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The Role of Bhlhe40 in Autoimmune Neuroinflammation and Mycobacterial Infection

Chih-Chung Lin
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The Role of Bhlhe40 in Autoimmune Neuroinflammation and Mycobacterial Infection
by
Chih-Chung Lin

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

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May 2017
Dedicated to My Grandparents and Parents.
The mammalian immune system is composed of innate and adaptive compartments, which cooperate with each other to maintain homeostasis and protect the host from the invasion by a variety of pathogens. The tight control of immune responses is extremely important for all individuals. Here, we discovered that the transcription factor basic helix-loop-helix family, member e40 (Bhlhe40) is a critical protein that regulates the autoimmune ("against self") and anti-microbial ("against non-self") responses of myeloid cells and T lymphocytes. Multiple sclerosis (MS) is a human neuroinflammatory disease in the central nervous system with an autoimmune etiology. We have reported that Bhlhe40 positively regulates the production of the pro-inflammatory cytokine, GM-CSF, but negatively regulates the production of the anti-
inflammatory cytokine, IL-10, by CD4+ T cells. The absence of Bhlhe40 abrogates T cell pathogenicity and thus Bhlhe40−/− mice were resistant to the animal model of MS. Tuberculosis, caused by Mycobacterium tuberculosis (Mtb), is one of the most serious infectious diseases in the world. We have demonstrated that Bhlhe40 suppresses IL-10 expression by myeloid cells and T cells to ensure protective immunity to Mtb. Bhlhe40-deficient immune cells produce high levels of IL-10 which dampens the immune responses to Mtb infection and therefore renders Bhlhe40−/− mice highly susceptible to this pathogen. Our findings have uncovered the critical roles of Bhlhe40 in regulating inflammation during an autoimmune disorder and an infectious disease. Bhlhe40, or pathways that regulate its expression or function, might represent therapeutic targets for researchers to manipulate immune responses in human autoimmunity and infection.
Chapter 1: Introduction
In 1989, Charles Janeway, Jr. proposed that the immune system has evolved to discriminate “infectious nonself from noninfectious self”\textsuperscript{1, 2}. He later described a "two-signal" system, whereby innate immune cells recognize microbial products and subsequently activate adaptive lymphocytes, as a reflection of the evolution of host defense\textsuperscript{2}. However, the immune system is not always perfect: autoimmune disorders still occur, and not all pathogens can be successfully controlled.

Adjuvants, nicknamed the "immunologist's dirty little secret" by Janeway, are potent reagents containing immunogenic materials which can activate the innate immune compartment and help generate antigen-specific adaptive immune responses\textsuperscript{3}. Complete Freund's adjuvant (CFA), consisting of heat-killed \textit{Mtb} in non-metabolizable oils, is a powerful adjuvant which induces fast and strong immune responses\textsuperscript{4}. In experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS), heat-killed \textit{Mtb} within CFA activates innate immune cells to present the co-injected myelin antigen to self-reactive T cells which have escaped from thymic negative selection. As a result, these activated autoreactive T cells infiltrate the central nervous system (CNS) and cause autoimmune neuroinflammation\textsuperscript{5}.

Despite having a variety of pathogen-associated molecular patterns (PAMPs), virulent \textit{Mtb} has co-evolved with humans for more than 70,000 years and has developed immune evasion strategies to avoid being eliminated by the host\textsuperscript{6, 7, 8}. It is estimated that one-third of the world's population is infected by \textit{Mtb}, with the majority having asymptomatic latent infection. In these individuals, innate and adaptive immune defenses keep \textit{Mtb} contained within the lung at a low level. In a small fraction of individuals, active tuberculosis (TB) develops, with the pathogen growing progressively in the lungs and in some cases disseminating to other organs, without control by the host immune system. In the mouse model of infection, virulent \textit{Mtb} also can
evade innate immunity, delay the initiation of adaptive host defenses, and persist as a chronic infection. Wild-type (WT) C57BL/6 mice do not show symptoms for the first several months after \textit{Mtb} infection, but eventually die of the disease between 8 and 12 months.

Accepting that the immune system is still in the slow process of evolution and is not yet perfect to maintain health at all times, biomedical scientists have come up with the idea of immune-directed therapies to treat autoimmune and infectious diseases. Immunotherapies include the administration of antibodies to target certain immune cell types or molecules. As an example, blockade of immune cell trafficking with a monoclonal antibodies recognizing alpha 4 integrin has been shown to be an effective treatment for relapsing-remitting MS (RRMS). Several host-directed therapies that aim to enhance antimicrobial mechanisms and reduce inflammation are currently being considered as adjunctive treatments for human TB. To advance the development of novel immune-directed therapies, it is fundamentally important to explore the immune reactions underlying MS and TB. A clearer understanding of the players and pathways used by the immune response during autoimmunity and infection may provide novel therapeutic targets.

In this dissertation, I will describe the cellular and molecular mechanisms whereby the transcription factor basic helix-loop-helix family, member e40 (Bhlhe40) and several cytokines regulate autoimmune and anti-microbial responses. The Edelson Lab has found that Bhlhe40, plays a critical role in controlling the balance of immune response against noninfectious self and infectious non-self. Bhlhe40 is essential for CD4$^+$ T cell pathogenicity and the induction of the murine autoimmune model, EAE. We found that myeloid cell-derived IL-1$\beta$ induces Bhlhe40 expression by CD4$^+$ T cells, and this factor promote neuroinflammation by enhancing the pro-inflammatory cytokine GM-CSF but suppressing the anti-inflammatory cytokine IL-10 (Chapter
2 and Chapter 3)\textsuperscript{11, 12}. While rendering mice susceptible to autoimmunity, Bhlhe40 is indispensable for both innate and adaptive compartments to engender protective host defense against \textit{Mtb}. Bhlhe40 in myeloid cells as well as T cells directly represses \textit{Il10} transcription to secure the protective host defense after \textit{Mtb} infection (Chapter 4; manuscript in preparation). We hope our findings in the mouse offer new insights for immune-directed therapies and can be translated to human medicine (Chapter 5).

In this chapter, I will first introduce the biology and function of the transcription factor, Bhlhe40. Second, I will describe the human neuroinflammatory disease, MS and its mouse model, EAE. I will also elaborate on the impact of IL-1\textbeta in MS and EAE. In the last section of this chapter, I will discuss human tuberculosis (TB) and its mouse model, followed by a discussion on the role of IL-10 in both human and mouse TB.

\section{1.1 The Transcription Factor Bhlhe40}

Between 1997 and 2002, the gene \textit{Bhlhe40} in mouse, rat, and human was discovered by several independent laboratories and given different names: \textit{Bhlhb2}, \textit{Stra13}, \textit{Sharp2}, \textit{Eip1}, \textit{Dec1}, \textit{CR-8}, and \textit{Clast5}\textsuperscript{13, 14, 15, 16, 17, 18}. The gene is located on chromosome 6 in mouse, chromosome 4 in rat, and chromosome 3 in human. \textit{Bhlhe40} is an evolutionarily-conserved gene which can be found not only in mammals but in fish, amphibians, reptiles, and birds. Murine \textit{Bhlhe40} encompasses five exons, encoding a transcription factor called the basic helix-loop-helix family, member e40. The protein is composed of 411-412 amino acids, with a molecular weight of 46 kD. Bhlhe40 belongs to the family of basic helix–loop–helix transcriptional regulators sharing structural features including a basic DNA-binding domain, a helix–loop–helix domain mediating
dimerization, and a 41 residue-long protein-protein interaction “orange domain” \(^{19,20}\). In 2002, researchers identified the class B E-box element (CACGTG) as the preferred binding site for Bhlhe40\(^{21}\).

Bhlhe40 is expressed in both the hematopoietic and non-hematopoietic compartments\(^{22}\). It has been shown to play roles in circadian rhythms, neuronal excitability, muscle repair, and responses to environmental stimuli such as light pulses, growth factors, and hypoxia\(^{23,24,25,26,27,28,29}\). Within immune cell populations, this factor can be found in many cell types, including granulocytes, macrophages, dendritic cells (DCs), natural killer cells (NK cells), and activated T cells\(^{12}\). However, the functions of Bhlhe40 in immune cells are not well appreciated. In 2001, Reshma Taneja’s group generated \(Bhlhe40^{-/-}\) mice and reported that they suffered from a non-fully penetrant, late-onset lymphoproliferative disease. The authors suggested that the autoimmune disorder might result from a failure of elimination of activated T and B cells\(^{30}\).

Bhlhe40 and its related transcription factor Bhlhe41, have been shown to regulate the maintenance and phenotype of peritoneal and splenic B-1a cells\(^{31}\). In T cells, Bhlhe40 is expressed upon T-cell receptor (TCR) stimulation and CD28 co-stimulation \(^{32,33}\). Miyazaki et al. suggested that Bhlhe40 is critical for the homeostasis of regulatory T cells (Tregs) both in vitro and in vivo \(^{32}\). Kanda et al. showed that Bhlhe40 served as a cofactor of T-bet and is required for full IFN-\(\gamma\) production by iNKT cells\(^{34}\). The importance of immune cell-expressed Bhlhe40 in disease models is just beginning to be appreciated. In this dissertation, I will describe the roles of this transcription factor in murine models of MS and TB.
1.2 Multiple Sclerosis and Its Mouse Model

1.2.1 Multiple sclerosis (MS)

MS is a chronic inflammatory disease of the brain, spinal cord, and optic nerves that presents clinically with different temporal and pathologic patterns and results in a variety of neurologic signs and symptoms. The symptoms of MS include fatigue, vision loss, numbness, muscle spasms, mobility problems, cognitive dysfunction, depression and anxiety, bladder problems, and autonomic dysfunction\(^{35}\). It is estimated that 2.5 million people in the world suffer from MS, with two-third being women. Most patients are diagnosed with MS between 20 and 45 years of age. The disease can last for decades, having a minimal impact on lifespan. The symptoms of MS hinder young adults from working and raising children, and as much MS poses a large socioeconomic burden. Although 13 MS-modifying medications have been approved by U.S. Food and Drug Administration (FDA), there is still no cure for MS. Current MS therapies mainly target the functions of lymphocytes, but are not universally-effective.

Although the autoantigens have not been identified, MS is thought to be an autoimmune disease. Studies in MS patients reveal that CNS-invading immune cells induce demyelination and axon loss, and communicate with resident astrocytes, glia, and neurons\(^{36}\). Pathogenic CD4\(^+\) T\(_H\) cells play an important role in driving MS pathology, although γδ T cells, CD8\(^+\) T cells, and B cells also appear to contribute to disease\(^{37, 38, 39, 40, 41}\). How autoreactive T\(_H\) cells acquire pathogenicity and how they mediate CNS damage remain important outstanding questions.
1.2.2 Experimental autoimmune encephalomyelitis (EAE)

Experimental autoimmune encephalomyelitis (EAE) serves as an animal model of MS and can be elicited in several species through active immunization with myelin antigen or via adoptive transfer of T lymphocytes (“passive EAE”)\(^\text{42, 43}\). In recent years, the C57BL/6 mouse model of EAE has become the most popular, as it has allowed the use of knockout and transgenic mouse strains on this genetic background. In the C57BL/6 EAE model, mice are immunized with a CFA-based emulsion containing a peptide from murine myelin oligodendrocyte glycoprotein (MOG\(_{35-55}\))\(^5\). Although this immunization elicits peptide-specific TH cells, mice must also be systemically injected with pertussis toxin (PTX) as a coadjuvant to induce a monophasic, paralytic clinical disease\(^44, 45\).

Following EAE induction, priming and differentiation of MOG-specific TH cells take place during the first week in secondary lymphoid organs. In the subsequent days, these TH cells and blood-derived myeloid cells traffic through the meninges and across the blood-brain barrier (BBB), with clinical signs of disease typically apparent by 10 days post-immunization. TH cells are thought to re-encounter their cognate antigen (MOG\(_{35-55}\)) in the context of MHC class II-expressing antigen presenting cells both in the meninges and the CNS parenchyma, with these interactions resulting in the production of pathogenic cytokines by the TH cells\(^46, 47\). In 2011, two groups reported that CD4\(^+\) T cells produce granulocyte macrophage colony-stimulating factor (GM-CSF) to initiate neuroinflammation in mice. Using several GM-CSF receptor conditional knockout mice, the group of Burkhard Becher demonstrated that the CCR2\(^+\) monocyte/monocyte-derived dendritic cells/macrophages (moDCs/Macs) must respond to T cell-derived GM-CSF to cause disease\(^48\). This same group recently showed that even transgenic overexpression of GM-CSF by myelin antigen non-specific CD4\(^+\) T cells can induce myeloid
cell infiltration to the CNS and lead to neurological deficits. These findings indicate that dysregulation of T cell-derived cytokines can influence autoimmune inflammation.

1.2.3 The associations between IL-1 and autoimmune neuroinflammation

Note: the contents of this section are modified from the manuscript “New Insights into the Role of IL-1β in EAE and MS” written by Chih-Chung Lin and Brian T. Edelson. This manuscript has been accepted for publication at the Journal of Immunology at the time of this dissertation.

The IL-1 family of cytokines displays pleiotropic effects on a variety of hematopoietic and non-hematopoietic cells relevant to neuroinflammation. IL-1α is generated constitutively by epithelial cells and induced upon stimulation of most immune cell types. IL-1β is produced via both inflammasome-dependent and -independent pathways upon activation of a variety of leukocytes. Both of these IL-1 family members bind to a single activating receptor complex, composed of the IL-1R1 and IL-1RAcP (the IL-1R accessory protein, also called IL-1R3) chains, each containing a cytosolic Toll/interleukin-1 receptor homology (TIR) domain. Cytokine binding by this receptor engages the MyD88 signaling cascade, including IRAK1/2/4, TRAF6, and TAK1, to ultimately result in AP-1 and NF-κB activation and pro-inflammatory gene transcription. Separately, another IL-1 family member, IL-1Ra (also called IL-1RN), functions as a soluble receptor antagonist, capable of binding to IL-1R1.

Several members of the IL-1 family of cytokines have been studied in the context of EAE and MS, with initial work in EAE beginning in the late 1980s. In 1987, Symons et al. found increased levels of IL-1 activity, measured at the time by a mouse thymocyte proliferation assay, in the plasma and cerebrospinal fluid (CSF) of guinea pigs immunized with spinal cord homogenates to induce a chronic relapsing form of EAE. In the same year, using a rat model...
of passive EAE, Mannie et al. showed lymph node cells from EAE-induced rats treated with human IL-1β were more encephalitogenic, and suggested that this effect was via the action of IL-1β on T lymphocytes. Two studies later found evidence for IL-1α within the spinal cord of mice with EAE, and one report showed that IL-1β protein could be detected in rats with EAE within meningeal macrophages, parenchymal infiltrating macrophages, and activated microglia. Subsequent studies in rats showed that recombinant human IL-1α treatment after EAE induction exacerbated clinical disease and that treatment with soluble recombinant murine IL-1 receptor or IL-1Ra could ameliorate disease. Schiffenbauer et al. first reported that IL-1R-deficient mice (on a mixed genetic background) were resistant to active EAE induction. Their results have been confirmed by several other groups using IL-1R-deficient mice on the C57BL/6 background, although the degree to which these mice were protected from clinical disease was somewhat variable. Despite one report to the contrary, IL-1β appears to be the critical mediator of EAE, rather than IL-1α, as IL-1β-deficient mice were seen to resist EAE by two groups, while IL-1α-deficient mice remained susceptible. Consistent with a critical requirement for IL-1β for EAE susceptibility, mice deficient in the inflammasome components NLRP3, ASC, caspase 1, and caspase 11 were also at least partially resistant to EAE, as were mice treated with inhibitors of NLRP3 or caspase 1. It is worth noting that in some reports, mice immunized with larger amounts of heat-killed Mtb (usually greater than 300 micrograms per mouse) as part of the MOG/CFA emulsion developed an NLPR3- and ASC-independent form of aggressive EAE. However, this form of EAE still appears to be IL-1β- and IL-1R-dependent, given that the experiments in IL-1β- and IL-1R-deficient mice which demonstrated EAE resistance were typically performed with large amounts of heat-killed Mtb.
Beginning in 1990, reports emerged showing that IL-1β protein or IL1B transcript could be detected in the CSF\textsuperscript{84, 85} or within CNS lesions of MS patients\textsuperscript{86, 87, 88, 89, 90, 91}. More recently, these findings were extended by Seppi et al. who showed that CSF levels of IL-1β correlate with the number and volume of brain cortical demyelinating lesions\textsuperscript{92}, and by Rossi et al. who showed that relapsing-remitting MS (RRMS) patients with detectable IL-1β in the CSF at the time of clinical remission had a more severe course of disease\textsuperscript{93}. In addition, transcript levels of IL1B and two inflammasome components (CASP1 and NLRP3) were more highly expressed by peripheral blood mononuclear cells from MS patients compared to healthy controls\textsuperscript{94, 95, 96}. Collectively, while these results indicate that IL-1β expression in the CNS and blood is associated with disease activity in MS, they do not establish a causal role for the cytokine in disease pathogenesis.
1.3 Immune responses to Mycobacterial infection

1.3.1 Human tuberculosis

Tuberculosis (TB) is one of the top 10 causes of death in the world. In 2015, it was estimated that 1.8 million people died from the disease, with more than one-fifth of these patients being co-infected with HIV (WHO Fact Sheet 2017 http://www.who.int/mediacentre/factsheets/fs104/en/). Sub-Saharan Africa, South Asia, and East Asia have the highest TB burden; about 60% of the TB cases were reported in India, Indonesia, China, Nigeria, Pakistan and South Africa (WHO Fact Sheet 2017 http://www.who.int/mediacentre/factsheets/fs104/en/)\textsuperscript{97}. TB remains one of the most important infectious diseases worldwide.

Human tuberculosis is caused by \textit{Mtb}. While latent infection is the most common form, 5-10% of infected individuals develop active TB with clinical symptoms (such as chronic cough, fever, and weight loss). These patients become the contagious sources of \textit{Mtb}. \textit{Mtb} can be spread through the air after an active TB patient coughs, sneezes or spits. After entering a new host, the bacteria deposit in the lung and replicate in macrophages and epithelial cells. Recruited myeloid cells and lymphocytes later surround infected cells and form granulomas, a hallmark feature of human TB. This compact aggregate of immune cells prevents the further spread of \textit{Mtb} but also allows the bacteria to persist in the lungs\textsuperscript{98}. \textit{Mtb} primarily infects the lung but it can disseminate other tissues (such as lymph nodes, bones, and the central nervous system) and cause extrapulmonary disease\textsuperscript{99,100}. 
1.3.2 Mouse models of tuberculosis

The German microbiologist, Robert Koch, identified *Mtb* as the causative agents for TB in 1882, and he was also the first to use the mouse as an experimental model. Subsequently, scientists have utilized a variety of animals for TB research: guinea pigs, rats, rabbits, and non-human primates\textsuperscript{101}. Each model has its advantages for studying TB but mice are the most widely used animal model mainly because of the relatively low cost and a broad availability of inbred and genetically-modified strains as well as research reagents\textsuperscript{102}. In addition, mouse models of human mycobacterial diseases have been suggested to be genetically relevant to human TB. The intensity of immune responses and disease prognosis is determined by several experimental conditions: (1) the species and strain of the infectious pathogen, (2) the route of infection, and (3) the genetic background of the host.

*Mtb* is the cause of human TB. Virulent strains include H37Rv, Erdman, NYH-27, and the Beijing strain. In contrast, H37Ra is an attenuated strain derived from H37Rv encompassing 76 strain-specific single nucleotide variations that affect 32 genes\textsuperscript{103, 104}. Another member of the *Mycobacterium* genus, *M. bovis*, is the causative agents of bovine TB. The Ravenel and the Branch strains of *M. bovis* are virulent while *M. bovis* Bacille Calmette-Guerin (BCG) is not\textsuperscript{103, 105}. *M. bovis* BCG is the only licensed vaccine used against human TB (the other vaccine modified vaccinia Ankara 85A, MVA85A, is in clinical trials.) *M. avium* is another pathogen used in TB animal models. This mycobacterial species infects cows, dogs, cats, and birds, and is a threat to immunocompromised individuals.

Both intravenous and aerosol routes are used to infect mice with mycobacteria. To infect mice intravenously, a large number of mycobacteria (eg. 1-10 x10\textsuperscript{5}) are delivered. Within one or two
weeks, the pathogen can infect splenic and lung cells and result in systemic infection\textsuperscript{102}.

Infecting animals with mycobacteria using small aerosol droplets mimics the natural infection route of human TB. Unlike intravenous infection, a low number of bacteria (50-200 CFU per lung) are sufficient to infect a mouse, with the bacteria undergoing a three-week phase of logarithmic growth in the lungs. Compared to mice infected through the intravenous route, it can take three weeks to observe an immune response in mice infected via the aerosol route\textsuperscript{102}.

It is known that mice of different genetic backgrounds show different degree of resistance to mycobacterial challenge\textsuperscript{106, 107, 108}. For instance, C57BL/6 and BALB/c mice showed higher \emph{M. bovis} BCG loads than DBA/2 or C3H/He mice did in the spleens at 28 days post-intravenous infection\textsuperscript{106}. Nevertheless, C57BL/6 and BALB/c mice are more resistant to intravenous inoculation of \emph{Mtb} when compared to DBA/2, C3H, or CBA mice, which all succumb to this route of infection and die around 100 days post infection (p.i.)\textsuperscript{108}. A variety of genes have been knocked out in mice and tested for their functions during mycobacterial infection. Mice that are unable to produce certain pro-inflammatory mediators (eg. \textit{Il1\alpha}\textsuperscript{-/-}, \textit{Il1\beta}\textsuperscript{-/-}, \textit{Nos2}\textsuperscript{-/-}) or have no or impaired T cell responses (eg. \textit{Rag1}\textsuperscript{-/-}, \textit{Tbx21}\textsuperscript{-/-}, \textit{Stat1}\textsuperscript{-/-}, \textit{Ifng}\textsuperscript{-/-} mice) are susceptible to TB\textsuperscript{109, 110, 111, 112, 113, 114}.

\subsection*{1.3.3 IL-10 in \emph{M. tuberculosis} infection}

IL-10 was discovered almost three decades ago and has been recognized as an anti-inflammatory cytokine in autoimmune and infectious diseases\textsuperscript{115, 116, 117}. IL-10 can be secreted by a variety of leukocytes including monocytes, macrophages, DCs, and T cells. Multiple cytokines such as IL-4, IL-12, and IL-27 signal through STATs to induce IL-10 production by T cells\textsuperscript{118}. Myeloid cells secrete this cytokine after sensing certain microbial products through pathogen recognition.
receptors. TLR2 agonists are potent inducers of IL-10 expression by macrophages and DCs. *Mtb* possesses a large repertoire of TLR2 ligands including lipomannan and lipoproteins, and thus can stimulate IL-10 production from myeloid cells. IL-10 can act on nearly all immune cell subsets via binding to its receptor. IL-10 receptor is composed of two chains, IL-10R1 and IL-10R2, which are associated with Jak1 and Tyk2, respectively. Upon ligand binding, the Jak kinases phosphorylate and activate STAT3 as well as STAT1 and STAT5 to initiate suppressive functions.

A meta-analysis including 31 studies revealed that polymorphisms in the *Il10* gene associate with TB susceptibility in Asians, Europeans, and Americans. Several reports showed that IL-10 could be detected in the serum, PBMCs, sputum, and bronchoalvelar lavage (BAL) fluid of active TB patients, and the blood levels of IL-10 are decreased after TB treatment. In TB patients, IL-10 is secreted by monocytes, macrophages, DCs, and T cells, and this cytokine can potentially dampen both myeloid cell and T cell responses to *Mtb* infection. In vitro studies using human mononuclear phagocytes showed that IL-10 inhibited phagosome maturation in *Mtb*-infected macrophages, down-regulated MHC and costimulatory molecules of *Mtb*-infected monocytes, drove an "M2-like" phenotype of *Mtb*-activated monocytes/macrophages, inhibited monocyte-DC differentiation after *Mtb* stimulation, and prevented the fusion of monocytes to become multi-nuclear giant cells. IL-10 also negative controls IL-12 production from human macrophages and DCs infected with *Mtb* and thus possibly impairs Th1 cell priming. IL-10 has been reported to suppress IFN-γ, IL-4, and IL-17 production from PBMCs and inhibit *Mtb*-reactive CD4+ T cell proliferation in TB patients. Although numerous in vitro and ex vivo studies suggest an adverse effect of IL-10 on immunity to *Mtb*, the definite role of IL-10 in TB patients remains unresolved.
Despite the fact that two studies demonstrated comparable susceptibility to \textit{Mtb} between WT and \textit{Il10}^{-/-} mice\textsuperscript{150, 151}, more recent reports have proposed that endogenous IL-10 restrains immune responses to \textit{Mtb} infection. Anti-IL-10R-treated or IL-10-deficient CBA/J, BALB/c, and C57BL/6 mice showed stronger T\textsubscript{H}1 responses, decreased bacterial burdens in the lungs, and even prolonged survival after infection\textsuperscript{152, 153, 154, 155}. IL-10 deficiency is also able to completely rescue \textit{Mtb}-infected WT mice from a type I-driven exacerbation of tuberculosis induced by polyinosinic-polycytidylic acid (Poly-IC) treatment\textsuperscript{156, 157}.

In 1991, the group of Anne O'Garra reported that IL-10 inhibited pro-inflammatory cytokine production from antigen-presenting cells (APCs) and IFN-\(\gamma\) expression from T\textsubscript{H}1 cells\textsuperscript{158, 159}. In the context of mycobacterial infection, Hickman et al. found that \textit{Mtb}-infected bone marrow-derived macrophages secreted IL-10 to suppress T\textsubscript{H}1 cell polarization\textsuperscript{160}. Demangel et al. showed that the autocrine IL-10 represses IL-12 expression by BCG-infected DCs and inhibits the generation of IFN-\(\gamma\)-producing T cells\textsuperscript{161}. McNab et al. reported that IFN\(\beta\)-induced IL-10 suppressed IL-12 expression and microbial killing of \textit{Mtb}-infected macrophages\textsuperscript{162}. Recently, Jeyanathan et al. found that DAP12 knockout mice were more resistant to \textit{Mtb} infection because this immunoadaptor promoted IL-10 production by APCs and delayed protective T\textsubscript{H}1 immune response to \textit{Mtb} infection\textsuperscript{163}. Collectively, these studies concluded that IL-10 could impair protective immune responses to mycobacteria through inhibiting intracellular bacterial killing, IL-12 production, and T\textsubscript{H}1 cell priming.
Chapter 2: Bhlhe40 controls cytokine production by T cells and is essential for pathogenicity in autoimmune neuroinflammation

The contents of this chapter have been modified from the following published article:

Bhlhe40 controls cytokine production by T cells and is essential for pathogenicity in autoimmune neuroinflammation.

2.1 Abstract

TH1 and TH17 cells mediate neuroinflammation in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Pathogenic TH cells in EAE must produce the pro-inflammatory cytokine granulocyte-macrophage colony stimulating factor (GM-CSF). TH cell pathogenicity in EAE is also regulated by cell-intrinsic production of the immunosuppressive cytokine interleukin 10 (IL-10). Here we demonstrate that mice deficient for the basic helix-loop-helix (bHLH) transcription factor Bhlhe40 (Bhlhe40−/−) are resistant to the induction of EAE. Bhlhe40 is required in vivo in a T cell-intrinsic manner, where it positively regulates the production of GM-CSF and negatively regulates the production of IL-10. In vitro, GM-CSF secretion is selectively abrogated in polarized Bhlhe40−/− TH1 and TH17 cells, and these cells show increased production of IL-10. Blockade of IL-10 receptor in Bhlhe40−/− mice renders them susceptible to EAE. These findings identify Bhlhe40 as a critical regulator of autoreactive T-cell pathogenicity.
2.2 Introduction

Experimental autoimmune encephalomyelitis (EAE) serves as an animal model for the human neuroinflammatory disease multiple sclerosis. Encephalomyelitis in C57BL/6 mice in response to immunization with a peptide from myelin oligodendrocyte glycoprotein (MOG) is driven by cytokine-producing autoreactive T helper (T_{H1}) cells$^{164}$. Historically, interferon-γ (IFN-γ)-producing T_{H1} cells were thought to be responsible for EAE, but recent evidence has revealed a more important role for interleukin 17 (IL-17)-producing T_{H17} cells$^{165, 166}$. Nevertheless, neither T_{H1} (IFN-γ) nor T_{H17} (IL-17A, IL-17F, IL-21 and IL-22) signature cytokines are required for the development of EAE$^{167, 168, 169, 170, 171}$. Instead, the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) has proven to be a critical factor in the encephalitogenicity of both T_{H1} and T_{H17} cells$^{172, 173, 174, 175, 176}$. IL-1 receptor and IL-23 receptor signaling are required for T_{H} cell production of GM-CSF during EAE$^{68, 174, 175}$. GM-CSF production by T cells is regulated cell-intrinsically through the action of transcription factors. Factors that regulate its transcription include AP-1 family members, NFAT, NF-kB family members, including c-Rel and NF-kB1, and RUNX1$^{177, 178, 179, 180, 181, 182}$. Recently, Codarri et al. reported a requirement for RORγt for maximal production of GM-CSF by T_{H17} cells, although a second report observed no reduction in GM-CSF secretion by Rorc$^{-/-}$ T_{H} cells in vitro$^{174, 175}$. T_{H1} cells also produce GM-CSF during EAE, yet specific transcription factors regulating their production of GM-CSF have not been identified$^{176}$.

Immunoregulatory cytokines, including IL-10, modulate the development of EAE$^{183}$. IL-10 is produced by several cell types during EAE, including autoreactive non-pathogenic T_{H} cells$^{184, 185, 186, 187, 188, 189, 190}$. IL-10 suppresses effector T cell responses and limits inflammation. IL-10-
deficient mice develop more severe EAE than wild-type (WT) mice\textsuperscript{191, 192, 193}. Expression of IL-10 in T\textsubscript{H} cells can be regulated by several transcription factors, depending on the T\textsubscript{H} cell subset and the cytokine environment\textsuperscript{119, 194, 195, 196, 197}. Relevant to EAE is the production of IL-10 by T\textsubscript{H}1, T\textsubscript{H}17, Tr1 cells and T regulatory cells (Tregs).

Bhlhe40 (also known as Dec1, Stra13, Sharp2 or Bhlhb2) belongs to a family of basic helix–loop–helix transcriptional regulators sharing structural features including a basic DNA binding domain, a helix–loop–helix domain mediating dimerization and a protein–protein interaction ‘Orange domain’\textsuperscript{19, 20}. Members of this family are known to respond to environmental stimuli and regulate several physiological processes in diverse cell types, including the cell cycle, apoptosis and differentiation via their actions as both transcriptional activators and repressors. Bhlhe40 is expressed in T cells upon T-cell receptor (TCR) stimulation\textsuperscript{38}. Bhlhe40\textsuperscript{-/-} mice develop a non-fully penetrant late-onset (age greater than ~8 months) lymphoproliferative disease associated with autoantibodies\textsuperscript{30, 32}, which may be due to a requirement for Bhlhe40 for Treg maintenance during ageing. Penetrance of this age-related disease appears to be influenced by genetic background. How Bhlhe40 regulates effector T-cell responses is incompletely characterized.

In this study, we show that Bhlhe40\textsuperscript{-/-} mice are resistant to EAE induction due, at least in part, to a T\textsubscript{H} cell-intrinsic defect. T\textsubscript{H} cells from these mice display nearly normal IFN-\gamma and IL-17A production in T\textsubscript{H}1 and T\textsubscript{H}17 cultures, respectively, and produce these cytokines after immunization, but fail to mediate EAE. Instead, transcriptional and phenotypic analyses of Bhlhe40\textsuperscript{-/-} T\textsubscript{H} cells reveal that they fail to produce GM-CSF. We also demonstrate a role for Bhlhe40 in GM-CSF production by \gamma\delta T cells. Bhlhe40\textsuperscript{-/-} T\textsubscript{H}1 and T\textsubscript{H}17 cells produce increased amounts of IL-10, consistent with their nonencephalitogenic phenotype. Moreover, blockade of
IL-10 signaling renders $Bhlhe40^{-/}$ mice susceptible to EAE. Chromatin immunoprecipitation sequencing (ChIP-Seq) reveals binding of Bhlhe40 to the $Il3$/$Csf2$ and $Il10$ loci at several sites bound by other transcriptional regulators. Overall, our results identify Bhlhe40 as a novel factor regulating GM-CSF and IL-10 production by T cells, and one that is required for autoimmune neuroinflammation.
2.3 Results

2.3.1 Bhlhe40-deficient mice are protected from EAE

To examine the role of Bhlhe40 in autoimmunity, we immunized *Bhlhe40<sup>−/−</sup>* mice with MOG peptide (amino acids 35–55 (MOG<sub>35–55</sub>). *Bhlhe40<sup>−/−</sup>* mice were markedly resistant to EAE (Figure. 2-1A), showing no leukocyte infiltration on histological examination of spinal cord sections (Figure. 2-1B). Bhlhe40 was required by the hematopoietic compartment for EAE susceptibility, since reconstitution of irradiated *Bhlhe40<sup>−/−</sup>* mice with WT bone marrow (BM) caused susceptibility to EAE, whereas reconstitution of WT mice with *Bhlhe40<sup>−/−</sup>* BM engendered EAE resistance (Figure. 2-1C). Bhlhe40 is expressed in activated T<sub>H</sub> cell subsets (Figure. 2-1D), as previously reported<sup>32, 198</sup>. Transfer of WT and *Bhlhe40<sup>−/−</sup>* T<sub>H</sub> cells each reconstituted the T-cell compartment of recipient *Rag1<sup>−/−</sup>* mice equally (Figure. 2-1E), but only WT cells, and not *Bhlhe40<sup>−/−</sup>* cells, allowed development of EAE after immunization (Figure. 2-1F). The central nervous system (CNS) from MOG<sub>35–55</sub>-immunized *Bhlhe40<sup>−/−</sup>* mice had reduced infiltrating myeloid cells (CD45<sup>HI</sup>CD11b<sup>+</sup>) relative to WT mice. In *Bhlhe40<sup>−/−</sup>* mice, these cells lacked MHC class II expression, as did resident microglia (CD45<sup>IM</sup>CD11b<sup>+</sup>) (Figure. 2-2, A and B). Immunized *Bhlhe40<sup>−/−</sup>* mice also harbored fewer CNS-infiltrating CD4<sup>+</sup> T cells (Fig. 2-2C). CNS-infiltrating CD4<sup>+</sup> T cells in WT mice produced IFN-γ and IL-17A, while the few CNS-infiltrating CD4<sup>+</sup> T cells in *Bhlhe40<sup>−/−</sup>* mice had reduced IFN-γ and IL-17A production (Figure. 2-2, D and E). Notably, WT CD4<sup>+</sup> T cells produced GM-CSF, but this was reduced by ~90% in *Bhlhe40<sup>−/−</sup>* mice (Figure. 2-2, D, E, and F). WT and *Bhlhe40<sup>−/−</sup>* mice showed a similar low frequency of IL-10-producing CD4<sup>+</sup> T cells in the CNS at the peak of disease (Figure. 2-2G). *Bhlhe40<sup>−/−</sup>* mice showed a slight reduction in the frequency of CNS-infiltrating Foxp3<sup>+</sup> CD4<sup>+</sup> T cells (analyzed at
day 29 after immunization, Figure. 2-2H). γδ T cells are known to infiltrate the CNS during EAE, and promote neuroinflammation via their production of IL-17 and GM-CSF. The role of IFN-γ production by these cells in this setting is unclear. Immunized Bhlhe40−/− mice harbored ~30-fold fewer CNS-infiltrating γδ T cells compared with WT mice. Bhlhe40−/− γδ T cells produced similar levels of IFN-γ and partially reduced levels of IL-17A, but more markedly reduced levels of GM-CSF (Figure. 2-2, I and J). Together with our observations on significantly reduced GM-CSF production by Bhlhe40−/− CD4+ T cells, these results suggest that Bhlhe40 is required for GM-CSF production by pathogenic T cells during neuroinflammation.

We also tested an adoptive transfer EAE system in which a WT T11-polarized MOG35–55-specific T cell line was transferred to WT or Bhlhe40−/− recipients. These T cells produced IFN-γ and GM-CSF, but not IL-17A, upon stimulation (Figure. 2-3A). When 5 million T cells were transferred, Bhlhe40−/− mice were protected from EAE, although when 10 million T cells were transferred, Bhlhe40−/− and WT mice both showed a similar incidence and severity of EAE (Figure. 2-3, B and C). We speculate that these results are the result of a requirement for Bhlhe40 in host T cells during the induction of neuroinflammation by low numbers of adoptively transferred MOG-specific T cells. Overall, our results indicate a role for Bhlhe40 in both immunization-induced and passive EAE.

2.3.2 Bhlhe40 regulates T-cell cytokine production after immunization

Draining lymph nodes (DLNs) from immunized Bhlhe40−/− mice had reduced cellularity relative to WT mice (Figure. 2-4A). Flow cytometry of these DLNs indicated normal frequencies of lymphoid and myeloid cell populations, indicating that the decreased cellularity was not due to
the reduction in a particular cell type (Figure. 2-4B). Similar frequencies of interleukin 2 (IL-2)-
and IFN-γ-producing T cells and a modest reduction in the frequency of IL-17A-producing T
cells were observed in DLNs from Bhlhe40−/− mice relative to WT mice in response to stimulation
with MOG35−55 or concanavalin A (ConA) as measured by ELISPOT assays (Figure. 2-4C).
Consistent with their decreased IL-17A production, the frequency of RORγt+ CD4+ T cells was
moderately decreased in DLNs of Bhlhe40−/− mice (Figure. 2-4, D and E). DLNs from Bhlhe40−/−
mice showed a markedly reduced frequency of MOG35−55-specific GM-CSF-producing T cells
relative to WT mice, and an increased frequency of MOG35−55-specific IL-10-producing T cells
(Figure. 2-5A). Intracellular cytokine staining (ICS) of DLN cells also showed diminished GM-
CSF (Figure. 2-5, B & C) and increased IL-10 production (Figure. 2-5, D and E) from Bhlhe40−/
−/− CD4+ T cells. The defect in GM-CSF production by Bhlhe40−/− CD4+ T cells could not be fully
restored in vitro by culture with IL-1β or IL-23, cytokines that are reported to promote GM-CSF
secretion68, 174, 175, 176 (Figure. 2-5, F and G). The increased IL-10 production by Bhlhe40−/− CD4+
T cells was significantly augmented by in vitro culture with IL-12 (Figure. 2-5, H and I). We
analyzed cytokine production by Bhlhe40−/− T cells in three additional settings. First, instead of
using the self-peptide MOG35−55, we immunized mice with the foreign, MHC class II-restricted
peptide of chicken ovalbumin (OVA323−339). Again, Bhlhe40−/− CD4+ T cells failed to produce
GM-CSF following this immunization, with intact IL-2 and IFN-γ production, and partially
decreased IL-17A production (Figure. 2-6A). Second, we analyzed CD4+ T-cell cytokine
responses in immunized mixed BM chimeric mice. Bhlhe40−/− T cells showed a T cell-intrinsic
defect in GM-CSF secretion in this setting, with normal IL-17A secretion (Figure. 2-6, B and
C). Third, we analyzed cytokine responses in immunized Rag1−/− mice that had received transfers
of purified WT or Bhlhe40−/− CD4+ T cells (Figure. 2-6D). Bhlhe40−/− CD4+ T cells showed
reduced GM-CSF and increased IL-10 production in this setting, with normal IFN-γ production. For unclear reasons, IL-17A secretion by $Bhlhe40^{-/-}$ CD4$^+$ T cells appeared decreased in the spleen, but increased in the DLN in this setting. $\gamma\delta$ T cells in the DLNs of immunized $Bhlhe40^{-/-}$ mice also showed a selective loss of GM-CSF production that could not be restored by in vitro culture with IL-1β and/or IL-23 (Figure 2-7, A and B). Naive splenic $\gamma\delta$ T cells from non-immunized $Bhlhe40^{-/-}$ mice were capable of responding to IL-1β, IL-23 and IL-12$^{199,201}$, as demonstrated by their increased size and granularity, but failed to produce normal levels of GM-CSF in these cultures (Figure 2-7, C and D). Overall, these results assessing T-cell responses in the periphery in combination with our findings in the CNS indicate that both CD4$^+$ and $\gamma\delta$ T cells require Bhlhe40 to produce GM-CSF.

### 2.3.3 Bhlhe40 regulates cytokine production by in vitro polarized T$_H$ cells

We tested whether $Bhlhe40^{-/-}$ CD4$^+$ T cells responded normally to in vitro activation. Stimulation with plate-bound anti-CD3 alone or anti-CD3 and anti-CD28 led to normal IL-2 secretion, reduced GM-CSF secretion and increased IL-10 secretion by $Bhlhe40^{-/-}$ CD4$^+$ T cells (Figure 2-8A). We next examined cytokine production by in vitro polarized T$_H$ cells from WT and $Bhlhe40^{-/-}$ mice. In general, polarized T$_H$ cells from $Bhlhe40^{-/-}$ mice showed normal production of their signature cytokines, although $Bhlhe40^{-/-}$ T$_H$1 cells showed a ~40% decrease in IFN-γ production, consistent with a previous report$^{30}$ (Figure 2-8, B and C). It is possible that Bhlhe40 served as a cofactor for T-bet to enhance IFN-γ production in T$_H$1 cells, like its role in iNKT cells$^{34}$. Consistent with these cytokine results, we found decreased expression of the transcription factor T-bet in $Bhlhe40^{-/-}$ T$_H$1 cells, with nearly normal expression of Gata-3 and RORγt in $Bhlhe40^{-/-}$ T$_H$2 and T$_H$17 cells, respectively (Figure 2-8D). GM-CSF production varied between WT T$_H$1, T$_H$2 and T$_H$17 cells, with the least production of GM-CSF found in T$_H$1 cultures.
(Figure. 2-8, B, C and E), consistent with an earlier report\textsuperscript{174}. \textit{Bhlhe40}^{-/-} T cells showed markedly reduced production of GM-CSF under all conditions of differentiation (Figure. 2-8, B, C and E). This lack of GM-CSF production by \textit{Bhlhe40}^{-/-} T cells was also seen in \textit{T}_{H}\textsubscript{17} cultures that utilized TGF-\textit{\beta}3 in place of TGF-\textit{\beta}1 (data not shown)\textsuperscript{189}. \textit{Bhlhe40}^{-/-} \textit{T}_{H}\textsubscript{1} and \textit{T}_{H}\textsubscript{17} cells showed increased IL-10 production relative to WT cells, while WT and \textit{Bhlhe40}^{-/-} \textit{T}_{H}\textsubscript{2} cells showed similar, high-level IL-10 production (Figure. 2-8, B and C). The increased IL-10 production in \textit{Bhlhe40}^{-/-} \textit{T}_{H}\textsubscript{1} cells came largely from IFN-\gamma^{+} cells, while in \textit{T}_{H}\textsubscript{17} cultures it came from both IL-17A^{+} and IL-17A^{-} populations (Figure. 2-8F). We further tested whether the increased IL-10 production by \textit{Bhlhe40}^{-/-} \textit{T}_{H} cells could contribute to their diminished GM-CSF production. Co-culture of congenically marked WT and \textit{Bhlhe40}^{-/-} \textit{T}_{H} cells under non-polarizing conditions showed that increased IL-10 secretion and decreased GM-CSF secretion were both cell intrinsic properties of \textit{Bhlhe40}^{-/-} \textit{T}_{H} cells (Figure. 2-8G). We tested whether abnormal GM-CSF and IL-10 production by \textit{Bhlhe40}^{-/-} \textit{CD4}^{+} T cells could be restored by the reintroduction of \textit{Bhlhe40}. Retrovirally transduced \textit{Bhlhe40}^{-/-} \textit{T}_{H}\textsubscript{1} and \textit{T}_{H}\textsubscript{17} cells overexpressing \textit{Bhlhe40} produced increased levels of GM-CSF and decreased levels of IL-10 compared with non-transduced \textit{Bhlhe40}^{-/-} T cells or \textit{Bhlhe40}^{-/-} T cells transduced by an empty retrovirus (Figure. 2-9). Overexpressing \textit{Bhlhe40} modestly increased GM-CSF and decreased IL-10 production by WT \textit{T}_{H}\textsubscript{17} cells, but had little effect on WT \textit{T}_{H}\textsubscript{1} cells.

### 2.3.4 Molecular analysis of \textit{Bhlhe40}-regulated target genes

We performed expression microarray analysis on WT and \textit{Bhlhe40}^{-/-} \textit{CD4}^{+} T cells cultured in \textit{T}_{H}\textsubscript{1}, \textit{T}_{H}\textsubscript{2} and \textit{T}_{H}\textsubscript{17} conditions and validated these results by quantitative RT-PCR for selected transcripts (Figure. 2-10). This analysis confirmed that \textit{Bhlhe40}^{-/-} T cells were able to acquire characteristics largely reflective of normal \textit{T}_{H}\textsubscript{1}, \textit{T}_{H}\textsubscript{2} or \textit{T}_{H}\textsubscript{17} differentiation, including their
expression of signature cytokines and transcription factors (Figure. 2-10, A and C). However, a common set of genes was affected by the loss of Bhlhe40 across two or more Th lineages (Figure. 2-10, B, D and E), including reduced expression of Csf2, Il3, Il1a, Ccl1, Ifitm3 and Ptgs2. Notably, expression levels of Il10, Ikzf3 (Aiolos) and Xcl1 were increased in Bhlhe40−/− T cells. We confirmed reduced secretion of IL-3 by Bhlhe40−/− CD4+ T cells following stimulation with anti-CD3 alone or anti-CD3 and anti-CD28 (Figure. 2-10F). To examine whether the Il3/Csf2 and Il10 loci were directly bound by Bhlhe40, we performed an analysis of Bhlhe40 ChIP-Seq data generated from CH12 cells by the Mouse Encyclopedia of DNA Elements (ENCODE) Consortium202. RNA sequencing (RNA-Seq) performed on this B-cell lymphoma line by the same Consortium showed these cells to express Bhlhe40, Il3, Csf2 and Il10. Within the Il3/Csf2 locus, Bhlhe40 seemed to bind multiple sites, including a previously identified distal enhancer located ~30kb downstream of Csf2 that regulates the expression of both cytokine genes in this locus (Figure. 2-11A)203. Within the Il10 locus, Bhlhe40 also seemed to bind multiple sites, including two known regulatory elements (Figure. 2-11B)204, 205. In both loci, many regions bound by Bhlhe40 were also bound by the general transcriptional regulators p300206, 207 and BRG1203 and the Th cell subset-specific transcription factors T-bet208 and RORγt207. Bhlhe40 is likely one of many transcriptional regulators present at these regions working in concert to control gene expression.

2.3.5 IL-10R blockade renders Bhlhe40−/− mice susceptible to EAE

As our expression microarray experiments demonstrate that Bhlhe40 deficiency leads to the dysregulation of hundreds of genes in polarized Th cells, we expect that the summation of these changes in gene expression underlies the T cell-intrinsic requirement for Bhlhe40 in T-cell encephalitogenicity. Nevertheless, we sought to test whether the administration of exogenous
GM-CSF could render Bhlhe40−/− mice susceptible to EAE. In two individual experiments, Bhlhe40−/− mice treated daily with either 10 or 100 ng per day recombinant murine GM-CSF intraperitoneally beginning on the day of immunization did not develop clinical EAE (n = 3–5 mice per experiment). In both of these experiments, control WT mice experienced a typical course of EAE, indicating successful immunization. Systemic GM-CSF treatment at a dose of 10 ng per day was reported to replace endogenous GM-CSF in Csf2−/− mice, such that these mice developed EAE when GM-CSF was administered after immunization209. Therefore, we take our results to indicate that Bhlhe40−/− mice resist EAE for reasons beyond just their defective production of GM-CSF by T cells. We also tested whether blockade of IL-10 signaling could affect the development of EAE in Bhlhe40−/− mice. Groups of WT or Bhlhe40−/− mice were treated biweekly with control rat IgG or anti-IL-10R blocking antibody (clone 1B1.3A), beginning 1 day prior to immunization and continuing throughout the course of the experiment. Anti-IL-10R antibody treatment of immunized WT mice led to the high incidence of early morbidity and a shock-like syndrome, characterized by ruffled fur, weight loss and a hunched posture, with rapid death prior to the onset of clinical EAE (Figure. 2-12A). This unexpected mortality, which we attribute to acute IL-10R blockade in the setting of a strong immune stimulus, was not apparent in immunized WT mice treated with control antibody, which experienced a typical course of EAE (Figure. 2-12B and Table 1). Immunized Bhlhe40−/− mice treated with anti-IL-10R antibody experienced only infrequent early morbidity. Those WT mice treated with anti-IL-10R antibody that did not succumb to an early death developed very severe EAE. Immunized Bhlhe40−/− mice treated with control antibody resisted EAE, while nearly half of all Bhlhe40−/− mice treated with anti-IL10R antibody developed severe clinical EAE, like that of control
antibody-treated WT mice. Overall, these results reveal that the pathogenicity of $Bhlhe40^{-/-}$ autoreactive T cells is at least partially, regulated by IL-10.
2.4 Discussion

Here we have shown that Bhlhe40−/− mice are protected from immunization-induced autoimmune neuroinflammation. TH cells have a cell-intrinsic requirement for Bhlhe40 during encephalomyelitis, and in these cells Bhlhe40 transcriptionally regulates the production of GM-CSF and IL-10. In vivo blockade of IL-10R allows immunized Bhlhe40−/− mice to develop EAE, indicating the important role of this cytokine in regulating autoreactive T-cell pathogenicity.

Bhlhe40−/− mice were also protected from adoptively transferred EAE using an encephalitogenic TH1 cell line, but only when a low number of pathogenic T cells were transferred (Figure. 2-3, B and C). In this system, cytokine-producing host TH cells infiltrate the CNS and participate in regulating neuroinflammation. Large numbers of adoptively transferred, autoreactive WT TH1 cells can overcome a requirement for Bhlhe40 in host cells (Figure. 2-3C). Overall, these results are reminiscent of experiments in which low numbers of an encephalitogenic TH1 cell line were unable to induce disease in IL-17A-deficient mice, but in which higher cell numbers could initiate neuroinflammation. We favor a model in which the explanation for our adoptive transfer results stems from a requirement for Bhlhe40 in host TH and γδ T cells, although it remains a possibility that Bhlhe40 also functions in non-T cells during adoptively transferred EAE.

The characteristics that define pathogenic TH cells in EAE have been a subject of recent debate. Neither IFN-γ nor IL-17, the signature cytokines of TH1 and TH17 cells, respectively, are required for the development of EAE, yet in vitro derived TH1 and TH17 cells specific for myelin antigens both can induce encephalomyelitis. These seemingly conflicting observations have been reconciled by the finding that GM-CSF, produced by both
T\textsubscript{H}1 and T\textsubscript{H}17 cells, serves a non-redundant pro-inflammatory role in EAE development\textsuperscript{172, 173, 174, 175, 176}. Control of Csf2 expression involves AP-1, NFAT, Runx1, NF-kB, histone acetylation and BRG1 recruitment\textsuperscript{177, 178, 179, 180, 181, 182, 203}. c-Rel- and NF-kB1 deficient T cells produce less GM-CSF, but these factors may be non-selective in their impact on cytokine production\textsuperscript{180, 181}. Malt1, a protease regulating the NF-kB pathway, was reported to be required for GM-CSF production by T\textsubscript{H}17 cells, but not T\textsubscript{H}1 cells\textsuperscript{211}, implying that the pathways that control GM-CSF production may not be the same in all T\textsubscript{H} cell lineages. In fact, we find that Bhlhe40 appears to play a less important role in regulating GM-CSF production in T\textsubscript{H}2 cells compared with its role in T\textsubscript{H}1 or T\textsubscript{H}17 cells, as GM-CSF secretion is only partly abrogated in Bhlhe40\textsuperscript{-/-} T\textsubscript{H}2 cells. Similarly, our observation that Bhlhe40\textsuperscript{-/-} mice can infrequently develop clinical EAE with some T-cell production of GM-CSF (Figure 2-2F) in the CNS indicates that Bhlhe40 is not absolutely required for Csf2 expression in T cells. Bhlhe41 (also known as Dec2, Sharp1 or Bhlhb3), the closest homologue to Bhlhe40, is expressed most highly in T\textsubscript{H}2 cells among activated T\textsubscript{H}-cell subsets\textsuperscript{198, 212}, and perhaps this factor can substitute for Bhlhe40 in some settings in the regulation of Csf2 transcription.

The role of ROR\textgreek{gamma} in GM-CSF production by CD4\textsuperscript{+} T cells is controversial\textsuperscript{174, 175, 176}. One study found that Rorc\textsuperscript{-/-} CD4\textsuperscript{+} T cells activated in the presence of anti-IFN-\gamma and anti-IL-12 neutralizing antibodies produced less GM-CSF than WT CD4\textsuperscript{+} T cells, but Rorc\textsuperscript{-/-} T\textsubscript{H}1 and T\textsubscript{H}17 cells produced normal or even greater GM-CSF than WT T cells\textsuperscript{174}. These investigators found that retroviral ROR\textgreek{gamma} overexpression increased GM-CSF production, so despite these incongruous results, they suggested that ROR\textgreek{gamma} drives GM-CSF production. A second study also showed that Rorc\textsuperscript{-/-} T\textsubscript{H}17 cells produced an increased amount of GM-CSF relative to WT T\textsubscript{H}17 cells\textsuperscript{175}, leading this group to suggest that ROR\textgreek{gamma} is not required for GM-CSF production. We
observed normal \textit{Rorc} expression in \textit{Bhlhe40}^- T_{H17} cells in our microarrays (\textbf{Figure. 2-10A}), and speculate that Bhlhe40 directly regulates \textit{Csf2} expression.

The recently described molecular signatures of pathogenic versus non-pathogenic T_{H17} cells\textsuperscript{189}\textsuperscript{195} includes four genes that we find to be selectively regulated by Bhlhe40 (\textit{Csf2} and \textit{Il3} for pathogenic T_{H17} cells; \textit{Il10} and \textit{Ikzf3} for non-pathogenic T_{H17} cells) (\textbf{Figure. 2-10}). Given these data, we believe that Bhlhe40 serves as a transcription factor that shapes the encephalitogenicity of T_{H17} cells. Considering IL-10 production in particular, we find the largest difference between WT and \textit{Bhlhe40}^- T_{H17} cells to be in T_{H1} cultures or in cells from immunized mice cultured with IL-12. This type of IL-10 production by T_{H1} cells has been reported to involve signals through ERK, STAT4 and e-Maf\textsuperscript{195}. Our data point to a cell-intrinsic role for Bhlhe40 in regulating IL-10 production in T cells, and we speculate that Bhlhe40 serves as a direct negative regulator of \textit{Il10}. Bhlhe40 could be acting in T_{H} cells in some cases as a transcriptional activator and in others as a transcriptional repressor on different direct target genes, perhaps based on its interactions with other transcription factors. Examples exist for both activating and repressive functions of Bhlhe40\textsuperscript{213, 214}. It also remains possible that some genes whose expression is abnormal in \textit{Bhlhe40}^- T_{H1} cells are indirect targets of Bhlhe40.

Supporting the importance of IL-10 in EAE pathogenesis, we found that nearly half of all \textit{Bhlhe40}^- mice treated with an anti-IL-10R blocking antibody developed EAE. Although IL-10-deficient mice are known to develop more severe EAE than WT\textsuperscript{191, 192, 193}, they have not been reported to exhibit early mortality prior to the onset of encephalomyelitis. We speculate that the sudden inhibition of IL-10 signaling by anti-IL-10R antibody in our experiments is fundamentally different than the lifelong absence of this signaling, such that antibody-mediated blockade resulted in a pro-inflammatory cytokine-driven shock-like syndrome. Antibody-treated
Bhlhe40\textsuperscript{−/−} mice were largely protected from this syndrome, implying that pro-inflammatory cytokines in these mice, perhaps including GM-CSF, were produced at lower levels.

Interestingly, some Bhlhe40\textsuperscript{−/−} mice were fully protected from EAE even in the setting of IL-10R blockade, and we speculate that this was also due to their decreased levels of pro-inflammatory cytokines. Nevertheless, our results support the notion that Bhlhe40\textsuperscript{−/−} T\textsubscript{H} cells can be encephalitogenic in the setting of IL-10R blockade. Further experiments will be performed to determine whether Bhlhe40\textsuperscript{−/−} T\textsubscript{H} cells are themselves the critical source of IL-10 preventing autoimmunity in these mice.

While we were preparing this manuscript, a separate study also identified Bhlhe40 as a transcription factor required for autoreactive CD4\textsuperscript{+} T-cell responses using the EAE system\textsuperscript{33}. Martínez-Llordella et al. identified Bhlhe40 as a gene whose expression was induced in activated CD4\textsuperscript{+} T cells in a CD28-dependent manner. These authors found Bhlhe40-deficient mice (a separately generated line than was used in our study\textsuperscript{26} to be resistant to the induction of EAE, and found decreased CD4\textsuperscript{+} T cell production of several cytokines, including GM-CSF. Both our study and theirs found decreased IL-17A responses in immunized Bhlhe40\textsuperscript{−/−} mice, with both identifying this defect as cell-extrinsic based on immunization experiments in mixed bone marrow chimeras. Our in vitro experiments showing normal Il17a transcript and IL-17A production by Bhlhe40\textsuperscript{−/−} T\textsubscript{H17} cells also suggest no direct role for Bhlhe40 in controlling this cytokine.

There are some key differences between our study and the work of Martínez-Llordella et al. They observed MOG\textsubscript{35-55}-specific CD4\textsuperscript{+} T-cell responses in Bhlhe40\textsuperscript{−/−} mice to be only slightly reduced in frequency at day 7 after immunization as determined by tetramer staining, but to be completely absent when assessed with a proliferation assay or by ELISAs for IL-17A, IFN-\gamma and
GM-CSF. In our assessment of CD4+ T-cell priming by ELISPOT assays using DLN cells at day 7 after immunization, we found essentially intact IL-2 and IFN-γ responses, partially decreased IL-17A responses and absent GM-CSF responses (Figure. 2-4C and Figure. 2-5A). We speculate that technical differences in the assay formats, cell culture densities or assay times could explain these discrepancies. An additional difference between our study and that of Martínez-Llordella et al. is that we found normal IL-2 production after in vitro stimulation of Bhlhe40−/− CD4+ T cells (Figure. 2-8A), while they saw a ~50% reduction in IL-2 secretion. Our results are consistent with those of Miyazaki et al.32 who reported normal IL-2 secretion by Bhlhe40−/− CD4+ T cells, and we again speculate that differences in cell culture conditions may explain these discrepancies. Finally, our expression microarrays and phenotypic analysis of polarized Bhlhe40−/− T₃H cells identified increased transcription and secretion of IL-10 by these cells compared with WT T₃H cells. Il10 was not identified as being differentially expressed in the transcriptional analysis performed by Martínez-Llordella et al., although we speculate that this may be related to their analysis of naive T cells activated under non-polarizing conditions at early time points (4 and 24h).

In conclusion, we have demonstrated that Bhlhe40 is required for the encephalitogenicity of CD4+ T cells, likely through its regulation of GM-CSF and IL-10 production. Bhlhe40 is also required for γδ T-cell production of GM-CSF. Bhlhe40 controls these cytokines transcriptionally, but further investigation will be required to understand the molecular details of this regulation. Moreover, the question of whether regulation of these cytokines by Bhlhe40 in T cells influences pathogenesis in other autoimmune or infectious diseases is intriguing. The role of GM-CSF in autoimmune disease has prompted clinical interest in therapeutics to target this cytokine in
rheumatoid arthritis and multiple sclerosis\textsuperscript{215}. Bhlhe40, or pathways that regulate its expression or function, could also represent therapeutic targets in human autoimmunity.
2.5 Methods

Mice. C57BL/6 (Taconic), B6.SJL (Taconic), B6.PL-Thy1a/CyJ (Jackson), Rag1\(^{-/-}\) (on a C57BL/6 background, Jackson) and Bhlhe40\(^{-/-}\) mice\(^{30}\) (backcrossed 10 generations to the C57BL/6 background) were maintained in our SPF facility. Experiments were performed with mice of either sex (groups were sex matched within individual experiments) at 8–24 weeks of age. No Bhlhe40\(^{-/-}\) mice on the C57BL/6 background displayed lymphoproliferative disease in our colony throughout this age range, in keeping with a previous report of C57BL/6 Bhlhe40\(^{-/-}\) mice\(^{32}\). Flow cytometry of splenocytes and bone marrow from Bhlhe40\(^{-/-}\) mice in our colony at the ages used in these experiments showed normal leukocyte populations (Figure. 2-13). All animal experiments were approved by the Animal Studies Committee of Washington University.

Bone marrow transplantation and T\(_H\) cell transfers. Bone marrow cells were collected from femurs and tibias of donor mice, and 10–20 million cells were injected intravenously into recipient mice following irradiation (1,200 rads). Mixed chimeras were prepared by mixing donor bone marrow cells at a 1:1 ratio before injection. Mice were used in experiments 13–16 weeks following transplantation. For cell transfers, total CD4\(^{+}\) T cells were magnetically purified from donor splenocytes (Invitrogen Dynabeads FlowComp Mouse CD4 kit, typical purity ~90–95%). CD4\(^{+}\) T cells (7 to 10 million) were injected intravenously into Rag1\(^{-/-}\) mice 1 day prior to the induction of EAE or immunization for the assessment of T-cell responses.

Induction of EAE and immunizations. For active EAE induction, mice were immunized subcutaneously with 100mg MOG(35–55) (C S Bio Co.) emulsified in CFA (made with 5 mg/ml heat-killed Mycobacterium tuberculosis H37Ra (BD Difco) in incomplete Freund’s adjuvant
Pertussis toxin (List Biological Laboratories) was injected intraperitoneally (300ng) on days 0 and 2. Mice were observed for signs of EAE and graded on a standard 0–5 scale as described\textsuperscript{175}. In some experiments, mice were treated daily with recombinant murine GM-CSF (Peprotech) at a dose of 10 ng per day or 100 ng per day intraperitoneally, beginning on the day of immunization and continuing throughout the course of the experiment. In some experiments, mice were treated with control rat IgG (Sigma) or anti-IL-10R monoclonal antibody (clone 1B1.3A, BioXcell)\textsuperscript{216}. Mice were given 250 mg of antibody intraperitoneally two times per week, beginning 1 day prior to immunization. For histology experiments, spinal cords were dissected, fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E).

For adoptively transferred EAE, MOG\textsubscript{35–55}-specific T\textsubscript{H1} cell lines were generated by culturing CD4\textsuperscript{+} T cells from MOG\textsubscript{35–55}-immunized CD90.1 congenic B6.PL-Thy1a/CyJ mice with MOG\textsubscript{35–55}, irradiated C57BL/6 splenocytes, IL-2 and IL-12 for two or four rounds of stimulation\textsuperscript{200}. Four days after the last stimulation, 5–10 million live T cells were intravenously transferred to recipient mice.

For analysis of T-cell responses in DLNs, mice were immunized in hind footpads with 10 nanomoles MOG\textsubscript{35–55} or OVA\textsubscript{323–339} emulsified in CFA. Popliteal lymph nodes were collected on day 7.

**Cell preparation.** DLNs from immunized mice were digested with 250 mg/ml collagenase B (Roche) and 30 U/ml Dnase I (EMD) for 40–60 min at 37°C with stirring in Iscove’s modified Dulbecco’s media containing 10% FCS, L-glutamine, sodium pyruvate, non-essential amino
acids, penicillin/streptomycin and 2-mercaptoethanol (cIMDM). EDTA (5 mM final) was added and cells were incubated on ice for 5 min. Cells were passed through a 70-mm strainer before cell counting with trypan blue. Spleens were mashed between frosted glass slides and made into a single-cell suspension. Red blood cells were lysed with ACK lysis buffer. Cells were passed through a 70-mm strainer before cell counting with acetic acid.

Brains and spinal cords were removed following perfusion with 30 ml PBS via cardiac puncture of the left ventricle. Organs were minced and digested with 500 mg/ml Type I collagenase (Sigma) and 10 mg/ml DNAse I (Sigma) in the presence of 0.1 mg/ml TLCK trypsin inhibitor (Sigma) and 10 mM Hepes (pH 7.4) in HBSS at room temperature for 1h. Following centrifugation, cells were resuspended in 70% Percoll (Sigma) in HBSS and additional 37% and 30% layers were added above the cells. Gradients were centrifuged for 30 min at 1,200g. The 30% Percoll layer containing debris was discarded, and the cells from the 37% layer were collected and washed in HBSS before stimulation or flow cytometry.

Cell culture. DLN cells from MOG35–55-immunized mice were cultured in cIMDM and stimulated with 10mM MOG35–55 for 4 days before supernatants were collected for ELISAs or before ICS. In some cases, IL-1β (10 ng/ml, BioLegend), IL-23 (20 ng/ml, BioLegend) or IL-12 (2 ng/ml, BioLegend) was added to the culture at set-up.

Splenocytes or magnetically purified total splenic CD4\(^+\) T cells (Invitrogen Dynabeads FlowComp Mouse CD4 kit) from untreated mice cultured in cIMDM were stimulated with plate-bound anti-CD3 antibody (10 mg/ml, clone 1452C11, BioLegend) with or without plate-bound anti-CD28 antibody for 48–72h (5 mg/ml, clone 37.51, BioLegend). Supernatants were collected
for ELISAs or ICS was performed on cells. In some experiments, splenocytes were cultured for 3 days with IL-1β and/or IL-23 without TCR stimulation. ICS was subsequently performed on these cells, gating on γδ T cells.

For T<sub>H</sub> cell polarization, magnetically purified total CD4<sup>+</sup> T cells (Invitrogen Dynabeads FlowComp Mouse CD4 kit) or naive CD4<sup>+</sup> T cells (Stemcell Technologies Easysep Mouse Naive CD4<sup>+</sup> T Cell Isolation kit, typical purity ~90–96%) were cultured in cIMDM with plate-bound anti-CD3 and anti-CD28 antibodies in the presence of cytokines (BioLegend except TGF-β3) and neutralizing antibodies (BioLegend except anti-IL-12) as follows. T<sub>H</sub>1 conditions: IFN-γ (200 ng/ml), IL-12 (2 ng/ml), anti-IL-4 (5 mg/ml, clone 11B11). T<sub>H</sub>2 conditions: IL-4 (10 ng/ml), anti-IFN-γ (5 mg/ml, clone XMG1.2 or clone H22), anti-IL-12 (10 mg/ml, clone Tosh, a gift from Emil R. Unanue, Washington University). T<sub>H</sub>17 conditions: TGF-β1 (2 ng/ml) or TGF-β3 (2 ng/ml) (Miltenyi), IL-6 (25 ng/ml), IL-1β (10 ng/ml), IL-23 (10 ng/ml), anti-IL-4 and anti-IFN-γ. Cells were split at day 3, and on days 4–5 cells were either used for ICS or stimulated for 24h with plate-bound anti-CD3 and anti-CD28 for assessment of cytokine secretion by ELISA.

**ELISPOT and ELISA assays.** ELISPOT assays were performed on Mutiscreen Filter Plates (Millipore). DLN cells from immunized mice were plated at 5×10<sup>5</sup>–1×10<sup>6</sup> cells per well in cIMDM and stimulated with 10 mM peptide or 1 mg/ml ConA overnight at 37°C. IL-2, IFN-γ and IL-10 ELISPOT antibody pairs were from BD Bioscience. IL-17 and GM-CSF ELISPOT antibody pairs and streptavidin-alkaline phosphatase were from BioLegend. Plates were developed with NBT/BCIP substrate (Roche) and spots were counted on an Immunospot counter (Cellular Technology Ltd.). ELISA assays were performed on Nunc Maxisorp plates using the
same antibody pairs as above but were developed with streptavidin-HRP (BioLegend) and TMB substrate (BioLegend). Standard curves for these ELISAs were generated with purified cytokines. The Mouse IL-3 ELISA MAX kit was used to measure IL-3 (BioLegend).

**Flow cytometry.** All antibodies were used at a dilution of 1/200 except where noted.

Streptavidin conjugates were used at a dilution of 1/400. The following anti-mouse antibodies were from BioLegend: FITC, APC or biotin anti-CD3e (1452C11), PB anti-CD4 (RM4-5), APC-Cy7 or PerCP-Cy5.5 anti-CD8a (53-6.7), APC or biotin anti-TCR γδ (GL3), FITC anti-CD25 (PC61), PB anti-CD44 (IM7), FITC anti-B220 (RA3-6B2), FITC anti-CD45.1 (A20), APC or biotin anti-CD45.2 (104), PerCP-Cy5.5 anti-Siglec H (551), FITC anti-Ly6C (HK1.4), PE anti-Ly6G (1A8), PE-Cy7 anti-CD11b (M1/70), APC-Cy7 anti-CD11c (N418), PB anti-I-A/I-E (M5/114.15.2, used at 1/500), PE-Cy7 anti-IFN-γ (XMG1.2), PerCP-Cy5.5 anti-IL-17A (TC11-18H10.1), PE anti-GM-CSF (MP1-22E9), PE anti-KLH (RTK2758, isotype rat IgG2a, k), APC or biotin anti-IL-10 (JES5-16E3) and streptavidin-APC. The following anti-mouse antibodies were from BD Biosciences: V450 anti-CD4 (RM45) and V500 anti-B220 (RA3-6B2). APC anti-CD62L (MEL-14), PE anti-CD25 (PC61.5), APC-e780 anti-CD45 (30-F11, used at 1/600), PE anti-Foxp3 (FJK-16s), eFluor660 anti-T-bet (eBio4B10), PerCP-eFluor710 anti-Gata-3 (TWAJ), APC anti-RORγt (AFKJS-9) and streptavidin-APC-e780 were from eBioscience.

For ICS, cells were stimulated with PMA (50 ng/ml, Enzo Life Sciences) and ionomycin (1 µM, Enzo Life Sciences) in the presence of brefeldin A (1 mg/ml, Enzo Life Sciences) for 4–5 hours. Surface staining was performed in FACS buffer (0.5% BSA, 2 mM EDTA, 0.02% sodium azide in PBS) in the presence of Fc receptor blocking antibody (clone 93, BioLegend) for 20 min at
4°C followed by streptavidin staining if necessary. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 20 min at room temperature followed by permeabilization (0.1% BSA, 0.5% saponin in PBS) or with the BD Cytofix/Cytoperm Fixation and Permeabilization Kit. Cytokine staining was performed in permeabilization buffer for 20 min at 4°C. Staining for intracellular Foxp3, T-bet, Gata-3 and RORγt was performed in the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Flow cytometry was performed on a FACSCanto II (BD Bioscience) and data was analyzed with FlowJo (Tree Star Inc.).

For microarray experiments, naive CD4+ T cells (CD4+B220CD25CD44loCD62Lhi) were purified (98% purity) by cell sorting on a FACSAria II (BD Biosciences) after B-cell depletion using anti-B220 magnetic beads (Invitrogen).

**Expression microarrays.** Naive CD4+ T cells were cultured in Th0, Th1, Th2 or Th17 conditions for 4 days and stimulated for 4h with PMA and ionomycin before total RNA was isolated (EZNA MicroElute Total RNA Kit, Omega Bio-Tek). RNA was amplified (Ovation PicoSL WTA System V2, Nugen), labelled (Encore Biotin Module, Nugen), fragmented and hybridized to Affymetrix Gene 1.0ST arrays. Array data were imported into Arraystar 5 software (DNAstar) and then normalized using the robust multi-array analysis method with quantile normalization. Probesets were curated according to a validated gene annotation, and only these probesets were utilized in subsequent analyses. Correlation analysis was performed using Arraystar 5. Heat maps were generated using GENE-E software (Broad Institute, http://www.broadinstitute.org/cancer/software/GENE-E/). For analysis of GSE14308217, which
was performed on Affymetrix Mouse Genome 430 2.0 arrays, we also used the robust multi-array analysis method with quantile normalization using Arraystar 5 software.

**Quantitative RT-PCR.** Magnetically purified total splenic CD4$^+$ T cells (Invitrogen Dynabeads FlowComp Mouse CD4 kit) were cultured in T$_{h1}$1, T$_{h2}$ or T$_{h17}$ conditions for 4 days and stimulated for 4h with plate-bound anti-CD3 and anti-CD28 before total RNA was isolated (EZNA MicroElute Total RNA Kit, Omega Bio-Tek). cDNA was synthesized (High Capacity RNA-to-cDNA Kit, Invitrogen) and quantitative real-time PCR was performed according to the manufacturer’s instructions with Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Expression of genes was determined relative to *Hprt* by the DCT method.

The following primers were used for quantitative RT-PCR. *Ccl1*, forward 5’-

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GGCTGCCGTGTGGGATAACG-3’, reverse 5’-AGGTGATTTTGAACCCACGT-3’; Cs2, forward 5’-GCCATCAAAGAAAGGCCTGAA-3’, reverse 5’-GCCGGTCTGCACACATGT-3’; Hprt, forward 5’-TCAGTCAACGCGGCCGACATAAA-3’, reverse 5’-

GGGGCTGTACCTGCTTAACCAG-3’, Ifitm3, forward 5’-CCCCAAACTACGAAAGAATCA-3’, reverse 5’- ACCATCTTCCGATCCCTAGAC-3’; Ifng, forward 5’-

GGATGCATTTCATGAGATGTCC-3’, reverse 5’-CCTTTTCCGCTTCCTGAG-3’; Ikzf3, forward 5’-

GCTCCGCTCATAATTGTCT-3’, Il1a, forward 5’-CGAAGACTACAGTCTGAGTGG-3’, Il4a, forward 5’-

GGGATACCCACACGTTAACCA-3’, reverse 5’-AGGTATTGTCGATCAGGCTCT-3’; Il4,
forward 5’-ATCATCGGCATTTTGAACGAGG-3’, reverse 5’-
TGCAGCTCCATGAGAACACTA-3’; II10, forward 5’-AGCCTTATCGGAAATGTCCAGT-
3’, reverse 5’-GGCCTTGTAGACACCTTGGT-3’; II17a, forward 5’-
TTTAATCCTTTGGCCAAAAA-3’, reverse 5’-CTTTTCCCTCCGCATTGACAC-3’; Ptgs2,
forward 5’-TGCACTATGGTTACAAAAGCTGG-3’, reverse 5’-
TCAGGAAGCTCCTATTTCCCT-3’; Xcl1, forward 5’-
TAGCTGTGTGAACCTAACAACCC-3’, reverse 5’-ACAGTCTTGATCGCTGCTTC-3’.

ChIP-Seq data analysis. Bhlhe40 ChIP-Seq data and RNA-Seq data performed by
ENCODE/Stanford/Yale using CH12 cells were directly viewed in the University of California
Santa Cruz (UCSC) Genome Browser on the NCBI37/mm9 mouse genome assembly.
Discriminative DNA Motif Discovery (DREME) software218 was used as part of the online
version of MEME-ChIP (http://meme.nbcr.net/meme/cgi-bin/meme-chip.cgi)219 to validate that
Bhlhe40 ChIP-Seq data identified the expected consensus motif for Bhlhe40 (CACGTG)21 with a
significant E-value (4.8x10⁻¹³⁹⁰).

Raw data for the following ChIP-Seq data sets were downloaded from the Gene Expression
Omnibus of the NCBI: GSE40463 (p300 in T_h1 and T_h2 cells)206, GSE33802 (T-bet in T_h1
cells)208, GSE20898 (Gata-3 in T_h2 cells)220, GSE40918 (RORγt and p300 in T_h17 cells)207, and
GSE23719 (BRG1 in stimulated T_h1, T_h2, and T_h17 cells)221. Each of these data sets was put
through the following pipeline of analysis within Galaxy (http://usegalaxy.org/)222, 223, 224:
FASTQ files were groomed, mapped to the NCBI37/mm9 mouse genome assembly using
Bowtie, converted from SAM to BAM files and peak called using MACS software. Tracks were then viewed in the UCSC Genome Browser.

**Cloning of Bhlhe40 and retroviral transduction.** The murine *Bhlhe40* full-length cDNA (coding for amino acids 2–411) was cloned with the addition of 50 and 30 BglII sites, and a Kozak sequence upstream of an N-terminal Myc tag from cDNA derived from GM-CSF-derived bone marrow dendritic cells. The following primers were used: forward, 5’-

AATAAGATCTCCACCATGGCAGAACAGAAGCTCATTTCTGAAGAAGACTTGAACGA
AGGATCCCATCAGGC

ACGGATCCAGCGC-3’, reverse, 5’-

ATAAAGATCTCCCCCTCCAGAGTTTAGTCTTTTGGTTTCT-3’. The resulting fragment was digested with BglII and cloned into the BglII site of the GFP-RV vector which contains an internal ribosome entry sequence upstream of GFP70. Retroviral vectors were transfected into Phoenix E cells by calcium phosphate coprecipitation\(^{225}\), and viral supernatants were collected after 2 days of culture. Magnetically purified total CD4\(^+\) T cells (Invitrogen Dynabeads FlowComp Mouse CD4 kit) were stimulated in T\(_{\text{H}1}\) or T\(_{\text{H}17}\) conditions, and viral supernatants were used to infect T cells by spin infection at 2,000 r.p.m. in the presence of 2 mg/ml polybrene for 1h on day 1 after activation. Cells were used for intracellular flow cytometry on day 4 of culture.

**Statistical analysis.** Differences between groups were analyzed by an unpaired, two-tailed Student’s t-test (Prism; GraphPad Software, Inc.), with P ≤ 0.05 considered significant.
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2.7 Author contributions

C.-C.L. planned and performed experiments and wrote the manuscript. T.R.B., E.A.S., J.S. and L.E.C. performed experiments. J.A.C. analyzed microarray data. C.C. and T.E. helped with the purification of T cells. R.T. provided $Bhlhe40^{-/-}$ mice and made helpful suggestions. T.L.M. and J.H.R. provided advice and edited the manuscript. B.T.E. supervised the study and wrote the manuscript.
Figure 2-1. Bhlhe40-deficient mice are protected from EAE.

(A) Mean clinical scores of EAE in immunized WT (n=18) or Bhlhe40<sup>−/−</sup> mice (n=18). Data are combined from four independent experiments. Incidence of disease: WT mice 17/18, Bhlhe40<sup>−/−</sup> mice 2/18. (B) H&E staining of spinal cord sections from WT and Bhlhe40<sup>−/−</sup> mice at day 13 after EAE induction. Scale bars: 200 mm in left images, 50 mm in insets. (C) Mean clinical scores of EAE in BM chimeric mice 13 weeks after bone marrow reconstitution (n=5 per group). (D) Relative expression of Bhlhe40 determined by expression microarrays performed by Wei et al. (GSE14308). (E) Spleens from Rag1<sup>−/−</sup> mice at day 38 after CD4<sup>+</sup> T cell transfer (day 37 following EAE induction) were analyzed by flow cytometry. (F) Mean clinical scores of EAE in Rag1<sup>−/−</sup> mice that received CD4<sup>+</sup> T cells from either WT or Bhlhe40<sup>−/−</sup> mice 1 day before immunization (n=4 per group). For all figures throughout, error bars show mean ± SEM.
Figure 2-2. Diminished neuroinflammation in Bhlhe40<sup>-/-</sup> mice.
Figure 2-2. Diminished neuroinflammation in Bhlhe40<sup>−/−</sup> mice.

(A) Flow cytometry of CNS myeloid cells from WT and Bhlhe40<sup>−/−</sup> mice (n=3 per group) on day 16 after immunization. (B) Percentage of MHC class II<sup>+</sup> microglia and infiltrating myeloid cells. (C-E) Flow cytometry of CD4<sup>+</sup> T cells in the CNS of WT and Bhlhe40<sup>−/−</sup> mice (n=3 per group) on day 16 after immunization. (C) CD4<sup>+</sup> T cell number. (D) Representative ICS for the indicated cytokines. (E) Frequency of CD4<sup>+</sup> T cells secreting each of the indicated eight possible combinations of GM-CSF, IL-17A and IFN-γ. (F) Infiltrating CNS cells were prepared from WT and Bhlhe40<sup>−/−</sup> mice on day 16 after EAE induction and ICS was performed. Four mice are shown. The first WT mouse achieved a peak EAE clinical score of 3 on day 11, and maintained this score through day 16. The second WT mouse achieved a peak EAE clinical score of 3 on days 12 and 13, but EAE had resolved to a clinical score of 1 on day 15 and 16. The first Bhlhe40<sup>−/−</sup> mouse showed mild clinical EAE, with a peak clinical score of 1 on days 11 and 12 with resolution to a score of 0 on days 13 through 16. The second Bhlhe40<sup>−/−</sup> mouse showed no evidence of disease through day 16 (clinical score of 0). (G) Flow cytometry of CD4<sup>+</sup> T cells in the CNS of WT and Bhlhe40<sup>−/−</sup> mice on day 14 after immunization. Representative ICS for IFN-γ and IL-10. (H) Flow cytometry of CD4<sup>+</sup> T cells in the CNS of WT and Bhlhe40<sup>−/−</sup> mice on day 29 after immunization. Representative staining for CD25 and Foxp3 to identify Tregs. (I, J) Flow cytometry of γδ T cells in the CNS of WT and Bhlhe40<sup>−/−</sup> mice (n=3 per group) on day 14 after immunization. (I) Representative ICS for IFN-γ, IL-17A and GM-CSF. (J) Frequency of γδ T cells secreting IFN-γ, IL-17A or GM-CSF.
Figure 2-3. High but not low number of autoreactive T_H1 cells induce passive EAE in 
*Bhlhe40^{−/−}* mice.

(A) ICS of our WT MOG\textsubscript{35-55}-specific T\textsubscript{H}1 cell line upon PMA/ionomycin stimulation tested on the day of adoptive transfer. (B, C) Mean clinical scores of EAE in WT or *Bhlhe40^{−/−}* mice after receipt of (B) 5 million or (C) 10 million adoptively transferred MOG\textsubscript{35-55}-specific WT T\textsubscript{H}1 cells. Incidence of disease: (B) WT mice 5/5, *Bhlhe40^{−/−}* mice 0/5, (C) WT mice 5/5, *Bhlhe40^{−/−}* mice 5/5.
Figure 2-4. Decreased cellularity of DLNs and T\textsubscript{H}17 responses in immunized \textit{Bhlhe40}\textsuperscript{-/-} mice.
Figure 2-4. Decreased cellularity of DLNs and TH17 responses in immunized Bhlhe40<sup>−/−</sup> mice.

(A) WT (n=33) and Bhlhe40<sup>−/−</sup> mice (n=32) were immunized with MOG<sub>35-55</sub>/CFA, and DLNs were collected at day 7. Live cell numbers per DLN were determined. Data are compiled from 10 independent experiments. (B) Frequencies of the indicated cell types in DLNs from immunized mice at day 7 (n=3 mice per group). Cell types were identified by the following surface markers using flow cytometry: B cells, B220<sup>+</sup>MHC II<sup>+</sup>; CD4<sup>+</sup> T cells, B220<sup>−</sup>CD3<sup>ε</sup><sup>+</sup>CD4<sup>+</sup>CD8<sup>−</sup>; CD8<sup>+</sup> T cells, B220<sup>−</sup>CD3<sup>ε</sup><sup>+</sup>CD4<sup>−</sup>CD8<sup>+</sup>; γδ T cells, B220<sup>−</sup>CD3<sup>ε</sup><sup>+</sup>CD4<sup>−</sup>CD8<sup>−</sup> γδ TCR<sup>+</sup>; NK/NKT cells, B220<sup>−</sup>NK1.1<sup>+</sup>; Polymorphonuclear leukocytes (PMNs), B220<sup>−</sup>NK1.1<sup>−</sup>Ly6G<sup>high</sup>,CD11b<sup>+</sup>; Ly6C<sup>+</sup> monocytes, B220<sup>−</sup>NK1.1<sup>−</sup>Ly6G<sup>neg/low</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup>; conventional dendritic cells (cDCs), B220<sup>−</sup>NK1.1<sup>−</sup>Ly6G<sup>neg/low</sup>CD11c<sup>high</sup>MHC II<sup>+</sup>; plasmacytoid DCs (pDCs), B220<sup>−</sup>CD3<sup>ε</sup>Σiglec H<sup>+</sup>. (C) ELISPOT assays for the quantitation of cells secreting IL-2, IFN-γ, and IL-17A performed on DLN cells 7 days after immunization of WT and Bhlhe40<sup>−/−</sup> mice. Data are combined from three independent experiments (n=9 mice per group). (D-E) WT and Bhlhe40<sup>−/−</sup> mice were immunized with MOG<sub>35-55</sub>/CFA, and DLNs were collected at day 7. (D) Representative plots showing intracellular staining for RORγt gated on CD4<sup>+</sup> T cells. (E) Frequencies of RORγt<sup>+</sup>CD4<sup>+</sup> T cells (n=3 per group).
Figure 2-5. T cells require Bhlhe40 for normal cytokine production after immunization.
Figure 2-5. T cells require Bhlhe40 for normal cytokine production after immunization.

A-E, WT and Bhlhe40−/− mice were immunized with MOG35-55/CFA, and DLNs were collected at day 7. (A) ELISPOT assays for the quantitation of cells secreting GM-CSF and IL-10 performed on DLN cells 7 days after immunization of WT and Bhlhe40−/− mice. Data for GM-CSF is combined from three independent experiments (n=9 mice per group). Data for IL-10 is from one representative experiment of two (n=4 mice per group). (B) Representative plots gated on CD4+ T cells, showing ICS data for the indicated cytokines. Data 5 from stains in which a PE-isotype control antibody (anti-KLH) was included in place of PE anti-GM-CSF are also presented. (C) Frequencies of IFN-γ+, IL-17A+, and GM-CSF+ CD4+ T cells (n=6 per group). (D) Representative plots gated on CD4+ T cells, showing ICS data for IFN-γ and IL-10. (E) Frequencies of IFN-γ+ IL-10+ and IFN-γ− IL-10+ CD4+ T cells (n=3-4 per group). (F,H) DLN cells from immunized WT and Bhlhe40−/− mice (n=14 per group) were cultured with or without MOG35-55 and with or without IL-1β, IL-23 and/or IL-12 as indicated. (F) GM-CSF or (H) IL-10 was measured in the supernatant at day 4. Data are combined from five independent experiments. Cells from all mice were not used in all conditions in each of the four experiments. (G) DLN cells from immunized WT and Bhlhe40−/− mice were cultured with MOG35-55 with or without IL-1β for 4 days, followed by ICS. Representative plots are gated on CD4+ T cells. (I) DLN cells from immunized WT and Bhlhe40−/− mice were cultured with MOG(35–55) with or without IL-12 for 4 days, followed by ICS. Representative plots are gated on CD4+ T cells.
Figure 2-6. *Bhlhe40*<sup>−/−</sup> CD4<sup>+</sup> T cells display a cell-intrinsic defect in GM-CSF and IL-10 production.
Figure 2-6. *Bhlhe40*<sup>−/−</sup> CD4<sup>+</sup> T cells display a cell-intrinsic defect in GM-CSF and IL-10 production.

(A) ELISPOT assays for the quantitation of cells secreting GM-CSF, IL-2, IFN-γ, and IL-17 performed on DLN cells 7 days after immunization of WT and *Bhlhe40*<sup>−/−</sup> mice with OVA<sub>323-339</sub> (n=4 per group). (B-C) ICS on DLN cells from the indicated MOG<sub>35-55</sub>/CFA-immunized mixed BM chimeric mice (n=3 per group). (B) Representative plots gated on CD4<sup>+</sup>CD45.1<sup>+</sup> or CD4<sup>+</sup>CD45.2<sup>+</sup> T cells. (C) Frequencies of IL-17A<sup>+</sup> and GM-CSF<sup>+</sup> CD4<sup>+</sup> T cells (either CD45.1<sup>+</sup> or CD45.2<sup>+</sup>). (D) *Rag1*<sup>−/−</sup> mice were immunized with MOG<sub>35-55</sub>/CFA one day after receiving transfers of 7 million purified WT or *Bhlhe40*<sup>−/−</sup> CD4<sup>+</sup> T cells. At day 7, responses in the spleen and DLN were analyzed by ICS for the indicated cytokines, gating on CD4<sup>+</sup> T cells.
Figure 2-7. γδ T cells require Bhlhe40 for GM-CSF production in response to cytokines.
Figure 2-7. γδ T cells require Bhlhe40 for GM-CSF production in response to cytokines.

(A) Frequencies of GM-CSF+ and IL-17A+ γδ T cells in DLNs 7 days after immunization of WT and Bhlhe40−/− mice (n=3 per group) as determined by ICS. (B) DLN cells from immunized WT and Bhlhe40−/− mice were cultured with or without MOG35-55 and with or without IL-1β and/or IL-23 as indicated for 4 days. Cells were stimulated with PMA/ionomycin in the presence of brefeldin A for 4 h and then analyzed for IL-17A and GM-CSF by intracellular staining (that is, our normal ICS protocol). (C) WT and Bhlhe40−/− splenocytes were cultured with the indicated cytokines for 3 days in the absence of TCR stimulation. Cells were analyzed by flow cytometry for forward scatter (FSC) and side scatter (SSC) after PMA/ionomycin stimulation. Plots are gated on γδ T cells. (D) WT and Bhlhe40−/− splenocytes were cultured with the indicated cytokines for 3 days in the absence of TCR stimulation. Cells were stimulated with PMA/ionomycin in the presence of brefeldin A for 4 hours and analyzed for GM-CSF, IL-17A (top), and IFN-γ (bottom) by intracellular staining. Representative plots are gated on γδ T cells.
Figure 2-8. T<sub>H</sub> cells require Bhlhe40 for normal cytokine production in vitro.
Figure 2-8. T<sub>H</sub> cells require Bhlhe40 for normal cytokine production in vitro.

(A) CD4<sup>+</sup> T cells from WT and Bhlhe40<sup>−/−</sup> mice (n=4 per group) were stimulated for 48 hours with plate-bound anti-CD3 and anti-CD28 antibodies as indicated. IL-2, GM-CSF, and IL-10 were measured in the supernatant by ELISA. (B-C) CD4<sup>+</sup> T cells from WT and Bhlhe40<sup>−/−</sup> mice (n=3 per group) were polarized in T<sub>H</sub>1, T<sub>H</sub>2 or T<sub>H</sub>17 culture conditions for 4 days. (B) Representative ICS for the indicated cytokines. (C) Frequencies of IFN-γ<sup>+</sup>, IL-4<sup>+</sup>, IL-17A<sup>+</sup>, GM-CSF<sup>+</sup> and IL-10<sup>+</sup> cells in T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 cultures. (D) CD4<sup>+</sup> T cells from WT and Bhlhe40<sup>−/−</sup> mice (n=2-4 per group) were polarized in T<sub>H</sub>1, T<sub>H</sub>2, or T<sub>H</sub>17 culture conditions for 4 days and intracellularly stained for expression of T-bet, Gata-3, and RORγt. Representative histograms are shown. (E) T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 cells were stimulated for 24 h with anti-CD3 and anti-CD28. GM-CSF was measured in the supernatant by ELISA. (F) Representative ICS for the indicated cytokines. (G) CD4<sup>+</sup> T cells from the indicated congenic mice were mixed prior to culturing in non-polarizing conditions for 4 days, followed by ICS. Cells were gated based on their expression of CD45.1 or CD45.2. Representative ICS for IL-10 and GM-CSF is shown.
Figure 2-9. Retroviral transduction of Bhlhe40 corrects the GM-CSF and IL-10 production by Bhlhe40−/− T_{H} cells.

ICS on CD4⁺ T cells from WT or Bhlhe40−/− mice following T_{H}1 or T_{H}17 polarization and retroviral transduction with control empty retrovirus (RV) or Bhlhe40 RV. Plots are gated on GFP⁺CD4⁺ T cells.
Figure 2-10. Transcriptional analysis of Bhlhe40-deficient T2 cells.
Figure 2-10. Transcriptional analysis of Bhlhe40-deficient TH cells.

(A) Heat map of gene expression for selected TH1, TH2 and TH17 lineage-specific genes in polarized WT and Bhlhe40<sup>−/−</sup> TH cells. (B) Pairwise comparison of WT and Bhlhe40<sup>−/−</sup> TH1, TH2 and TH17 cells. Probes that are overexpressed ≥2-fold in the indicated populations are shown in red or blue. Numbers in corners indicate the total number of probes meeting these conditions. Probes for selected relevant genes are shown in green. Coefficients of determination (R<sup>2</sup>) are indicated for each comparison. (C) Quantitative RT-PCR analysis of Ifng, Il4 and Il17a expression in WT and Bhlhe40<sup>−/−</sup> TH1, TH2 and TH17 cells (n=3 per group). (D) Quantitative RT-PCR analysis of the expression of the indicated nine genes in WT and Bhlhe40<sup>−/−</sup> TH1, TH2 and TH17 cells (n=3 per group). (E) Venn diagrams showing the overlap of probes differentially expressed by WT and Bhlhe40<sup>−/−</sup> (KO) TH cell lineages. Numbers in regions indicate the number of probes with greater than or equal to a twofold difference in expression (WT>KO or WT<KO). (F) CD4<sup>+</sup> T cells from WT and Bhlhe40<sup>−/−</sup> mice (n=4 per group) were stimulated for 48 hours with plate-bound anti-CD3 and anti-CD28 antibodies as indicated. IL-3 was measured in the supernatant by ELISA.
Figure 2-11. Bhlhe40 binds multiple sites within the \( \text{II3/Csf2} \) and \( \text{II10} \) loci.

(A-B) ChIP-Seq binding tracks derived from \( \text{T}_{\text{h}} \) cells for p300, BRG1, T-bet, Gata3, and ROR\( \gamma \)t, and from CH12 cells for Bhlhe40 at the (A) \( \text{II3/Csf2} \) and (B) \( \text{II10} \) loci. Boxes indicate regions previously identified as enhancers for \( \text{II3/Csf2} \) or \( \text{II10} \).
Figure 2-12. IL-10R blockade renders Bhlhe40<sup>−/−</sup> mice susceptible to EAE.

(A) Survival of WT or Bhlhe40<sup>−/−</sup> mice after EAE induction and biweekly treatment with control rat IgG or anti-IL-10R antibody. Data are combined from three independent experiments. (B) Mean clinical scores of EAE in diseased WT or Bhlhe40<sup>−/−</sup> mice treated biweekly with either control rat IgG or anti-IL-10R antibody. Data are combined from three independent experiments. Error bars are not shown for clarity. All SEM values were ≤1.
Figure 2-13. Flow cytometry of splenocytes and bone marrow cells from WT and $Bhlhe40^{-/-}$ mice.
Figure 2-13. Flow cytometry of splenocytes and bone marrow cells from WT and Bhlhe40−/− mice.

(A) Representative flow cytometry on splenocytes from 15 week-old WT and Bhlhe40−/− mice showing populations of B cells (B220+CD3ε−), CD4+ T cells (B220−CD3ε+CD4+CD8−), CD8+ T cells (B220−CD3ε−CD4−CD8+), and γδ T cells (B220−CD3ε+CD4−CD8−TCR γδ+). (B) Percentages of splenic T cell populations in 12 (n=1 mouse per group), 15 (n=3 mice per group), and 18 week-old (n=2 mice per group) WT and Bhlhe40−/− mice. (C) Representative flow cytometry on splenocytes from 15 week-old WT and Bhlhe40−/− mice showing populations of PMNs (B220−Siglec H Ly6G+CD11b+), cDCs (B220−Siglec H Ly6G−CD11chighMHC II+), and Ly6C+ monocytes (B220−Siglec H Ly6G−CD11b+ Ly6Chigh). (D) Representative flow cytometry on bone marrow cells from 15 week-old WT and Bhlhe40−/− mice showing populations of B cells (B220−Siglec H), pDCs (B220−Siglec H+), PMNs (B220−Siglec H CD11b−Ly6Cmid), and Ly6C+ monocytes (B220−Siglec H CD11b+Ly6Chigh).
Table 1 | Clinical EAE data in WT and Bhlhe40<sup>−/−</sup> mice treated with rat IgG or anti-IL-10R antibody.

<table>
<thead>
<tr>
<th></th>
<th>Day of onset</th>
<th>Incidence</th>
<th>Maximum score</th>
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<tbody>
<tr>
<td>WT + rat IgG</td>
<td>11 ± 0.4</td>
<td>100% (7/7)</td>
<td>3.43 ± 0.20</td>
</tr>
<tr>
<td>Bhlhe40&lt;sup&gt;−/−&lt;/sup&gt; + rat IgG</td>
<td>9.0</td>
<td>11.1% (1/9)</td>
<td>2.00</td>
</tr>
<tr>
<td>WT + anti-IL-10R</td>
<td>10 ± 1.0</td>
<td>100% (2/2)</td>
<td>4.00 ± 0.00</td>
</tr>
<tr>
<td>Bhlhe40&lt;sup&gt;−/−&lt;/sup&gt; + anti-IL-10R</td>
<td>12 ± 1.5</td>
<td>44.4% (4/9)</td>
<td>3.25 ± 0.25</td>
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*Mean ± s.e.m. of diseased mice.
Chapter 3: IL-1-Induced Bhlhe40 Identifies Pathogenic $T_H$ Cells in a Model of Autoimmune Neuroinflammation

The contents of this chapter have been previously published in the Journal of Experimental Medicine.

**IL-1-Induced Bhlhe40 Identifies Pathogenic T helper Cells in a Model of Autoimmune Neuroinflammation**

3.1 Abstract

The features that define autoreactive T helper (T_H) cell pathogenicity remain obscure. We have previously shown that T_H cells require the transcription factor Bhlhe40 to mediate experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Here, using Bhlhe40 reporter mice and analyzing both polyclonal and TCR transgenic T_H cells, we found that Bhlhe40 expression was heterogeneous after EAE induction, with Bhlhe40-expressing cells displaying marked production of IFN-γ, IL-17A, and granulocyte-macrophage colony-stimulating factor. In adoptive transfer EAE models, Bhlhe40-deficient T_H 1 and T_H 17 cells were both nonencephalitogenic. Pertussis toxin (PTX), a classical co-adjuvant for actively induced EAE, promoted IL-1β production by myeloid cells in the draining lymph node and served as a strong stimulus for Bhlhe40 expression in T_H cells. Furthermore, PTX co-adjuvanticity was Bhlhe40 dependent. IL-1β induced Bhlhe40 expression in polarized T_H 17 cells, and Bhlhe40-expressing cells exhibited an encephalitogenic transcriptional signature. In vivo, IL-1R signaling was required for full Bhlhe40 expression by T_H cells after immunization. Overall, we demonstrate that Bhlhe40 expression identifies encephalitogenic cells and defines a PTX–IL-1–Bhlhe40 pathway active in EAE.
3.2 Introduction

Autoreactive CD4+ T helper (T\(_H\)) cells specific for components of myelin drive experimental autoimmune encephalomyelitis (EAE), a widely-used animal model of the human neuroinflammatory disease multiple sclerosis (MS). In the active EAE model in C57BL/6 mice, naive T\(_H\) cells are primed by subcutaneous immunization with a peptide derived from myelin oligodendrocyte glycoprotein (MOG\(_{35-55}\)) emulsified in CFA\(^5\). Along with MOG/CFA, mice are treated systemically with the co-adjuvant pertussis toxin (PTX), an ADP-ribosylating exotoxin derived from \textit{Bordetella pertussis} that has been proven necessary for clinical disease in this model\(^{44,45}\). Although the target cell types and mechanisms of action of PTX are not fully understood, PTX has been shown to increase blood–brain barrier permeability\(^{226,227}\) and promote the maturation and cytokine production of antigen-presenting cells\(^{228,229}\). Several studies have shown PTX treatment or \textit{B. pertussis} infection to induce IL-1\(\beta\) and IL-6 production by myeloid cells\(^{230,231,232,233}\), which, during EAE, could contribute to PTX-mediated effects on regulatory T (Treg) cells\(^{234,235}\) and T\(_H\)17 cells\(^{230,236}\).

We and others have previously demonstrated that the transcription factor basic helix–loop–helix family member e40 (Bhlhe40; also known as Dec1, Stra13, Sharp2, and Bhlhb2) is required in a T\(_H\) cell–intrinsic fashion for susceptibility to EAE\(^{11,33}\). Bhlhe40 is a member of the basic helix–loop–helix–Orange subfamily of transcription factors with a recognized role in regulating circadian rhythms, cellular differentiation, and immune cell function\(^{22}\). Bhlhe40-deficient (Bhlhe40\(^{-/-}\)) mice resist EAE, and although their T\(_H\) cells are capable of mounting largely normal antigen-specific responses to immunization, Bhlhe40\(^{-/-}\) T\(_H\) cells show markedly decreased secretion of GM-CSF, an effector cytokine required for EAE\(^{174,175}\), and increased secretion of
IL-10, a cytokine with immunoregulatory properties\textsuperscript{11,192}. In vitro, \textit{Bhlhe40}\textsuperscript{-/-} T\textsubscript{H} cells differentiate normally in appropriate polarizing conditions into T\textsubscript{H}1, T\textsubscript{H}2, and T\textsubscript{H}17 cells subsets, although in each case Bhlhe40 deficiency results in the abnormal expression of \~200–300 genes, including \textit{Csf2} (encoding GM-CSF) and \textit{Il10}\textsuperscript{11}. Bhlhe40 is expressed in all subsets of polarized T\textsubscript{H} cells in vitro, and is known to be regulated in part through a signal provided by CD28 in combination with TCR signaling\textsuperscript{33}. Nevertheless, the pathways that regulate Bhlhe40 expression in T\textsubscript{H} cells in vivo during an immune response and the features of Bhlhe40-expressing T\textsubscript{H} cells during EAE remain unknown.
3.3 Results

3.3.1 Bhlhe40\textsuperscript{GFP} Tg mice show Bhlhe40 expression in immune cells

We used bacterial artificial chromosome (BAC) transgenic (Tg) reporter mice generated by the Gene Expression Nervous System Atlas (GENSAT) Project\textsuperscript{237} to identify and study Bhlhe40 expression in \( T_H \) cells in vivo. Cells from these Bhlhe40\textsuperscript{GFP} mice show Bhlhe40 expression through enhanced GFP in the context of a BAC transgene spanning the 205-kb genomic DNA segment containing Bhlhe40. To determine the steady-state expression of Bhlhe40, we performed flow cytometry on tissues obtained from these reporter mice. Thymocytes of all developmental stages did not express GFP (Figure. 3-1A). Splenic CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells expressed GFP in <0.5% of cells (Figure. 3-1B). These rare GFP\textsuperscript{pos} cells stained as CD44\textsuperscript{−}CD62L\textsuperscript{−}, indicating prior antigen experience. Other immune cells showed a range of GFP expression (Figure. 3-1, C and D). An overall comparison of our flow cytometry results with the expression of Bhlhe40 in immune cells based on expression microarray datasets from the Immgen Consortium\textsuperscript{238} showed excellent agreement (Figure. 3-1, E and F). These data indicate that Bhlhe40\textsuperscript{GFP} mice faithfully reveal Bhlhe40 expression.

3.3.2 Bhlhe40-expressing \( T_H \) cells are enriched in the CNS and are robust cytokine producers during EAE

Based on the known cell-intrinsic requirement for Bhlhe40 expression in \( T_H \) cells for EAE susceptibility\textsuperscript{11, 33}, we expected that Bhlhe40 would be expressed in effector T cells during EAE. Flow cytometry of Bhlhe40\textsuperscript{GFP} mice at day 13–14 after EAE induction showed that \( \sim 1–10\% \) of splenic and \( \sim 10–70\% \) of CNS-infiltrating CD4\textsuperscript{+} T cells expressed GFP at this time (Figure. 3-2, \textit{cont.})
A and B), notably more than in naïve mice. In general, the frequency of GFP<sup>pos</sup> CD4<sup>+</sup> T cells in the CNS correlated with disease severity at the day of sacrifice. GFP expression was also notable in CD8<sup>+</sup> and γδ T cells during EAE, again at an increased frequency in the CNS relative to the spleen, consistent with these cell types participating in disease pathogenesis<sup>199, 239</sup>. Some GFP expression was also observed in CD11b<sup>+</sup> infiltrating myeloid cells during EAE, but not in microglia (unpublished data). Histological examination of spinal cord sections from diseased mice confirmed GFP expression within CD4<sup>+</sup> T cells (Figure. 3-2C) and CD11b<sup>+</sup> myeloid cells (unpublished data) present in inflammatory lesions. Strikingly, intracellular cytokine staining (ICS) showed that Bhlhe40-expressing GFP<sup>pos</sup> CD4<sup>+</sup> T cells produced almost all of the IFN-γ, IL-17A, and GM-CSF in both the spleen and CNS (Figure. 3-2, D and E), suggesting that Bhlhe40 expression could serve as an identifying feature of encephalitogenic Th<sub>1</sub> cells.

### 3.3.3 PTX serves as an essential co-adjuvant by inducing Bhlhe40 expression in Th<sub>1</sub> cells

One week after immunization of Bhlhe40<sup>GFP</sup> mice with MOG/CFA, we observed only a very small increase in the abundance of GFP-expressing CD4<sup>+</sup> T cells in the DLNs relative to naïve mice (Figure. 3-3, A and B), with almost all GFP<sup>pos</sup> cells being CD44<sup>+</sup>. Because the same immunization given with systemic administration of PTX had resulted in robust GFP expression by CD4<sup>+</sup> T cells during EAE, we reasoned that the co-adjuvant PTX might serve as a stimulus for Bhlhe40 expression. Indeed, DLNs from mice immunized with MOG/CFA and treated with PTX contained significantly increased numbers of GFP<sup>pos</sup> CD4<sup>+</sup> T cells relative to naïve mice, PTX-only–treated mice, or non-PTX–treated MOG/CFA-immunized mice (Figure. 3-3, A and B), with these GFP<sup>pos</sup> T cells again bearing CD44. To mediate this effect, the ADP-ribosylating
activity of PTX was necessary, as treatment of MOG/CFA-immunized mice with mutant PTX (mPTX) lacking this activity did not result in an increase in the frequency or number of GFP<sup>pos</sup> CD4<sup>+</sup> T cells relative to MOG/CFA-immunized mice given no PTX. In immunized mice followed for the development of clinical EAE, only PTX, but not mPTX, was able to serve as a co-adjuvant for disease induction (Figure. 3-3C). ICS showed PTX treatment to be a strong inducer of IFN-γ, IL-17A, and GM-CSF secretion, particularly among GFP<sup>pos</sup> CD4<sup>+</sup> T cells (Figure. 3-3, D and E), again suggesting that Bhlhe40 expression serves to identify encephalitogenic T<sub>H</sub> cells. In the setting of MOG/CFA immunization given with PTX treatment, GFP<sup>pos</sup> CD4<sup>+</sup> T cells showed higher expression of Ki-67, indicating that these cells were more proliferative than their GFP<sup>neg</sup> counterparts (Figure. 3-3F). qRT-PCR analysis comparing GFP<sup>neg</sup> and GFP<sup>pos</sup> CD4<sup>+</sup> T cells from these mice showed that GFP<sup>pos</sup> cells expressed higher levels of Egfp, Bhlhe40, and Csf2 transcripts (Figure. 3-3G).

We next tested the hypothesis that PTX co-adjuvanticity was Bhlhe40 dependent. We previously showed by ELISPOT assays that antigen/CFA immunization in the absence of PTX primed generally normal frequencies of antigen-specific IFN-γ and IL-17A responses in the DLNs of Bhlhe40<sup>-/-</sup> mice<sup>11</sup>. These mice, however, showed a markedly decreased frequency of antigen-specific GM-CSF–producing T cells, and an increased frequency of IL-10–producing cells relative to WT mice. We repeated these ELISPOT assays in WT and Bhlhe40<sup>−/−</sup> mice, but included additional groups of mice that were treated with PTX (Figure. 3-4). In WT mice, PTX treatment significantly increased the frequency of MOG-specific cells producing all four cytokines. Importantly, PTX treatment showed no ability to augment MOG-specific responses in Bhlhe40<sup>−/−</sup> mice. These data indicate that PTX co-adjuvanticity is Bhlhe40 dependent, and
explain, at least in part, why PTX is a required co-adjuvant for clinical EAE induction in C57BL/6 mice.

3.3.4 Bhlhe40 expression identifies the cytokine-producing fraction of autoreactive T\(_H\) cells

We have shown that GFP expression in CD\(_4^+\) T cells of Bhlhe40\(^{GFP}\) mice identifies cytokine-producing T\(_H\) cells, whereas GFP\(^{neg}\) CD\(_4^+\) T cells largely lack cytokine production. These results could be explained by either of two non-mutually exclusive possibilities. In one scenario, GFP\(^{neg}\) cells may represent CD\(_4^+\) T cells with TCR specificities unrelated to the ongoing myelin-specific response. Bystander CD\(_4^+\) T cells have been described in the CNS during EAE\(^{240}\), so it is likely that at least some GFP\(^{neg}\) CD\(_4^+\) T cells in this compartment are bystanders. Alternatively, Bhlhe40 expression may be heterogeneous among autoreactive CD\(_4^+\) T cells. In this scenario, only those self-reactive T cells expressing Bhlhe40 would serve as encephalitogenic, cytokine-producing effectors.

We tested these possibilities by two approaches. First, we identified MOG-specific CD\(_4^+\) T cells using MOG\(_{38-49}\)–I\(^b\) tetramers at day 15 after EAE induction in Bhlhe40\(^{GFP}\) mice. Although we failed to detect tetramer\(^{pos}\) cells in the spleen, ~7% of CNS-infiltrating CD\(_4^+\) T cells were stained by tetramer, and nearly half of them were GFP\(^{pos}\) (Figure. 3-5, A and B). Among tetramer\(^{pos}\) cells, those expressing GFP showed the highest level of IFN-\(\gamma\) production (Figure. 3-5C). Interestingly, however, most CNS-infiltrating GFP\(^{pos}\) cells did not stain with tetramer.

Although this may suggest bystander activation by non-MOG–specific T cells, we feel that this is more consistent with the findings of Sabatino et al., where most MOG-specific cells after EAE induction had a low affinity for MOG/I\(^b\), such that they were negative for tetramer staining\(^{241}\).
These low-affinity cells were reported to contribute significantly to the pool of IFN-γ–producing T cells in the CNS. Consistent with this, we also found that among tetramer\textsuperscript{neg} cells, a higher frequency of GFP\textsuperscript{pos} cells produced IFN-γ (Figure. 3-5C).

In our second approach, we crossed 2D2 TCR Tg mice, bearing T cells with a TCR specific for MOG\textsubscript{35-55} \textsuperscript{45} to Bhlhe40\textsuperscript{GFP} mice and crossed in one allele of the congenic marker CD45.1 (resulting in hematopoietic cells with co-expression of CD45.1 and CD45.2). Purified CD4\textsuperscript{+} T cells from these mice (henceforth termed 2D2 Bhlhe40\textsuperscript{GFP} CD45.1/CD45.2) were transferred to WT recipients (bearing only CD45.2), and one day later these mice were immunized with MOG/CFA with or without PTX treatment (Figure. 3-5, D–G). To control for the possible effects of microbial ligands in CFA or the effects of PTX that are independent of cognate antigen stimulation, a separate group of mice that received 2D2 Bhlhe40\textsuperscript{GFP} CD45.1/CD45.2 CD4\textsuperscript{+} T cells was immunized with an irrelevant peptide antigen (OVA\textsubscript{323-339})/CFA and treated with PTX. Immunization with MOG resulted in an expansion of the population of transferred T cells in the DLN one week later, with additional PTX treatment resulting in a markedly increased frequency of GFP\textsuperscript{pos} T cells (Figure. 3-5, D and E). In MOG/CFA-immunized mice treated with PTX, ICS (Figure. 3-5, F and G) on DLN cells showed that host-derived polyclonal CD4\textsuperscript{+} T cells secreted minimal amounts of IFN-γ, IL-17A, and GM-CSF. 2D2 Bhlhe40\textsuperscript{GFP} CD45.1/CD45.2 CD4\textsuperscript{+} T cells produced all of these cytokines, although cytokine production was significantly increased among 2D2 GFP\textsuperscript{pos} T cells compared with 2D2 GFP\textsuperscript{neg} T cells, particularly for IFN-γ and GM-CSF. Thus, there exists heterogeneity among clonal autoreactive T cells in their expression of Bhlhe40, and expression of this transcription factor identifies those cells with encephalitogenic features. In a variation of the aforementioned experiment, we transferred 2D2 Bhlhe40\textsuperscript{GFP} CD45.1/CD45.2 CD4\textsuperscript{+} T cells to Bhlhe40\textsuperscript{GFP} reporter mice (CD45.2) to allow for the
simultaneous analysis of GFP\(^{\text{neg}}\) and GFP\(^{\text{pos}}\) host CD4\(^{+}\) T cells and GFP\(^{\text{neg}}\) and GFP\(^{\text{pos}}\) 2D2 CD4\(^{+}\) T cells after EAE induction (Figure. 3-5, H and I). In these mice, ICS staining showed significantly increased cytokine production by both GFP\(^{\text{pos}}\) host and GFP\(^{\text{pos}}\) 2D2 CD4\(^{+}\) T cells, relative to their GFP\(^{\text{neg}}\) counterparts, supporting the notion that Bhlhe40 expression identified both monoclonal and polyclonal encephalitogenic T cells.

3.3.5 Bhlhe40 is essential for the encephalitogenicity of TH cells in adoptive transfer models of EAE

To further test the notion that Bhlhe40-expressing CD4\(^{+}\) T cells are encephalitogenic, we used an adoptive transfer system of EAE that allows in vitro polarization of 2D2 cells without a requirement for immunization to generate MOG-specific cells\(^{210}\). Because in our immunizations with MOG/CFA + PTX, we found GFP\(^{\text{pos}}\) CD4\(^{+}\) T cells to produce both IFN-\(\gamma\) and IL-17A, we separately tested both TH1 and TH17 adoptive transfer models, comparing the encephalitogenicity of 2D2 WT (CD45.1) and 2D2 \(\text{Bhlhe}40^{-/-}\) (CD45.2) cells after transfer to WT recipients (CD45.1/CD45.2). Despite normal TH1 or TH17 polarization before transfer (Figure. 3-6 A), 2D2 \(\text{Bhlhe}40^{-/-}\) cells were completely nonencephalitogenic in vivo (Figure. 3-6, B and C). Nevertheless, 2D2 \(\text{Bhlhe}40^{-/-}\) cells could be identified in recipient mice at 28–34 days after transfer. Stimulation of these cells recovered from spleens showed that they maintained production of their hallmark cytokines at levels similar to 2D2 WT cells recovered from diseased mice (Figure. 3-6, D–G). Notably, GM-CSF production by polarized 2D2 \(\text{Bhlhe}40^{-/-}\) cells was decreased relative to 2D2 WT cells both before transfer and upon recovery, consistent with our previous experiments using polyclonal CD4\(^{+}\) T cells\(^{11}\). These findings confirm a cell-intrinsic requirement for Bhlhe40 in both encephalitogenic TH1 and TH17 cells.
3.3.6 Bhlhe40 expression negatively correlates with Foxp3 and IL-10 expression during EAE

Foxp3\textsuperscript{pos} Treg cells and the suppressive cytokine IL-10 have been recognized to ameliorate disease during EAE \cite{242,243,244}. Given our findings that Bhlhe40-expressing CD4\textsuperscript{+} T cells were robust producers of inflammatory cytokines and that expression of Bhlhe40 was required in adoptive transfer models of EAE, we hypothesized that Bhlhe40-expressing cells in the CNS during EAE would be unlikely to express Foxp3 or IL-10. We performed intracellular staining for Foxp3 on CNS-infiltrating immune cells after EAE induction in \textit{Bhlhe40\textsuperscript{GFP}} mice, but found that this protocol significantly diminished our ability to discriminate GFP\textsuperscript{pos} CD4\textsuperscript{+} T cells. To circumvent this problem, we sorted three populations of CNS-infiltrating CD4\textsuperscript{+} T cells at day 16 after EAE induction in reporter mice based on their expression of CD44 and GFP, and performed subsequent intracellular staining for Foxp3 (\textbf{Figure. 3-7A}). Many Foxp3\textsuperscript{pos} CD4\textsuperscript{+} T cells were present in the CD44\textsuperscript{−}GFP\textsuperscript{neg} (R1) and CD44\textsuperscript{+}GFP\textsuperscript{neg} (R2) gates, whereas within the population of CD44\textsuperscript{+}GFP\textsuperscript{pos} (R3) cells, only a small fraction of T cells were Foxp3\textsuperscript{pos} (\textbf{Figure. 3-7, A and B}). We are unsure of the identity of these Bhlhe40 and Foxp3 double-positive cells, but they may be akin to populations of Treg cells that express Foxp3 and a second transcription factor, as has been reported for T-bet\textsuperscript{pos}Foxp3\textsuperscript{pos} Treg cells in EAE \cite{245,246}. We also analyzed IL-10 production by CNS-infiltrating CD4\textsuperscript{+} T cells in reporter mice that received congenically-marked 2D2 \textit{Bhlhe40\textsuperscript{GFP}} cells to allow us to track both GFP\textsuperscript{neg} and GFP\textsuperscript{pos} host and TCR Tg T cells. Although a high frequency of GFP\textsuperscript{pos} host and GFP\textsuperscript{pos} 2D2 cells produced IFN-\(\gamma\), IL-10 production came almost entirely from host GFP\textsuperscript{neg} cells (\textbf{Figure. 3-7, C and D}). These IL-10–producing GFP\textsuperscript{neg} host cells likely represent a mixture of both Treg cells and IFN-\(\gamma\)\textsuperscript{+}IL-10\textsuperscript{+} type 1 regulatory T cells (Tr1) cells \cite{243,247,197,248}, whereas GFP\textsuperscript{pos} host and 2D2 cells represent autoreactive effectors. At
day 29, during the resolving phase of EAE, GFP\textsuperscript{neg} CD4\textsuperscript{+} T cells in the CNS continued to express higher levels of IL-10 than GFP\textsuperscript{pos} T cells, with the latter continuing to robustly produce IFN-\(\gamma\) (Figure. 3-7, E and F).

3.3.7 Bhlhe40-expressing T\textsubscript{H}17 cells exhibit a pathogenic molecular signature

Next, we polarized naive CD4\textsuperscript{+} T cells from Bhlhe40\textsuperscript{GFP} mice in T\textsubscript{H}1 (IL-12) or T\textsubscript{H}17 (TGF-\(\beta\)1, IL-6, IL-23, and IL-1\(\beta\)) conditions in vitro. On day 4, \(\sim5–10\%\) of T\textsubscript{H}1 cells and \(\sim30–40\%\) of T\textsubscript{H}17 cells expressed GFP (Figure. 3-8A). Because of significant GFP expression by T\textsubscript{H}17 cells and their relevance to the EAE model, we sorted GFP\textsuperscript{neg} and GFP\textsuperscript{pos} T\textsubscript{H}17 cells for transcriptional analysis. Immunoblotting confirmed that GFP\textsuperscript{pos} cells expressed \(\sim2.5\) times more Bhlhe40 than GFP\textsuperscript{neg} cells (Figure. 3-8, B and C). Expression microarrays were performed on these cells and differentially expressed transcripts were identified (Figure. 3-8D). Strikingly, GFP\textsuperscript{pos} T\textsubscript{H}17 cells exhibited a pathogenic signature, with higher expression of many genes previously identified as being expressed by encephalitogenic T\textsubscript{H}17 cells, including Bhlhe40, Csf2, Tgfb3, Il7r, Icos, and several chemokines\textsuperscript{174, 175, 249, 11, 33, 189}. We confirmed differential expression for four transcripts that encoded surface proteins by flow cytometry (Figure. 3-8E), two with higher expression in GFP\textsuperscript{pos} cells (CD93 and IL-7R) and two with higher expression in GFP\textsuperscript{neg} cells (Slamf6 and CD62L). Lastly, we compared our microarray data to a publicly available Gene Expression Omnibus (GEO) dataset (GSE23505) in which microarrays were performed on T\textsubscript{H}17 cells derived from cultures using a variety of cytokine conditions (Figure. 3-8D)\textsuperscript{188}. Using gene set analysis, we tested whether the 700 most differentially expressed genes with higher expression in Bhlhe40-expressing (GFP\textsuperscript{pos}) cells in our experiment (Figure. 3-8D, yellow box) showed differential expression in T\textsubscript{H}17 cells across culture conditions. Interestingly, as a group, these 700 genes showed an overall pattern to suggest that their expression was
specifically increased by culture with IL-1β (Figure. 3-8D, red line), independent of whether
TH17 cells were derived by culture with TGF-β1 and/or IL-23. In these data, Bhlhe40 was also
expressed more highly in conditions that included IL-1β (Figure. 3-8D, green line). This finding
suggested a model in which IL-1β acted through Bhlhe40 to promote encephalitogenicity of
TH17 cells and led us to explore whether IL-1β directly regulated the expression of Bhlhe40.

3.3.8 IL-1β increases Bhlhe40 expression in TH17 and γδ T cells

We used our TH17 cell culture system with purified CD4+ T cells from Bhlhe40GFP mice to test
whether IL-1β induced the expression of Bhlhe40. Indeed, IL-1β, but not IL-23, induced strong
expression of GFP in this system (Figure. 3-9A). Expression of GFP was notable by day 2 in
these cultures, and increased steadily through day 7 (Figure. 3-9B). By performing TH17
cultures with irradiated splenocytes from Il1r1−/− mice, we confirmed that IL-1β acted directly on
CD4+ T cells to increase their GFP expression (Figure. 3-9C). Immunoblotting for Bhlhe40 also
confirmed induction of Bhlhe40 by IL-1β (Figure. 3-9D). IL-1β increased both IL-17A and GM-
CSF production by TH17 cells, with GFPpos cells displaying the highest production of these
cytokines (Figure. 3-9E). In contrast with our in vivo results (Figure. 3-7, C–F), in vitro, GFPpos
cells also produced slightly higher levels of IL-10 (Figure. 3-9E). IL-10 production derived
mainly from IL-17A+ cells (unpublished data). Consistent with the fact that IL-1R is expressed at
low levels on TH1 cells250,251, IL-1β only minimally increased GFP expression when added to
TH1 cultures (Figure. 3-9, F–H). Nevertheless, a higher frequency of GFPpos TH1 cells expressed
IFN-γ, GM-CSF, and IL-10 (Figure. 3-9I), with GM-CSF and IL-10 production deriving mainly
from IFN-γ+ cells (not depicted). γδ T cells are known to produce GM-CSF in response to IL-
1β199, and we have previously shown that γδ T cells require Bhlhe40 to produce GM-CSF11.
Similar to T_{h}17 cells, TCR-activated γδ T cells from Bhlhe40^{GFP} mice also increased their expression of GFP in response to IL-1β (Figure. 3-9, J and K).

### 3.3.9 IL-1R signaling is required in vivo for full Bhlhe40 expression by T_{h} cells

To test whether IL-1 played a role in the induction of Bhlhe40 in CD4^{+} T cells in vivo, we crossed Bhlhe40^{GFP} reporter mice with Il1r1^{-/-} mice. We immunized these mice with MOG/CFA, along with PTX treatment, and compared their CD4^{+} T cell responses to control Bhlhe40^{GFP} mice. In the absence of IL-1R signaling, fewer CD4^{+} T cells in the DLN expressed GFP (Figure. 3-10, A and B). Similarly, in vivo IL-1 blockade by antibodies in Bhlhe40^{GFP} mice resulted in fewer GFP^{pos} CD4^{+} T cells after immunization and PTX treatment (Figure. 3-10, C and D). Consistent with previous studies^{67, 71, 252 47, 48, 68}, and the notion that Bhlhe40 expression is partially dependent on IL-1R signaling, we observed a lower incidence of clinical EAE in Il1r1^{-/-} mice compared with WT mice, with a later onset and less severe disease (Figure. 3-10E). Bhlhe40^{-/-} mice showed complete EAE resistance, indicating that Bhlhe40 serves a more essential role in the development of EAE than IL-1 does. To define a cell-intrinsic role for IL-1R signaling in the induction of Bhlhe40, we co-transferred CD4^{+} T cells from Bhlhe40^{GFP} (CD45.1/CD45.2) or Il1r1^{-/-} Bhlhe40^{GFP} (CD45.2) mice that had previously been immunized with MOG/CFA + PTX to WT (CD45.1) recipients. The next day, these mice were immunized with MOG/CFA + PTX, and at day 7 DLNs were examined for GFP expression on the transferred CD4^{+} T cells, which were found to uniformly express the activation marker CD44 (Figure. 3-10F). Within the population of GFP^{pos} cells, IL-1R–sufficient Bhlhe40^{GFP} cells were
found at a ∼3:1 ratio over Il1r1+/− Bhlhe40GFP cells (Figure. 3-10, F and G), confirming a cell-intrinsic role for IL-1R signaling in Bhlhe40 induction.

3.3.10 Systemic PTX treatment induces IL-1β production by lymph node cells

Because both PTX treatment and IL-1β regulated Bhlhe40 expression by CD4+ T cells after immunization, we next asked whether PTX could regulate IL-1β production in vivo. DLN cells from MOG/CFA + PTX-immunized mice at day 7 secreted IL-1β in culture (Figure. 3-10H). Treatment with heat-killed Mycobacterium tuberculosis (Mtb), a component of CFA, could further augment this secretion. mPTX, which failed to induce Bhlhe40 expression in CD4+ T cells in vivo (Figure. 3-3, A and B), also was unable to drive IL-1β secretion by lymph node cells when combined with MOG/CFA immunization. Pro–IL-1β was detectable in lymph node cells only in mice that received PTX with immunization (Figure. 3-10, I and J), and DLNs from these mice contained sizeable populations of Ly6G+ neutrophils and Ly6G−Ly6C+MHC class II+ monocytes/moDCs (Figure. 3-10K). Neutrophils and Ly6G−Ly6C+MHC class II+ cells, but not migratory or resident DCs, were the major source of PTX-induced IL-1 (Figure. 3-10L).
3.4 Discussion

We and others have previously demonstrated that the transcription factor Bhlhe40 is required for autoimmune neuroinflammation through its action in $T_H$ cells$^{11,33}$. Here, we have used novel $Bhlhe40^{GFP}$ reporter mice to study Bhlhe40-expressing cells during EAE. Bhlhe40-expressing $T_H$ cells were abundant in the CNS, where they secreted IFN-$\gamma$, IL-17A, and GM-CSF. PTX, when administered systemically with immunization, served as a strong stimulus for Bhlhe40 expression by $T_H$ cells. PTX treatment enhanced antigen-specific $T_H$ cell cytokine responses, and this effect was abrogated in $Bhlhe40^{-/-}$ mice. IL-1$\beta$ production by lymph node myeloid cells was markedly augmented by systemic PTX treatment administered with MOG/CFA immunization. IL-1$\beta$ acted directly on $T_H$17 cells to positively regulate the expression of Bhlhe40, with Bhlhe40-expressing $T_H$17 cells exhibiting an encephalitogenic transcriptional signature.

We find that Bhlhe40 expression is nonuniform in autoreactive CD4$^+$ T cells during EAE. Bhlhe40 is not expressed in naive CD4$^+$ T cells, but is induced upon T cell activation$^{30,32}$. Polyclonal T cells with a spectrum of TCR affinities for MHC class II peptide could tune Bhlhe40 expression levels, although in experiments with monoclonal TCR Tg CD4$^+$ T cells, we also found nonuniform Bhlhe40 expression. Recently, Helmstetter et al. described intraclonal heterogeneity in the production of IFN-$\gamma$ by $T_H$1 cells, explained by intrapopulation differences in the expression of the transcription factor T-bet$^{253}$. These authors also studied monoclonal T cells, and concluded that a diverse TCR repertoire was not essential to generate heterogeneity in effector cell responses. Previous work has described a range of TCR signal strength, even in stimulated monoclonal T cells, determined by the stochastic expression of proximal signaling components downstream of the TCR$^{254}$. Therefore, it remains possible that TCR signal strength
could contribute to establishing a range of Bhlhe40 expression in activated T cells. Numerous non-TCR signals also likely act in combination to establish a population of T cells with a wide spectrum of transcription factor and effector molecule expression levels. CD28 signaling is known to induce Bhlhe40 expression in CD4\(^+\) T cells\(^{33}\), and other positive or negative co-stimulatory signals may also participate. In naive PD-1–deficient mice, for example, CD8\(^+\) T central memory phenotype cells express higher levels of Bhlhe40 than the same cells derived from naive WT mice\(^{255}\). Diverse cytokine receptor signals also likely integrate to shape T cell responses. We found IL-1R signaling to strongly impact on Bhlhe40 expression levels in T\(_H\)17 cells. IL-1 has been shown to induce Bhlhe40 in primary human gingival epithelial cells through a PI-3K–Akt pathway\(^{256}\), and in primary human amnion mesenchymal cells\(^{257}\). Recently, IL-1R signals were shown to modulate T\(_H\)17 cell responses through the repression of SOCS3 and the subsequent strengthening of STAT3 activity\(^{258}\). It is interesting to consider whether these pathways are involved in the induction of Bhlhe40 by IL-1 in T\(_H\)17 cells.

The development of autoimmune diseases is thought to involve both genetic and environmental factors. In the EAE model, roles for both pathogenic and commensal microbes have been considered to regulate effector and regulatory T\(_H\) cell responses\(^{259}\). Active EAE induction protocols involve immunization with myelin antigens administered with CFA to prime dormant autoreactive T\(_H\) cells, although in C57BL/6 mice this strategy is insufficient to induce EAE. In these mice, PTX is a required co-adjuvant\(^{44,45}\), perhaps functioning as a surrogate for the environmental factors that trigger MS in genetically susceptible hosts. Our work shows that PTX co-adjuvanticity, as read out by an increase in antigen-specific CD4\(^+\) T cell cytokine responses, requires Bhlhe40 expression in vivo. Immunization combined with PTX treatment induces Bhlhe40 expression in CD4\(^+\) T cells, and this induction of Bhlhe40 is partially IL-1 dependent.
PTX was unable to act directly on T cells in vitro to induce Bhlhe40 expression (unpublished data), suggesting that PTX acts indirectly in vivo to promote CD4+ T cell responses.

We have found that immunization combined with PTX treatment induces IL-1β production by neutrophils and monocytes/moDCs. Our data are consistent with two non-mutually exclusive actions for PTX in this process. Evidence exists for the ability of PTX to directly stimulate myeloid cell production of IL-1β. Zhang et al. showed that macrophages infected with PTX-deficient B. pertussis produced less IL-1β than those infected with WT bacteria231. More recently, Dumas et al. showed that PTX administered by a protocol akin to its use in EAE rapidly triggered IL-1β production by peritoneal macrophages and neutrophils in a pathway dependent on the ADP-ribosylating activity of the toxin and on a pyrin-containing inflammasome233. Alternatively, PTX may induce initial GM-CSF production (and perhaps Bhlhe40 expression) by antigen-stimulated T\textsubscript{H} cells through an undefined mechanism. This GM-CSF may then act directly on lymph node myeloid cells to induce their production of IL-1β, which could feed forward to direct full Bhlhe40 expression and encephalitogenicity of T\textsubscript{H} cells. Consistent with this proposed positive-feedback loop, a cell-intrinsic role for GM-CSF receptor signaling on CCR2\textsuperscript{+} monocytes after EAE induction was recently described by Croxford et al.47, 48, who concluded that T cell–derived GM-CSF served to regulate IL-1β production by Ly6C\textsuperscript{+}MHC class II\textsuperscript{+} cells, and that this signaling was required for the development of EAE. Khameneh et al. have also reported that GM-CSF can synergize with a TLR ligand to enhance IL-1β secretion by bone marrow–derived DCs/macrophages via increased NF-κB activation260. In MS, the detection of IL-1 in the cerebrospinal fluid at disease onset has been linked with cortical pathology92, whereas its detection during remission in relapsing-remitting MS has been shown to correlate with a more
severe disease course. The role of IL-1 in directly promoting T<sub>H</sub> cell pathogenicity in MS remains unknown.

Our study demonstrates that among autoreactive T<sub>H</sub> cells, Bhlhe40 expression identifies those with encephalitogenic features. No data exists on whether human BHLHE40 is expressed in T cells in MS lesions. Some studies have focused on the role of IFN-γ/IL-17 double-producing T<sub>H</sub>1/17 cells as being the bona fide pathogenic cells in EAE or MS, which in some cases have also been reported to produce GM-CSF. A requirement for GM-CSF in EAE is well established, and newer studies have suggested a role for this cytokine in MS. IL-1β in conjunction with IL-12 can direct human T<sub>H</sub>17 cells to differentiate into IFN-γ/IL-17/GM-CSF triple-producing cells. We have demonstrated that Bhlhe40 expression in murine T<sub>H</sub> cells controls GM-CSF production, identifies encephalitogenic T<sub>H</sub>17 cells, and is regulated by IL-1. Our study provides impetus for exploring the role of BHLHE40 in autoreactive T<sub>H</sub> cells in human autoimmune disease.
3.5 Methods

Mice. C57BL/6 (Taconic), B6.SJL (CD45.1; Taconic), 2D2 TCR transgenic (The Jackson Laboratory), Il1r1\textsuperscript{-/-} (on a C57BL/6 background; The Jackson Laboratory), and Bhlhe40\textsuperscript{-/-30} (backcrossed 10 generations to the C57BL/6 background) mice were maintained in our SPF facility. The Bhlhe40\textsuperscript{GFP} BAC Tg mouse strain, STOCKTg(Bhlhe40-EGFP)PX84Gsat/Mmucd, identification number 034730-UCD, was obtained from the Mutant Mouse Regional Resource Center (MMRRC), a National Center for Research Resources-National Institutes of Health (NCRR-NIH) funded strain repository, and was donated to the MMRRC by the National Institute of Neurological Disorders and Stroke (NINDS) funded Gene Expression Nervous System Atlas (GENSAT) BAC transgenic project. Frozen spermatozoa from hemizygotes were obtained and mice were reanimated and backcrossed to the C57BL/6 background (hemizygous mice at backcross 7–11 were used). A strain-specific PCR with primers suggested by the MMRRC was used to type for the presence of the transgene (forward, 5’-GGGCAGCCCTTTCTCACACTCTAC-3’; reverse, 5’-GGTCGGGGTAGCGGCTGAA-3’). 2D2 TCR transgenic mice were crossed to B6.SJL mice to generate 2D2 CD45.1 mice that were subsequently crossed to Bhlhe40\textsuperscript{GFP} mice or Bhlhe40\textsuperscript{-/-} mice for some experiments. Il1r1\textsuperscript{-/-} mice were crossed to Bhlhe40\textsuperscript{GFP} mice for some experiments. Experiments were performed with mice at 8–20 weeks of age. All animal experiments were approved by the Animal Studies Committee of Washington University in St. Louis.

Induction of active EAE and immunizations. For EAE induction, mice were immunized subcutaneously with 100 µg MOG\textsubscript{35-55} (CS Bio Co.) emulsified in CFA (made with 5 mg/ml
heat-killed *Mtb* H37Ra [BD] in incomplete Freund’s adjuvant [BD]). PTX (List Biological Laboratories) or mutant PTX (mPTX; mutated at two positions in the S1 subunit [R9K and E129A]; List Biological Laboratories) was injected i.p. (300 ng) on days 0 and 2. Mice were monitored for signs of classical EAE for at least 28 days and graded on a standard 0–5 scale, as previously described. For analysis of T cell responses in DLNs, mice were immunized in hind footpads with 10 nM MOG_{35-55} or OVA_{323-339} peptide emulsified in CFA. Some mice were injected with PTX i.p. (300 ng) on days 0 and 2. To analyze MOG-specific T cell responses, 2 × 10^6 magnetically purified 2D2 *Bhlhe40*{\textsuperscript{GFP}} CD45.1/CD45.2 CD4\textsuperscript{+} T cells (Dynabeads FlowComp Mouse CD4 kit; Invitrogen) were transferred i.p. to *Bhlhe40*{\textsuperscript{GFP}} or WT recipient mice (CD45.2) one day before MOG/CFA ± PTX immunization. In some experiments, 1.5 × 10^6 magnetically purified CD4\textsuperscript{+} T cells from the DLN of MOG/CFA + PTX-immunized (day 7) *Bhlhe40*{\textsuperscript{GFP}} (CD45.1/CD45.2) mice and 1.5 × 10^6 magnetically purified CD4\textsuperscript{+} T cells from the DLN of MOG/CFA + PTX-immunized (day 7) *Il1r1*{\textsuperscript{-/-}} *Bhlhe40*{\textsuperscript{GFP}} (CD45.2) mice were co-transferred into WT (CD45.1) recipients one day before immunization with MOG/CFA + PTX. At day 7 after this immunization, DLN cells were collected and analyzed for GFP expression.

**In vivo IL-1 blockade.** Anti–IL-1\(\beta\) antibody (200 \(\mu\)g/dose; clone B122; Bio X Cell or Leinco Technologies, Inc.) and anti–IL-1R antibody (200 \(\mu\)g/dose; clone JAMA-147; Bio X Cell or Leinco Technologies, Inc.) were combined (IL-1 blockade) in 200 \(\mu\)l of PBS and injected i.p. into mice on days −1, 1, and 4. Controls received 400 \(\mu\)g/dose isotype control Armenian hamster IgG (clone PIP; a gift from R.D. Schreiber, Washington University, St. Louis, MO) on the same days. Mice were left unimmunized or were immunized with MOG/CFA or MOG/CFA + PTX on day 0 and sacrificed on day 7.
**Cell preparation.** Cells from bone marrow, peritoneum, thymus, inguinal lymph node, and spleen were collected from naive WT and Bhlhe40GFP mice for analysis of GFP expression in immune cells. Lungs were perfused with 10 ml PBS via injection into the right ventricle. Lungs were minced and digested with 4 mg/ml collagenase D (Roche) at 37°C for 40–60 min with stirring, followed by addition of EDTA (5 mM final concentration) with incubation on ice for 5 min. Brains and spinal cords from naive mice, immunized mice, or mice that received adoptive transfers of 2D2 cells were collected after perfusion of the left ventricle with 30 ml PBS, and were digested with 500 µg/ml type I collagenase (Sigma-Aldrich) and 10 µg/ml DNase I (Sigma-Aldrich) in the presence of 0.1 µg/ml TLCK trypsin inhibitor (Sigma-Aldrich) and 10 mM Hepes (pH 7.4) in HBSS at room temperature for one hour with shaking. Centrifuged cells were resuspended in a 70%/37%/30% Percoll gradient (Sigma-Aldrich) in HBSS and centrifuged for 30 min at 1,200g. The 30% Percoll layer containing debris was discarded and the cells from the interface of the 30% and 37% layers were collected for subsequent use. From naive, PTX-treated, and immunized mice, DLNs were digested with 250 µg/ml collagenase B (Roche) and 30 U/ml DNase I (EMD) for 50–60 min at 37°C with stirring in Iscove’s modified Dulbecco’s media (IMDM) containing 10% FCS, l-glutamine, sodium pyruvate, nonessential amino acids, penicillin/streptomycin, and 2-mercaptoethanol (cIMDM). EDTA (5 mM final concentration) was added, and cells were incubated on ice for 5 min. Spleens were mashed and filtered through 70-µm strainers to make single-cell suspensions. From all cell suspensions, erythrocytes were lysed with ACK lysis buffer if necessary, followed by filtration through a 70-µm strainer.
In vitro TH1 and TH17 polarization and adoptive transfer EAE models. Naive CD62L⁺ CD4⁺ T cells were magnetically purified from spleens and inguinal lymph nodes using EasySep mouse naive CD4⁺ T cell isolation kits (StemCell Technologies). TH1 and TH17 cells were polarized by the method of Jäger et al. with minor modifications. In brief, naive CD4⁺ T cells were cultured in cIMDM with WT, Bhlhe40⁻/⁻ or Il1r1⁻/⁻ irradiated splenocytes (3,400 rads), 2.5 µg/ml soluble anti-CD3 (clone 145-2C11; Bio X Cell or Leinco Technologies, Inc.), and anti–IL-4 (20 µg/ml; clone 11B11; Bio X Cell or Leinco Technologies, Inc.). TH1 cell cultures also included IL-12 (10 ng/ml; BioLegend). TH17 cells were polarized with the addition of anti–IFN-γ (20 µg/ml; clone XMG1.2 [Bio X Cell] or clone H22 [Leinco Technologies, Inc.]), recombinant human TGF-β1 (3 ng/ml; BioLegend), and recombinant mouse IL-6 (30 ng/ml; BioLegend). In some experiments, recombinant mouse IL-1β (10 ng/ml; BioLegend) or anti–IL-1R (20 µg/ml; clone JAMA-147; Bio X Cell or Leinco Technologies, Inc.) plus anti–IL-1β (20 µg/ml; clone B122; Bio X Cell or Leinco Technologies, Inc.; IL-1 blockade) was added to the culture. Cells were split on day 2 with or without the addition of recombinant mouse IL-2 (10 ng/ml; BioLegend) for TH1 cells or IL-23 (10 ng/ml; BioLegend or R&D Systems) for TH17 cultures. For analysis by flow cytometry or immunoblotting, TH17 cells were harvested on day 4–7. For generation of effector 2D2 T cells, naive WT 2D2 (CD45.1) and Bhlhe40⁻/⁻ 2D2 (CD45.2) cells were magnetically purified and polarized in TH1 or TH17 conditions. Cells were split on day 2 and cultured with IL-2 (TH1) or IL-23 (TH17) for 4 more days. Polarized cells were harvested, counted, and restimulated with plate bound anti-CD3 (2 µg/ml) and anti-CD28 (2 µg/ml; clone 37.51; Bio X Cell or BioLegend) at a concentration of 2 × 10⁶/ml for 48 hours. After restimulation, cells were harvested, counted, and washed. 2 × 10⁶ TH1 or TH17 cells were injected i.p. into WT recipients. Mice were monitored for signs of EAE for at least 28 days.
**Lymph node γδ T cell stimulation.** Inguinal lymph nodes were collected from naive Bhlhe40\(^\text{GFP}\) mice and cell suspensions were cultured at 5 × 10\(^5\) cells/well in 200 µl cIMDM in the presence or absence of 2.5 µg/ml soluble anti-CD3 and/or 10 ng/ml IL-1β. At day 3, flow cytometry was performed to analyze GFP expression by γδ T cells.

**Flow cytometry.** The following anti–mouse antibodies were obtained from BioLegend: FITC (17A2), APC, or APC-Cy7 (145-2C11) anti-CD3e, BV510 or PE-Cy7 anti-CD4 (RM45), APC-Cy7 or PerCP-Cy5.5 anti-CD8α (53-6.7), APC or PE-Cy7 anti-CD11b (M1/70), APC-Cy7 anti-CD11c (N418), APC anti-CD25 (PC61), PB anti-CD44 (IM7), APC anti-CD45.2 (104), PE anti-CD62L (MEL-14), APC anti–IL7Rα (CD127; SB/199), Alexa Fluor 488 anti-B220 (RA3-6B2), FITC, PB, or PerCP-Cy5.5 anti-Ly6C (HK1.4), PE anti-Ly6G (1A8), BV510 or PB anti–I-A/I-E (M5/114.15.2), A647 anti-ICAM2 (3C4), APC anti-NK1.1 (PK136), PerCP-Cy5.5 anti-NKp46 (29A1.4), PerCP-Cy5.5 anti–Siglec H (551), PB anti-TCRβ (H57-597), APC anti-TCR γδ (GL3), PE-Cy7 anti–IFN-γ (XMG1.2), PerCP-Cy5.5 anti–L-17A (TC1118H10.1), and PE anti–GM-CSF (MP1-22E9). The following anti–mouse antibodies were purchased from BD: V450 anti-CD4 (RM4-5), APC-Cy7, V500, or BV510 anti-CD45 (30F11), V500 anti-B220 (RA3-6B2), PE anti–Ki-67 (B56), PE anti-NK1.1 (PK136), and PE anti–Siglec F (E50-2440). The following anti–mouse antibodies were obtained from eBioscience: PE-Cy7 anti-CD93 (AA4.1), PB anti-CD49b (DX5), APC anti-Slamf6 (eBio13G3-19D), APC-e780 anti-CD45 (30-F11), PE anti-Foxp3 (FJK-16s), and PerCP-eFluor710 anti–pro–IL-1β (NJT EN3). V450 or PerCP-Cy5.5 anti-CD45.1 (A20), APC-Cy7 anti-CD45.2, and PE-Cy7 anti-Ly6G (1A8) were purchased from
Tonbo Biosciences. PO-PRO-1 and 7-AAD obtained from Life Technologies were used for flow cytometry performed on lungs to discriminate live and dead cells. Fc receptor blocking was performed (clone 93; BioLegend or clone 2.4G2; Bio X Cell) in FACS buffer (0.5% BSA, 2 mM EDTA, and 0.02% sodium azide in PBS) for 5–10 min at 4°C before surface staining (4°C for 20 min). ICS was performed after a 3–4 hour stimulation of T cells with PMA (50 ng/ml; Enzo Life Sciences) and ionomycin (1 μM; Enzo Life Sciences) with brefeldin A (1 μg/ml; Enzo Life Sciences). Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) at room temperature for 20 min, followed by permeabilization with the Cytofix/Cytoperm Fixation and Permeabilization kit (BD). ICS was performed in permeabilization buffer at 4°C for 20 min. APC MOG\textsubscript{38-49-I-A\textsuperscript{b}} tetramers were obtained from the National Institutes of Health Tetramer Core Facility. Splenic or CNS cells were incubated with 4.7 μg/ml (1:300) tetramers for 30 min at 37°C. Cells were then used for surface staining. For Ki-67 staining, cells were initially fixed with 2% paraformaldehyde (4°C for 20 min), and then fixed and permeabilized with the eBioscience Foxp3/Transcription Factor Staining Buffer Set. Ki-67 staining was performed for one hour at 4°C. For Foxp3 staining, sorted CD4+GFP\textsuperscript{neg}, CD4+GFP\textsuperscript{neg}, and CD4+GFP\textsuperscript{pos} CD4+ T cells from the CNS of EAE-induced mice were fixed and permeabilized with the eBioscience Foxp3/Transcription Factor Staining Buffer Set. Foxp3 staining was performed for 30–60 minutes at room temperature. Flow cytometry was performed on a FAC SCount II (BD) or a FACS Aria II (BD). Flow cytometry data were analyzed with FlowJo software (Tree Star).

**Fluorescent microscopy.** Spinal cords from EAE-induced Blhle40\textsuperscript{GFP} mice were dissected, fixed in formalin followed by sucrose, frozen in OCT media, and cryosectioned as 10-μm sections. Tissues were blocked with 0.4% Triton-X 100 in 10% FBS, and then in 10% FBS.
Sections were then stained with primary antibody (biotin anti–mouse CD4; clone RM4-5; Bio X Cell) or biotin anti–mouse CD11b (clone M1/70; Bio X Cell; both biotinylated in house) diluted in 10% FBS, washed, and then stained with goat anti–rat IgG (H+L) Alexa Flour 555 (Invitrogen). Sections were mounted with Abcam Fluoroshield Mounting Medium with DAPI and viewed on a Nikon Eclipse E800 epifluorescence scope equipped with a QImaging EXi Blue camera and QCapture software (QImaging).

**Enzyme-linked ImmunoSpot assay (ELISPOT).** DLN cells from immunized mice were cultured in Mutiscreen Filter Plates (EMD Millipore) at 0.5–1×10^6 cells/well in cIMDM and stimulated with media, 10 µM MOG35-55 peptide, or 1 µg/ml concanavalin A (ConA; Sigma-Aldrich) at 37°C overnight. IFN-γ and IL-10 ELISPOT pairs were obtained from BD. IL-17A and GM-CSF ELISPOT pairs and streptavidin-alkaline phosphatase were purchased from BioLegend. An Immunospot counter (Cellular Technology Ltd.) was used for spot counting after plate development with NBT/BCIP substrate (Roche).

**ELISA.** DLN cells from naive, PTX-treated, or immunized mice were cultured at 10^6 cells/well in 200 µl cIMDM and left unstimulated or stimulated with 50 µg/ml heat-killed *Mtb* at 37°C for 48 hours. Cell-free supernatants were analyzed for mature IL-1β by ELISA (BioLegend). Assays were performed on Nunc Maxisorp plates and developed with streptavidin-HRP (BioLegend) plus TMB substrate (BioLegend). Standard curves were generated with purified IL-1β (BioLegend).
**Immgen data analysis and expression microarrays.** Data from the Immgen Consortium (GEO accession nos. GSE15907 [phase 1] and GSE37448 [phase 2]) were downloaded and used to determine Bhlhe40 expression in a variety of immune cell types. Arraystar 5 software (DNASTar) was used to normalize data using the robust multiarray analysis method with quantile normalization. Naive CD4+ T cells from Bhlhe40GFP mice were cultured in Th17 conditions (TGF-β1+IL-6+IL-23+IL-1β) for 4 days and stimulated for 3 hours with PMA and ionomycin. GFPneg and GFPpos Th17 cells (CD45.2+CD4+CD8−) were purified (95% purity) by cell sorting before total RNA was isolated (E.Z.N.A. MicroElute Total RNA kit; Omega Bio-Tek). RNA was amplified using the Ovation PicoSL WTA System V2 (Nugen), biotin labeled with the Encore Biotin Module (Nugen), fragmented, and hybridized to Affymetrix Mouse Gene 1.0 ST arrays. Arraystar 5 software was used as described above. Data have been deposited in GEO under accession no. GSE75407. GENE-E software (Broad Institute) was used to determine differential gene expression and generate a heat map for the most expressed 14,000 probesets. The 700 genes (5%) that were most highly expressed in GFPpos Th17 cells compared with GFPneg Th17 cells were identified, and GENE-E software was used to determine the relative expression of these 700 genes in GEO accession no. GSE23505 (arrays performed on Affymetrix Mouse Genome 430 2.0 arrays) in which Th cells had been cultured in 6 different cytokine conditions.

**Quantitative RT-PCR.** GFPneg and GFPpos CD4+ T cells (gated on CD3−B220−CD4+ cells) were sorted from DLNs on day 7 after immunization with MOG/CFA + PTX. RNA was isolated (E.Z.N.A. MicroElute Total RNA kit; Omega Bio-Tek), and then cDNA was synthesized (High Capacity RNA-to-cDNA kit; Invitrogen). Quantitative real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus Real-Time PCR
machine (Applied Biosystems). Gene expression was determined relative to *Hprt* by the ΔCT method. The following primers were used: *Hprt*, forward 5’-TCAGTCAACGGGGGACATAAA-3’, reverse 5’-GGGGCTGACTGCTTAAACCAG-3’; *Egfp*, forward 5’-CCTACGGCGTGAGCTTCAGC3’, reverse 5’-CGGCGAGCTGCACGCTGCCTC-3’; *Bhlhe40*, forward 5’-ACGGAGACCTGCAAGGGATG-3’, reverse 5’-GCGAGTTTGAAGTTTCCCTTG-3’; *Csf2*, forward 5’-GCCATCAAAGAAGCCCTGA-3’, reverse 5’-GCGGGTCTGCACACATGTTAC-3’.

**Immunoblotting.** T<sub>H1</sub> or T<sub>H17</sub> cells were counted and lysed at 10<sup>6</sup>/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-PVDF membranes (Costar). Blots were incubated with anti-Bhlhe40 (Novus Biologicals; used at 1:1,000) or anti-HDAC1 (Abcam; used at 1:2,000) primary antibodies at 4°C overnight with shaking. Blots were washed at least four times before incubation with anti–rabbit IgG-HRP (clone 5A6-1D10 [light chain specific]; Jackson ImmunoResearch Laboratories) at room temperature for 60 min with shaking. After five washes, Clarity Western ECL substrate (Bio-Rad Laboratories) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) was applied, and blots were placed on Blue basic autoradiography film (GeneMate). Film was developed with a Medical Film Processor (model SRX-101A; Konica Minolta), and scanned films were analyzed with ImageJ software (NIH).
**Statistical analysis.** Data were analyzed by paired or unpaired two-tailed Student’s t tests (Prism; GraphPad Software, Inc.) as indicated in the figure legends, with $P \leq 0.05$ considered significant.
3.6 Acknowledgements

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The authors declare no competing financial interests.
3.7 Author contributions

C.-C. Lin planned and performed experiments and wrote the manuscript. T.R. Bradstreet, E.A. Schwarzkopf, N.N. Jarjour, C. Chou, A.S. Archambault, and J. Sim performed experiments. B.H. Zinselmeyer analyzed microscopy data. J.A. Carrero and M.N. Artyomov analyzed microarray data. G.F. Wu and J.H. Russell provided advice and edited the manuscript. R. Taneja provided $Bhlhe40^{−/−}$ mice and made helpful suggestions. B.T. Edelson supervised the study and wrote the manuscript.
Figure 3-1. **Bhlhe40**$^{\text{GFP}}$ mice show Bhlhe40 expression in immune cells.
**Figure 3-1.** *Bhlhe40*<sup>GFP</sup> mice show *Bhlhe40* expression in immune cells.

(A–D) GFP (*Bhlhe40*) expression in multiple immune cell types in thymus (A), spleen (B and D), and bone marrow, peritoneum, lung, and brain (C) from naive nontransgenic (*Tg<sup>−</sup>*) or *Bhlhe40*<sup>GFP</sup> (*Tg<sup>+</sup>*) reporter mice. DN, double-negative cells; DP, double-positive cells; CD4SP, CD4 single-positive cells; CD8SP, CD8 single-positive cells; mac, macrophages; mono, monocytes; pDC, plasmacytoid DCs. All cell types were analyzed at least two times in at least two independent experiments. (B) GFP (*Bhlhe40*) expression by splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells from naive *Bhlhe40*<sup>GFP</sup> mice. CD44 and CD62L expression by GFP<sup>neg</sup> and GFP<sup>pos</sup> T cells were analyzed. (E and F) *Bhlhe40* expression in immune cells based on microarray datasets from the Immgen Consortium, datasets GSE15907 (E; phase 1) and GSE37448 (F; phase 2). BM, bone marrow; PMN, polymorphonuclear cells; MO, monocytes; DC, dendritic cells; FO B, follicular B cells; GC B, germinal center B cells; MZ B, marginal zone B cells; Eos, eosinophils. Data are mean ± SEM with dots representing individual microarrays performed by Immgen.
Figure 3-2. Bhlhe40-expressing $T_h$ cells are enriched in the CNS and are robust cytokine producers during EAE.
Figure 3-2. Bhlhe40-expressing T_H cells are enriched in the CNS and are robust cytokine producers during EAE.

(A) GFP (Bhlhe40) expression by CD4^+ T cells in the spleen and CNS of Bhlhe40^GFP mice on day 14 after EAE induction. Clinical scores of individual mice on the day of sacrifice are indicated. One experiment out of four is shown (n = 10 mice total). (B) Quantitation of the percentage of GFP^{pos} cells (of CD4^+ T cells) in spleen and CNS from A. Each line represents an individual mouse (n = 10). Data are combined from four independent experiments performed at day 13 or 14 after EAE induction. Clinical scores on the day of sacrifice are indicated by color. (C) Immunofluorescent staining of a spinal cord section at day 15 after EAE induction in a Bhlhe40^GFP mouse (clinical score of 2). Dashed line represents the edge of the tissue. Bottom right quadrant is the overlapping image of DAPI (blue), CD4 (red), and GFP (green). High magnification images of selected CD4^+ T cells expressing GFP are shown. Data are representative of two independent experiments (n = 2 mice total). (D) ICS plots for IFN-γ, IL-17A, and GM-CSF for GFP^{neg} and GFP^{pos} CD4^+ T cells in the spleen and CNS at day 14 after EAE induction in Bhlhe40GFP mice. One experiment out of two is shown (n = 5 mice). (E) Quantitation of the frequency of GFP^{neg} or GFP^{pos} CD4^+ T cells secreting IFN-γ, IL-17A, or GMCSF from D. Each line represents an individual mouse (n = 5). Data are combined from two experiments. Paired two-tailed Student's t test was performed to determine significance. ***, P ≤ 0.001.
Figure 3-3. PTX serves as an essential co-adjuvant by inducing Bhlhe40 expression in $T_H$ cells.
Figure 3-3. PTX serves as an essential co-adjuvant by inducing Bhlhe40 expression in T cells.

(A) GFP (Bhlhe40) and CD44 expression by CD4⁺ T cells from the DLN of Bhlhe40GFP mice on day 7. Groups of mice were either unimmunized or immunized with MOG/CFA. PTX or mPTX was administered i.p. on days 0 and 2. One experiment out of five is shown (n = 4–10/group).

(B) Quantitation of the percentage (left) of GFP⁺ cells (of CD4⁺ T cells) and the absolute number (right) of GFP⁺ CD4⁺ T cells in the DLNs from (A). Dots represent individual mice. Data are combined from five experiments (n = 4–10/group). (C) Mean EAE scores of WT mice immunized with MOG/CFA. PTX or mPTX, where indicated, were administered i.p. on days 0 and 2. Data are combined from two experiments (n = 8–9). Incidence of clinical disease is indicated. (D) ICS plots for IFN-γ, IL-17A, and GM-CSF for GFP⁻ cells and GFP⁺ CD4⁺ T cells in the DLN in naive mice or at day 7 after immunization as indicated in Bhlhe40GFP mice. One experiment out of three is shown (n = 9 mice). (E) Quantitation of the frequency of GFP⁻ and GFP⁺ CD4⁺ T cells from MOG/CFA + PTX-immunized mice producing each of the eight combinations of IFN-γ, IL-17A, and GM-CSF. Data are combined from three experiments (n = 9). (F) Mean fluorescence intensity (MFI) of Ki-67 by GFP⁻ or GFP⁺ CD4⁺ T cells from the DLN of Bhlhe40GFP mice on day 7 after immunization with MOG/CFA + PTX. Data are combined from two experiments (n = 7). (G) Quantitative RT-PCR analysis of Egfp, Bhlhe40, and Csf2 expression by sorted GFP⁻ and GFP⁺ CD4⁺ T cells from the DLN of Bhlhe40GFP mice on day 7 after MOG/CFA + PTX immunization (n = 3 mice from a single experiment). Data are mean ± SEM. Unpaired (B) or paired (E–G) two-tailed Student's t tests were performed to determine significance. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Figure 3-4. PTX co-adjuvanticity is Bhlhe40 dependent.

ELISPOT assays for the quantitation of cells secreting IFN-γ, IL-17A, GM-CSF, or IL-10 performed on DLN cells 7 d after MOG/CFA immunization of WT or Bhlhe40<sup>−/−</sup> mice treated with or without PTX. Data are combined from four experiments (n = 7–13 mice/group). Dots represent individual mice. For clarity, only mean values (lines) are shown. Unpaired two-tailed Student's t test was performed to determine significance. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Figure 3-5. Bhlhe40 expression identifies the cytokine-producing fraction of autoreactive T<sub>H</sub> cells.
Figure 3-5. Bhlhe40 expression identifies the cytokine-producing fraction of autoreactive Th cells.

(A) Plots of MOG38-49–I-Ab tetramer staining and GFP (Bhlhe40) expression by CD4+ T cells from the spleen and CNS of naive non-Tg and EAE-induced Bhlhe40GFP mice (day 15). One experiment out of two is shown (n = 10 mice). (B) Quantitation of the percentage of CNS CD4+ T cells positive or negative for GFP (Bhlhe40) and/or MOG38-49–I-Ab tetramer staining in the CNS at day 15 after EAE induction. Data are combined from two experiments (n = 10). (C) Quantitation of the frequency of IFN-γ+ cells within the indicated CD4+ T cell subsets (as shown in B). One experiment out of two is shown (n = 6). (D) Plots for (left) CD4 and CD45.1 and (right) CD4 and GFP (Bhlhe40) on DLN cells after the transfer of T cells from 2D2 Bhlhe40GFP CD45.1/CD45.2 mice into WT (CD45.2) recipients. 1 d after cell transfer, mice were left unimmunized or immunized with the indicated peptide (MOG35-55 or OVA323-339) in CFA. Some mice were treated with PTX on days 0 and 2. DLNs were harvested on day 7 after immunization. One experiment out of three is shown (n = 3–10/group). (E) Quantitation of the percentage of (top) CD45.1+ 2D2 cells (of CD4+ T cells) and (bottom) CD45.1+ 2D2 GFPpos cells (of CD45.1+ 2D2 cells) in the DLNs from (D). Dots represent individual mice. Data are combined from three experiments (n = 3–10 mice/group). (F) ICS plots for IFN-γ, IL-17A, and GM-CSF for host CD4+ T cells, GFPneg, or GFPpos 2D2 cells in the DLN from MOG/CFA + PTX-immunized mice from (D). One experiment out of three is shown (n = 10 mice). (G) Quantitation of the frequency of host CD4+ T cells or GFPneg or GFPpos 2D2 cells secreting IFN-γ, IL-17A, or GM-CSF from F. Lines connect data from individual mice (n = 10). Data are combined from three experiments. (H) ICS plots for IFN-γ, IL-17A, and GM-CSF for host GFPneg or GFPpos CD4+ T cells, or GFPneg or GFPpos 2D2 cells in the CNS at day 14 after EAE induction. T cells from 2D2
Bhlhe40\textsuperscript{GFP} CD45.1/CD45.2 mice were transferred into Bhlhe40\textsuperscript{GFP} (CD45.2) recipients 1 d before immunization with MOG/CFA + PTX. One experiment out of two is shown (n = 6 mice).

(I) Quantitation of the frequency of host GFP\textsuperscript{neg} or GFP\textsuperscript{pos} CD4\textsuperscript{+} T cells, GFP\textsuperscript{neg}, or GFP\textsuperscript{pos} 2D2 cells secreting IFN-\textgamma, IL-17A, or GM-CSF from H. Lines connect data from individual mice (n = 3). One experiment out of two is shown. Data are mean ± SEM. Unpaired (E) or paired (C and G) two-tailed Student's t tests were performed to determine significance. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Figure 3-6. Bhlhe40 is essential for the encephalitogenicity of $\mathrm{T}_{\mathrm{H}}$ cells in adoptive transfer models of EAE.
Figure 3-6. Bhlhe40 is essential for the encephalitogenicity of T_H cells in adoptive transfer models of EAE.

(A) ICS plots for the indicated cytokines from polarized WT and Bhlhe40−/− 2D2 T_H1 or T_H17 cells at day 4 of culture. One experiment out of three is shown (n = 10–14 mice/group). (B and C) Mean EAE scores of WT mice after receiving transfers of WT or Bhlhe40−/− 2D2 T_H1 (B) or T_H17 (C) cells. Data are combined from four (T_H1, n = 10–14) or two (T_H17, n = 10–11) experiments. Incidence of clinical disease is indicated. (D–G) ICS plots for the indicated cytokines (D and F) and quantitation of the frequency (E and G) of cytokine-producing WT and Bhlhe40−/− 2D2 T_H1 (D and E) or T_H17 (F and G) cells in the spleen and CNS on days 28–34 after immunization. (D and E) Data are combined from two independent experiments (n = 7/group). (F and G) One experiment of two is shown (n = 3–4/group). Data are mean ± SEM. Unpaired two-tailed Student's t test was performed to determine significance. **, P ≤ 0.01; ***, P ≤ 0.001.
Figure 3-7. Bhlhe40 expression negatively correlates with Foxp3 and IL-10 expression.
Figure 3-7. Bhlhe40 expression negatively correlates with Foxp3 and IL-10 expression.

(A) Strategy for sorting CD44^-GFPneg (R1), CD44^+ GFPneg (R2), and CD44^+ GFPpos (R3) CD4^+ T cells in the CNS at day 16 after EAE induction. Foxp3 and CD25 expression on sorted cells from each population is shown. One experiment out of two is shown (n = 5–7 mice). (B) Quantitation of the frequency of Foxp3pos cells within each of the three populations as shown in A. Data are combined from two experiments (n = 5–7). Data are mean ± SEM. (C) ICS plots for IFN-γ and IL-10 for host GFPneg or GFPpos CD4^+ T cells, or GFPneg or GFPpos 2D2 cells in the CNS at day 14 after EAE induction. T cells from 2D2 Bhlhe40GFP CD45.1/ CD45.2 mice were transferred into Bhlhe40GFP (CD45.2) recipients 1 d before immunization with MOG/CFA + PTX. One experiment out of two is shown (n = 6 mice). (D) Quantitation of the frequency of host GFPneg or GFPpos CD4^+ T cells, GFPneg, or GFPpos 2D2 cells secreting IFN-γ or IL-10 from (C). Lines connect data from individual mice (n = 3). One experiment out of two is shown. (E) ICS plots for IFN-γ and IL-10 for GFPneg and GFPpos CD4^+ T cells in the CNS at day 29 after EAE induction in Bhlhe40GFP mice. One experiment out of two is shown (n = 5 mice). (F) Quantitation of the frequency of GFPneg or GFPpos CD4^+ T cells secreting IFN-γ (left) or IL-10 (right) from E. Lines connect data from individual mice (n = 5). Data are combined from two experiments. Unpaired (B) or paired (F) two-tailed Student's t tests were performed to determine significance. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Figure 3-8. Bhlhe40-expressing TH17 cells exhibit a pathogenic molecular signature.
Figure 3-8. Bhlhe40-expressing TH17 cells exhibit a pathogenic molecular signature.

(A) CD4 and GFP (Bhlhe40) expression by polarized Bhlhe40GFP TH1 or TH17 cells at day 4 of culture. Data are representative of at least five experiments with at least eight mice per condition. (B) Strategy for sorting GFP<sup>neg</sup> and GFP<sup>pos</sup> TH17 cells at day 4 of culture. Sorted cells were subjected to immunoblots for Bhlhe40 and HDAC1 (loading control). One experiment out of two is shown (n = 5 mice). (C) Quantitation of the band intensity of Bhlhe40 relative to HDAC1 in sorted GFP<sup>neg</sup> and GFP<sup>pos</sup> TH17 cells from (B). Data are expressed as a fold change in intensity comparing sorted cells from the same culture. Data are combined from two experiments (n = 5). Mean ± SEM. (D) Heat map comparing gene expression between sorted GFP<sup>neg</sup> and GFP<sup>pos</sup> TH17 cells from three mice. Listed genes have been associated with EAE or represent surface markers amenable to flow cytometry (Cd93, Il7r, Slamf6, and Sell). Shown are the 14,000 probesets with the highest mean expression across all six arrays. The yellow box identifies the 700 genes (5%) that were most highly expressed in GFP<sup>pos</sup> TH17 cells compared with GFP<sup>neg</sup> TH17 cells. The relative expression of these 700 genes was analyzed in GEO DataSet GSE23505<sup>188</sup> in which TH cells had been cultured in different cytokine conditions as indicated (right). The red line indicates the mean expression of these genes. The green line represents the expression of Bhlhe40. (E) CD4 and CD93, IL-7R, Slamf6, or CD62L (encoded by Sell) expression by GFP<sup>neg</sup> and GFP<sup>pos</sup> TH17 cells cultured as in A. One experiment out of two is shown (n = 4 mice).
Figure 3-9. IL-1β increases Bhlhe40 expression in T\textsubscript{H17} and γδ T cells.
Figure 3-9. IL-1β increases Bhlhe40 expression in T<sub>H17</sub> and γδ T cells.

(A) CD4 and GFP (Bhlhe40) expression by Bhlhe40<sup>GFP</sup> T<sub>H17</sub> cells at day 4 of culture in the indicated conditions. One experiment out of three is shown (n = 4 mice). (B) Kinetics of GFP (Bhlhe40) expression by Bhlhe40<sup>GFP</sup> T<sub>H17</sub> cells cultured in the presence of IL-1 blockade (anti–IL-1β and anti–IL-1R) or IL-1β. Data are combined from two experiments (n = 3–5 mice/condition). (C) CD4 and GFP (Bhlhe40) expression by Bhlhe40<sup>GFP</sup> T<sub>H17</sub> (TGF-β1 + IL-6 + IL-23) cells at day 4 of culture in the presence of IL-1 blockade or IL-1β and with WT or Il1r1<sup>−/−</sup> irradiated splenocytes. One experiment out of two is shown (n = 3 mice). (D) Immunoblots of Bhlhe40 and HDAC1 in T<sub>H17</sub> cells cultured with the indicated cytokines for 4 d (bottom). Quantitation of the band intensity of Bhlhe40 relative to HDAC1. Data are representative of four experiments (n = 2 mice/condition). (E) Quantitation of the frequency of GFP<sup>neg</sup> or GFP<sup>pos</sup> T<sub>H17</sub> (TGF-β1 + IL-6 + IL-23) cells secreting IL-17A, GM-CSF, or IL-10 cultured in the absence or presence of IL-1β or IL-1 blockade. Data are combined from four experiments (n = 4–5 mice/condition). (F) CD4 and GFP (Bhlhe40) expression by Bhlhe40<sup>GFP</sup> T<sub>H1</sub> cells at day 4 of culture with IL-1β or IL-1 blockade. One experiment out of three is shown (n = 5 mice). (G) Quantitation of the percentage of GFP (Bhlhe40)-expressing T<sub>H1</sub> cells cultured in the presence (IL-1β) or absence of IL-1β (no IL-1β or IL-1 blockade). Data are combined from four experiments (n = 5). (H) Immunoblots of Bhlhe40 and HDAC1 in T<sub>H1</sub> cells cultured with or without IL-1β for 4 d. The fold change of band intensity of Bhlhe40 relative to HDAC1 is indicated. Data are representative of two experiments (n = 3 mice). (I) Quantitation of the frequency of GFP<sup>neg</sup> or GFP<sup>pos</sup> T<sub>H1</sub> cells secreting IFN-γ, GM-CSF, or IL-10 cultured in the absence or presence of IL-1β or IL-1 blockade. Data are combined from four experiments (n = 3–5/condition). (J) CD44 and GFP (Bhlhe40) expression by lymph node γδ T cells from
Bhlhe40<sup>GFP</sup> mice cultured for 3 d in the indicated conditions. One experiment out of three is shown (n = 5 mice). (K) Quantitation of the percentage of GFP (Bhlhe40)-expressing γδ T cells from (J). Data are combined from three experiments (n = 5 mice/condition). Data are mean ± SEM. Unpaired (B, D, and K) or paired (G) two-tailed Student's t tests were performed to determine significance. For E and I, p-values represent paired, two-tailed Student's t test when comparing GFP<sup>neg</sup> to GFP<sup>pos</sup> cells from the same culture, and represent unpaired two-tailed Student's t test when comparing cultures with no IL-1β or IL-1 blockade vs. IL-1β. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Figure 3-10. IL-1R signaling is required in vivo for full Bhlhe40 expression by T<sub>H</sub> cells, and systemic PTX induces IL-1β production by lymph node cells.
Figure 3-10. IL-1R signaling is required in vivo for full Bhlhe40 expression by Th cells, and systemic PTX induces IL-1β production by lymph node cells.

(A) GFP (Bhlhe40) expression by CD4⁺ T cells from the DLNs of Bhlhe40GFP or Il1r1⁻/⁻ Bhlhe40GFP mice on day 7 after immunization with MOG/CFA + PTX. One representative experiment out of three is shown (n = 9–10 mice/group). (B) Quantitation of the percentage (left) of GFP⁺ cells (of CD4⁺ T cells) and the (right) number of GFP⁺ CD4⁺ T cells in the DLNs from (A). Data are combined from three experiments (n = 9–10/group). (C) GFP (Bhlhe40) expression by CD4⁺ T cells from the DLNs of Bhlhe40GFP mice treated with control Ab or IL-1 blockade in vivo on days −1, 1, and 4. Mice were left unimmunized or were immunized with MOG/CFA ± PTX treatment (days 0 and 2). One experiment out of two is shown (n = 1–3 mice/group). (D) Quantitation of the percentage (left) of GFP⁺ cells (of CD4⁺ T cells) and the (right) number of GFP⁺ CD4⁺ T cells in the DLNs from (C). One experiment out of two is shown (n = 1–3 mice/group). (E) Mean EAE scores of WT, Bhlhe40⁻/⁻, and Il1r1⁻/⁻ mice immunized with MOG/CFA + PTX. Data are combined from two experiments (n = 6–12). Incidence of clinical disease is indicated. (F) CD4⁺ T cells from the DLN of MOG/CFA + PTX-immunized Bhlhe40GFP (CD45.1/CD45.2) mice and Il1r1⁻/⁻ Bhlhe40GFP (CD45.2) mice were purified on day 7 and co-transferred to WT (CD45.1) recipients 1 d before immunization with MOG/CFA + PTX. 7 d after this immunization, transferred Bhlhe40GFP or Il1r1⁻/⁻ Bhlhe40GFP GFP⁺ cells were identified. One experiment out of two is shown (n = 6). (G) Quantitation of the percentage of transferred Bhlhe40GFP or Il1r1⁻/⁻ Bhlhe40GFP GFP⁺ cells (of GFP⁺ cells) in the DLNs from (F). One experiment out of two is shown (n = 6). (H) Groups of WT mice were either unimmunized or immunized with MOG/CFA. PTX or mPTX was administered i.p. on days 0 and 2. DLN cells, collected on day 7, were cultured with or without
50 µg/ml heat-killed *Mtb* for 2 d and supernatants were analyzed for IL-1β. Data are combined from three experiments (n = 3–7 mice/group). (I) Quantitation of the percentage of pro–IL-1β⁺ cells (of B220⁻ DLN cells) from WT mice treated as in (H). Data are combined from four experiments (n = 3–12/group). (J) ICS for pro–IL-1β expression by B220⁻ DLN cells from representative mice shown in I. (K; left) Ly6G and Ly6C expression on B220⁻ DLN cells from the mice shown in (J). Numbers show the percentage of Ly6G⁺Ly6C⁺ neutrophils (right). MHC class II and Ly6C expression by Ly6G⁻B220⁻ cells. Numbers show the percentage of monocytes/moDCs. One experiment out of four is shown (n = 3–12/group). (L) Quantitation of the number of pro–IL-1β⁺ neutrophils, MHC II⁺Ly6C⁺ monocytes/moDCs, migratory DCs, and resident DCs in the DLNs from (I-K). Data are combined from two experiments (n = 2–7/group). Data are mean ± SEM. Dots represent individual mice except in (E). Unpaired (B, D, H, I, and L) or paired (G) two-tailed Student's t tests were performed to determine significance. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Chapter 4: Bhlhe40 regulates IL-10 production by innate immune cells and is critical for control of *Mycobacterium tuberculosis*.

*Note: A manuscript related to the content of this chapter is currently in preparation.*
4.1 Abstract

Mononuclear phagocytes and T cells control bacterial growth and limit pathology during pulmonary *Mycobacterium tuberculosis* (*Mtb*) infection. IL-10 is an immunosuppressive cytokine generated by several cell types during *Mtb* infection and is known to impair protective responses to this pathogen. IL-10 can inhibit intracellular bacterial killing, antigen presentation, IL-12 production, and T\(_H\)1 cell priming. How IL-10 production is regulated by both innate and adaptive immune cells during *Mtb* infection is unclear. In Chapter 2, we showed that the transcription factor Bhlhe40 repressed IL-10 production by CD4\(^+\) T cells during EAE. In this chapter, I describe our experiments in which we further uncovered that Bhlhe40 also negatively regulates IL-10 expression by myeloid cells during *Mtb* infection in mice. In vivo, *Bhlhe40\(^{-/-}\)* mice rapidly succumbed to pulmonary *Mtb* infection with uncontrolled bacterial growth and large neutrophil-rich lesions. This increased susceptibility was also seen in *Rag1\(^{-/-}\)* *Bhlhe40\(^{-/-}\)* mice when compared to *Rag1\(^{-/-}\)* mice, indicating that the innate compartment required Bhlhe40 for *Mtb* resistance. Using IL-10 reporter mice crossed to *Bhlhe40\(^{-/-}\)* mice, we identified *Bhlhe40\(^{-/-}\)* lung CD11b\(^+\) DCs and monocyte-derived cells as sources of abundant IL-10 during *Mtb* infection. Genetic deletion of *Il10* in Bhlhe40-deficient mice (*Il10\(^{-/-}\)* *Bhlhe40\(^{-/-}\)*) reversed the susceptibility of *Bhlhe40\(^{-/-}\)* mice to *Mtb* infection, confirming that negative regulation of IL-10 was an essential function for Bhlhe40. ChIP-qPCR and ChIP-Seq performed in myeloid cells identified a putative regulatory element within the *Il10* locus. Our results established a critical role for Bhlhe40 in innate immune cells and provided novel insights into *Il10* gene regulation within these populations during mycobacterial infection.
4.2 Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*Mtb*). This single pathogen is responsible for more deaths than HIV and malaria. In 2015, an estimated 10.4 million new TB cases were reported and about 1.8 million people died of this disease (WHO Fact Sheet 2017 http://www.who.int/mediacentre/factsheets/fs104/en/), making TB one of the top ten causes of human mortality in the world. However, the only vaccine for TB, Bacille Calmette-Guerin (BCG), is not effective for adults\(^2\). In addition, control of the global TB epidemic is challenged by the emergence of multidrug-resistant TB (MDR TB)\(^2\). To develop new vaccines and treatments for this disease, it is important to understand the immune response to *Mtb* infection.

Studies from experimental mouse models showed that TB is controlled by both innate and adaptive immune responses. Upon *Mtb* infection, lung macrophages and DCs phagocytose the pathogen and secrete antimicrobial molecules, including TNF, IL-1, IL-6, IL-12, and several chemokines\(^1\). Mice deficient in TNF, TNF receptor p55, IL-1 receptor (IL-1R), IL-\(\alpha\), IL-1\(\beta\), or the adaptor for IL-1R signaling Myd88 are highly susceptible to *Mtb*\(^1\). A recent study showed that the hydrolases within the alveolar lining fluid (ALF) released *Mtb* cell wall components, which enhanced the anti-microbial properties of human macrophages\(^\)\(^2\). Nevertheless, even in WT mice, infected phagocytes fail to efficiently eliminate the bacteria\(^2\). Eight to 12 days after *Mtb* infection, pulmonary CCR7\(^+\) DCs transport antigens to lung-draining mediastinal lymph nodes guided by the chemokines CCL19 and CCL21\(^2\). In DLNs, mature DCs provide TCR stimulation and secrete IL-12 to direct T\(_{H1}\) cell differentiation. About 14-17 days after infection, *Mtb* antigen-specific T\(_{H1}\) cells migrate to the lung and secrete IFN-\(\gamma\) to activate phagocytes as well as lung epithelial and
endothelial cells\textsuperscript{284}. Activated macrophages then produce reactive oxygen species (ROS) and nitric oxide (NO) to kill \textit{Mtb} or restrict its replication in the lungs\textsuperscript{110, 285, 286}. Not surprisingly, mice lacking T cells (eg. \textit{Rag1}\textsuperscript{+/−}, TCRαβ\textsuperscript{+/−}, MHC-II\textsuperscript{+/−}) or with impaired T\text{H}1 cell-mediated responses (eg. \textit{Tbx21}\textsuperscript{−/−}, \textit{Stat1}\textsuperscript{−/−}, \textit{Ifng}\textsuperscript{−/−}, \textit{Ifngr}\textsuperscript{−/−}, \textit{Nos2}\textsuperscript{−/−} mice) all fail to generate protective immunity to \textit{Mtb}\textsuperscript{112, 113, 114 110, 111}.

The anti-inflammatory cytokine IL-10 has been shown to limit the immune responses in mouse models of mycobacterial infections\textsuperscript{287}. Several studies observed that IL-10-deficient CBA/J, BALB/c, and C57BL/6 mice showed stronger T\text{H}1 responses and enhanced protection from aerosol \textit{Mtb} infection\textsuperscript{152, 154, 155, 287}. Others reported that transgenic overexpression of IL-10 by immune cells in FVB, BALB/c, and C57BL/6 mice rendered the animals more susceptible to chronic mycobacterial infection\textsuperscript{288, 289, 290, 291, 292}. Turner et al. overexpressed IL-10 under the \textit{Il2} promoter in C57BL/6 mice and found that these mice had higher bacterial loads after four months of \textit{Mtb} infection\textsuperscript{292}. Schreiber et al. generated transgenic FVB mice with macrophage-specific overexpression of IL-10, which failed to control \textit{Mtb} replication and died significantly earlier than their non-transgenic counterparts\textsuperscript{290}. These studies indicated that the hyperproduction of IL-10 from T cells and phagocytes could increase \textit{Mtb} susceptibility.

In the previous two chapters, we showed that \textit{Bhlhe40}\textsuperscript{+/−} T cells produce enhanced amounts of IL-10, especially from IL-12-polarized T\text{H}1 cells\textsuperscript{11, 12}. Although WT T\text{H}1 cells can restrict \textit{Mtb} replication, IL-10-hyperproducing T\text{H}1 cells might fail to do so and thus be detrimental. We originally hypothesized that mice lacking Bhlhe40 would be susceptible to \textit{Mtb} infection due to impaired immune responses caused by higher levels of IL-10 production from CD4\textsuperscript{+} T cells. In this chapter, we found that \textit{Bhlhe40}\textsuperscript{−/−} mice indeed succumbed to aerosol \textit{Mtb} infection, and that genetic deletion of \textit{Il10} in Bhlhe40-deficient mice (\textit{Il10}\textsuperscript{−/−}/\textit{Bhlhe40}\textsuperscript{−/−}) significantly prolonged their
survival. Nevertheless, our $\text{Rag} 1^{-/-}\text{Bhlhe} 40^{-/-}$ mice data suggested that Bhlhe40 is actually essential for the innate immune compartment to control $Mtb$ infection in the early stage. Using IL-10 reporter mice, we pinpointed several myeloid cell subsets that expressed higher levels of IL-10 in infected $\text{Bhlhe} 40^{-/-}$ lungs. Furthermore, ChIP-Seq and ChIP-qPCR performed in GM-CSF-cultured bone marrow cells identified a putative regulatory element within the $\text{Il} 10$ locus. These results suggested a critical role for Bhlhe40 in $Mtb$ resistance through regulating IL-10 expression by innate immune cells.
4.3 Results

4.3.1 Bhlhe40-deficient mice succumb to *Mtb* infection

To examine the role of Bhlhe40 in immune responses to bacterial infections, we infected *Bhlhe40*<sup>−/−</sup> mice via the aerosol route with the virulent Erdman strain of *Mtb*. While WT and *Bhlhe40*<sup>+/−</sup> mice survived longer than 100 days after infection, *Bhlhe40*<sup>−/−</sup> mice were highly susceptible to *Mtb*. Knockout mice started losing weight at day 21 and all died by seven weeks post infection (p.i.) (Figure 4-1A and data not shown). Gross pathology showed several white abscesses on the lung surfaces of *Bhlhe40*<sup>+/−</sup> mice at day 33 after infection (Figure 4-1B). Compared to WT mice, *Bhlhe40*<sup>−/−</sup> mice had higher mycobacterial burdens in both lungs and spleens at week three after infection (Figure 4-1, C and D). Our results showed that Bhlhe40 is required for effective control of *Mtb* replication.

4.3.2 Anti-Ly6G treatment fails to rescue *Bhlhe40*<sup>−/−</sup> mice from *Mtb*-induced lethality

Histology of lung sections showed that the lesions in infected *Bhlhe40*<sup>−/−</sup> mice were dominated by granulocytes (Figure 4-2A). Using flow cytometry, we confirmed that the majority of CD45<sup>+</sup> hematopoietic cells in the *Bhlhe40*<sup>−/−</sup> lungs were Ly6C<sup>+</sup>Ly6G<sup>+</sup> neutrophils after three weeks of infection (Figure 4-2, B and C). Exacerbated neutrophil recruitment is commonly associated with poor outcomes after TB infection<sup>293</sup>. Massive neutrophilia was observed in several *Mtb*-susceptible mutant mice such as *Card9*<sup>−/−</sup> mice, *Ifngr*<sup>−/−</sup> mice, and *Lyz2-Cre x Atg5<sup>fl/fl</sup>* conditional knockout mice, and all of these strains quickly succumb to *Mtb* infection<sup>112,294,295</sup>. Notably, the survival of these mutant mice could be prolonged by treating them with anti-Ly6G antibody
(clone 1A8), which specifically deletes neutrophils. We utilized the antibody treatment protocol reported by Kimmey et al. to test if neutrophil depletion affected the survival of \textit{Bhlhe40}⁻/⁻ mice after exposure to \textit{Mtb}. Anti-Ly6G treatments largely reduced Gr-1⁺Ly6C⁺ neutrophils in both naïve WT and \textit{Bhlhe40}⁻/⁻ mice, indicating that the antibody-mediated phagocytosis machinery was functioning in the absence of Bhlhe40 (Figure 4-2D). Nevertheless, the injections of anti-Ly6G antibody did not impact the susceptibility of \textit{Bhlhe40}⁻/⁻ mice to \textit{Mtb}. \textit{Bhlhe40}⁻/⁻ mice treated with control or 1A8 antibody lost weight at the same kinetics and died at nearly the same time (Figure 4-2E and data not shown). Despite the absence of Gr-1⁺Ly6C⁺ cells after anti-Ly6G injections, we observed a less frequent Gr-1⁺Ly6C⁺ population that resembled granulocytes in the lungs of \textit{Bhlhe40}⁻/⁻ mice (Figure 4-2F). We concluded that anti-Ly6G treatment at least partially depleted the neutrophil subset in \textit{Bhlhe40}⁻/⁻ mice but was unable to improve their resistance to \textit{Mtb}.

4.3.3 Innate immune cells require \textit{Bhlhe40} to control \textit{Mtb} infection

To test if \textit{Bhlhe40} is required by the hematopoietic compartment to restrict \textit{Mtb} replication, we generated bone marrow chimeras by reconstituting irradiated WT or \textit{Bhlhe40}⁻/⁻ mice with \textit{Bhlhe40}⁻/⁻ or WT bone marrow cells. We found that \textit{Bhlhe40} was essential in the hematopoietic compartment for the control of \textit{Mtb} infection, since \textit{Bhlhe40}⁻/⁻ bone marrow-reconstituted WT or \textit{Bhlhe40}⁻/⁻ recipient mice phenocopied global \textit{Bhlhe40}-deficient mice upon \textit{Mtb} infection (Figure. 4-3A).

Next, we examined the requirements for \textit{Bhlhe40} in the innate and adaptive immune compartments. \textit{Bhlhe40}⁻/⁻ mice were crossed to \textit{Rag1}⁻/⁻ mice and infected with \textit{Mtb}. Interestingly, we found that \textit{Rag1} deficiency did not affect the susceptibility of \textit{Bhlhe40}⁻/⁻ mice.
Bhlhe40<sup>−/−</sup> mice and Rag1<sup>−/−</sup> Bhlhe40<sup>−/−</sup> mice died at the same time, about one week earlier than Bhlhe40-sufficient Rag1<sup>−/−</sup> mice (Figure. 4-3B). Also, Rag1<sup>−/−</sup> Bhlhe40<sup>−/−</sup> mice showed increased lung bacterial loads compared to Rag1<sup>−/−</sup> mice (Figure. 4-3C). These results led us to conclude that Bhlhe40 was critical for Rag1-independent innate immune cells to restrain Mtb infection.

### 4.3.4 Bhlhe40<sup>−/−</sup> myeloid cells express higher levels of IL-10 in vivo

We next analyzed myeloid cell responses in Mtb-infected mixed bone marrow chimeras. Irradiated mice reconstituted with only WT cells (but from two different donors) showed minimal signs of lung inflammation at 3 wk p.i. In contrast, recipients reconstituted with mixed WT and Bhlhe40<sup>−/−</sup> bone marrow cells exhibited an increased total proportion of neutrophils (Figure. 4-4A) which originated equally from WT and Bhlhe40<sup>−/−</sup> progenitors (Figure. 4-4B). Furthermore, both neutrophil populations, regardless of genotype, were highly infected by GFP-labeled Mtb (Figure. 4-4C). These results suggested that cell-extrinsic factors secreted from Bhlhe40<sup>−/−</sup> bone marrow-derived cells remodeled the lung environment and diminished the ability of WT cells to control Mtb infection.

Detection of cytokine and chemokine expression by WT and Bhlhe40<sup>−/−</sup> total lung cells after infection revealed that Il10 transcripts and protein was upregulated in Bhlhe40<sup>−/−</sup> mice (Figure. 4-5, A and B). Stimulation of total lung cells from infected Bhlhe40<sup>−/−</sup> mice with heat-killed Mtb also showed higher IL-10 production but a comparable amount of TNFα (Figure. 4-5C). IL-10 is an immunosuppressive cytokine secreted by multiple cell types during Mtb infection<sup>268</sup>. It has been proposed that IL-10 impairs immune responses by inhibiting macrophage maturation, DC antigen presentation and trafficking, and Th1 cell polarization, migration, and function<sup>139, 144, 268</sup>. Monocytes from TB patients and the human mononuclear phagocytes infected with Mtb in vitro
have been reported to secrete IL-10\textsuperscript{140, 141, 142}. Since we have previously shown that Bhlhe40 negatively regulates IL-10 production by immune cells\textsuperscript{11}, we hypothesized that Bhlhe40 might repress IL-10 expression by myeloid cells to assure their ability to control infection.

To identify the cellular sources of IL-10 in the steady state and during \textit{Mtb} infection, we crossed \textit{Bhlhe40}\textsuperscript{−/−} mice to IL-10-Thy1.1 BAC transgenic (“10BiT”) reporter mouse\textsuperscript{296}. No or very low IL-10 expression was observed in naïve WT and \textit{Bhlhe40}\textsuperscript{−/−} animals, but its expression increased after \textit{Mtb} infection (Figure. 4-5D). Particularly, several immune cell subsets from infected \textit{Bhlhe40}\textsuperscript{−/−} lungs, including CD11b\textsuperscript{+} DCs, monocyte-derived cells, and CD4\textsuperscript{+} T cells, showed higher levels of IL-10 production in comparison to their WT counterparts (Figure. 4-5D). In human patients, phagocytes rather than lymphocytes seem to be the major IL-10 producers during TB infection\textsuperscript{297}. At least at early time points after infection, we felt that the myeloid cells rather than the T cells were the primary sources of the excess amounts of IL-10 observed in \textit{Mtb}-infected \textit{Bhlhe40}\textsuperscript{−/−} mice, since \textit{Rag1}\textsuperscript{−/−}\textit{Bhlhe40}\textsuperscript{−/−} total lung cells also secreted more IL-10 when compared to \textit{Rag1}\textsuperscript{−/−} cells after ex vivo heat-killed \textit{Mtb} stimulation (Figure. 4-5E).

\textbf{4.3.5 Bhlhe40 directly binds to the \textit{Il10} locus in bone marrow-derived myeloid cells}

In Chapter 2, we analyzed publicly-available ChIP-Seq data for Bhlhe40 performed in CH12 cells (a B cell lymphoma line) by the Mouse ENCODE consortium (Figure. 2-11)\textsuperscript{11}. Our analysis showed that Bhlhe40 might bind to the \textit{Il10} gene locus at cis regulator elements (Figure. 2-11B). To test if Bhlhe40 was a direct transcriptional repressor of \textit{Il10} in myeloid cells, we performed ChIP followed by quantitative PCR in WT and \textit{Bhlhe40}\textsuperscript{−/−} (served as negative control) GM-CSF-cultured bone marrow cells. We chose these cells because they
express Bhlhe40 and were easy to obtain in abundance. In addition, these cells can potentially sense \textit{Mtb} via TLR2 and respond to the stimulation by secreting IL-10\textsuperscript{123,126}. IL-10 concentrations were significantly higher in \textit{Bhlhe40}\textsuperscript{-/-} cultures than in WT cultures after heat-killed \textit{Mtb} stimulation of these cells, suggesting that the system was relevant for studying how Bhlhe40 regulates IL-10 secretion in myeloid cells (\textbf{Figure. 4-6A}).

WT and \textit{Bhlhe40}\textsuperscript{-/-} GM-CSF-cultured bone marrow cells were stimulated with heat-killed \textit{Mtb} and subjected to ChIP-qPCR. As expected, Bhlhe40 bound to its own promoter as previously described (\textbf{Figure. 4-6B})\textsuperscript{298}. We then designed nine pairs of primers that generated amplicons that span sites of Bhlhe40 binding found by ChIP-Seq in CH12 cells (\textbf{Figure. 2-11B}), and tested if any of these sites were bound directly by Bhlhe40 in myeloid cells (\textbf{Figure. 4-6C}). Among all the potential binding sites we examined, Bhlhe40 specifically bound to a novel regulatory region encompassing the E-box motif CACGTG at +6.1 kilobase (kb) downstream of the \textit{Il10} transcription start site (\textbf{Figure. 4-6C}). To identify Bhlhe40 binding sites across the genome by an unbiased method, we performed ChIP-Sequencing (ChIP-Seq) with heat-killed \textit{Mtb}-stimulated WT and \textit{Bhlhe40}\textsuperscript{-/-} GM-CSF-cultured bone marrow cells (\textbf{Figure. 4-6, D-G}). The Model-based Analysis of ChIP-Seq (MACS) peak-calling method identified \textasciitilde900 potential Bhlhe40-binding sites, with greater than 1/3 of the peaks located in promoter regions (\textbf{Figure. 4-6D}). Comprehensive motif analysis performed by MEME-ChIP found that the E-box motif CACGTG was the most common sequence within these sites (\textbf{Figure. 4-6E}), consistent with St-Pierre et al.'s report that CACGTG is the preferred binding motif for Bhlhe40\textsuperscript{21}. Our ChIP-Seq analysis identified two candidate Bhlhe40-binding sites within the \textit{Bhlhe40} promoter (\textbf{Figure. 4-6F}) and revealed the same +6.1kb site as the only Bhlhe40-binding site within the \textit{Il10} locus.
We concluded that Bhlhe40 directly binds to the \textit{Il10} locus and represses the cytokine's transcription in myeloid cells.

\textbf{4.3.6 IL-10 deficiency rescues Bhlhe40\textsuperscript{\textdagger} \textsuperscript{-/-} mice from early \textit{Mtb}-induced death}

To ask whether excessive IL-10 production renders \textit{Bhlhe40\textsuperscript{\textdagger} \textsuperscript{-/-}} mice highly susceptible to \textit{Mtb} infection, we generated \textit{Il10\textsuperscript{-/-} Bhlhe40\textsuperscript{-/-}} mice and tested their susceptibility. We found that genetically depleting \textit{Il10} was sufficient to protect \textit{Bhlhe40\textsuperscript{-/-}} mice from early fatal tuberculosis (\textbf{Figure. 4-6A}). In contrast to \textit{Bhlhe40\textsuperscript{-/-}} mice, \textit{Il10\textsuperscript{-/-} Bhlhe40\textsuperscript{-/-}} mice had lower mycobacterial burdens (\textbf{Figure. 4-6B}), and milder neutrophil infiltration (\textbf{Figure. 4-6C}) in the lungs at day 21 p.i., indicating that Bhlhe40 must repress \textit{Il10} production to allow protective immunity to \textit{Mtb}. Nevertheless, when compared to WT or \textit{Il10\textsuperscript{-/-}} mice, \textit{Il10\textsuperscript{-/-} Bhlhe40\textsuperscript{-/-}} mice still showed impaired survival (\textbf{Figure. 4-6A}) and slightly higher lung CFUs as well as neutrophil proportions (\textbf{Figure. 4-6, B and C}). These results suggested that while it is critical for Bhlhe40 to suppress the expression of IL-10 in innate immune cells to generate early protection, this transcription factor regulates other as yet unidentified targets to promote fully effective immune responses to \textit{Mtb}.
4.4 Discussion

In this chapter, we established that Bhlhe40 has an essential role in murine host resistance against *Mtb*. The absence of Bhlhe40 resulted in a lethal lung inflammation dominated by neutrophils, and all *Bhlhe40<sup>−/−</sup>* mice died by seven weeks after aerosol *Mtb* infection. We found that Bhlhe40 repressed IL-10 expression by innate immune cells, particularly CD11b<sup>+</sup> DCs and monocyte-derived cells. ChIP-qPCR and ChIP-Seq data revealed a putative Bhlhe40-binding site within the *Il10* locus in myeloid cells. Importantly, genetic deletion of *Il10* significantly decreased the bacterial loads and prolonged the survival of *Bhlhe40<sup>−/−</sup>* mice after *Mtb* infection, confirming that negative regulation of IL-10 is a critical function for Bhlhe40. These findings on Bhlhe40-dependent immune regulations might provide new insights for the development of vaccines and treatments for human TB.

In active TB patients, neutrophils are usually associated with disease pathogenesis and poor prognosis<sup>293</sup>. The neutrophil is the most abundant immune cell type and the predominant cell subset infected with *Mtb* in patients’ sputum and bronchoalveolar lavage (BAL) fluid<sup>299</sup>. In the blood of active TB patients, a neutrophil-driven interferon-inducible TB signature gene profile has been identified<sup>300, 301</sup>. Moreover, high percentages of blood neutrophils correlate with unfavorable outcomes in established TB patients<sup>302</sup>. Consistent with these observations in humans, several mouse studies also connect lung neutrophilia with high *Mtb* susceptibility<sup>112, 294, 295, 303</sup>. Massive and rapid neutrophil recruitment to the lung is a hallmark of severe pathology in many *Mtb*-susceptible mouse strains including *Card9<sup>−/−</sup>* mice, *Ifngr<sup>−/−</sup>* mice, and Lyz2-specific Atg5 conditional knockout mice. Lung-recruited neutrophils during infection are detrimental because specific depletion of these cells by administrating anti-Ly6G antibody (clone 1A8) is
able to prolong host survival\textsuperscript{112, 294, 295}. Here, we found that \textit{Bhlhe40}\textsuperscript{−/−} mice were extremely susceptible to virulent \textit{Mtb} infection with remarkable granulocytic accumulation in the lungs by three weeks of infection (\textbf{Figure. 4-2, A-C}). However, it was somewhat to our surprise that anti-Ly6G treatment neither prevented weight loss nor prolonged the survival of \textit{Mtb}-infected \textit{Bhlhe40}\textsuperscript{−/−} mice (\textbf{Figure. 4-2E and data not shown}). We felt that the antibody treatment was effective because the same protocol successfully reduced the lung and splenic neutrophil populations in uninfected \textit{Bhlhe40}\textsuperscript{−/−} mice, and our precise regimen of injections was able to rescue Atg5 conditional knockout mice by Kimmey et al.\textsuperscript{295}. The unresponsiveness to anti-Ly6G antibody of infected \textit{Bhlhe40}\textsuperscript{−/−} mice indicated that the observed neutrophil accumulation was a side effect downstream of \textit{Bhlhe40}-dependent immune defects in response to \textit{Mtb} but not the main cause of the early lethality of these animals. To further confirm that \textit{Bhlhe40} is not required by neutrophils to control \textit{Mtb} infection, we are currently breeding \textit{Bhlhe40}\textsuperscript{−/−} mice to \textit{Csf3r}\textsuperscript{−/−} mice, which lack G-CSF signaling and have decreased numbers of neutrophils\textsuperscript{304}. Our preliminary results show that G-CSF receptor deficiency minimally affects the survival of \textit{Mtb}-infected \textit{Bhlhe40}\textsuperscript{−/−} mice (\textbf{data not shown}), supporting the notion that \textit{Bhlhe40} is required by non-neutrophils for effective protection.

Although endogenous IL-10 has been shown to impair the immune response in mice infected with \textit{M. bovis} BCG and \textit{M. avium}\textsuperscript{305, 306, 307 152, 308}, the role of IL-10 in immune resistance against \textit{M. tuberculosis} is inconclusive. This controversy might be explained by differences in the genetic backgrounds of the mice used and the stages of disease examined in various experimental studies. In \textit{Mtb}-infected CBA/J mice, anti-IL-10R treatment decreased lung bacterial loads at day 150 p.i. and modestly improved their survival\textsuperscript{309}. Consistent with this, \textit{Il10}\textsuperscript{−/−} CBA/J mice were more capable of controlling infections as evidenced by significantly lower \textit{Mtb} CFUs in the
lungs and spleens\textsuperscript{154}. In BALB/c background mice, IL-10 deficiency enhanced protection to aerosol \textit{Mtb} infection\textsuperscript{153}. However, one early study showed that the absence of IL-10 in C57BL/6 mice did not affect susceptibility to \textit{Mtb} within the first 100 days post infection\textsuperscript{150}. C57BL/6 mice are more resistant to mycobacterial infection than other strains commonly used for TB studies\textsuperscript{108, 308, 309}. Jung et al. found no differences in bacterial burden in the lungs of WT and \textit{Il10}\textsuperscript{-/-} C57BL/6 mice in the first 100 days after aerosol \textit{Mtb} infection\textsuperscript{151}. A more recent study, however, argued that although \textit{Il10}\textsuperscript{-/-} C57BL/6J mice showed normal resistance in the first five months, they failed to control \textit{Mtb} growth at day 185 p.i. and died by day 200 p.i.\textsuperscript{155}. In addition, Roach et al. observed a transiently increased resistance to \textit{Mtb} infection in \textit{Il10}\textsuperscript{-/-} C57BL/6 mice examined at 4 wk p.i.\textsuperscript{152}, and Redford et al. reported mild but significant \textit{Mtb} CFU reductions in the lungs and spleens of \textit{Il10}\textsuperscript{-/-} mice of the same background at days 56 and 128 p.i.\textsuperscript{153}. An interesting study from Anne O'Garra's group showed that blocking IL-10 signaling during BCG vaccination enhanced subsequent immune responses to \textit{Mtb} infection in both CBA/J and C57BL/6 mice. Their results indicated that one of the mechanisms by which IL-10 impaired immune responses to \textit{Mtb} was to interrupt T cell priming\textsuperscript{310}. Collectively, work performed in C57BL/6 mice suggested a negative but moderate role of endogenous IL-10 during \textit{Mtb} infection.

To explore the effects of IL-10 overexpression on immune responses to mycobacterial infections, several groups have generated transgenic mice that overexpress IL-10 in T cells or antigen-presenting cells in FVB, BALB/c, or C57BL/6 mice. IL-10 overexpression by T cells and CD68\textsuperscript{+} macrophages in FVB mice resulted in higher \textit{M. bovis} BCG burdens in the lungs\textsuperscript{288, 289}. BALB/c mice with increased IL-10 expression from MHC-II\textsuperscript{+} cells failed to control \textit{M. avium} growth and died by week 20 p.i.\textsuperscript{291}. Turner et al. overexpressed IL-10 by T cells in C57BL/6
mice and found that these mice had higher bacterial loads four months post \textit{Mtb} infection\textsuperscript{292}. In the study from Schreiber et al., FVB transgenic mice with macrophage-specific overexpression of IL-10 had increased lung \textit{Mtb} burdens and died by day 126 p.i.\textsuperscript{290}. In summary, these studies showed that extra IL-10 production from immune cells diminished immune responses to mycobacteria.

Here, we discovered that suppression of IL-10 by \textit{Bhlhe40} is absolutely required for protective immune resistance against \textit{Mtb}. In the absence of \textit{Bhlhe40}, several immune cell subsets including T cells and myeloid cells overexpressed IL-10 during infection. In TB patients, macrophages rather than T cells have been shown to be the major source of IL-10\textsuperscript{297}. Although \textit{Bhlhe40} negatively regulated this cytokine in CD4\textsuperscript{+} T cells\textsuperscript{11}, we concluded that \textit{Bhlhe40} must also repress \textit{Il10} expression by innate immune cells to allow effective protection, because \textit{Rag1}\textsuperscript{-/-} \textit{Bhlhe40}\textsuperscript{-/-} mice and \textit{Bhlhe40}\textsuperscript{-/-} mice were equally susceptible to \textit{Mtb}, and both strains were more vulnerable to this pathogen than \textit{Bhlhe40}-sufficient \textit{Rag1}\textsuperscript{-/-} mice were (\textbf{Figure. 4-3, B and C}). In the lungs of \textit{Mtb}-infected \textit{Bhlhe40}\textsuperscript{-/-} mice, we found that CD11b\textsuperscript{+} DCs and monocyte-derived cells expressed increased levels of IL-10 (\textbf{Figure. 4-5D, bottom}). Notably, \textit{Bhlhe40}\textsuperscript{-/-} lung-resident myeloid cells and T cells did not hyper-produce IL-10 in the steady state (\textbf{Figure. 4-5D, top}). The excess amounts of IL-10 only appeared after \textit{Mtb} infection and greatly increased with heat-killed \textit{Mtb} stimulation ex vivo (\textbf{Figure. 4-5, C and E}). We speculate that \textit{Bhlhe40} tightly controls IL-10 expression in multiple myeloid cell subsets at certain times and anatomical locations in order to initiate and/or sustain protective immune responses to \textit{Mtb}. This broad expression and function of \textit{Bhlhe40} in the innate immune compartment might explain why \textit{Bhlhe40}\textsuperscript{-/-} mice were more susceptible to mycobacterial infections than the aforementioned IL-10 transgenic mice.
While IL-10 deficiency significantly improved the survival of and largely decreased *Mtb* loads in *Bhlhe40*<sup>−/−</sup> mice, we observed that *Il10*<sup>−/−</sup>*Bhlhe40*<sup>−/−</sup> mice were still more susceptible to *Mtb* infection when compared to WT or *Il10*<sup>−/−</sup> mice (Figure. 4-7). These results indicated that *Bhlhe40* likely regulates other targets which are particularly responsible for late-stage protection to *Mtb*. To fully understand the complete role of *Bhlhe40* in immune responses to mycobacteria, future experiments should utilize the ChIP-Seq data generated in myeloid cells and compare *Il10*<sup>−/−</sup>*Bhlhe40*<sup>−/−</sup> mice with *Il10*<sup>−/−</sup> mice to identify other genes controlled by this transcription factor.
4.5 Methods

Mice. C57BL/6 (Taconic), B6.SJL (CD45.1; Taconic), IL-10-Thy1.1 BAC transgenic "10BiT" (on a C57BL/6 background; provided by Dr. Casey Weaver, University of Alabama at Birmingham), Il10−/− (on a C57BL/6 background; The Jackson Laboratory), Rag1−/− (on a C57BL/6 background; provided by Dr. Megan Cooper, Washington University in St. Louis), Bhlhe40+/−, and Bhlhe40−/− (Sun et al., 2001; backcrossed 10 generations to the C57BL/6 background) mice were maintained in the Edelson Lab’s SPF (BS2) facility or the Stallings' Lab BS3 animal facility. IL-10-Thy1.1 BAC transgenic mice, Il10−/− mice, and Rag1−/− mice were crossed to Bhlhe40−/− mice for some experiments. Adult mice (7–20 weeks of age) of both sexes were used in the experiments.

To generate bone marrow chimeras, bone marrow cells were collected from femurs and tibias of donor mice, and 10–20 million cells were injected intravenously into irradiated recipient mice (1,000 rads). Mixed chimeras were generated by mixing donor bone marrow cells at a 1:1 ratio before injection. Mice were used in experiments 13–20 weeks following transplantation.

All animal experiments were approved by the Animal Studies Committee of Washington University in St. Louis.

M. tuberculosis infection of mice. The maintenance and infection of Mycobacterium tuberculosis (Erdman strain) was described previously. The mycobacteria was cultured at 37 °C in 7H9 (broth) or 7H10 (agar) (Difco) medium in the presence of 10% oleic acid/albunin/dextrose/catalase (OADC), 0.5% glycerol, and 0.05% Tween 80 (for broth only).
In brief, mice were placed in an Inhalation Exposure System (Glas-Col) and ~100 bacteria were delivered to each animal. The exact bacterial titers in the lungs were determined in at least one mouse at 24 h after infection. Lung and spleen bacterial burdens were determined by plating serial dilutions of homogenates onto 7H10 agar plates and were incubated at 37 °C in 5% CO₂ for 3-4 weeks before counting colonies. Beginning one week after infection, infected mice were monitored for weight loss and survival.

**Lung harvest.** Lungs were perfused with 10 ml PBS via injection into the right ventricle and were collected for multiple analyses. For histology experiments, left lungs were dissected, fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E).

To generate homogenates, the right lobe of lungs were homogenized in 1 ml (uninfected mice) or 5 ml (*Mtb*-infected mice) PBS + 0.05% Tween 80. Supernatants from homogenized tissue were filtered (0.22 μm) and analyzed by ELISA. In some experiments, total RNA was isolated (RNeasy Kit, Qiagen). cDNA was synthesized with SuperScript III reverse transcriptase using oligo-dT primers (Life Technologies) and quantitative real-time PCR was performed according to the manufacturer’s instructions with iTAQ SYBR Green (BioRad). Expression of genes was determined relative to β-actin. The following primers were used: *Il10* forward, 5′-AGCCTTATCGGAAATGATCCAGT -3′; reverse, 5′-GGCCTTGTAGACACACCTTGGT -3′; *Actb* forward, 5′-ACCTTCTACAATGAGCTGCG -3′; reverse, 5′-CTGGATGGCTACGTACATGG -3′.

To generate single-cell suspension, lungs were minced and digested with 625 μg/ml collagenase D (Roche) and 75 U/ml DNase I (Sigma) at 37°C for 40–60 min. Total lung cells were counted
and some cells were used for flow cytometry analysis. In some experiments, 2x10^5 lung cells were placed in wells of 96-well plates and treated with or without 100 µg/ml heat-killed *Mtb* (H37Ra strain, Difco) overnight. Supernatants were collected for TNFα or IL-10 ELISA assays.

**In vivo neutrophil depletion.** Anti–Ly6G antibody (200 µg/dose; clone 1A8; Bio X Cell or Leinco Technologies, Inc.) or control rat IgG (Sigma) were diluted in 200 µl of PBS and injected i.p. into mice on every other day from days 10 and 30 p.i. Mice were monitored for weight loss and survival. Efficacy of neutrophil depletion in lungs and spleens was determined by flow cytometry at day 21 p.i.

**Bone marrow cell cultures and stimulation.** Bone-marrow cells were isolated from femurs and tibias of mice and treated with ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) to lyse red blood cells. To generate GM-CSF-cultured bone marrow-derived dendritic cells and macrophages, 6x10^5 bone marrow cells were cultured in complete RPMI (10% heat-inactivated FBS+ L-glutamine+ Penicillin/Streptomycin+ β-mercaptoethanol) in 6-well plates with the addition of 20 ng/ml GM-CSF (PeproTech). To generate M-CSF bone marrow-derived macrophages, 4x10^6 bone marrow cells were cultured in complete IMDM (10% heat-inactivated FBS + L-glutamine + Penicillin/Streptomycin + β-mercaptoethanol+ nonessential amino acid + sodium pyruvate) in petri dishes with the addition of 20 ng/ml M-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, suspension cells were seeded at the concentration of 5x10^5 cells/well in 96-well plates
and were stimulated with or without 50 µg/ml heat-killed *Mtb* (H37Ra strain, Difco) for 18 h (FACS) or 24 (ELISA) hours. In some experiments, suspension cells were stimulated in the presence of 10 µg/ml heat-killed *Mtb* (H37Ra strain, Difco) for 4 hours for ChIP assays.

**Flow cytometry.** Lung single-cell suspension and bone marrow-derived cells were stained with Fc receptor antibody (clone 2.4G2; Bio X Cell) for 5 min at 4°C and surface staining for 20 min at 4°C in FACS buffer (0.5% BSA, 2 mM EDTA, and 0.02% sodium azide in PBS). For samples from *Mtb*-infected mice, stained cells were fixed before analysis on FACSCanto II (BD) or FACSaria II (BD) flow cytometers. Flow cytometry data were analyzed with FlowJo software (Tree Star). The following anti–mouse antibodies were obtained from BioLegend: APC-Cy7 (17A2) anti-CD3e, PE-Cy7 or APC-Cy7 anti-CD4 (RM4-5), APC-Cy7 or PerCP-Cy5.5 anti-CD8α (53-6.7), APC or PE-Cy7 anti-CD11b (M1/70), APC-Cy7 or BV605 anti-CD11c (N418), APC-Cy7 or BV605 anti CD19 (6D5), PB anti-CD44 (IM7), APC anti-CD45.2 (104), PE anti-CD62L (MEL-14), FITC anti-CD90.1 (Thy-1.1) (OX-7), PerCP-Cy5.5 anti-CD103 (2E7), Alexa Fluor 488 anti-B220 (RA3-6B2), PB, PerCP-Cy5.5, or APC-Cy7 anti-Ly6C (HK1.4), PE anti-Ly6G (1A8), PB anti–I-A/I-E (M5/114.15.2), PB anti-TCRβ (H57-597), APC anti-TCR γδ (GL3). The following anti–mouse antibodies were purchased from BD Biosciences: V450 or BV510 anti-CD4 (RM4-5), BV510 anti-CD45 (30F11), and PE anti–Siglec F (E50-2440). The following anti–mouse antibodies were obtained from Tonbo Biosciences: PerCP-Cy5.5 anti-CD8α (53-6.7), 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). FITC anti-mouse Ly6B (7/4) was purchased from Bio-Rad. Fixable Viability Dye eFluor780 was obtained from eBioscience.
**Chromatin immunoprecipitation (ChIP).** Anti-Bhlhe40 ChIP was modified from the protocol published by Chou et al.\textsuperscript{311}. Bone marrow cells from WT and Bhlhe40\textsuperscript{-/-} mice were collected and cultured with GM-CSF at 20 ng/ml. Suspension cells were harvested after culturing with GM-CSF for 8-9 days and stimulated with 10 \( \mu \)g/ml heat-killed \textit{Mtb} (H37Ra strain, Difco) for 4 hours. The stimulated cells were fixed for 10 minutes at room temperature with 1% PFA with shaking. Cross-linked chromatin was fragmented by sonication and then immunoprecipitated with a commercially available polyclonal rabbit anti-Bhlhe40 antibody (Novus NB100-1800). After immunoprecipitation, DNA was purified by the GenElute PCR clean-up kit (Sigma). For ChIP-qPCR, we used the SYBR green method to determine the abundance of different candidate regions of the \textit{Il10} locus in the IP. The following primers were used for real-time PCR analysis: \textit{Il10} -25.7k, CCAGGCTATGAGTCAGATGACG and CTTTGGCCAGAGGCTGT; \textit{Il10} -20.1k, CCCTCCAGGTCTCGTCTCAAG and CTTTTGATTCATGCCTACC\textsuperscript{312}; \textit{Il10} -9k, CTTGAGGAAAAGCCAGCATCA and TTTGCGTGTTCACCTGTGT (modified from Lee et al.)\textsuperscript{204}; \textit{Il10} promoter (pro), GCCCATTTATCCACGTCACTATG and TGGTCTATGTACAGGCCCCCTCAC\textsuperscript{312}; \textit{Il10} +1.8k, GGTCCTCCAGCTCATCTGCTCTCG and AGGCTATGCGAAATCTTCTCC\textsuperscript{312}; \textit{Il10} +6.1k, GGATAAGGGGAATAATAGAGCT and CCTCCGATGGTTACTTTAACT (modified from Chang et al.)\textsuperscript{204}; \textit{Il10} +6.3k, GCAGAGCTGGGATGGCTCA and TCTCACTGGTGCCCGCA\textsuperscript{312}; \textit{Il10} +18.4k, AGGTTCAGGAGGGCATGGA and TCACCAGTGTTGGTAACAGC\textsuperscript{312}; \textit{Il10} +18.8k, ACTTGATTTTACCTTCCCGAGA and CTTTGCAAGGCGACGTGAG; \textit{Bhlhe40} promoter (pro) CAGCTGGGCAGGACTCTG and GGCTGGTTGAAGACTACGTG.
For ChIP-Seq, purified DNA was used for library construction, followed by 50–base pair single-read sequencing on a HiSeq3000 system (Illumina) at the Genome Technology Access Center (GTAC) at Washington University in St. Louis. Sequences were mapped to the NCBI37/mm10 mouse genome assembly by GTAC and peak calling was performed using MACS software within Galaxy (http://usegalaxy.org/). Tracks were viewed in the UCSC Genome Browser. Discriminative DNA Motif Discovery (DREME) software was used as part of the online version of MEME-ChIP (http://meme.nbcr.net/meme/cgi-bin/meme-chip.cgi) to validate that Bhlhe40 ChIP-Seq data identified the expected consensus motif for Bhlhe40 (CACGTG).

**ELISA.** TNFα or IL-10 ELISA assays were performed on Nunc Maxisorp plates using TNFα capture and detection antibodies (BioLegend) and IL-10 ELISPOT antibody pairs (BD Bioscience). The enzyme reaction was developed with streptavidin-HRP (BioLegend) and TMB substrate (BioLegend). Purified TNFα or IL-10 proteins were used to generate standard curves for these ELISAs.

**Immunoblotting.** Bone marrow-derived cells were counted and lysed at $10^6/40$ µl in Laemmlli 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-PVDF membranes (Costar). Blots were incubated with anti-Bhlhe40 (Novus Biologicals; used at 1:1,000) or anti-HDAC1 (Abcam; used at 1:2,000) primary antibodies at 4°C overnight with shaking. Blots were washed 4-5 times before incubation with anti–rabbit IgG-HRP (clone 5A6-
1D10 [light chain specific]; Jackson ImmunoResearch Laboratories) at room temperature for 60 min with shaking. After five washes, Clarity Western ECL substrate (Bio-Rad Laboratories) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) was applied, and blots were placed on Blue basic autoradiography film (GeneMate). Film was developed with a Medical Film Processor (model SRX-101A; Konica Minolta), and scanned films were analyzed with ImageJ software (NIH).

**Data and statistical analysis.**

All experiments were performed at least twice independent. Each sample represents biological replicates of mice randomly sorted into each experimental group. In some experiments, mice were excluded only when morbidity and mortality unrelated to *Mtb* infection was observed. Determination of statistical differences was performed with Prism 5 or 7 (Graphpad Software, Inc.) using unpaired two-tailed Student’s t-tests, one-way ANOVA with Tukey’s multiple comparisons tests. In some figures, central values and error bars represent the mean ± SEM.
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4.7 Author contributions

Chih-Chung Lin and Jeremy P. Huynh planned and performed experiments. Jacqueline M. Kimmey, Nicholas N. Jarjour, Elizabeth A. Schwarzkopf, and Tara R. Bradstreet performed experiments. Chun Chou helped with ChIP experiments. Maxim N. Artyomov helped analyzing ChIP-Seq data. Reshma Taneja provided Bhlhe40−/− mice, and Casey T. Weaver provided IL-10 reporter 10BiT mice. Brian T. Edelson and Christina L. Stallings supervised the study.
Figure 4-1. Bhlhe40-deficient mice succumb to *Mtb* infection.

(A) The survival of *Bhlhe40*^+/+*, *Bhlhe40*^+/−*, and *Bhlhe40*^−/−* mice infected with 100-200 CFU aerosolized *Mtb*. The number of biological replicates is indicated in parentheses. (B) Gross pathology of the left lungs of *Bhlhe40*^+/+*, *Bhlhe40*^+/−*, and *Bhlhe40*^−/−* mice at day 33 p.i. (C) Lung and (D) splenic *Mtb* CFU of *Bhlhe40*^+/+* and *Bhlhe40*^−/−* mice at week 2, 3, and 4 after aerosol *Mtb* infection. Data are mean ± SEM. Statistical differences were by two-tailed unpaired Student’s t-test. *p<0.05, **p<0.01.
Figure 4-2. Anti-Ly6G treatment fails to rescue Bhlhe40−/− mice from Mtb-induced lethality.
Figure 4-2. Anti-Ly6G treatment fails to rescue Bhlhe40\(^{+/+}\) mice from Mtb-induced lethality.

*Bhlhe40\(^{+/+}\) and Bhlhe40\(^{+/−}\) mice were infected with 100-200 CFU of aerosolized *Mtb*. (A) Lungs from naïve and infected mice (day 21 p.i.) were sectioned and stained by hematoxylin and eosin (H&E). (B) Representative FACS plots of lung cells gated on CD45\(^{+}\) cells. The frequency of Ly6C\(^{+}\)Ly6G\(^{+}\) neutrophils is shown prior to infection or at days 14, 22, and 29 p.i. (C) Total numbers of neutrophils in the left lungs of naïve or *Mtb*-infected Bhlhe40\(^{+/+}\) and Bhlhe40\(^{+/−}\) mice. Statistical differences were determined by two-tailed unpaired Student’s t-test. *p<0.05, ***p<0.001. (D) Representative FACS plots of lung cells (gated on non-B, non-T CD11b\(^{+}\) cells) from naïve Bhlhe40\(^{+/+}\) or Bhlhe40\(^{+/−}\) mice treated with control rat IgG or monoclonal anti-Ly6G (clone 1A8) antibody every other day for 5 injections. Lung cells were stained for neutrophil markers to assess depletion efficacy. (E) Bhlhe40\(^{+/+}\) (black symbols) and Bhlhe40\(^{+/−}\) (red symbols) mice were infected with 100-200 CFU aerosolized *Mtb* and treated every other day from day 10 to 30 p.i. with rat IgG (filled symbols) or anti-Ly6G (open symbols) antibody from days 10-30 p.i. Mice were monitored for changes in body weight. (F) Representative FACS plots of lung cells (gated on non-B, non-T CD11b\(^{+}\) cells) from *Mtb*-infected (day 21 p.i.) Bhlhe40\(^{+/+}\) or Bhlhe40\(^{+/−}\) mice treated with control rat IgG or anti-Ly6G antibody every other day starting at day 10 p.i.
Figure 4-3. Innate immune cells require Bhlhe40 to control Mtb infection.

(A, B) The survival of (A) the indicated bone marrow chimeric mice, or (B) Bhlhe40+/+, Bhlhe40−/−, Rag1−/−, or Rag1−/−Bhlhe40−/− mice infected with 100-200 CFU of aerosolized Mtb. (C) Right lung Mtb CFU of the mice in (B) at day 21 p.i. Statistical differences were determined by unpaired Kruskal-Wallis test with Dunn’s multiple comparison test. **p<0.01, ***p<0.001.
Figure 4-4. The presence of Bhlhe40<sup>−/−</sup> cell diminishes the ability of WT neutrophils to control Mtb infection.
Figure 4-4. The presence of Bhlhe40<sup>−/−</sup> cell diminishes the ability of WT neutrophils to control Mtb infection.

Congenically-marked WT irradiated recipient mice were reconstituted with CD45.1<sup>+</sup> Bhlhe40<sup>+/+</sup> and CD45.2<sup>+</sup> Bhlhe40<sup>+/+</sup> or with CD45.1<sup>+</sup> Bhlhe40<sup>+/+</sup> and CD45.2<sup>+</sup> Bhlhe40<sup>−/−</sup> bone marrow cells at a 1:1 ratio. After bone marrow transplantation, these mixed bone marrow chimeric mice and non-chimeric Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> mice were infected with GFP-labeled Mtb. (A) Representative FACS plots gated on lung CD45<sup>+</sup> cells. The frequency of Ly6C<sup>+</sup>Ly6G<sup>+</sup> neutrophils mixed bone marrow chimeric mice at day 21 p.i. is shown. (B) The ratios of grafted CD45.1<sup>+</sup> Bhlhe40<sup>+/+</sup>/CD45.2<sup>+</sup> Bhlhe40<sup>+/+</sup> (black) and CD45.1<sup>+</sup> Bhlhe40<sup>+/+</sup>/CD45.2<sup>+</sup> Bhlhe40<sup>−/−</sup> (red) neutrophils from (A). Data are mean ± SEM. These two groups are not statistically different (n.s.) determined by two-tailed unpaired Student’s t-test. (C) Representative FACS plots gating on neutrophils from non-chimeric and mixed bone marrow chimeric mice at day 21 post Mtb infection. The frequencies of Mtb-infected (GFP-positive) neutrophils in lungs are shown.
Figure 4-5. *Bhlhe40*−/− myeloid cells express higher levels of IL-10 in vivo.
Figure 4-5. *Bhlhe40*<sup>−/−</sup> myeloid cells express higher levels of IL-10 in vivo.

(A-C) *Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> mice were infected with *Mtb* and lungs were harvested at day 21 p.i. to analyze IL-10 expression. Total lung homogenates were used to detect (A) *Il10* RNA by qRT-PCR and (B) IL-10 protein by ELISA. (C) 2 x 10<sup>5</sup> lung cells were stimulated without (No stimulation) or with (*Mtb*) 100 µg/ml heat-killed *Mtb* overnight. Supernatants were collected for IL-10 (left) and TNFα (right) ELISA. (D) Representative FACS plots of lung immune cell populations from naïve (top) or *Mtb*-infected (day 21 p.i., bottom) *Bhlhe40<sup>+/+</sup>* or *Bhlhe40<sup>−/−</sup>* Thy1.1-IL-10 reporter (10BiT Tg<sup>+</sup>) mice. Thy1.1 (IL-10) expression from five indicated immune cell types are shown. (E) *Bhlhe40<sup>+/+</sup>, Bhlhe40<sup>−/−</sup>, Rag1<sup>−/−</sup>, or Rag1<sup>−/−</sup>Bhlhe40<sup>−/−</sup> mice were infected with *Mtb* for 21 days. 2 x 10<sup>5</sup> lung cells were stimulated without (No stimulation) or with (*Mtb*) 100 µg/ml heat-killed *Mtb* overnight. Supernatants were collected for IL-10 ELISA. Data are mean ± SEM. Statistical differences were determined two-tailed unpaired Student’s t-test. *p<0.05, **p<0.01, ***p<0.001.
Figure 4-6. Bhlhe40 directly binds to the \textit{Il10} locus in bone marrow-derived myeloid cells.
Figure 4-6. Bhlhe40 directly binds to the *Il10* locus in bone marrow-derived myeloid cells. (A) *Bhlhe40*/*+* and *Bhlhe40*/*−/* GM-CSF-cultured bone marrow cells were stimulated with heat-killed *Mtb* for 24 hours. Supernatants were analyzed for IL-10 (left) and TNFα (right) by ELISA. Data are mean ± SEM. Statistical differences were determined two-tailed unpaired Student’s t-test. *p<0.05, ***p<0.001. (B-G) *Bhlhe40*/*+*/ and *Bhlhe40*/*−/* GM-CSF-cultured bone marrow cells were stimulated with heat-killed *Mtb* for 4 hours and subjected to ChIP analysis. ChIP-qPCR analysis of Bhlhe40 binding to (B) the *Bhlhe40* promoter or to (C) multiple sites indicated within the *Il10* locus. (D-F) ChIP-seq analysis for Bhlhe40. (D) Pie chart showing genomic locations of Bhlhe40 binding sites. (E) Consensus Bhlhe40 binding motif identified by Discriminative DNA Motif Discovery (DREME)\textsuperscript{218}, used as part of the online version of MEME-ChIP. (F-G) ChIP-Seq analysis identifies the Bhlhe40 binding sites within (F) the promoter region of *Bhlhe40*, and (G) at the +6.1kb site within the *Il10* locus. The degree of vertebrate conservation is shown in the blue tracks.
Figure 4-7. IL-10 deficiency rescues Bhlhe40−/− mice from early Mtb-induced death.

(A) The survival of Bhlh40+/+, Bhlh40−/−, Il10−/−, and Il10−/−Bhlhe40−/− mice after infection with 100-200 CFU aerosolized Mtb. (B) Right lung Mtb CFU and (C) the frequency of lung-infiltrating neutrophils among total CD45+ cells of the mice in (A) were analyzed at day 21 p.i. Statistical differences were determined by one-way unpaired ANOVA with Tukey’s post-test.

**p<0.01, ****p<0.0001.
Chapter 5: Conclusions and Discussion
In this study, we have used an autoimmune (Chapter 2 and 3) and an infectious disease model (Chapter 4) to demonstrate that Bhlhe40 is a critical pro-inflammatory transcription factor. The presence of Bhlhe40 renders mice susceptible to T cell-mediated autoimmune neuroinflammation, but protects mice from Mtb infection. Bhlhe40 is required for normal cytokine production in a T cell-intrinsic manner, where it positively regulates the production of GM-CSF and negatively regulates the production of IL-10. PTX, serving as a co-adjuvant for EAE induction, induces IL-1β secretion by myeloid cells which enhance Bhlhe40 expression by CD4+ T cells. We define a PTX - IL-1β - Bhlhe40 axis that is essential for T cell pathogenicity in EAE. In contrast, Bhlhe40 assures protective innate and adaptive immune responses against Mtb by direct repression of Il10 in several immune cell types during infection. Bhlhe40−/− mice fail to control Mtb replication and die by seven weeks after exposure to Mtb, whereas IL-10 deficiency largely rescues these mice from mycobacteria-induced early death. Overall, by analyzing the immune response in the absence of Bhlhe40 in two different disease models, we showed that Bhlhe40 is harmful in terms of autoimmunity, but is indispensable for a protective immune response to mycobacterial infection.
5.1 The requirements for TH cell transcription factors during EAE induction

It has been debated which autoreactive TH cell subset, TH1 or TH17 cells, mediates neuroinflammation in mice and humans. Several studies have demonstrated that neither IFN-γ nor IL-17 is absolutely required for EAE development in C57BL/6 mice\textsuperscript{167, 168, 169}. Recent reports have generally agreed that self-reactive cells with features of both TH1 and TH17 cells may represent the most pathogenic subset (termed TH1/TH17 cells)\textsuperscript{266, 314, 315}. These cells likely mediate their pathogenicity through the production of GM-CSF\textsuperscript{174, 175}. Notably, utilizing an IL-17 fate mapping reporter system, Hirota et al. identified an “ex-TH17” population in the DLN and CNS during EAE that was positive for IFN-γ, but that had previously produced IL-17\textsuperscript{316}. These ex-TH17 cells produce GM-CSF, and seem to represent the pathogenic subset of TH cells.

A large body of research has been devoted to identifying the transcription factors that drive T cell pathogenicity during EAE. Early reports showed that T-bet was essential for EAE induction\textsuperscript{317, 318}. Using myelin-specific B10.PL transgenic mice, the group of Amy Lovett-Racke claimed that T-bet regulates the pathogenicity of both TH1 and TH17 cells\textsuperscript{319, 320}. When in vitro-polarized WT or Tbx21\textsuperscript{-/-} TH17 2D2 cells were adoptively transferred to C57BL/6 recipients, Wang et al. found that T-bet-deficient cells failed to induce disease by two weeks\textsuperscript{321}. However, the importance of T-bet for T-cell encephalitogenicity has been challenged by more-recent papers. Duhen et al. showed that selective deletion of T-bet in T cells did not prevent the generation and infiltration of IL-17\textsuperscript{+}IFN-γ\textsuperscript{+} or GM-CSF\textsuperscript{+} T cells into the CNS and did not protect mice from EAE induction\textsuperscript{262}. Two other studies simultaneously reported that Tbx21\textsuperscript{-/-} mice are nearly as susceptible as WT mice to active EAE induction\textsuperscript{322, 323}. O’Connor et al. found that IL-12-
polarized (T_H1 condition) myelin-reactive T cells from previously-immunized Tbx21^/-/ mice cannot initiate disease in recipient mice, while IL-23-treated (T_H17 condition) cells are still pathogenic even when T-bet is absent. In agreement with this finding, Grifka-Walk et al. showed that DLN cells from EAE-induced Tbx21^/-/ mice cultured under T_H17 polarization conditions (IL-23, IL-1β, anti-IFN-γ and anti-IL-4) are sufficient to cause neuroinflammation upon transfer to WT or Rag1^/-/ mice, despite a delayed onset and milder symptoms. Collectively, these results indicate that T-bet may not be universally-required for all T_H subsets to induce EAE.

The orphan nuclear receptors RORγt and RORα are two primary transcription factors regulating T_H17 differentiation. T cells deficient in either of these two proteins exhibit a significant reduction of IL-17 production both in vitro and in vivo. One paper proposed that RORγt might regulate T_H cell pathogenicity by directly binding to the Csf2 locus and promoting GM-CSF expression, whereas another report did not find changes in GM-CSF production by RORγt-deficient T_H cells. Rorc^/-/ mice were initially thought to be resistant to EAE, but later Yang et al. discovered that only mice lacking both Rorc and Rora are truly protected from this disease. An important observation regarding the requirements for T-bet and RORγt for T-cell pathogenicity was published by Brucklacher-Waldert et al, who genetically deleted Tbx21 and Rorc in IL-17-producing cells. They bred Il17-Cre mice to Tbx21^fl/fl or Rorc^fl/fl mice and found that the deficiencies of T-bet or RORγt specifically in IL-17-producing cells did not prevent EAE development. These authors concluded that T-bet and RORγt are largely dispensable for T_H17-associated autoimmunity.
5.2 The role of T cell-expressed Bhlhe40 in EAE induction

The transcription factor Bhlhe40 is not expressed by naïve T cells but is upregulated upon TCR ligation and CD28 costimulation\(^\text{32,33}\), and we found that myeloid cell-derived IL-1β can enhance its expression by CD4\(^+\) T cells\(^\text{12}\). Bhlhe40 can be found in both T\(_{\text{H1}}\) and T\(_{\text{H17}}\) cell subsets polarized in vitro, and loss of Bhlhe40 only has a limited impact on their hallmark cytokine production (i.e. IFN-γ and IL-17A, respectively), showing that Bhlhe40 is dispensable for T\(_{\text{H}}\) cell differentiation\(^\text{11}\). However, Bhlhe40 is absolutely required for T\(_{\text{H}}\) cell pathogenicity in the EAE model. Rag1\(^-/-\) mice reconstituted with Bhlhe40\(^-/-\) but not WT CD4\(^+\) T cells are completely resistant to active EAE induction (Figure. 2-1F)\(^\text{11,33}\). Moreover, we have shown that myelin-reactive, IFN-γ or IL-17A-producing T\(_{\text{H1}}\) or T\(_{\text{H17}}\) 2D2 cells polarized in vitro required Bhlhe40 to mediate passive EAE in WT recipients (Figure. 3-6, A-C)\(^\text{12}\). 2D2 Bhlhe40\(^-/-\) T\(_{\text{H}}\) cells could be recovered in the recipient mice more than a month after transfer. These cells persisted in the spleen and even in the CNS, maintaining expression of IFN-γ or IL-17A, but were unable to initiate EAE (Figure. 3-6, D-G)\(^\text{12}\).

To further examine if Bhlhe40 is essential specifically for the pathogenicity of IL-17-producing cells, we have generated Il17-Cre x Bhlhe40\(^{\text{fl/fl}}\) mice and actively induced EAE in these animals (Figure. 5-1A). These conditional knockout mice displayed a delayed onset and less severe clinical EAE symptoms compared to Bhlhe40\(^{\text{0/0}}\) mice. Interestingly, Il17-Cre x Bhlhe40\(^{\text{0/0}}\) mice appeared to almost completely recover from clinical disease by 25 days post-EAE induction. I interpret this result to mean that Bhlhe40 is required by T\(_{\text{H17}}\), T\(_{\text{H1/TH17}}\), or ex-T\(_{\text{H17}}\) cells to sustain neuroinflammation. Intrigued by the finding that Bhlhe40 is critical for T\(_{\text{H17}}\)-mediated immunopathology, we generated IL-17-tdTomato fate mapping Bhlhe40\(^{\text{GFP}}\) double reporter mice.
(Figure. 5-1, B-D). Our preliminary data showed that tdTomato⁺Bhlhe40-expressing T cells were not present in the DLN from naïve mice but appeared after immunization (Figure. 5-1B). ICS analysis further revealed that Bhlhe40 was expressed at the highest levels in tdTomato⁺IFN-γ⁺IL-17A⁻ “ex-TH17” cells and tdTomato⁺IFN-γ⁺IL-17A⁺ TTH1/TTH17 cells (Figure. 5-1, C and D). Overall, our results imply that Bhlhe40 expression occurs within the pathogenic TTH cells, and serves a more important role in the context of EAE than the traditional TTH1 and TTH17 master transcription factors.
5.3 Cell type-specific requirements for Bhlhe40 in EAE

We and others have provided strong evidence that Bhlhe40 is absolutely required for Th cell pathogenicity in both active and passive EAE models\textsuperscript{11, 12, 33}. Nevertheless, Bhlhe40 is also expressed by several other immune cell types including DCs, macrophages, Ly6\textsuperscript{Clow} monocytes, and perhaps neutrophils. Does the expression of Bhlhe40 in these immune cell subsets also contribute to neuroinflammation? To examine the cell-intrinsic requirements for Bhlhe40 in EAE development, we generated Bhlhe40 conditional knockout mice by crossing several cell type-specific Cre mice to Bhlhe40\textsuperscript{fl/fl} mice and induced EAE in the offspring. Deletion of Bhlhe40 in T cells (Cd4-Cre x Bhlhe40\textsuperscript{fl/fl}) largely decreased the incidence and severity of EAE (Figure. 5-2A), confirming the finding that Bhlhe40 is required for Th cell pathogenicity\textsuperscript{11, 12, 33}. We also tested the effect of depleting Bhlhe40 in neutrophils (MRP8-Cre x Bhlhe40\textsuperscript{fl/fl}) during EAE and found equal susceptibility between control mice and Bhlhe40 conditional knockout mice (Figure. 5-2B). Currently, we are breeding Bhlhe40\textsuperscript{fl/fl} mice to Itgax-Cre (CD11c-Cre) and Ly2-Cre (LysM-Cre) mice. In the near future, we will be able to test the roles of Bhlhe40 in DCs and monocytes/macrophages during EAE.
5.4 Cell type-specific requirements for Bhlhe40 in the mouse model of TB

Although we have shown that Bhlhe40−/− mice and Rag1−/−Bhlhe40−/− mice fail to control Mtb replication and rapidly succumb in an IL-10-dependent manner, we have not yet fully determined which cell type(s) requires Bhlhe40 to control Mtb infection. Using our Bhlhe40GFP mice, we found that several immune cell types in the lung express Bhlhe40 at different levels (Figure. 5-3, A and B). Alveolar macrophages and CD11b+ and CD103+ DCs showed high-to-medium levels of Bhlhe40 expression, while moDCs/Mac and neutrophils appeared to express Bhlhe40 at lower levels (Figure. 5-3A). The expression of Bhlhe40 by these myeloid cells seemed to be only mildly affected by Mtb infection (data not shown). In contrast, all three lung T cell subsets examined, in particular CD4+ T cells, increased their Bhlhe40 expression three weeks after Mtb infection (Figure. 5-3B). We speculated that at least some of these activated, Bhlhe40-expressing T_H cells were Mtb-specific. We used 10BiT reporter mice to explore IL-10 expression by lung immune cells before and after Mtb infection (Figure. 5-3 and Figure. 4-5D). We found that after Mtb infection, some Bhlhe40-expressing cell types including CD11b+ DCs, moDCs, and T cells expressed higher IL-10 levels when Bhlhe40 was absent (Figure 4-5D, bottom), implying that Bhlhe40 might repress Il10 in these cell types. However, even though alveolar macrophages express the highest level of Bhlhe40 in WT mice, we did not detect any IL-10 reporter signals from this phagocyte population during Mtb infection (Figure. 4-5D and Figure. 5-3A). In contrast, we did observe an increased amount of IL-10 from Bhlhe40−/− Ly6C<sup>hi</sup> monocytes, a population which does not express Bhlhe40 in naïve or Mtb-infected WT animals (Figure. 5-3A and data not shown). These results suggest that the function of Bhlhe40 might
be cell-type dependent, and the increased expression of IL-10 in \(Bhlhe40^{--}\) cell subsets during \(Mtb\) infection might not be entirely cell-intrinsic.

To identify the cell type(s) that requires \(Bhlhe40\) to control \(Mtb\) infection, we infected the aforementioned \(Bhlhe40\) conditional knockout mice (See Chapter 5.2) with \(Mtb\). Our preliminary results have shown that \(Bhlhe40\) deficiency in only neutrophils (\(MRP8\)-Cre x \(Bhlhe40^{fl/fl}\) mice) or monocytes/macrophages (\(Lyz2\)-Cre x \(Bhlhe40^{fl/fl}\) mice) does not impair the protective immune response to this pathogen. Both strains of mice survived significantly longer than global \(Bhlhe40\)-deficient mice (Figure. 5-3C and data not shown). Interestingly, deletion of \(Bhlhe40\) in CD11c\(^+\) cells (\(Itgax\)-Cre x \(Bhlhe40^{fl/fl}\)) rendered mice \(Mtb\)-susceptible (in a single pilot experiment at the time of this dissertation) (Figure. 5-3C). These \(Bhlhe40\) conditional knockout mice started losing weight at ~25 days post \(Mtb\) infection and died at about day 60 p.i., indicating that \(Bhlhe40\) was required by classical DCs, CD11c\(^+\) moDCs/Macs, or other CD11c\(^+\) subsets to control infection. Moreover, deletion of \(Bhlhe40\) in T cells (\(Cd4\)-Cre x \(Bhlhe40^{fl/fl}\)) also impaired host defense and immunity to \(Mtb\). Mice lacking \(Bhlhe40\) in T cells failed to resist \(Mtb\) infection and died in the same time frame as \(Itgax\)-Cre x \(Bhlhe40^{fl/fl}\) mice (Figure. 5-3C). These data suggest that \(Bhlhe40\) is essential for both CD11c\(^+\) cells and T cells to control \(Mtb\) infection. Notably, in our previous data showing a role for \(Bhlhe40\) in \(Mtb\) resistance in the absence of T and B cells (i.e. in Rag1-deficient mice, Figure. 4-3B), \(Rag1^{-/-} \text{Bhlhe40}^{-/-}\) succumbed to \(Mtb\) infection at approximately day 30-40 p.i. (the same time as \(Bhlhe40^{-/-}\) mice).

In the context of our experiments in cell type-specific conditional knockout mice, overall our data would suggest that \(Bhlhe40\)-deficiency in CD11c\(^+\) cells has a more profound impact on susceptibility when this cell type-specific deficiency is placed in the setting of absent adaptive immunity.
5.5 The relevance of the murine findings to human disease

One important question is whether the function of Bhlhe40 is relevant to human disease. 

*Bhlhe40* is an evolutionarily-conserved gene which can be found in fish, amphibians, reptiles, birds, and mammals. The human and mouse Bhlhe40 gene and protein show 87.9% and 90.5% identity, respectively (NCBI HomoloGene: [https://www.ncbi.nlm.nih.gov/homologene](https://www.ncbi.nlm.nih.gov/homologene)). The Human Protein Atlas demonstrates that BHLHE40 protein is expressed by many human tissues at various levels ([http://www.proteinatlas.org/ENSG00000134107-BHLHE40/tissue](http://www.proteinatlas.org/ENSG00000134107-BHLHE40/tissue)), and *BHLHE40* transcript can be detected in blood T_{H} cell subsets from healthy donors ([Figure. 5-4A](#)). Our preliminary immunoblotting data showed that cultured T_{H}17 cells from a health donor expressed BHLHE40 protein, and its level was enhanced with the addition of IL-1β and IL-12 to cultures ([Figure. 5-4B](#)). It will be worth examining if BHLHE40 expression by human T cells is critical for MS pathogenesis as observed in the mouse model. In chapter 3, we showed that IL-1R signaling strongly impacts on Bhlhe40 expression levels in murine T_{H}17 cells ([Figure. 3-8, 3-9, and 3-10](#)). IL-1 has been shown to induce Bhlhe40 in primary human gingival epithelial cells through a PI-3K–Akt pathway, and in primary human amnion mesenchymal cells. It will be interesting to study if IL-1β promotes T cell encephalitogenicity during MS.

In recent years, several studies have compared the whole blood transcriptomes of active TB patients to those from healthy individuals or latent TB patients. Examination of the blood transcriptional signature could provide information on the immune response occurring during active TB disease. Interestingly, our own analysis of publicly-available datasets generated from studies performed with human samples from the Gambia, South Africa, or the UK
revealed that *BHLHE40* expression was significantly lower in active TB patients as compared to healthy controls or latent TB patients (Figure 5-4C, left; GSE1949132 and data not shown), while the expression of *STAT1*, a signature gene of the T\(_{H1}\) response, was significantly higher in the blood of active TB patients (Figure 5-4C, right). Lower *BHLHE40* expression during active TB disease correlates with our findings that Bhlhe40-deficient mice are more susceptible to *Mtb* infection (Chapter 4). Future experiments would be required to examine the role of BHLHE40 in human anti-TB immunity.

A transcription factor could be targeted by direct inhibition of its expression, by disruption of its binding to DNA or other partner proteins, or by manipulation of chromosome accessibility\(^{330}\). A recent success story came from the group of Ari Melnick, who designed small-molecule inhibitors targeting the oncogene *BCL6* and found that these inhibitors could suppress mouse and human B cell lymphomas\(^{331}\). In addition, the expression and action of a transcription factor could be affected by its upstream and downstream signaling pathways. For instance, blocking IL-1\(\beta\) production might reduce BHLHE40 expression by human T cells, and blockade of IL-10 might overcome the adverse effects (if any) resulting from low BHLHE40 expression. Our study provides impetus and new insights for exploring the role of BHLHE40, or pathways that regulate its expression and function, as candidate therapeutic targets in human autoimmunity and infectious diseases.
Figure 5-1. Bhlhe40 is critical for IL-17-associated pathogenicity in EAE.
Figure 5-1. Bhlhe40 is critical for T\textsubscript{H}-17-associated pathogenicity in EAE.

(A) Mean clinical scores of EAE in immunized Bhlhe40\textsuperscript{fl/fl} (n=10) or Il17-Cre x Bhlhe40\textsuperscript{fl/fl} mice (n=7). Data are combined from three independent experiments. (B-D) IL-17-tdTomato fate mapping Bhlhe40\textsuperscript{GFP} double reporter mice were left untreated (naïve) or immunized with MOG\textsubscript{35-55}/CFA subcutaneously in the footpads with systemic PTX treatments on days 0 and 2. DLNs were harvested for flow cytometry analysis on day 7. (B) Representative FACS plots visualizing tdTomato (IL-17 fate mapping reporter) and GFP (Bhlhe40 reporter) in the indicated T cell subsets. (C) DLN cells were stimulated with PMA/ionomycin in the presence of brefeldin A for 4 hours. Representative ICS plots for IFN-\gamma and IL-17A expression by tdTomato\textsuperscript{+}CD4\textsuperscript{+} T cells were shown. (D) GFP (Bhlhe40) expression in each of the four cytokine-producing tdTomato\textsuperscript{+}CD4\textsuperscript{+} subsets shown in (C).
Figure 5-2. *Bhlhe40* is required by T cells but not neutrophils to induce EAE.

Mean clinical scores of EAE in immunized *Bhlhe40*^fl/fl^, (A) *Cd4-Cre x Bhlhe40*^fl/fl^ mice, or (B) *MRP8-Cre x Bhlhe40*^fl/fl^ mice. For (A), data are combined from three independent experiments. For (B), the data is from one experiment.
Figure 5-3. Bhlhe40 is required by CD11c⁺ cells and T cells to control *Mtb* infection.
Figure 5-3. Bhlhe40 is required by CD11c+ cells and T cells to control Mtb infection.

(A) GFP (Bhlhe40) expression in lung myeloid immune cell types from Mtb-infected nontransgenic (Tg-negative) or Bhlhe40GFP (Tg-positive) reporter mice at day 21 p.i.  (B) GFP (Bhlhe40) expression in lung T cell subsets from naïve or Mtb-infected Bhlhe40GFP (Tg+) reporter mice at day 21 p.i.  (C) The survival of WT (Bhlhe40+/+), Bhlhe40−/−, Bhlhe40fl/fl mice, and three strains of Bhlhe40 conditional knockout mice infected with 100-200 CFU aerosolized Mtb. The number of biological replicates is indicated in parentheses. Data is from one experiment.
Figure 5-4. BHLHE40 expression in human T cells and PBMCs.
Figure 5-4. BHLHE40 expression in human T cells and PBMCs.

(A) The relative expression of five transcription factors was analyzed in GEO DataSet GSE43005\textsuperscript{328} in human primary CD4\textsuperscript{+} T-cell subsets. \textit{GATA3}, \textit{RORC}, and \textit{FOXP3} have been shown in Figure 1 of the original report published by Zhang et al. in 2013. (B) Immunoblots of BHLHE40 and HDAC1 in FACS-sorted human blood T\textsubscript{H17} cells (CD161\textsuperscript{+}CCR6\textsuperscript{+}CXCR3\textsuperscript{+}) cultured with the indicated cytokines for 4 days (top). Quantitation of the band intensity of Bhlhe40 relative to HDAC1 is shown (bottom). (C) The relative expression of \textit{BHLHE40} and \textit{STAT1} was analyzed in GEO DataSet GSE43005\textsuperscript{300} in the blood of healthy control, latent or active TB patients recruited in the UK. Error bars show mean ± SEM. Statistical differences were determined two-tailed unpaired Student’s t-test. **p<0.01, ***p<0.001.
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