al., 2005; Villagra et al., 2009; Kang et al., 2010; Krausgruber et al., 2011), *Bhlhe40* is the first transcription factor that controls *Mtb* infection.
Figure 6. **Bhlhe40 directly binds the 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 Mtb for 4 h. CD4+ T cells were T₆₁ polarized in vitro for 4 d. 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 (3.5). (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 and Il10 loci in Bhlhe40⁺/⁺ 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.
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 TB disease in humans.

Survival experiments showed that about one quarter of Bhlhe40fl/fl-Cd11c-Cre and Bhlhe40fl/fl-Cd4-Cre mice recapitulated the very early susceptibility of Bhlhe40−/− mice (Fig. 3D), with the remainder of these Bhlhe40 conditionally deleted mice dying between 50 and 75 dpi. In the case of the 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, in Bhlhe40−/− mice, Mtb lung burdens are higher, and neutrophil-dominated inflammation is uncontrolled, ultimately resulting in susceptibility.

Figure 7. IL-10 deficiency protects Bhlhe40−/− mice from Mtb infection. (A and B) Bhlhe40+/+, Bhlhe40−/−, Il10−/−, and Il10−/−Bhlhe40−/− mice were monitored for survival after Mtb infection (A; n = 9–10 per group) or analyzed for pulmonary CFU at 21 dpi (B; n = 6–12 per group). The number of biological replicates is indicated in parentheses. (C) Representative flow cytometry plots for lung neutrophils as percentages of the total CD45+ population at 21 dpi. (D) The absolute neutrophil count and the frequency of neutrophils in the total lung CD45+ population at 21 dpi (n = 6–12 per group). (E) Cytokine transcript levels in total lung samples at 21 dpi (n = 4–5 per group). Each point represents data from one mouse. 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 nonnormally distributed groups (B, D, and E). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from two (A and E) or four (B and D) independent experiments. Data in C are representative of four independent experiments.
remained in the absence of Il10. For example, although the susceptibility of Bhlhe40+/+ and Il10−/− Bhlhe40+/+ mice was not significantly different, we did observe that ∼20% of the Il10−/− Bhlhe40+/+ mice succumbed before 100 dpi (Fig. 7A). Additionally, Il10−/− Bhlhe40+/+ mice had a significantly higher frequency of neutrophils in the lungs at 21 dpi compared with Bhlhe40+/+ mice (Fig. 7, C and 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 TB have increased levels of IL-10 in their serum (Verbon et al., 1999; Olobo et al., 2001) and lungs (Barnes et al., 1993; Huard et al., 2003; Bonecini-Almeida et al., 2004; Almeida et al., 2009), suggesting a link between increased IL-10 levels and TB disease (Redford et al., 2011). Although it is unclear whether the reduced expression of BHLHE40 in the blood of patients with active TB (Fig. 1A) is a cause or effect of their disease or related to the higher IL-10 levels found in this form of TB, this correlation agrees with the increased susceptibility of mice lacking Bhlhe40 and suggests that our experiments could be relevant to human Mtb infection.

Materials and methods
Bacterial 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, 0.5% glycerol, and 0.05% Tween-80 (broth only). GFP-expressing Mtb (GFP-Mtb) was generated by transformation of Mtb strain Erdman with a plasmid (pMV261-kan-GFP) that drives constitutive expression of GFP under the control of the mycobacterial hsp60 promoter. Cultures were grown in the presence of kanamycin to ensure plasmid retention.

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), Bhlhe40GFP BAC Tg (N10 to C57BL/6; Sun et al., 2001), Cd11c-Cre (B6. Cg-Tg(Itgax-cre)-1Reiz/J; 008068), and Cd4-Cre (B6.Cg-Tg(Cd4-cre)1Cwi/B6j; 022071), all from The Jackson Laboratory.

Age-matched littermate adult mice (9–23 wk 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 for 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 CO2 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 Bhlhe40+/+ or Bhlhe40−/− recipients and reconstitution with 1–2 × 106 bone marrow cells from Bhlhe40+/+ or Bhlhe40−/− donors. Mixed bone marrow chimeric mice were generated by lethal irradiation (1,000 rads) of Bhlhe40−/−/CD45.1/2 recipients and reconstitution with 1–2 × 106 bone marrow cells from Bhlhe40−/−/CD45.1/1 and either Bhlhe40+/− (CD45.1/2) or Bhlhe40−/− (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 wk after reconstitution and were allowed to reconstitute for at least 8 wk 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 NH4Cl, 10 mM KHCO3, 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 + 1× penicillin/streptomycin + 55 μM β-mercaptoethanol + 1× 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 + 1× penicillin/
streptomyacin + 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 d. 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 × 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) h. In some experiments, suspension cells were stimulated in the presence of 10 μg/ml heat-killed Mtb (H37Ra strain; Difco) for 4 h for ChIP assays.

To generate in vitro–polarized T helps 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 I45-2C11; 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 (250 U IFN-γ (BioLegend) for 12 h before infection.

were initially infected with a sublethal dose of 10⁵ Mtb (H37Ra strain; Difco) for 4 h.

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 (53–67), APC anti-CD11b (M1/70), BV605 anti-CD11c (N418), BV605 anti-CD19 (6D5), APCanti-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 (IA8), PB anti-TCRβ (H57-597), and APC anti-TCRγ (GL3). The following anti-mouse antibodies were purchased from BD: PB anti-CD3e (145-2C11), BV510 anti-CD45 (50F11), 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-Ly6-6 (IA8), and redFluor710 anti-I-A/I-E (MS5/114.15.2).

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.

For identification of antigen–specific T cells, APC-conjugated tetrabodies of Ag85B280–294 peptide (GQDNAYAGGHNAVF) or ESAT6.17 peptide (QQWNFAEIEAAA) bound to MHC-II (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⁺/CD3e⁺/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 Fig. S5 (C and D). Gates for Tcol (Bilhe40COMP and 10BiT) mice were set using nontransgenic mice to control for background staining.

Neutrophil depletion

Mice were intraperitoneally injected with 200 μg monoclonal anti-Ly6G antibody (clone IA8; BioXCell or Leinco) or 200 μg polyclonal rat serum IgG (Sigma-Aldrich) diluted in sterile PBS (HyClone) every 48 h beginning at 10 dpi and ending at 30 dpi.

Quantitative RT-PCR

Lung samples were lysed by bead-beating in TRIzol reagent (Invitrogen), pelleted to remove beads, and stored at ~80°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 OligoT primers (Thermo Fisher Scientific). Quantitative RT-PCR was performed using iTAQ SYBR green (Bio-Rad Laboratories) on a CI000 thermal cycler with the CFX96 real-time system (Bio-Rad Laboratories). Transcript levels were analyzed using the 2⁻ΔΔCt method normalized to Actb (β-actin) as the reference gene. The following primers were used: Actb forward, 5’-AGCTTCCTCATGAGCTGGG-3’; Actb reverse, 5’-CTGGATGCTACGTACATGG-3’; Ccl3 forward, 5’-AACACTCTGCACCAAGTCTTC-3’; Ccl3 reverse,

Huynh et al.
Bilhe40 represses IL-10 during M. tuberculosis infection

Journal of Experimental Medicine
https://doi.org/10.1084/jem.20171704
Bone marrow–derived cells were counted and lysed at 10^6/40 µl 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 BioBlot-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 (GeneRays, Inc.) at RT for 60 min with shaking. 

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.

ChIP-seq

Anti-Bhlhe40 ChIP was modified from the protocol published by Huynh et al. (2016). GM-CSF–cultured cells were harvested after 9 d and stimulated with 10 µg/ml heat-killed Mtb (strain H37Ra; Difco) for 4 h. In vitro–polarized T_hil cells were stimulated with PMA (50 ng/ml; Enzo Life Sciences) and ionomycin (1 µM; Enzo Life Sciences) for 1.5 h. 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_hil 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 Bhlhe40/− samples. Peak calling was performed using MACs v1.4 (Zhang et al., 2008) 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; Machanick and Bailey, 2011) 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 cis-regulatory regions for Gene Ontology Biological Process and MSigDB Pathway gene sets (McLean et al., 2010).

Links for raw sequencing data are available at GSE113054. All primary processed data (including mapped reads) for ChIP-seq experiments are also available there.

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 publically available GEO datasets (GSE19491 [Berry et al., 2010], GSE28623 [Maertzdorf et al., 2011], and GSE42834 [Bloom et al., 2013]) that compared the whole-blood transcriptomes of humans with active TB to other humans with either no disease, latent TB, or in some cases, lung cancer, pneumonia, or sarcoidosis. The following probesets were used to examine the expression of BHLHE40 (previously called BHLLHB2): for GSE19491 and GSE42834, ILMN_1768534; and for GSE28623, Agilent Technologies feature number 37838. The following probesets were used to examine the expression of 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

All data are from at least two independent experiments unless otherwise indicated. 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 nonnormal distributions), one-way ANOVA with Tukey’s multiple comparisons tests (to compare more than two groups with normal distributions), or unpaired Student’s t tests (to compare two groups with nonnormal distributions).
Kruskal–Wallis tests with Dunn’s multiple comparisons tests (to normality was determined using a D’Agostino–Pearson omnibus 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.

Online supplemental material
Fig. S1 provides additional data on pulmonary histology, hematopoietic cell population numbers, Mtb-specific T cell frequencies, the number and MFI of Mtb+ lung cells, and analysis of neutrophil-depleted mice. Fig. S2 contains in vivo expression patterns of Bhlhe40 before and after Mtb infection. Fig. S3 provides additional analysis of Mtb-infected Ragt−/−/Bhlhe40−/−, Bhlhe400/f/f Lysm-Cre, Bhlhe400/f/f Mrp8-Cre, and mixed bone marrow chimeric mice. Fig. S4 shows representative flow cytometry plots and absolute cell counts for lung immune cells from 10BiT IL-10 reporter mice. Fig. S5 shows that Bhlhe40−/− GM-CSF–cultured cells are not defective in control of Mtb infection, putative Bhlhe40 binding sites in the Ifng locus, and the flow cytometry gating strategies used throughout this study. Data on Bhlhe40 binding sites identified by ChIP-seq, predicted functions of associated cis-regulatory regions, and associated gene ontology analysis in GM-CSF–cultured cells and T1i-polarized T cells are available in Tables S1 and S2, respectively.

Acknowledgments
C.L. Stallings is supported by an Arnold and Mabel Beckman Foundation Beckman Young Investigator Award and a Burroughs Wellcome Fund Investigators in the Pathogenesis of Infectious Disease award. B.T. Edelson was supported by National Institutes of Health grant R01 AI113118 and a Burroughs Wellcome Fund Career Award for Medical Scientists. J.P. Huynh was supported by a National Science Foundation Graduate Research Fellowship (DGE-1143954). C.-C. Lin was supported by the McDonnell International Scholars Academy at Washington University in St. Louis. J.M. Kimmey was supported by a National Science Foundation Graduate Research Fellowship (DGE-1143954) and the National Institute of Medical General Sciences Cell and Molecular Biology Training grant GM007067. N.N. Jarjour was supported by National Institutes of Health grant ST3ZAI007163. The Bhlhe400/f/f mouse strain used, STOCK Tg(Bhlhe40-EGFP) PX84Gsat/Mmucd, identification number 034730-UCD, was obtained from the Mutant Mouse Regional Resource Center, a National Center for Research Resources–National Institutes of Health–funded strain repository, and was donated to the Mutant Mouse Regional Resource Center by the National Institute of Neurological Disorders and Stroke–funded Gene Expression Nervous System Atlas BAC transgenic project (The GENSAT Project; National Institute of Neurological Disorders and Stroke contract N01NS02331 to the Rockefeller University). Research reported in this publication was supported by the Washington University in St. Louis Institute of Clinical and Translational Sciences grant from the National Center for Advancing Translational Sciences of the National Institutes of Health (UL1 TR000448).

The authors declare no competing financial interests.

Author contributions: J.P. Huynh, C.-C. Lin, B.T. Edelson, and C.L. Stallings designed the experiments, analyzed data, and wrote the manuscript. J.P. Huynh and J.M. Kimmey performed Mtb infections. J.P. Huynh and C.-C. Lin performed flow cytometry. E.A. Schwarzkopf and T.R. Bradstreet performed L. monocytogenes infections. C.-C. Lin, E.A. Schwarzkopf, T.R. Bradstreet, and N.N. Jarjour generated bone marrow chimeras. E.A. Schwarzkopf performed ELISA. N.N. Jarjour performed Western blots. C.-C. Lin and T.R. Bradstreet performed ChIP experiments. I. Shchukina, O. Shpynov, and M.N. Artymov analyzed ChIP-seq data. C.T. Weaver and R. Taneja provided key mouse strains.

Submitted: 14 September 2017
Revised: 10 April 2018
Accepted: 9 May 2018

References
Demangel, C., P. Bertolino, and W.J. Britton. 2002. Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial

Huynh et al.
Bhlhe40 represses IL-10 during M. tuberculosis infection

Journal of Experimental Medicine
https://doi.org/10.1084/jem.20171704


Huynh et al. Blhle40 represses IL-10 during M. tuberculosis infection Journal of Experimental Medicine https://doi.org/10.1084/jem.20171704


Figure S1. **Myeloid cells are highly infected in Bhlhe40−/− mice.** (A) At 21 dpi, pulmonary histopathology and acid-fast bacilli were visualized by H&E and Ziehl-Neelsen staining, respectively. Bars: (H&E) 100 µm; (Ziehl-Neelsen) 50 µm. Images are representative of six mice per group. (B) Absolute cell counts for pulmonary immune cells before infection and at 21 dpi (n = 7 per group). MoDC counts in four naive Bhlhe40+/+ and three naive Bhlhe40−/− samples were below the limit of detection (~100 cells). (C) Frequency of Mtb antigen-specific CD4+ T cells at 21 dpi (n = 6 per group). (D) Absolute count for GFP+ (Mtb+) lung myeloid cells at 21 dpi (n = 10–12 per group). Four Bhlhe40−/− alveolar macrophage, four Bhlhe40−/− CD103+ DCs, and two Bhlhe40−/− eosinophils samples were below the limit of detection (~850 cells). (E) MFI for FITC in GFP+ (Mtb+) lung cell populations at 21 dpi (n = 10–12 per group). Mac., macrophage; Inf. Mono., inflammatory monocyte; Alv. Mac., alveolar macrophage; CD11b+, CD103+, CD4+ T cells, Eosinophils. (F–H) Mice were infected with Mtb and treated with rat IgG or anti-Ly6G mAb from 10–30 dpi. Mice were monitored for changes in body weight (F; n = 9 per group), survival (G; n = 6 per group), and pulmonary Mtb burden at 21 dpi (H; n = 10 per group). Ab, antibody. Each point represents data from one mouse. 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 (B–E), log-rank Mantel-Cox test (G), or unpaired Kruskal-Wallis test with Dunn’s multiple comparison test (H). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are from or representative of two (A, B, and E–G) or five (C and D) independent experiments.
Figure S2. Bhlhe40 is expressed in multiple myeloid and lymphoid populations in the lungs of naive and infected mice. (A and B) Representative flow cytometry plots for GFP (Bhlhe40) in pulmonary immune cells from the lungs of nontransgenic Bhlhe40−/− and Tg Bhlhe40GFP mice (n = 3–6 per group) before infection (A) or at 21 dpi (B). Bold numbers are the percentages of cells that fall within the gate. (C) Frequency of GFP+ cells for each lung cell population in nontransgenic mice at 21 dpi, naive Bhlhe40GFP mice, and Bhlhe40GFP mice at 21 dpi (n = 3–6 per group). *, P < 0.05; **, P < 0.01; *** P < 0.001; ****, P < 0.0001. Data are representative of three independent experiments. Mac., macrophage; Inf. Mono., inflammatory monocyte; Alv. Mac., alveolar macrophage.
Figure S3. *Bhlhe40 enables innate immune cell control of Mtb.* (A) The absolute number and frequency of neutrophils in the lungs of Rag1−/− and Rag1−/−; Bhlhe40−/− mice at 21 dpi (n = 10–11 per group). (B and C) Pulmonary myeloid cell populations in Rag1−/− and Rag1−/−; Bhlhe40−/− mice (n = 10–11 per group) were assessed for the frequency (B) and geometric MFI (C) for GFP in GFP+ (Mtb+) cells at 21 dpi. Mac., macrophage; Inf. Mono., inflammatory monocyte; Alv. Mac., alveolar macrophage. (D) Weight loss in Bhlhe40fl/fl, Bhlhe40fl/fl-Lysm-Cre, and Bhlhe40fl/fl-Mrp8-Cre mice after Mtb infection (n = 6–8 per group). (E) Mtb CFUs in the lungs of mixed bone marrow chimeric mice at 21 dpi (n = 3–4 per group). Each point represents data from one mouse. 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. Data are from two (D and E) or five (A–C) independent experiments.
Figure S4.  **Il10** expression in the lungs of 10BiT⁺ Bhlhe40⁺/⁺ and Bhlhe40⁻/⁻ mice. (A) Representative flow cytometry plots for Thy1.1⁺ (IL-10⁺) pulmonary immune cells from the lungs of naive 10BiT⁺ Bhlhe40⁺/⁺ and 10BiT⁺ Bhlhe40⁻/⁻ mice. (B) Representative flow cytometry plots for Thy1.1⁺ (IL-10⁺) pulmonary immune cells from the lungs of 10BiT⁺ Bhlhe40⁺/⁺ and Bhlhe40⁻/⁻ mice at 21 dpi. (C) Absolute counts for Thy1.1⁺ (IL-10⁺) pulmonary immune cells at 21 dpi (n = 5 per group). Statistical differences were determined by two-tailed unpaired Student’s t test. Bold numbers are the percentages of cells that fall within the indicated gate. Data are from or representative of one (A) or two (B and C) independent experiments. Mac., macrophage; Inf. Mono., inflammatory monocyte; Alv. Mac., alveolar macrophage.
Figure S5. In vitro GM-CSF–cultured cell infection, Ifng locus binding sites, and flow cytometry gating strategy. (A) CFU analysis of bone marrow–derived GM-CSF–cultured cells infected with Mtb and left untreated or treated with IFN-γ (n = 3 per group). (B) ChIP-seq binding tracks for the Ifng locus in Bhlhe40+/+ 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. (C and D) Representative flow cytometry plots for gating strategies used throughout this study to identify pulmonary myeloid (C) and lymphoid (D) cell populations in Mtb-infected mice. Plots are from a nontransgenic Bhlhe40+/+ mouse infected with GFP-Mtb at 21 dpi. Bold numbers are percentages of cells that fall within the indicated gate. Statistical differences (or lack thereof) were determined by one-way unpaired ANOVA with Tukey's post-test (A). Alv. Mac., alveolar macrophage; Inf. Mono., inflammatory monocytes; FSC-A, forward scatter–area; FSC-W, forward scatter–width; SSC-A, side scatter–area. (A and B) Data are from one experiment. (C and D) Data are representative of all flow cytometry experiments in this article.

Table S1 is a separate Excel file showing Bhlhe40 binding sites in GM-CSF–cultured cells.

Table S2 is a separate Excel file showing Bhlhe40 binding sites in T1/2-polarized T cells.