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The transcription factor Bhlhe40 in immunity and autoimmunity

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Abstract

The basic helix-loop-helix transcription factor Bhlhe40 is emerging as a key regulator of immunity during infection, autoimmunity, and inflammatory conditions. We describe roles of Bhlhe40 in the circulating and tissue-resident arms of the immune system, with emphasis on recent work on regulation of cytokine production and proliferation. We explore mechanisms behind these functions in mouse models and human cells, including interactions with other transcription factors, and propose that Bhlhe40 is both a central mediator of inflammation and pathogen control, as well as a crucial regulator for a growing number of tissue-resident leukocyte populations. Finally, we suggest areas for further study that we think will contribute to advances in our understanding of immunity and disease.

Bhlhe40: emerging regulator of immunity

Bhlhe40 (see Box 1) is a member of the basic helix-loop-helix transcription factor (TF) family. Bhlhe40 and its most related family member, Bhlhe41, bind to class B E-box DNA sequences with the consensus motif CACGTG [1, 2]. These proteins oligomerize both as homodimers and heterodimers [1–3]. Many functions for mammalian Bhlhe40 have been identified, including regulating cell cycling, cell death, and differentiation, although these studies were predominantly conducted in cell lines [4]. Bhlhe40/41 are unique from other helix-loop-helix Orange domain-containing transcriptional repressors in that they do not interact with the corepressor Groucho, but instead, repress transcription both in cooperation with histone deacetylases (HDACs) and in an HDAC- independent manner [2, 4]. Bhlhe40 also has a poorly understood potential for transcriptional activation. [5–8]. *Bhlhe40* is

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expressed in both hematopoietic and nonhematopoietic mammalian cell types, with early studies focusing on its role beyond the immune system [4, 9].

Bhlhe40$^{-/-}$ mice were initially generated on a mixed genetic background and developed a late onset lymphoproliferative disorder characterized by enlargement of lymphoid organs and immune cell infiltration into peripheral tissues [10]. This phenotype was largely due to a failure to eliminate activated T and B cells. We and others have observed a less severe, partially penetrant form of this phenotype in C57BL/6 *Bhlhe40*$^{-/-}$ mice, predominantly in older females [8, 11]. Because *Bhlhe40* expression is widespread, *Bhlhe40* deletion in specific immune cell lineages has been essential to elucidating its functions. Recent work has demonstrated that Bhlhe40 regulates cytokine production in mouse and human **CD4$^+$ T cells** (see Glossary) as well as regulating proliferation and/or metabolism in mouse myeloid, B, and tissue-resident **CD8$^+$ T cells**, sometimes in partnership with Bhlhe41. Here, we review these advances and speculate on molecular mechanisms explaining these factors' functions in specific cell types.

Bhlhe40 in circulating lymphocytes

After activation, circulating B and T cells are licensed for effector functions including proliferation, cytokine secretion, and migration into tissues. CD4$^+$ helper T (T_H) cells are specialized to regulate other cells, in part via cytokines. While a substantial body of literature has revealed the transcriptional regulators controlling T_H cell subset differentiation, less is known about common regulators of cytokine production across T_H cell subsets. Recent work has established a key role for Bhlhe40 in this process.

Bhlhe40 in CD4$^+$ T cells

Autoimmune and alloimmune models.

Experimental autoimmune encephalomyelitis (EAE) is the major animal model of multiple sclerosis (MS). In EAE, mice immunized with myelin antigens develop ascending paralysis and neuroinflammation [12]. EAE induction in *Bhlhe40*$^{\mathbf{GFP}}$ **bacterial artificial chromosome** reporter mice showed that *Bhlhe40* was heterogeneously expressed by CD4$^+$ T cells [13]. Pro-inflammatory cytokine-producing CD4$^+$ T cells were more frequent in the Bhlhe40-positive subset, while Bhlhe40-negative CD4$^+$ T cells were enriched for Foxp3 and IL-10 [13]. These data suggested that *Bhlhe40* expression correlated with CD4$^+$ T cell encephalitogenicity. Indeed, *Bhlhe40*$^{-/-}$ mice were protected from disease in both **active and passive EAE models**, primarily because *Bhlhe40*$^{-/-}$ CD4$^+$ T cells were non-pathogenic [11, 13, 14]. *In vitro*, Bhlhe40 regulated cytokine production in polarized T_H cells (see Box 2 for details). *In vivo*, *Bhlhe40*$^{-/-}$ mouse CD4$^+$ T cells were impaired in their expression of *Csf2* (encoding GM-CSF), a pro-inflammatory cytokine, crucial for T cell-mediated EAE [15, 16]. Additionally, relative to *Bhlhe40*$^{+/+}$ cells, *Bhlhe40*$^{-/-}$ CD4$^+$ T cells produced an increased amount of IL-10 [11, 17] -- known to restrain T_H cell effector functions and to protect mice from autoimmunity [18]. IL-10 receptor blockade rendered *Bhlhe40*$^{-/-}$ mice susceptible to EAE, indicating that excessive IL-10 contributed to EAE resistance in *Bhlhe40*$^{-/-}$ mice [11]. Nevertheless, hundreds of genes were dysregulated in *Bhlhe40*$^{-/-}$ T_H

cells, suggesting that other Bhlhe40-dependent genes contributed to EAE susceptibility [11, 14].

In the T cell transfer mouse model of **colitis**, naïve $CD4^+$ T cells are transferred into immunocompromised mice, causing intestinal inflammation and weight loss resembling human colitis [19]. Transferred $CD4^+$ T cells secrete inflammatory cytokines including interferon-γ (IFN-γ) [20], while IL-10 can play a protective role [21]. A study using this model suggested that Bhlhe40 could regulate colitis by controlling the balance between cytokines. Specifically, *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ naïve $CD4^+$ T cells transferred into *Rag1*$^{-/-}$ recipient mice failed to induce weight loss [17]. Compared with control *Bhlhe40*$^{fl/fl}$ $CD4^+$ T cells, *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ $CD4^+$ T cells showed low IFN-γ, but high IL-10 expression. Thus, Bhlhe40 expression might directly influence colitis by regulating these cytokines, although further robust experiments are needed to show this conclusively.

In contrast to the autoimmune models of EAE and colitis, an alloimmune mouse model of **graft-versus-host disease** (GvHD) can be induced by transferring donor splenocytes to MHC-mismatched recipient mice [21]. Donor **allogeneic** $CD4^+$ T cells secrete IFN-γ and GM-CSF -- the latter being an essential driver of inflammation in this model [22–24]. When *Bhlhe40*$^{-/-}$ donor $CD4^+$ T cells were tested in this model, they showed reduced IFN-γ and GM-CSF expression and caused less weight loss and mortality after transfer, relative to control $CD4^+$ T cells [24]. Notably, similar numbers of IL-10-producing *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ $CD4^+$ T cells were observed, suggesting that Bhlhe40 could promote GvHD primarily through upregulation of GM-CSF (and perhaps IFN-γ) [24].

Infection models.

Bhlhe40-mediated inflammatory responses can be beneficial in host defense against pathogens. T_H1 responses and the balance of IFN-γ and IL-10 are crucial for protective immunity in animal models of toxoplasmosis (caused by *Toxoplasma gondii*; a protozoan) and tuberculosis (caused by *Mycobacterium tuberculosis*; a bacterium) [25–28]. A study showed that *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice were highly susceptible to *T. gondii* and *M. tuberculosis* infections [17, 29]. Compared with *Bhlhe40*$^{fl/fl}$ $CD4^+$ T cells, *Cd4- Cre*$^+$ *Bhlhe40*$^{fl/fl}$ $CD4^+$ T cells harvested from *T. gondii*-infected mice secreted less IFN-γ, but more IL-10, after antigen stimulation *in vitro* [17]. Moreover, in *M. tuberculosis*-infected *Bhlhe40*$^{-/-}$ mice, decreased *Ifng* and increased *Il10* transcripts were observed in lung homogenates when compared with infected *Bhlhe40*$^{+/+}$ mice [29]. In addition, disrupting IL-10 signaling, by either administering an IL-10R blocking antibody or by generating *Bhlhe40*$^{-/-}$ *Il10*$^{-/-}$ mice, rescued the lethal phenotypes resulting from Bhlhe40 deficiency in both disease models; this suggested that these phenotypes were driven by excessive IL-10 production [17, 29]. Of note, Bhlhe40 may also be required in **dendritic cells** (DC), as *Cd11c-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice were more susceptible to lethal *M. tuberculosis* infection than *Bhlhe40*$^{fl/fl}$ mice [29]. This could be related to Bhlhe40-mediated regulation of *Il10* in DCs[29].

Recent work suggests that Bhlhe40 also regulates **type 2 immunity**. Using *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice, Bhlhe40 was found to be crucial for $CD4^+$ T cell-mediated control of the intestinal **helminth** *Heligmosomoides polygyrus bakeri* (*H. polygyrus*) [30]. Bhlhe40-

sufficient mice are able to control helminth infection in part by generating type 2 **granulomas,** at the expense of damage to their intestinal architecture [30]. *Bhlhe40*$^{-/-}$ and *Cd4-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice are unable to generate a protective immune response and granulomas, resulting in impaired helminth control but protection from excessive tissue damage, suggesting that Bhlhe40 mediates immunopathology in this model [30]. Moreover, gene expression analysis of lamina propria CD4^{+} T cells indicated that the presence of Bhlhe40 was required for normal expression of many helminth-elicited genes [30]. This suggested that, *in vivo*, the T_H2 cell program and related cytokine production was Bhlhe40-dependent, consistent with a CRISPR-Cas9 screen which assessed regulators of *in vitro*-derived T_H2 cells and showed that Bhlhe40 was required to regulate *Irf4* [31]. The related TF Bhlhe41 is also required in T_H2 cells. Previous work described defects in IL-4, IL-5, and IL-13 production from *Bhlhe41*$^{-/-}$ mouse T_H2 cells *in vitro* and *in vivo* after ovalbumin treatment (allergy/asthma model) or after treatment with *Schistosoma mansoni* eggs (a parasite eliciting potent type 2 immunity) (see Box 2 for further discussion of Bhlhe40/Bhlhe41 *in vitro*) [32].

In addition, during secondary *H. polygyrus* infection, Bhlhe40 was required for normal CD4^{+} T cell production of IL-5 and GM-CSF [30]. While GM-CSF had not been connected to helminth infection, exploring the effects of Bhlhe40 deficiency revealed a combinatorial role for IL-5 and GM-CSF in anti-helminth responses [30]. *Csf2rb*$^{-/-}$ mice, lacking the common beta chain cytokine receptor component (and therefore, responsiveness to IL-5 and GM-CSF), or combined antibody blockade of IL-5 and GM-CSF, demonstrated impaired recall responses to *H. polygyrus* [30]. This redundancy could have unexpected implications for **atopic diseases**. Taken together, we argue that Bhlhe40 can be a double-edged sword, acting to promote inflammatory responses (Figure 1), but sometimes at the cost of increased tissue damage. Investigation into these activities merits further attention.

BHLHE40 in human CD4^{+} T cells.

BHLHE40 is evolutionarily conserved [4, 33]. Similar to findings in murine CD4^{+} T_H cells [13, 14], overexpression and knockout experiments with human circulating CD4^{+} T cells demonstrated that *BHLHE40* expression positively correlated with expression of GM-CSF and other pro-inflammatory cytokines, including IL-17A, IL-22, and TNF [34]. In colorectal cancer patients, *BHLHE40* was highly expressed by T_H1-like tumor-infiltrating CD4^{+} T cells, a population enriched in microsatellite-unstable, **checkpoint blockade**-sensitive tumors, compared to microsatellite-stable, checkpoint-insensitive tumors [35]. Furthermore, anti-**CD40 ligand** (CD40L) agonist therapy in the MC38 mouse colon cancer model stimulated conventional type 1 DCs to elicit a similar *Bhlhe40*-expressing T_H1-like population in tumors, as identified by single cell RNA-sequencing [36]. These studies support the notion that BHLHE40 is functionally conserved between mice and humans.

Bhlhe40/BHLHE40 in mouse and human B cells

Data regarding expression of *Bhlhe40* in B cells have been conflicting, with reports indicating that *Bhlhe40* expression is repressed [37–39] or induced upon B cell activation [40]. Variables ranging from B cell type (marginal zone versus follicular B cells in lymphoid

organs), or stimulus, might explain to a certain extent these discordant findings. BHLHE40 has been implicated as a regulator of human germinal center B cells based on gene expression analyses of activated human B cells [41, 42], but its function remains untested. Much of the literature on Bhlhe40 and B cells concerns proliferation. Overexpression of Bhlhe40 in a mouse B cell line resulted in less G0/G1-to-S-phase transition relative to controls [37]. In addition, transgenic mice overexpressing Bhlhe40 (*Clast5*-Tg) exhibited reduced splenic and bone marrow (BM) B cell numbers with impaired proliferative responses to B cell receptor (BCR) crosslinking compared with non-transgenic mice [38]. This study also suggested that Bhlhe40 may be repressed early in B cell development, perhaps to allow proliferation in response to IL-7. Recently, Ikaros was identified as an important regulator of **anergic** B cells, as *Cd23*-Cre^{+} *Ikaros*$^{fl/fl}$ mice (exhibiting loss of Ikaros in splenic immature B cells) developed an autoimmune syndrome [43]. Of note, Ikaros was proposed to activate expression of Bhlhe40 in anergic B cells, suggesting that this regulation and Bhlhe40 itself may be important for maintenance of anergy [43]. It would therefore be informative to test whether *Cd23*-Cre^{+} *Bhlhe40*$^{fl/fl}$ mice might phenocopy the autoimmunity seen in *Cd23*-Cre^{+} *Ikaros*$^{fl/fl}$ mice. One could speculate that the B cell expansion seen in *Bhlhe40*$^{-/-}$mice on a non-C57BL/6 background might be caused by loss of anergy, but this phenotype is confounded by potential B cell-extrinsic effects, namely on T cells [10]. Therefore, Bhlhe40's function in **B2 B cells** remains poorly understood, especially concerning its regulation of proliferation and anergy. Further investigation is warranted to understand its functional role in a context- and species-dependent manner.

Of note, B cells are pathogenic in hepatitis C virus-associated mixed cryoglobulinemia (HCV-MC) -- a disease driven by potentially HCV cross-reactive $V_H1–69^{+}$ B cells [44]. In patients presenting with HCV-MC, $V_H1–69^{+}$ B cells have been reported to proliferate less and to express higher amounts of *BHLHE40* than B cells from healthy controls; however, whether BHLHE40 can actually regulate the proliferation of these cells remains unclear [45, 46]. Nevertheless, these findings might be clinically relevant as HCV-MC patients are at an elevated risk of developing non-Hodgkin's lymphoma [47]. Thus, it could be informative to investigate whether B cells from such patients have altered *BHLHE40* expression which might contribute to proliferative expansion of malignant clones.

Bhlhe40 in tissue-resident leukocytes

A crucial feature of resident leukocytes is their capacity to locally proliferate for self-renewal and population expansion during an immune response [48, 49]. The transcriptional control of this process is not well understood. Until recently, it remained unclear whether tissue-specific TFs govern this process.

Macrophages

Bhlhe40 is expressed in several mouse myeloid cell subsets, including certain tissue-resident macrophage populations (notably **alveolar macrophages (AMs)** and **peritoneal macrophages**), **granulocytes**, and DCs [13].Natural mutations in E-box motifs bound by Bhlhe40 have correlated with altered binding of the macrophage-lineage specifying transcription factor PU.1 in large peritoneal macrophages (LPMs) [50]. *Bhlhe40*$^{-/-}$ or

LysM-Cre^{+} *Bhlhe40*$^{fl/fl}$ conditional knockout mice showed reduced peritoneal macrophage populations in the steady state compared to *Bhlhe40*$^{+/+}$ or *LysM Cre*$^{-}$ *Bhlhe40*$^{fl/fl}$ mice [51]. Together with models of IL-4 cytokine complex (IL-4c) treatment or helminth infection in mixed BM chimeras and *LysM-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice, these data indicated that Bhlhe40 was cell-intrinsically required for normal proliferation of tissue-resident LPMs and large pleural macrophages in homeostasis and during type 2 immunity, while dispensable in other macrophage populations [51–53]. A high proportion of Bhlhe40-deficient LPMs expressed Ki67, a marker of proliferating cells, suggesting that **cell cycling** was dysregulated relative to Bhlhe40-sufficient LPMs [51]. In this study, proliferation of Bhlhe40-deficient LPMs (determined by pHH3 staining and BrdU incorporation) was severely affected during type 2 immunity, despite normal acquisition of alternative activation markers (CD301 and RELMα staining) [51]. Furthermore, **ChIP-Seq** and gene expression microarray analysis indicated that one function of Bhlhe40 was to transcriptionally repress the TF c-Maf, which antagonizes macrophage proliferation, to allow normal cell cycling [48, 51, 54]. Additionally, **thioglycolate**-elicited macrophages responding to IL-4c (a mouse model of **alternative activation** of **monocyte**-derived macrophages [55, 56]), also exhibited Bhlhe40-dependent cell cycling (BrdU incorporation), suggesting that the peritoneal environment could induce a Bhlhe40-dependent proliferative program in both monocyte-derived and tissue-resident macrophages in these models [51].

A recent report demonstrated that *Bhlhe40*$^{-/-}$ *Bhlhe41*$^{-/-}$ mice (but not mice singly deficient for either factor) exhibited pronounced cell-intrinsic defects in AMs, including impaired proliferation (EdU incorporation) and overaccumulation of intracellular lipid stores (Oil Red O and BODIPY staining) [57]. Moreover, in contrast to Bhlhe40-deficient

LPMs, which largely maintained their tissue-specific gene expression program, Bhlhe40/41-deficient AMs exhibited reduced expression of AM-specific genes and increased expression of genes characteristic of other macrophage lineages [57]. Another study reported that Bhlhe41 was expressed at steady state in murine microglia [40]. Furthermore, Bhlhe40 was upregulated in microglia in a mouse model of Alzheimer's disease induced by overexpression of mutated human APP-PS1 [58], though the functional importance and possible redundancy of these factors in microglia remains unknown. Taken together, these data suggest that Bhlhe40 plays important roles in homeostatic and inflammatory macrophages and is a tissue-specific regulator of macrophage proliferation in mice.

Lymphocytes

B1a cells.

Bhlhe41 is also a key regulator of splenic and peritoneal **B1a cells** in partnership with Bhlhe40 [39]. *Bhlhe40*$^{-/-}$ *Bhlhe41*$^{-/-}$ (and often *Bhlhe41*$^{-/-}$) B1a cells exhibit profound defects, including an altered BCR repertoire, impaired signaling, and an increased proportion of Ki67^{+} cells relative to *Bhlhe40*$^{+/+}$ *Bhlhe41*$^{+/+}$ B1a cells [39]. In contrast to Bhlhe40-deficient LPMs -- not exhibiting an increase in cell cycle progression beyond G1 phase as compared to Bhlhe40-sufficient LPMs [51] -- a greater proportion of Bhlhe40/41-deficient B1a cells successfully complete cell cycling compared to Bhlhe40/41-sufficient B1a cells [39]. Despite their increased proliferation, Bhlhe40/41-deficient B1a cells are

impaired in self-renewal due to increased cell death relative to Bhlhe40/41-sufficient B1a cells, resulting in fewer B1a cells in *Bhlhe40*$^{-/-}$ *Bhlhe41*$^{-/-}$ mice [39].

Tissue-resident memory CD8$^+$ T cells.

Transcriptomes of **tissue-resident memory CD8$^+$ T cells** (T_{RM}) have identified Bhlhe40 as part of the core signature of mouse T_{RM} [59]. Bhlhe40 is required by lung T_{RM} to support survival, normal amounts of effector molecules (including cytokines, chemokines, and granzyme B), and normal mitochondrial function and acetyl coenzyme A (AcCoA) concentration [60]. It is unclear whether Bhlhe40 regulates mitochondrial metabolism in other immune cells. However, lipid accumulation or **vacuolation** of Bhlhe40/41- (or Bhlhe40-) deficient macrophages has been observed in mice [51, 57]. This is relevant as fatty acids are metabolized to AcCoA within mitochondria; thus, these data might suggest a role for Bhlhe40 in mitochondrial function in other hematopoietic lineages. In an influenza virus infection model, antigen-specific lung CD8$^+$ T_{RM} were reduced in number in *Bhlhe40*$^{-/-}$ and *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice relative to *Bhlhe40*$^{+/+}$ and *Cd4-Cre*$^-$ *Bhlhe40*$^{fl/fl}$ mice, respectively [60]. This correlated with increased apoptosis in antigen-specific Bhlhe40-deficient lung CD8$^+$ T_{RM} relative to Bhlhe40-sufficient lung CD8$^+$ T_{RM} [60], but whether or not a defect in proliferation also contributed to reduced cell numbers is unclear (see Box 3). Furthermore, *Bhlhe40*$^{-/-}$ and *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice showed impaired T_{RM}-dependent protective immunity upon influenza virus reinfection, demonstrating the physiologic importance of Bhlhe40 in lung T_{RM} [60].

Bhlhe40 also plays an important role in tumor-infiltrating CD8$^+$ T cells (TILs). In murine transplantable tumor models (B16-OVA, MC-38, and LLC lines), *Bhlhe40*$^{-/-}$ mice displayed increased tumor growth relative to *Bhlhe40*$^{+/+}$ mice and *Bhlhe40*$^{-/-}$ TILs displayed decreased production of multiple effector molecules compared to *Bhlhe40*$^{+/+}$ TILs [60]. Additionally, PD-1 signaling cell-intrinsically repressed *Bhlhe40* expression in TILs in the B16-OVA model, and checkpoint blockade therapy with anti-PD-L1 antibody in this model was ineffective in controlling tumor growth in *Bhlhe40*$^{-/-}$ and *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice relative to *Bhlhe40*$^{+/+}$ and *Cd4-Cre*$^-$ *Bhlhe40*$^{fl/fl}$ mice [60].

Invariant NKT cells and innate lymphoid cells.

Whether Bhlhe40 is required in other tissue-resident lymphocytes is of considerable interest. In a mouse B16 melanoma model, IFN-γ production from *Bhlhe40*$^{-/-}$ iNKT cells was impaired, and these cells provided less protection against lung metastases compared to *Bhlhe40*$^{+/+}$ iNKT cells [7]. Of note, *Bhlhe40* expression appears to be widespread across multiple mouse **innate lymphoid cell** (ILC) populations. For instance, transcriptional profiling of mouse ILCs in multiple tissues has identified a signature of small intestinal lamina propria ILCs which includes *Bhlhe40* [61]. Moreover, single cell RNA-sequencing has revealed *Bhlhe40*/*BHLHE40* expression in both murine and human splenic NK cells [62]. Also, *BHLHE40* DNA demethylation has been proposed as a putative biomarker of NK cell activation [63]. However, a functional role for Bhlhe40 in NK cells or other ILCs remains to be investigated. We speculate that Bhlhe40 has a function in ILCs akin to that in mouse T_H cells, where it contributes to the regulation of cytokine production [11, 14]. Figure 2 summarizes Bhlhe40/41's known roles in tissue-resident leukocytes, but in our

opinion, the full extent of Bhlhe40's (and Bhlhe41's) role in tissue residency remains to be revealed, and careful assessment of individual and shared roles of Bhlhe40 and Bhlhe41 is required.

Mechanisms of Bhlhe40 activity

Upstream regulation of Bhlhe40.

Different stimuli likely induce *Bhlhe40* expression in different cell types (Key Figure, Figure 3). In non-hematopoietic cells, Bhlhe40 can be induced during many physiologic responses, including hypoxia [64, 65] and upon circadian clock oscillation in rodents [66, 67], but these specific inducers have not been investigated in immune cells. *Bhlhe40* was first identified as a retinoic acid (RA)-inducible gene [4], and RA maintained *Bhlhe40* expression *ex vivo* in cultured LPMs [50]. As RA supports LPM differentiation [68], it is likely that LPM expression of *Bhlhe40* is RA-dependent. However, whether expression of *Bhlhe40* in peritoneal B1a cells is likewise RA-elicited is unclear. In AMs, one possible regulator may be GM-CSF, as it is crucial for differentiation of this population [69], and induces *Bhlhe40* in cultured murine BM cells [29]. Alternatively, TGF-β might stimulate *Bhlhe40* expression in mouse AMs [57], but this remains to be further assessed.

Reports demonstrate that in mouse $CD4^+$ and $CD8^+$ T cells, Bhlhe40 is partially induced by T cell receptor (TCR) stimulation, but robust expression seems to require CD28-dependent costimulation and cytokine signaling [13, 14, 60]. After T cell activation, IL-1β can enhance Bhlhe40 transcript and protein expression in murine T_H17 cells [13, 70, 71]. Following EAE induction, myeloid cells in murine draining lymph nodes can secrete IL-1β, which acts on polarized $CD4^+$ T cells to ensure full *Bhlhe40* expression [13, 72, 73]. Other cytokines also likely modulate *Bhlhe40* expression. For instance, in a GvHD model, IL-1 receptor (IL-1R) deficiency (in *Il1r1*$^{-/-}$ mice) or treatment with the IL-1R antagonist Anakinra did not change *Bhlhe40* mRNA expression in donor mouse $CD4^+$ T cells, possibly because these IFN-γ-producing cells lacked IL-1R [24]. Regulation of Bhlhe40 differs between diseases, and different factors might stimulate *Bhlhe40* expression during type 1, 2, and 3 immunity. It remains to be investigated which signaling pathways (e.g. RAR/RXR, JAK/STAT, SMADs, MAPK, PI3K/Akt) can actually regulate Bhlhe40, and if regulation via these signaling pathways varies between different immune cells.

Mechanisms of regulation by Bhlhe40

Activation vs. repression.

Bhlhe40 can function as both a transcriptional activator and repressor (Key Figure, Figure 3) [5–7, 74]. In murine immune cells, its role as a direct transcriptional activator is poorly understood and may be independent of its binding to E-box motifs; instead, it may depend on interactions with other TF partners at their DNA-binding motifs (possibly T-bet, Runx1, or PU.1) [7, 8, 50]. Thus, studies are needed to establish the basis of Bhlhe40-mediated transcriptional activation in immune cells.

As a transcriptional repressor in cell lines, Bhlhe40 sometimes outcompetes transcriptional activators at target genes [2, 74]. In immune cells, one possible example of this is at the +6

kb region of the *Il10* locus, where Bhlhe40 has bound in mouse T_H1 cells, GM-CSF-cultured BM cells, and LPMs, as demonstrated by ChIP-Seq [29, 51]. In other work, this enhancer region bound transcriptional activators AP-1, IRF4, and c-Maf in mouse $CD4^+$ T cells [75–78], and we speculate that Bhlhe40 might function to disrupt their binding. Moreover, whether BHLHE40 transcriptionally represses the *IL10* locus in human T cells remains unclear [34].

In other instances of repression, Bhlhe40 recruits HDAC1 to silence gene transcription in cell lines [74]. While Bhlhe40 and HDAC1 have not been shown to interact in immune cells, two Bhlhe40-dependent histone acetylation patterns recently emerged. Specifically, genes repressed by Bhlhe40/41 (e.g. *Maf*, *Mafb*) exhibit increased H3 acetylation in *Bhlhe40*$^{-/-}$ *Bhlhe41*$^{-/-}$ mouse AMs, consistent with HDAC-dependent repression [57]. Conversely, genes activated by Bhlhe40 (e.g. *Ifng*) exhibit decreased H3 acetylation in *Bhlhe40*$^{-/-}$ mouse iNKT and $CD8^+$ T cells [7, 60]. In mouse T_{RM}, Bhlhe40 can indirectly promote global histone acetylation (flow cytometric analysis of acetylated H3) by maintaining AcCoA amounts [60] -- an essential substrate for histone acetyl transferases. We speculate that in instances when Bhlhe40 partners with transcriptional activators, Bhlhe40's interactions with HDACs might be prohibited, eliminating its repressive function.

Select targets of Bhlhe40.

Bhlhe40 and Bhlhe41 bind to their own promoter regions and auto- and cross-repression are seen in murine non-hematopoietic cell lines [2, 29, 51, 74]. Bhlhe40 ChIP-Seq in LPMs has indicated that Bhlhe40 binds multiple proximal sites in the *Bhlhe40* and *Bhlhe41* loci, and transcriptional data have shown that *Bhlhe40*$^{-/-}$ mouse LPMs exhibit higher *Bhlhe41* expression than *Bhlhe40*$^{+/+}$ LPMs [51]. Whether loss of auto- or cross-repression would significantly affect immunity is unknown. *Maf* has been identified as another target gene that is repressed by Bhlhe40 in T_H1 cells and LPMs, as well as by Bhlhe40/41 in mouse AMs [17, 51, 57]. Of note, *Bhlhe40* may also be repressed by c-Maf in mouse $CD4^+$ T cells, though the mechanism by which this occurs has not been established [75, 78]. This is a potential instance of complex cross-regulation of Bhlhe40 with another TF.

In multiple studies, GM-CSF production by mouse $CD4^+$ T cells is promoted by Bhlhe40 [11, 13, 14, 30], and candidate binding sites for Bhlhe40 have been identified in the mouse *Csf2* locus [11, 30]. In human $CD4^+$ T cells, BHLHE40 supports GM-CSF production, as evidenced by both CRISPR-guided deletion and lentiviral-driven overexpression [34]. However there is no evidence for direct interactions between BHLHE40 and the human *CSF2* gene, as tested via BHLHE40 ChIP-Seq analysis in Jurkat T cells [34]; this suggests that BHLHE40 might promote GM-CSF expression indirectly. Two novel immunoregulatory targets are suppressed by BHLHE40 in human $CD4^+$ T cells: miR-146a and ZC3H12D [34]. miR-146a prevents NF-kB activation [79] -- a pathway that induces *CSF2*-- and the RNase ZC3H12D degrades the mRNAs of several pro-inflammatory cytokines, including GM-CSF [34, 80, 81]. Specifically, ChIP-Seq and luciferase assays have revealed that BHLHE40 can regulate miR-146a indirectly, but can repress *ZC3H12D* gene expression directly in human $CD4^+$ T cells [34]. Collectively, BHLHE40 appears to function as a direct and indirect transcriptional regulator in mammals (Figure 3). We expect that novel target genes of

BHLHE40/41 may be identified when these factors are studied in additional immune cell types.

Cell cycling.

While Bhlhe40 has been linked to proliferation in some cancers and B cells, it has been recently shown to regulate proliferation of several mouse tissue-resident hematopoietic lineages as described above (LPMs, AMs, and B1a cells) [4, 37, 39, 51, 57]. In a seeming contradiction, older studies have linked Bhlhe40 with suppressed cell cycling, while others have linked it to promoting proliferation, particularly in the context of cancer, as recently reviewed [4, 82]. In light of the more recent data in mouse tissue-resident leukocytes [39, 51, 57], we think that these findings might be reconciled if one assumes that Bhlhe40 can enforce normal proliferation in multiple cellular lineages, perhaps inhibiting proliferation and/or preserving proliferative capacity depending on the cell type [4]. Presumably, when Bhlhe40 expression is lost, some populations of cells may inappropriately enter the cell cycle and fail to efficiently complete mitosis, or may undergo cell death [39, 51, 57].

Key downstream targets of Bhlhe40 and Bhlhe41 in cycling immune cells remain unknown. In murine macrophages, beyond Bhlhe40's (and perhaps Bhlhe41's) role in repressing *Maf*, Bhlhe40/41 has also repressed *Mafb* by as-yet unknown mechanisms [51, 57]. Bhlhe40/41 are suggested to repress *E2f* genes and support normal expression of common beta chain cytokine receptor components in mouse B1a cells [39]. Moreover, Bhlhe40 can bind loci encoding cell cycle genes whose expression is decreased in *Bhlhe40*$^{-/-}$ LPMs relative to *Bhlhe40*$^{+/+}$ LPMs in mice [51], suggesting possible direct activation. Aside from these targets, identifying other genes controlled by Bhlhe40 might reveal novel regulators of tissue-resident leukocyte proliferation.

Cell identity.

Bhlhe40/41 are variably required for proper expression of lineage identity-related genes in different murine immune cell populations [30, 51, 57], and are likely dependent on their binding partners in specific cell types. In mouse AMs, analysis of binding motifs and gene expression data have suggested that Bhlhe40 possibly cooperates with peroxisome proliferator activated receptor γ (PPARγ) [57]. In addition, given that lineage-specific gene expression programs of *H. polygyrus*-elicited *Bhlhe40*$^{-/-}$ mouse CD4^{+} T cells and homeostatic *Bhlhe40*$^{-/-}$ *Bhlhe41*$^{-/-}$ mouse AMs are particularly disrupted, we suspect that in both cases, these factors have possible connections to PPARγ, warranting further investigation [30, 57, 83]. We speculate that there may be crosstalk between Bhlhe40/41 and PPARγ that is required in some lineages for transcriptional activation and cellular identity. Thus, this may be a fruitful area for future study.

Concluding remarks

Bhlhe40 is now established as an essential regulator of mouse and human CD4^{+} T cells, in addition to a growing number of tissue-resident leukocyte populations; it contributes to controlling cytokine secretion, cell cycling, and metabolism. Bhlhe40 tilts the balance towards inflammation over disease tolerance, suggesting therapeutic potential for

stimulating or inhibiting Bhlhe40 in the appropriate setting. Key areas for future research (see Outstanding Questions) include determining the factors and signaling pathways stimulating *Bhlhe40* expression in different contexts. Why does Bhlhe40 alone regulate some resident populations while Bhlhe40 and Bhlhe41 jointly control others? Is this division of labor functionally important? What other key partners does Bhlhe40 interact with in immune cells? Bhlhe40 is expressed in multiple other hematopoietic lineages, including ILCs, granulocytes, and DCs, suggesting further interesting biology. Given the rapid pace of research showing Bhlhe40's importance in anti-tumor, anti-pathogen, autoimmune, and alloimmune responses, we are hopeful that BHLHE40 might be leveraged either as a putative biomarker, or as a candidate target for therapy in a variety of human diseases.

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Glossary

Active and passive EAE models
EAE induced by immunization with myelin antigen (active model) or adoptive transfer of myelin-specific $CD4^+$ T cells (passive model). Allogeneic: T cell response in the context of transplantation enabled by non-self MHC of either donor (by host T cells) or host (by donor T cells).

Alternative activation
a broad term for the transcriptional and proteomic changes that occur in macrophages and monocytes during a type 2 immune response.

Alveolar macrophages (AMs)
lung-resident; responsible for clearing debris and surfactants.

Anergic
quiescent, hyporesponsive state of T and B cells generally induced by exposure to cognate antigen in the absence of costimulation as a mechanism of tolerance.

Atopic diseases
allergic conditions such as allergic rhinitis, asthma, and eczema (atopic dermatitis) characterized by inappropriate immune responses to typically innocuous antigens.

Bacterial artificial chromosome (BAC)
used to stably insert large portions of DNA into the genome, such as fluorescent reporter constructs.

B1a B cells
one of two subtypes of innate-like, antibody-producing B1 lymphocytes (T cell-independent).

B2 B cells
adaptive, antibody-producing lymphocytes (T cell-dependent).

$CD4^+$ T cells
helper T cells regulating the activity of other immune cells. Divided into several subsets including T helper (T_H) type 1, T_H2, T_H17, and regulatory T cells (T_{REG}).

$CD8^+$ T cells
cytotoxic T cells capable of killing target cells and producing cytokines.

Cell cycle
process of cell division. Resting (G0 phase) cells proliferate by sequentially passing through G1, S, G2, and M phases.

Checkpoint blockade
use of biologic agents to block inhibitory immunoreceptors.

ChIP-Seq
method to determine transcription factor binding or chromatin modifications throughout the genome.

Colitis
inflammation of the colon.

Dendritic cells
professional antigen presenting cells encompassing several subsets, including cDC1, cDC2, and plasmacytoid DC.

Experimental autoimmune encephalomyelitis (EAE)
animal model of multiple sclerosis; results in immune cell infiltration into the central nervous system, demyelination, and ascending paralysis.

Granulocytes
immune cells characterized by prominent cytoplasmic granules (neutrophils, eosinophils, and basophils).

Granuloma
structure formed by immune cells to encase pathogens/antigens during certain infections.

Graft-versus-host disease
allogeneic reaction occurring post-transplant when donor (graft) lymphocytes attack recipient (host) tissue.

Helminths
parasitic worms which establish chronic infections.

Innate lymphoid cells

lack rearranged antigen-specific receptors capable of rapid cytokine production; include ILC1 (T_H1-like), ILC2 (T_H2-like), and ILC3 (T_H17-like) subsets, as well as ILC1-like NK cells and lymphoid tissue inducer cells.

Monocytes
circulating myeloid cells generated from primitive and definitive hematopoiesis. Fetal liver monocytes give rise to most tissue-resident macrophage populations. Adult bone marrow monocytes are recruited to peripheral tissues in homeostasis and differentiate into macrophages and so-called monocyte-derived dendritic cells during inflammation.

Peritoneal macrophages
embryonically-derived large serous cavity and monocyte-derived small serous cavity macrophages found in the peritoneum.

Thioglycolate broth
media preparation eliciting robust recruitment of monocyte-derived macrophages to the peritoneum.

Tissue-resident memory T cells
frontline mediators of protective memory responses stably occupying tissue sites without exchange with the circulation.

Type 2 immunity
characterized by T_H2 cell responses and cytokines including IL-4, IL-5, and IL-13 observed in helminth infections and allergy.

Vacuolation
the appearance of numerous membrane-enclosed fluid-filled vesicles within cells.

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Box 1:

Nomenclature and available tools.

What's in a name?

Both Bhlhe40 and Bhlhe41 have had many names over the years. Bhlhe40 was first identified as Stimulated with retinoic acid 13 (Stra13) in P19 embryonal carcinoma cells [82] and Bhlhe41 was initially identified in rat brain and called enhancer of Split and HAiry Related Protein-1 (SHARP-1) [83]. Other names for Bhlhe40 include Deleted in esophageal cancer 1 (Dec1), SHARP-2, Basic helix-loop-helix domain containing, class b, 2 (Bhlhb2), and Clast5, while other names for Bhlhe41 include Dec2 and Bhlhb3. Table I shows the names of mouse strains used to study Bhlhe40 and Bhlhe41. For clarity, here, Bhlhe40 denotes the murine protein and BHLHE40 denotes the human protein.

Table I.
Mouse Strains Useful for the Study of Bhlhe40 and Bhlhe41[a]

Mouse strain	Notes	Ref.
Bhlhe40$^{-/-}$	Global deletion of *Bhlhe40*	[8,10,86]
Bhlhe41$^{-/-}$	Global deletion of *Bhlhe41*	[32,87]
Cd4-Cre^{+} *Bhlhe40*$^{fl/fl}$	Deletion of *Bhlhe40* in T cells	[17,29,60]
LysM-Cre^{+} *Bhlhe40*$^{fl/fl}$	Deletion of *Bhlhe40* in macrophages, monocytes, and neutrophils	[29, 51]
Cd11c-Cre^{+} *Bhlhe40*$^{fl/fl}$	Deletion of *Bhlhe40* in DCs and alveolar macrophages	[29]
Mrp8-Cre^{+} *Bhlhe40*$^{fl/fl}$	Deletion of *Bhlhe40* in neutrophils	[29]
Clast5-Tg	Overexpression of *Bhlhe40* in B and T cells	[38]
Dec1-Tg	Overexpression of *Bhlhe40* in T cells	[8]
Dec2-Tg	Overexpression of *Bhlhe41* in T cells	[88]
Bhlhe40^{GFP}	*Bhlhe40* BAC transgenic reporter strain	[13]
Bhlhe41-Cre-hCD2	cre-IRES-hCD2 gene expression under control of *Bhlhe4l* BAC	[39]
Bhlhe41^{Tag}	*Bhlhe41* with multiple tag epitopes (FLAG and V5)	[39]
FLAG-Dec2	*Bhlhe41* with FLAG tag expressed under *hCD2* promoter control	[32]

[a] *HHCD2* denotes human *CD2*.

Box 2.

Bhlhe40 and in vitro T helper cell polarization.

T cell receptor (TCR) and costimulation together with cytokine signals instruct T_H cell differentiation and proliferation. Differentiated T_H cells secrete a variety of cytokines acting on other immune cells and non-hematopoietic cells during infection and autoimmunity. *In vitro*, naïve $CD4^+$ T cells can be differentiated into T_H1, T_H2, T_H17, and induced regulatory T (iT_{REG}) cells with surrogate TCR stimulation and co-stimulation in the presence of polarizing cytokines. Bhlhe40/BHLHE40 is absent in naïve mouse and human T cells but is upregulated after anti-CD3 and anti-CD28 stimulation [11, 14]. *In vitro*-polarized mouse T_H1 and T_H17 cells have shown higher Bhlhe40 expression than T_H2 cells [11]. Although *in vitro* expression of classical lineage-specifying transcription factors (TF) and most signature genes of each T_H lineage do not require Bhlhe40, this TF is essential for normal cytokine production [11]. IFN-γ secretion is reduced in mouse *Bhlhe40*$^{-/-}$ and *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ T_H1 cells [11, 17]; this phenotype may be due to Bhlhe40's proposed role as a co-factor for T-bet, the master regulator of T_H1 cells [7], although this interaction has not been observed in T_H1 cells [17]. Moreover, mouse *Bhlhe40*$^{-/-}$ T_H1, T_H2, and T_H17 lineages show reduced GM-CSF but enhanced IL-10 production relative to *Bhlhe40*$^{+/+}$ cells [11, 17]. Bhlhe41 is specifically expressed in T_H2 cells, and mouse *Bhlhe41*$^{-/-}$ T_H2 cells exhibit impaired proliferation and production of IL-4, IL-5, and IL-13 *in vitro* [32]. Bhlhe41 shares some overlapping functions with Bhlhe40, and thus, may compensate in *Bhlhe40*$^{-/-}$ T_H2 cell cultures. Upregulated Bhlhe40 was reported in mouse iT_{REG} in an expression microarray analysis [11]. An older study using *Bhlhe40*$^{-/-}$ and transgenic mice claimed that Bhlhe40 in complex with Runx1 activated the *Il2ra* locus (encoding CD25) and maintained T_{REG}, suggesting a role for Bhlhe40 in mouse iT_{REG} cells [8].

Box 3.

The role of Bhlhe40 in T cell proliferation.

A still-unresolved question is the role that Bhlhe40 plays in the proliferation of $CD4^+$ and $CD8^+$ T cells. An early study showed impaired *in vitro* proliferation of mouse *Bhlhe40*$^{-/-}$ $CD4^+$ T cells under limited anti-CD3/anti-CD28 antibody stimulation which could be restored upon IL-2 addition [10]. Lymph node cells from *Bhlhe40*$^{-/-}$ mice immunized with keyhole limpet hemocyanin (KLH) and complete Freund's adjuvant were less proliferative *in vitro* after KLH restimulation than cells from *Bhlhe40*$^{+/+}$ mice [10]. Others found that without added IL-2, mouse *Bhlhe40*$^{-/-}$ $CD4^+$ T cells exhibited reduced proliferation *in vitro* compared to *Bhlhe40*$^{+/+}$ $CD4^+$ T cells [14]. In contrast, we and others showed that mouse Bhlhe40-deficient T_H-neutral (with or without IL-2), T_H1, T_H2, and T_H17 cells proliferated normally *in vitro* [17, 30]. Also, encephalitogenic Bhlhe40-expressing $CD4^+$ T cells (assessed via GFP expression in *Bhlhe40*^{GFP} reporter mice) in the mouse EAE model exhibited a greater frequency of Ki67 positivity relative to GFP-non-expressing $CD4^+$ T cells, suggesting that Bhlhe40 might correlate with cell cycling *in vivo*, yet awaiting further investigation [13]. Bhlhe41 might also play a role in T_H2 cell cycling in light of impaired *Bhlhe41*$^{-/-}$ T_H2 cell proliferation *in vitro*, introducing additional complexity to this issue [32]. One group recently demonstrated that Bhlhe40 was essential for mouse T_{RM} and tumor-infiltrating lymphocyte accumulation in influenza virus infection and transplantable tumor models, respectively [60]. This was at least in part due to a survival defect of Bhlhe40-deficient cells as demonstrated by increased apoptosis [60], but there might also be a role for Bhlhe40 in $CD8^+$ T cell proliferation. Taken together, these data indicate that the effects of Bhlhe40 on T cell proliferation are complex and may be context-dependent. In light of the importance of the tissue environment in eliciting Bhlhe40-dependent proliferation in other lineages [51, 57], carefully controlled *in vivo* studies are needed to address the biological importance of Bhlhe40 and Bhlhe41 in T cell proliferation.

Outstanding Questions

- There are conflicting reports on whether Bhlhe40 regulates T cell proliferation *in vitro*. Does Bhlhe40 regulate T cell proliferation *in vivo*?
- Does Bhlhe40 predominantly regulate cytokine production by direct transcriptional control or indirectly through other regulators such as c-Maf? Uncovering novel targets of Bhlhe40 could lead to discovery of molecules with unappreciated roles in controlling cytokine production.
- Is Bhlhe40 an important regulator of non-hematopoietic cell cytokine production? Bhlhe40 has traditionally had roles in cell proliferation and differentiation in non hematopoietic cells, but its role as a cytokine regulator in these cells is unknown.
- Are the roles for Bhlhe40 in $CD4^+$ T helper cell subsets mirrored by key roles in ILCs? As ILCs are tissue-resident sentinels of immunity, understanding the transcriptional pathways that regulate their cytokine production could have important implications for pathogen control early in infection.
- In light of Bhlhe40's role in mitochondrial metabolism in T_{RM}, is Bhlhe40 an important regulator of immunometabolism in other tissue-resident leukocytes?
- While Bhlhe40 is a key regulator of specific tissue-resident macrophages in homeostasis, its expression can be induced in monocytes recruited during a type 2 immune response. Is Bhlhe40 important to monocyte-derived, alternatively-activated macrophages during pathological conditions such as fibrosis?

Highlights

- Bhlhe40 is a key regulator of cytokine production by human and mouse T cells.
- Bhlhe40 can drive inflammation and enables typical immune responses in autoimmunity, transplantation, cancer, and infection.
- Bhlhe40 is a tissue-specific regulator of murine tissue-resident macrophage proliferation.
- Bhlhe40 and Bhlhe41 are required in mouse B1a cells for normal proliferation, survival, and BCR repertoire.
- Bhlhe40 is essential for mitochondrial metabolism of mouse tissue-resident memory CD8+ T cells and tumor-infiltrating lymphocytes.
- Bhlhe40 contributes to controlling the balance between inflammation and disease tolerance, promoting highly inflammatory responses.

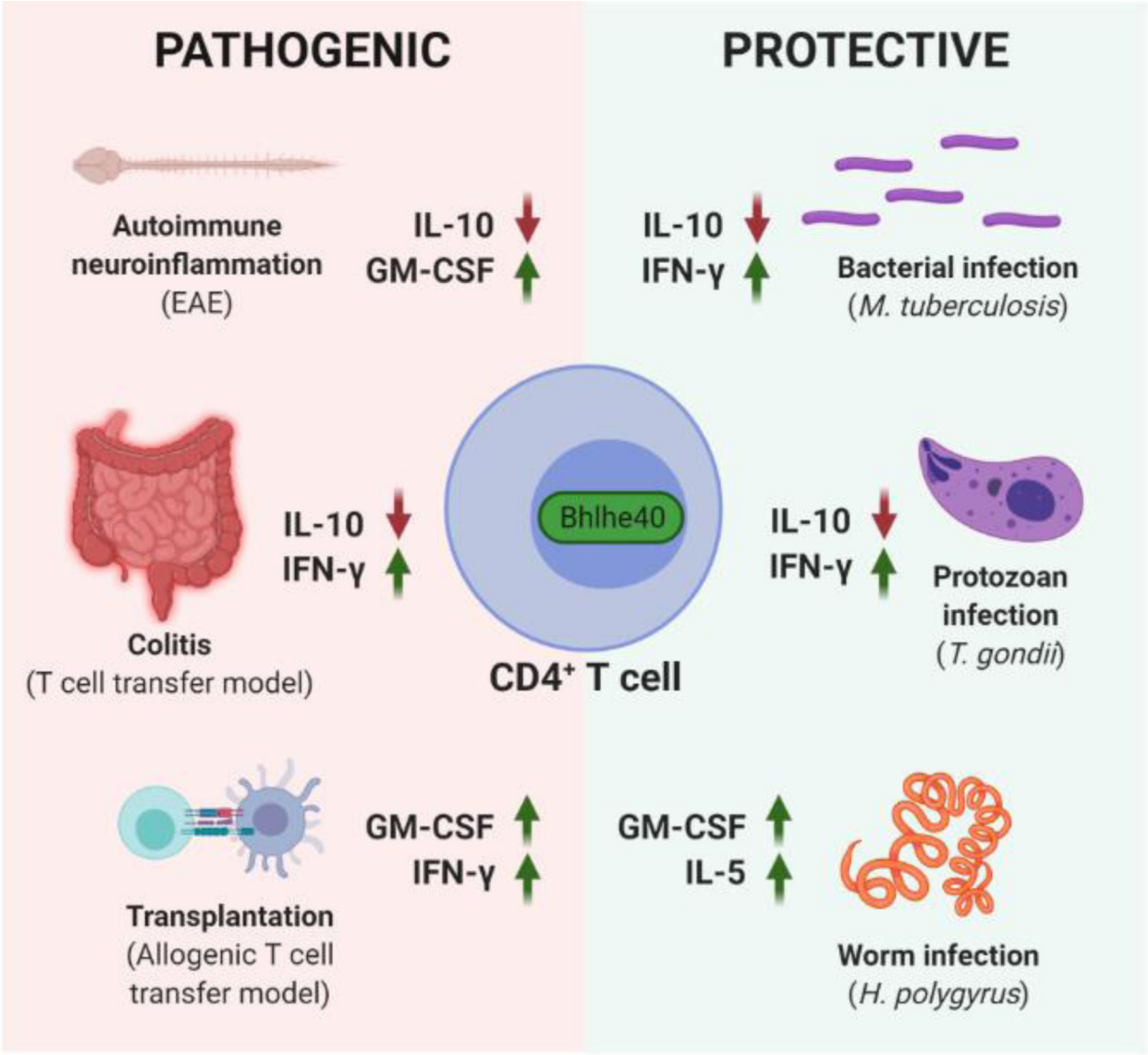


Figure 1. Bhlhe40 regulates CD4$^+$ T cell cytokines and shapes disease outcomes in mouse models.

Bhlhe40 is detrimental in murine autoimmune and alloimmune models, mainly because Bhlhe40 drives CD4$^+$ T cell-mediated inflammation by negatively regulating the antiinflammatory cytokine IL-10 and/or positively regulating the proinflammatory cytokines GM-CSF and IFN-γ (Left, "PATHOGENIC") [11, 14, 17, 24]. However, such Bhlhe40-mediated inflammatory responses can be beneficial in host defense against *Mycobacterium tuberculosis* (*M. tuberculosis*), *Toxoplasma gondii* (*T. gondii*), and *Heligmosomoides polygyrus bakeri* (*H. polygyrus*), in some cases at the cost of increased tissue damage (not shown) (Right, "PROTECTIVE") [17, 29, 30]. This figure was created using BioRender (https://biorender.com/).

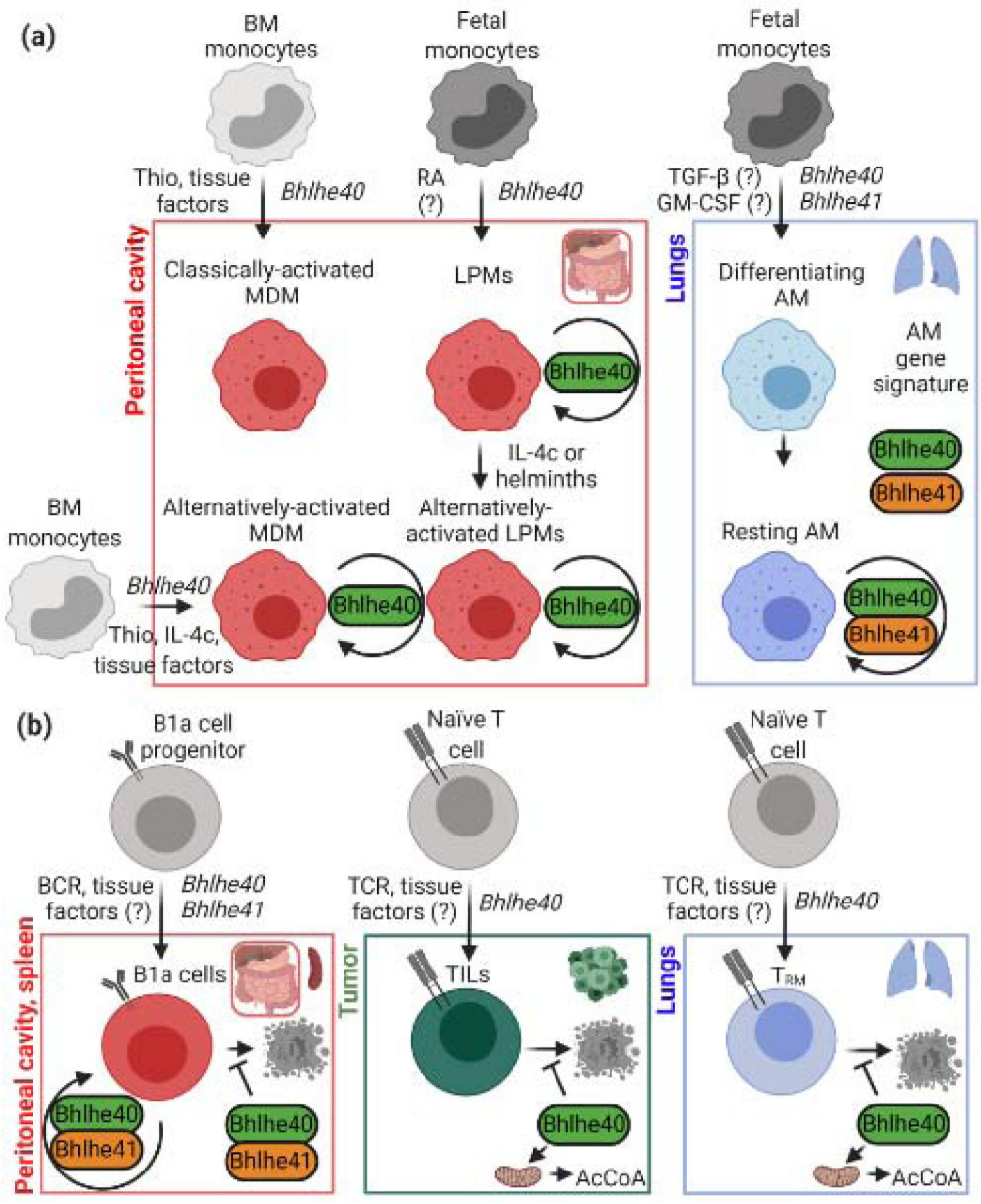
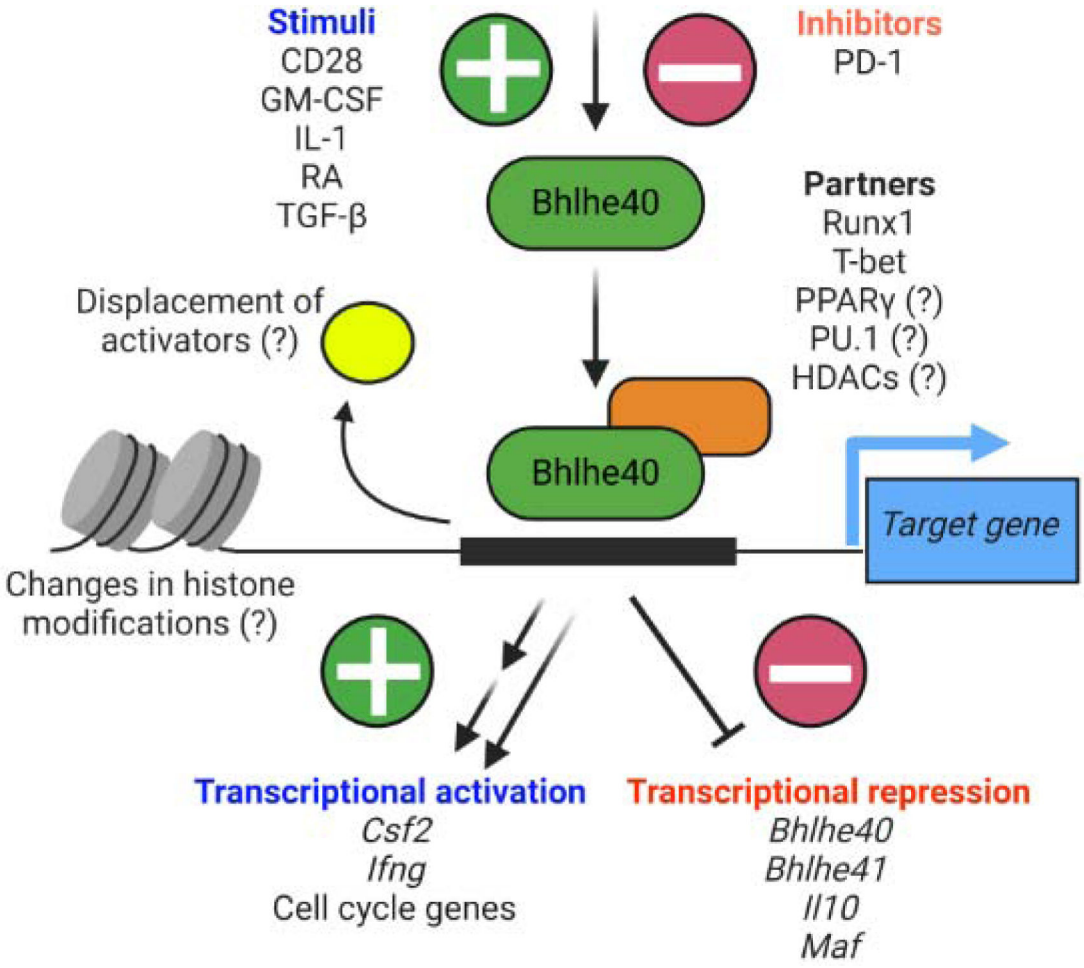


Figure 2. Roles of Bhlhe40 and Bhlhe41 in murine tissue-resident immune cells.

Bhlhe40 plays essential roles in tissue-resident macrophages **(a)** and lymphocytes **(b)**, cooperating with Bhlhe41 in B1a cells and alveolar macrophages (AM) [39, 51, 57, 60]. In many cases, unknown tissue factors are thought to be important for *Bhlhe40* expression, including retinoic acid [50]. These transcription factors regulate the proliferation of several resident leukocyte populations. In macrophages, this involves repression of c-Maf and MafB, but further mechanisms are thought to be involved [51, 57]. Recent work in T_{RM} suggests that Bhlhe40 is also an important regulator of mitochondrial metabolism [60], though it is unclear whether Bhlhe40-deficient T_{RM} are also deficient in proliferation. BM, bone marrow; Thio, thioglycolate; AM, alveolar macrophages; MDM, monocyte-derived macrophages; LPMs, large peritoneal macrophages; IL-4c, IL-4 cytokine complex; TILs,

tumor-infiltrating lymphocytes; AcCoA, acetyl coenzyme A; RA, retinoic acid. This figure was created using BioRender (https://biorender.com/).



Key Figure, Figure 3. Regulation and functions of Bhlhe40 in mouse hematopoietic cells.

Multiple stimuli induce *Bhlhe40* expression in immune cells, including IL-1, retinoic acid (RA), and GM-CSF. IL-1 is partially responsible for Bhlhe40 expression in T_H17 cells [13, 70], retinoic acid (RA) can sustain *Bhlhe40* expression by *in vitro* cultured LPMs [50], and GM-CSF can elicit *Bhlhe40* expression from bone marrow monocytes *in vitro* [29]. However, the factors responsible for *Bhlhe40* expression in immune cells *in vivo* are generally poorly understood. The activity of Bhlhe40 is likely dependent on its cell-specific binding partners. Bhlhe40 interacts with HDACs in cell lines and this may be important for its function *in vivo* [74]. Bhlhe40 is primarily a transcriptional repressor (represented by the single bar-headed line connecting Bhlhe40 to downstream targets), though some targets may be indirectly repressed. A potential mechanism of repression might be that Bhlhe40 displaces transcriptional activators from binding target loci. Bhlhe40 also possesses activating function at some loci. Therefore, while many transcripts that are reduced in the absence of Bhlhe40 are likely indirectly regulated (represented by the two staggered arrows), there are likely some loci directly activated by Bhlhe40 (represented by the single arrow). Human gene targets, including *CSF2*, *MIR146A*, and *ZC3H12D* are not depicted [34]. This figure was created using BioRender (https://biorender.com/).