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

Chih-Chung Lin¹, Tara R. Bradstreet¹, Elizabeth A. Schwarzkopf¹, Julia Sim², Javier A. Carrero¹, Chun Chou¹, Lindsey E. Cook¹, Takeshi Egawa¹, Reshma Taneja³, Theresa L. Murphy¹, John H. Russell², and Brian T. Edelson¹

¹Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA

²Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA

³Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Abstract

T_H1 and T_H17 cells mediate neuroinflammation in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Pathogenic T_H cells in EAE must produce the pro-inflammatory cytokine granulocyte-macrophage colony stimulating factor (GM-CSF). T_H 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*^{-/-} T_H1 and T_H17 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.

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Corresponding Author: Brian T. Edelson, M.D., Ph.D., Washington University School of Medicine, Dept. of Pathology and Immunology, Div. of Laboratory and Genomic Medicine, 660 S. Euclid Ave., Box 8118, St. Louis, MO 63110 USA, Phone: 314-362-4427, Fax: 314-747-0809, bedelson@path.wustl.edu.

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.

Competing Financial Interests

The authors declare no competing financial interests.

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Microarray datasets have been deposited in GEO DataSets under accession code GSE49166.

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_H) cells¹. 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^{2,3}. Nevertheless, neither T_H1 (IFN- γ) nor T_H17 (IL-17A, IL-17F, IL-21, IL-22) signature cytokines are required for the development of EAE⁴⁻⁷. Instead, the cytokine GM-CSF has proven to be a critical factor in the encephalitogenicity of both T_H1 and T_H17 cells⁸⁻¹². IL-1 receptor and IL-23 receptor signaling are required for T_H cell production of GM-CSF during EAE^{10,11,13}.

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- κ B family members, including c-Rel and NF- κ B1, and RUNX1¹⁴⁻¹⁹. Recently, Codarri et al. reported a requirement for ROR γ t 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^{10,11}. T_H1 cells also produce GM-CSF during EAE, yet specific transcription factors regulating their production of GM-CSF have not been identified¹².

Immunoregulatory cytokines, including IL-10, modulate the development of EAE²⁰. IL-10 is produced by several cell types during EAE, including autoreactive non-pathogenic T_H cells²¹⁻²⁷. IL-10 suppresses effector T cell responses and limits inflammation. IL-10-deficient mice develop more severe EAE than wild-type (WT) mice²⁸⁻³⁰. Expression of IL-10 in T_H cells can be regulated by several transcription factors, depending on the T_H cell subset and the cytokine environment³¹⁻³⁵. Relevant to EAE is the production of IL-10 by T_H1, T_H17, 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"^{36,37}. 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) stimulation³⁸. *Bhlhe40*^{-/-} mice develop a non-fully penetrant late-onset (age greater than ~8 months) lymphoproliferative disease associated with autoantibodies^{38,39}, that may be due to a requirement for Bhlhe40 for Treg maintenance during aging. 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*^{-/-} mice are resistant to EAE induction due, at least in part, to a T_H cell-intrinsic defect. T_H cells from these mice display nearly normal IFN- γ and IL-17A production in T_H1 and T_H17 cultures, respectively, and produce these cytokines

after immunization, but fail to mediate EAE. Instead, transcriptional and phenotypic analyses of *Bhlhe40*^{-/-} T_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*^{-/-} T_{H1} and T_{H17} 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.

Results

Bhlhe40-deficient mice are protected from EAE

To examine the role of Bhlhe40 in autoimmunity, we immunized *Bhlhe40*^{-/-} mice with MOG peptide (amino acids 35-55 (MOG(35-55))). *Bhlhe40*^{-/-} mice were markedly resistant to EAE (Fig. 1a), showing no leukocyte infiltration on histological examination of spinal cord sections (Fig. 1b). Bhlhe40 was required by the hematopoietic compartment for EAE susceptibility, since reconstitution of irradiated *Bhlhe40*^{-/-} mice with WT bone marrow (BM) caused susceptibility to EAE, whereas reconstitution of WT mice with *Bhlhe40*^{-/-} BM engendered EAE resistance (Fig. 1c). *Bhlhe40* is expressed in activated T_H cell subsets (Supplementary Fig. 1a), as reported^{38,40}. Transfer of WT and *Bhlhe40*^{-/-} T_H cells each reconstituted the T cell compartment of recipient *Rag1*^{-/-} mice equally (Supplementary Fig. 1b), but only WT cells, and not *Bhlhe40*^{-/-} cells, allowed development of EAE after immunization (Fig. 1d).

The central nervous system (CNS) from MOG(35-55)-immunized *Bhlhe40*^{-/-} mice had reduced infiltrating myeloid cells (CD45^{hi}CD11b⁺) relative to WT mice. In *Bhlhe40*^{-/-} mice, these cells lacked MHC class II expression, as did resident microglia (CD45^{int}CD11b⁺) (Fig. 2a, b). Immunized *Bhlhe40*^{-/-} mice also harbored fewer CNS-infiltrating CD4⁺ T cells (Fig. 2c). CNS-infiltrating CD4⁺ T cells in WT mice produced IFN- γ and IL-17A, while the few CNS-infiltrating CD4⁺ T cells in *Bhlhe40*^{-/-} mice had reduced IFN- γ and IL-17A production (Fig. 2d, e). Notably, WT CD4⁺ T cells produced GM-CSF, but this was reduced by ~90% in *Bhlhe40*^{-/-} mice (Fig. 2d, e and Supplementary Fig. 2a). WT and *Bhlhe40*^{-/-} mice showed a similar low frequency of IL-10-producing CD4⁺ T cells in the CNS at the peak of disease (Supplementary Fig. 2b). *Bhlhe40*^{-/-} mice showed a slight reduction in the frequency of CNS-infiltrating Foxp3⁺ CD4⁺ T cells (analyzed at day 29 after immunization, Supplementary Fig. 2c).

$\gamma\delta$ T cells are known to infiltrate the CNS during EAE, and promote neuroinflammation via their production of IL-17 and GM-CSF^{13,41}. The role of IFN- γ production by these cells in this setting is unclear. Immunized *Bhlhe40*^{-/-} mice harbored ~30-fold fewer CNS-infiltrating $\gamma\delta$ T cells compared to WT mice. *Bhlhe40*^{-/-} $\gamma\delta$ T cells produced similar levels of IFN- γ and partially reduced levels of IL-17A, but more markedly reduced levels of GM-CSF (Fig. 2f, g). 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 T_H1-polarized MOG(35-55)-specific T cell line was transferred to WT or *Bhlhe40*^{-/-} recipients⁴². These T cells produced IFN- γ and GM-CSF, but not IL-17A, upon stimulation (Supplementary Fig. 2d). 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 (Supplementary Fig. 2e, f). 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.

Bhlhe40 regulates T cell cytokine production after immunization

Draining lymph nodes (DLNs) from immunized *Bhlhe40*^{-/-} mice had reduced cellularity relative to WT mice (Supplementary Fig. 3a). 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 (Supplementary Fig. 3b). 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 MOG(35-55) or concanavalin A (ConA) as measured by ELISPOT assays (Fig. 3a). Consistent with their decreased IL-17A production, the frequency of ROR γ t⁺ CD4⁺ T cells was moderately decreased in DLNs of *Bhlhe40*^{-/-} mice (Supplementary Fig. 4a, b). DLNs from *Bhlhe40*^{-/-} mice showed a markedly reduced frequency of MOG(35-55)-specific GM-CSF-producing T cells relative to WT mice, and an increased frequency of MOG(35-55)-specific IL-10-producing T cells (Fig. 3b). Intracellular cytokine staining (ICS) of DLN cells also showed diminished GM-CSF (Supplementary Fig. 4c, d) and increased IL-10 production (Supplementary Fig. 4e, f) 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-1 β or IL-23, cytokines that are reported to promote GM-CSF secretion¹⁰⁻¹³ (Fig. 3c, d). The increased IL-10 production by *Bhlhe40*^{-/-} CD4⁺ T cells was significantly augmented by in vitro culture with IL-12 (Fig. 3e, f).

We analyzed cytokine production by *Bhlhe40*^{-/-} T cells in three additional settings. First, instead of using the self-peptide MOG(35-55), we immunized mice with the foreign, MHC class II-restricted peptide of chicken ovalbumin (OVA(323-339)). 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 (Supplementary Fig. 4g). 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 (Supplementary Fig. 4h, i). Third, we analyzed cytokine responses in immunized *Rag1*^{-/-} mice that had received transfers of purified WT or *Bhlhe40*^{-/-} CD4⁺ T cells (Supplementary Fig. 5). *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-1 β and/or IL-23 (Fig. 3g, h). Naïve splenic $\gamma\delta$ T cells from non-immunized *Bhlhe40*^{-/-} mice were capable of responding to IL-1 β , IL-23, and IL-12^{41,43}, as demonstrated by their increased size and granularity, but failed to produce normal levels of GM-CSF in these cultures (Supplementary Fig. 6a, b). 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.

***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 (Supplementary Fig. 7a-c). 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_{H1} cells showed a ~40% decrease in IFN- γ production, consistent with a previous report³⁹ (Fig. 4a, b). Consistent with these cytokine results, we found decreased expression of the transcription factor T-bet in *Bhlhe40*^{-/-} T_{H1} cells, with nearly normal expression of Gata-3 and ROR γ t in *Bhlhe40*^{-/-} T_{H2} and T_{H17} cells, respectively (Supplementary Fig. 7e).

GM-CSF production varied between WT T_{H1}, T_{H2}, and T_{H17} cells, with the least production of GM-CSF found in T_{H1} cultures (Fig. 4a-c), consistent with an earlier report¹⁰. *Bhlhe40*^{-/-} T cells showed markedly reduced production of GM-CSF under all conditions of differentiation (Fig. 4a-c). This lack of GM-CSF production by *Bhlhe40*^{-/-} T cells was also seen in T_{H17} cultures that utilized TGF- β 3 in place of TGF- β 1²⁶ (Supplementary Fig. 7f).

Bhlhe40^{-/-} T_{H1} and T_{H17} cells showed increased IL-10 production relative to WT cells, while WT and *Bhlhe40*^{-/-} T_{H2} cells showed similar, high-level IL-10 production (Fig. 4a, b). The increased IL-10 production in *Bhlhe40*^{-/-} T_{H1} cells came largely from IFN- γ ⁺ cells, while in T_{H17} cultures it came from both IL-17A⁺ and IL-17A⁻ populations (Fig. 4d). We further tested whether the increased IL-10 production by *Bhlhe40*^{-/-} T_H cells could contribute to their diminished GM-CSF production. Co-culture of congenically marked WT and *Bhlhe40*^{-/-} T_H cells under non-polarizing conditions showed that increased IL-10 secretion and decreased GM-CSF secretion were both cell-intrinsic properties of *Bhlhe40*^{-/-} T_H cells (Fig. 4e).

We tested whether abnormal GM-CSF and IL-10 production by *Bhlhe40*^{-/-} CD4⁺ T cells could be restored by the reintroduction of *Bhlhe40*. Retrovirally transduced *Bhlhe40*^{-/-} T_{H1} and T_{H17} cells overexpressing *Bhlhe40* produced increased levels of GM-CSF and decreased levels of IL-10 compared to non-transduced *Bhlhe40*^{-/-} T cells or *Bhlhe40*^{-/-} T cells transduced by an empty retrovirus (Supplementary Fig. 8). Overexpressing *Bhlhe40* modestly increased GM-CSF and decreased IL-10 production by WT T_{H17} cells, but had little effect on WT T_{H1} cells.

Molecular analysis of *Bhlhe40*-regulated target genes

We performed expression microarray analysis on WT and *Bhlhe40*^{-/-} CD4⁺ T cells cultured in T_H1, T_H2, and T_H17 conditions and validated these results by quantitative RT-PCR for selected transcripts (Fig. 5). This analysis confirmed that *Bhlhe40*^{-/-} T cells were able to acquire characteristics largely reflective of normal T_H1, T_H2, or T_H17 differentiation, including their expression of signature cytokines and transcription factors (Fig. 5a, d). However, a common set of genes was affected by the loss of *Bhlhe40* across two or more T_H lineages (Fig. 5b, c, 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 (Supplementary Fig. 7d).

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) Consortium⁴⁴. 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* bound multiple sites, including a previously identified distal enhancer located ~30 kb downstream of *Csf2* that regulates the expression of both cytokine genes in this locus⁴⁵ (Supplementary Fig. 9a). Within the *Il10* locus, *Bhlhe40* also bound multiple sites, including two known regulatory elements^{46,47} (Supplementary Fig. 9b). In both loci, many regions bound by *Bhlhe40* were also bound by the general transcriptional regulators p300^{48,49} and BRG1⁴⁵ and the T_H cell subset-specific transcription factors T-bet⁵⁰ and RORγt⁴⁹. *Bhlhe40* is likely one of many transcriptional regulators present at these regions working in concert to control gene expression.

IL-10R blockade renders *Bhlhe40*^{-/-} mice susceptible to EAE

Because our expression microarray experiments demonstrate that *Bhlhe40* deficiency leads to the dysregulation of hundreds of genes in polarized T_H 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/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/day was reported to replace endogenous GM-CSF in *Csf2*^{-/-} mice, such that these mice developed EAE when GM-CSF was administered after immunization⁵¹. 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, beginning one 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 (Fig. 6a). 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 (Fig. 6b 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-IL-10R 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 regulated by IL-10.

Discussion

Here, we have shown that *Bhlhe40*^{-/-} mice are protected from immunization-induced autoimmune neuroinflammation. T_H 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 T_H1 cell line, but only when a low number of pathogenic T cells were transferred (Supplementary Fig. 2e). In this system, cytokine-producing host T_H cells infiltrate the CNS and participate in regulating neuroinflammation⁴². Large numbers of adoptively-transferred, autoreactive WT T_H1 cells were capable of overcoming a requirement for Bhlhe40 in host cells (Supplementary Fig. 2f). Overall, these results are reminiscent of experiments in which low numbers of an encephalitogenic T_H1 cell line were unable to induce disease in IL-17A-deficient mice, but in which higher cell numbers could initiate neuroinflammation⁴². We favor a model in which the explanation for our adoptive transfer results stems from a requirement for Bhlhe40 in host T_H and $\gamma\delta$ T cells, although it remains a possibility that Bhlhe40 also functions in non-T cells during adoptively transferred EAE.

The characteristics that define pathogenic T_H cells in EAE have been a subject of recent debate²⁴⁻²⁶. Neither IFN- γ nor IL-17, the signature cytokines of T_H1 and T_H17 cells, are required for the development of EAE^{4,5}, yet in vitro derived T_H1 and T_H17 cells specific for myelin antigens both can induce encephalomyelitis^{8,52}. These seemingly conflicting observations have been reconciled by the recent finding that GM-CSF, produced by both T_H1 and T_H17 cells, serves a non-redundant pro-inflammatory role in EAE development⁸⁻¹². Control of *Csf2* expression involves AP-1, NFAT, Runx1, NF- κ B, histone acetylation, and BRG1 recruitment^{14-19,45}. c-Rel- and NF- κ B1-deficient T cells produce less GM-CSF, but these factors may be non-selective in their impact on cytokine production^{17,18}. Malt1, a

protease regulating the NF- κ B pathway, was reported to be required for GM-CSF production by T_H17 cells, but not T_H1 cells⁵³, implying that the pathways that control GM-CSF production may not be the same in all T_H cell lineages. In fact, we find that Bhlhe40 appears to play a less important role in regulating GM-CSF production in T_H2 cells compared to its role in T_H1 or T_H17 cells, as GM-CSF secretion is only partly abrogated in *Bhlhe40*^{-/-} T_H2 cells. Similarly, our observation that *Bhlhe40*^{-/-} mice can infrequently develop clinical EAE with some T cell production of GM-CSF (Supplementary Fig. 2a) 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 homolog to Bhlhe40, is expressed most highly in T_H2 cells amongst activated T_H cell subsets^{40,54}, and perhaps this factor can substitute for Bhlhe40 in some settings in the regulation of *Csf2* transcription.

The role of ROR γ T in GM-CSF production by CD4⁺ T cells is controversial¹⁰⁻¹². One study found that *Rorc*^{-/-} CD4⁺ T cells activated in the presence of anti-IFN- γ and anti-IL-12 neutralizing antibodies produced less GM-CSF than WT CD4⁺ T cells, but *Rorc*^{-/-} T_H1 and T_H17 cells produced normal or even greater GM-CSF than WT T cells¹⁰. These investigators found that retroviral ROR γ T overexpression increased GM-CSF production, so despite these incongruous results, they suggested that ROR γ T drives GM-CSF production. A second study also showed that *Rorc*^{-/-} T_H17 cells produced an increased amount of GM-CSF relative to WT T_H17 cells¹¹, leading this group to suggest that ROR γ T is not required for GM-CSF production. We observed normal *Rorc* expression in *Bhlhe40*^{-/-} T_H17 cells in our microarrays (Fig. 5a), and speculate that Bhlhe40 directly regulates *Csf2* expression.

The recently described molecular signatures of pathogenic versus non-pathogenic T_H17 cells²⁶ includes four genes that we find to be selectively regulated by Bhlhe40 (*Csf2* and *Il3* for pathogenic T_H17 cells; *Il10* and *Ikzf3* for non-pathogenic T_H17 cells) (Fig. 5). 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 *Bhlhe40*^{-/-} T_H 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 c-Maf³³. 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 *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^{55,56}. It also remains possible that some genes whose expression is abnormal in *Bhlhe40*^{-/-} T_H cells are indirect targets of Bhlhe40.

Supporting the importance of IL-10 in EAE pathogenesis, we found that nearly half of all *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 mice²⁸⁻³⁰, 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*^{-/-} 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*^{-/-} 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*^{-/-} T_H cells can be encephalitogenic in the setting of IL-10R blockade. Further experiments will be performed to determine whether *Bhlhe40*^{-/-} T_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⁺ T cell responses using the EAE system⁵⁷. Martínez-Llordella et al. identified *Bhlhe40* as a gene whose expression was induced in activated CD4⁺ T cells in a CD28-dependent manner. These authors found *Bhlhe40*-deficient mice (a separately generated line than was used in our study⁵⁸) to be resistant to the induction of EAE, and found decreased CD4⁺ T cell production of several cytokines, including GM-CSF. Both our study and theirs found decreased IL-17A responses in immunized *Bhlhe40*^{-/-} 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*^{-/-} T_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.⁵⁷. They observed MOG(35-55)-specific CD4⁺ T cells responses in *Bhlhe40*^{-/-} 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- γ , 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 (Fig. 3a, b). 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 (Supplementary Fig. 7a), while they saw an approximately 50% reduction in IL-2 secretion. Our results are consistent with those of Miyazaki et al.³⁸ 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. Lastly, our expression microarrays and phenotypic analysis of polarized *Bhlhe40*^{-/-} T_H cells identified increased transcription and secretion of IL-10 by these cells compared to WT T_H cells. *Il10* was not identified as being differentially expressed in the transcriptional analysis performed by Martínez-Llordella, although we speculate that this may be related to their analysis of naïve T cells activated under non-polarizing conditions at early time points (4 and 24 hours).

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 $\gamma\delta$ 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 sclerosis⁵⁹. Bhlhe40, or pathways that regulate its expression or function, could also represent therapeutic targets in human autoimmunity.

METHODS

Mice

C57BL/6 (Taconic), B6.SJL (Taconic), B6.PL-*Thy1^a/CyJ* (Jackson), *Rag1^{-/-}* (on a C57BL/6 background, Jackson), and *Bhlhe40^{-/-}* mice³⁹ (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^{-/-}* mice³⁸. Flow cytometry of splenocytes and bone marrow from *Bhlhe40^{-/-}* mice in our colony at the ages used in these experiments showed normal leukocyte populations (Supplementary Fig. 10). 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 (1200 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%). 7 to 10 million CD4⁺ T cells 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 100 µg MOG(35-55) (C S Bio Co.) emulsified in CFA (made with 5 mg/ml heat killed *M. tuberculosis* H37Ra (BD Difco) in incomplete Freund's adjuvant (BD Difco)). Pertussis toxin (List Biological Laboratories) was injected intraperitoneally (300 ng) on days 0 and 2. Mice were observed for signs of EAE and graded on a standard 0-5 scale as described¹¹. In some experiments, mice were treated daily with recombinant murine GM-CSF (Peprotech) at a dose of 10 ng/day or 100 ng/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)⁶⁰. Mice were given 250 µg of antibody intraperitoneally two times per week, beginning one 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(35-55)-specific T_H1 cell lines were generated by culturing CD4⁺ T cells from MOG(35-55)-immunized CD90.1 congenic B6.PL-*Thy1^a/CyJ* mice with MOG(35-55), irradiated C57BL/6 splenocytes, IL-2, and IL-12 for 2-4 rounds of stimulation⁴². 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(35-55) or OVA(323-339) emulsified in CFA. Popliteal lymph nodes were collected on day 7.

Cell preparation

DLNs from immunized mice were digested with 250 µg/ml collagenase B (Roche) and 30 U/ml Dnase I (EMD) for 40-60 minutes 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-µm 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-µm 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 µg/ml Type I collagenase (Sigma) and 10 µg/ml DNase I (Sigma) in the presence of 0.1 µg/ml TLCK trypsin inhibitor (Sigma) and 10 mM Hepes (pH 7.4) in HBSS at room temperature for 1 hour. 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 minutes at 1200×g. 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 MOG(35-55) immunized mice were cultured in cIMDM and stimulated with 10 micromolar MOG(35-55) for 4 days before supernatants were collected for ELISAs or before intracellular cytokine staining. 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 setup.

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 µg/ml, clone 145-2C11, BioLegend) with or without plate-bound anti-CD28 antibody for 48-72 hours (5 µg/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_H cell polarization, magnetically purified total CD4⁺ T cells (Invitrogen Dynabeads FlowComp Mouse CD4 kit) or naïve CD4⁺ T cells (Stemcell Technologies Easysep Mouse

Naïve CD4⁺ 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_H1 conditions: IFN-γ (200 ng/ml), IL-12 (2 ng/ml), anti-IL-4 (5 μg/ml, clone 11B11). T_H2 conditions: IL-4 (10 ng/ml), anti-IFN-γ (5 μg/ml, clone XMG1.2 or clone H22), anti-IL-12 (10 μg/ml, clone Tosh, a gift from Emil Unanue, Washington University). T_H17 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, anti-IFN-γ. Cells were split at day 3, and on day 4-5 cells were either used for intracellular cytokine staining or stimulated for 24 hours with plate-bound anti-CD3 and anti-CD28 for assessment of cytokine secretion by ELISA.

ELISPOT and ELISA assays

ELISPOT assays were performed on Multiscreen Filter Plates (Millipore). DLN cells from immunized mice were plated at 5×10^5 - 1×10^6 cells/well in cIMDM and stimulated with 10 micromolar peptide or 1 μg/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 (145-2C11), PB anti-CD4 (RM4-5), APC-Cy7 or PerCP-Cy5.5 anti-CD8α (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, κ), APC or biotin anti-IL-10 (JES5-16E3), and streptavidin-APC. The following anti-mouse antibodies were from BD Biosciences: V450 anti-CD4 (RM4-5) 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 nanomolar, Enzo Life Sciences) in the presence of brefeldin A (1 μg/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 minutes at 4°C followed by streptavidin staining if necessary. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 20 minutes at room temperature followed by permeabilization (0.1% BSA, 0.5% saponin in PBS) or with the BD Cytotfix/Cytoperm Fixation and Permeabilization Kit. Cytokine staining was performed in permeabilization buffer for 20 minutes at 4°C. Staining for intracellular Foxp3, T-bet, Gata-3, and ROR γ t 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, naïve CD4⁺ T cells (CD4⁺B220⁻CD25⁻CD44^{lo}CD62L^{hi}) were purified (98% purity) by cell sorting on a FACS Aria II (BD Biosciences) after B cell depletion using anti-B220 magnetic beads (Invitrogen).

Expression microarrays

Naïve CD4⁺ T cells were cultured in T_H0, T_H1, T_H2, or T_H17 conditions for 4 days and stimulated for 4 hours with PMA and ionomycin before total RNA was isolated (E.Z.N.A. MicroElute Total RNA Kit, Omega Bio-Tek). RNA was amplified (Ovation PicoSL WTA System V2, Nugen), labeled (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 GSE14308⁶⁴, 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, T_H2, or T_H17 conditions for 4 days and stimulated for 4 hours with plate-bound anti-CD3 and anti-CD28 before total RNA was isolated (E.Z.N.A. 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 C_T method.

The following primers were used for quantitative RT-PCR. *Ccl1*, forward 5'-GGCTGCCGTGTGGATACAG-3', reverse 5'-AGGTGATTTTGAACCCACGTTT-3'; *Csf2*, forward 5'-GCCATCAAAGAAGCCCTGAA-3', reverse 5'-GCGGGTCTGCACACATGTTA-3'; *Hprt*, forward 5'-TCAGTCAACGGGGGACATAAA-3', reverse 5'-GGGGCTGTACTGCTTAACCAG-3'; *Ifitm3*, forward 5'-CCCCAAACTACGAAAGAATCA-3', reverse 5'-ACCATCTTCCGATCCCTAGAC-3'; *Ifng*, forward 5'-GGATGCATTCATGAGTATTGC-3', reverse 5'-CCTTTTCCGCTTCCTGAGG-3'; *Ikzf3*,

forward 5'-CTGAATGACTACAGCTTGCCC-3', reverse 5'-GCTCCGGCTTCATAATGTTCT-3'; *Il1a*, forward 5'-CGAAGACTACAGTTCTGCCATT-3', reverse 5'-GACGTTTCAGAGGTTCTCAGAG-3'; *Il13*, forward 5'-GGGATACCCACCGTTTAACCA-3', reverse 5'-AGGTTTACTCTCCGAAAGCTCTT-3'; *Il14*, forward 5'-ATCATCGGCATTTTGAACGAGG-3', reverse 5'-TGCAGCTCCATGAGAACACTA-3'; *Il10*, forward 5'-AGCCTTATCGGAAATGATCCAGT-3', reverse 5'-GGCCTTGTAGACACCTTGGT-3'; *Il17a*, forward 5'-TTTAACTCCCTTGGCGCAAAA-3', reverse 5'-CTTCCCTCCGCATTGACAC-3'; *Ptgs2*, forward 5'-TGCAGTATGGTTACAAAAGCTGG-3', reverse 5'-TCAGGAAGCTCCTTATTTCCCTT-3'; *Xcl1*, forward 5'-TAGCTGTGTGAACTTACAAACCC-3', reverse 5'-ACAGTCTTGATCGCTGCTTTC-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) software⁶² was used as part of the online version of MEME-ChIP(<http://meme.nbcr.net/meme/cgi-bin/meme-chip.cgi>)⁶³ to validate that Bhlhe40 ChIP-Seq data identified the expected consensus motif for Bhlhe40 (CACGTG)⁶⁴ with a significant E-value (4.8×10^{-1391}).

Raw data for the following ChIP-Seq datasets were downloaded from the Gene Expression Omnibus of the NCBI: GSE40463 (p300 in T_H1 and T_H2 cells)⁴⁸, GSE33802 (T-bet in T_H1 cells)⁵⁰, GSE20898 (Gata3 in T_H2 cells)⁶⁵, GSE40918 (ROR γ t and p300 in T_H17 cells)⁴⁹, and GSE23719 (BRG1 in stimulated T_H1, T_H2, and T_H17 cells)⁶⁶. Each of these datasets was put through the following pipeline of analysis within Galaxy (<http://usegalaxy.org/>)⁶⁷⁻⁶⁹: 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 5' and 3' 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'-AATAAGATCTCCACCATGGCAGAACAGAAGCTCATTCTGAAGAAGACTTGAACGAACGGA TCCCCAGCGC-3', reverse, 5'-ATAAAGATCTCCCTCCAGAGTTTAGTCTTTGGTTTCT-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 (IRES) upstream of GFP⁷⁰. Retroviral vectors were transfected into Phoenix E cells by calcium phosphate coprecipitation⁷⁰, 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_H1 or T_H17 conditions, and viral supernatants were used to infect T cells by spin infection at 2000 rpm

in the presence of 2 µg/ml polybrene for 1 hour 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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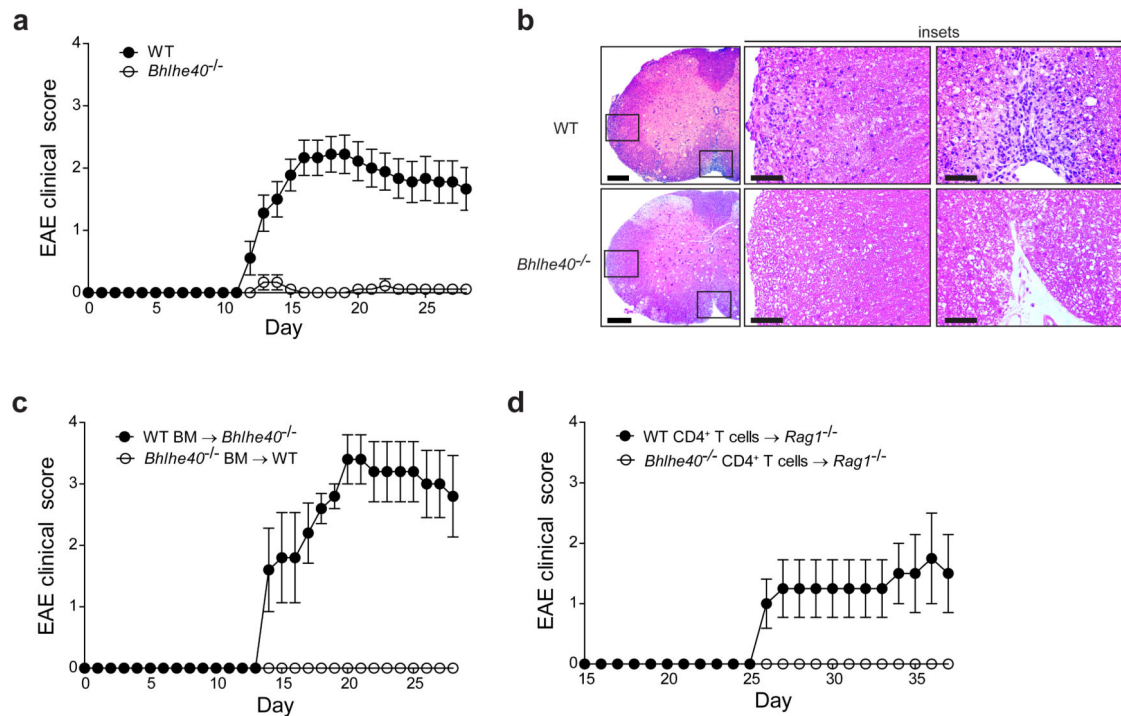


Figure 1. *Bhlhe40*-deficient mice are protected from EAE

a, Mean clinical scores of EAE in immunized WT (n=18) or *Bhlhe40*^{-/-} mice (n=18). Data are combined from 4 independent experiments. Incidence of disease: WT mice 17/18, *Bhlhe40*^{-/-} mice 2/18. **b**, H&E staining of spinal cord sections from WT and *Bhlhe40*^{-/-} mice at day 13 after EAE induction. Scale bars 200 μ m in left images, 50 μ m in insets. **c**, Mean clinical scores of EAE in BM chimeric mice 13 weeks after bone marrow reconstitution (n=5 per group). **d**, Mean clinical scores of EAE in *Rag1*^{-/-} mice that received CD4⁺ T cells from either WT or *Bhlhe40*^{-/-} mice one day prior to immunization (n=4 per group). For all figures throughout, error bars show mean \pm s.e.m.

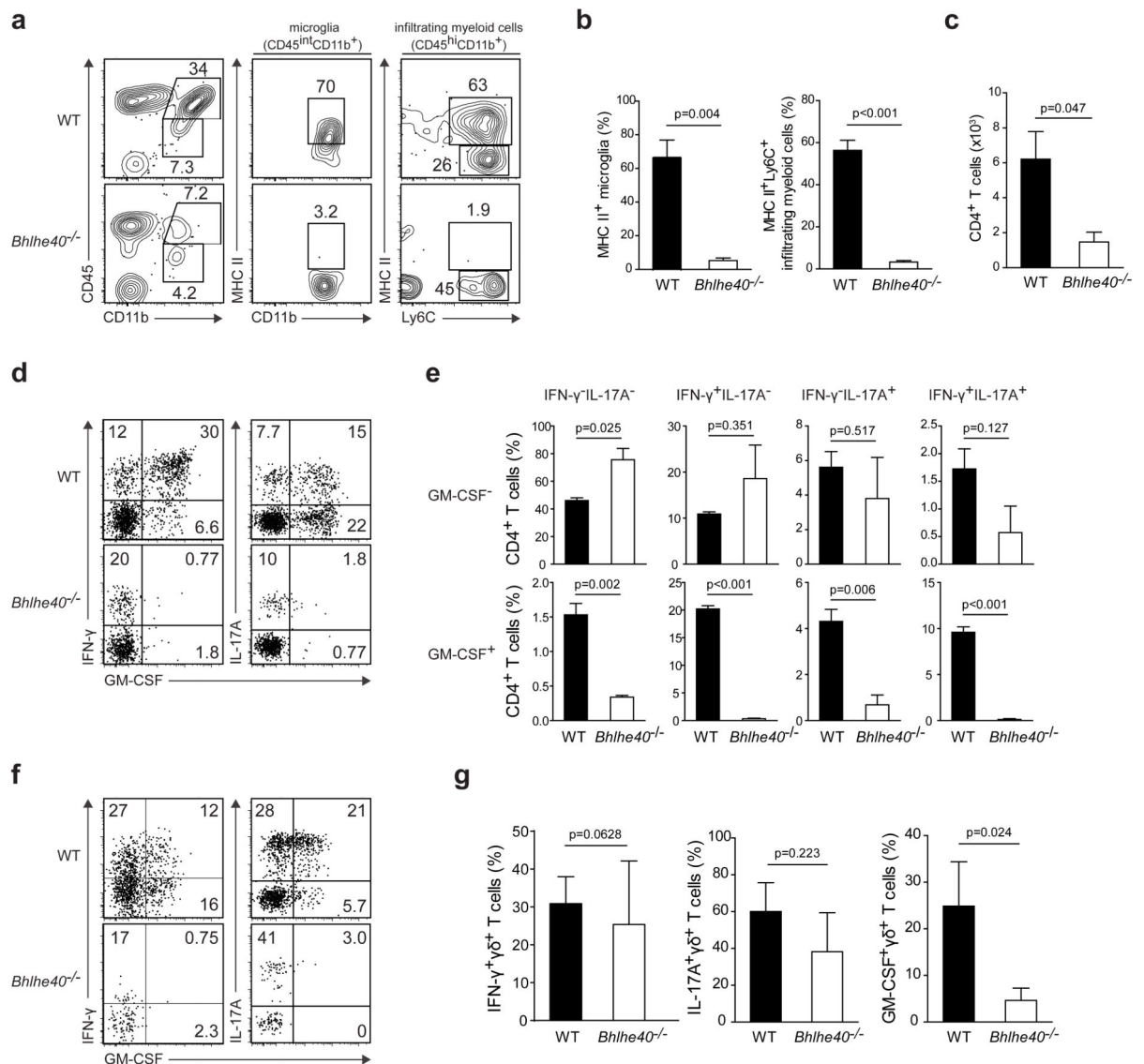


Figure 2. Diminished neuroinflammation in *Bhlhe40*^{-/-} mice

a, Flow cytometry of CNS myeloid cells from WT and *Bhlhe40*^{-/-} mice (n=3 per group) on day 16 after immunization. **b**, Percentage of MHC class II⁺ microglia and infiltrating myeloid cells. **c-e**, Flow cytometry of CD4⁺ T cells in the CNS of WT and *Bhlhe40*^{-/-} mice (n=3 per group) on day 16 after immunization. **(c)** CD4⁺ T cell number. **(d)** Representative ICS for the indicated cytokines. **(e)** Frequency of CD4⁺ T cells secreting each of the indicated 8 possible combinations of GM-CSF, IL-17A, and IFN- γ . **f, g**, Flow cytometry of $\gamma\delta$ T cells in the CNS of WT and *Bhlhe40*^{-/-} mice (n=3 per group) on day 14 after immunization. **(f)** Representative ICS for IFN- γ , IL-17A, and GM-CSF. **(g)** Frequency of $\gamma\delta$ T cells secreting IFN- γ , IL-17A, or GM-CSF.

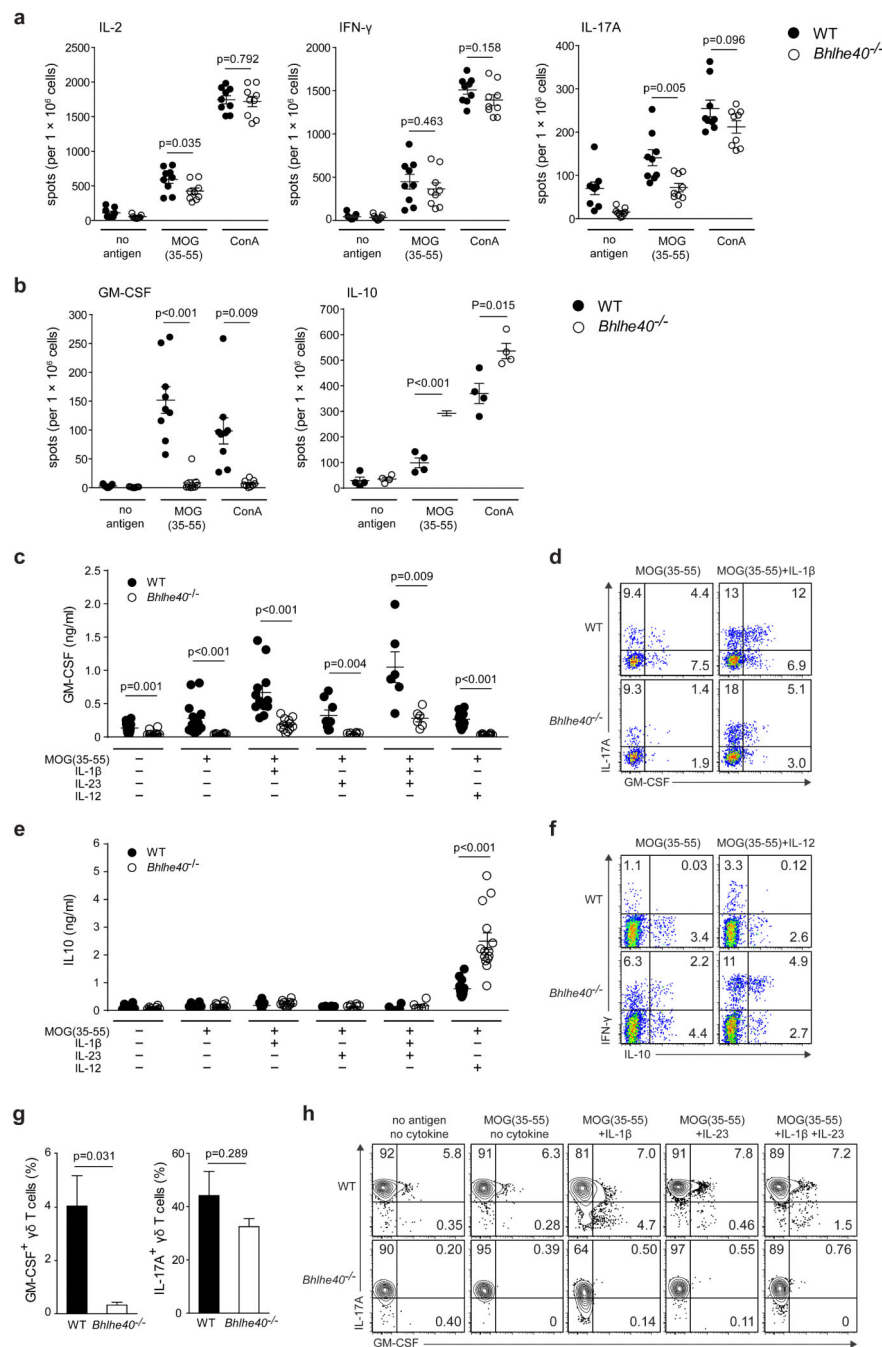


Figure 3. T cells require *Bhlhe40* for normal cytokine production after immunization
a, b, ELISPOT assays for the quantitation of cells secreting **(a)** IL-2, IFN- γ , and IL-17A or **(b)** GM-CSF and IL-10 performed on DLN cells 7 days after immunization of WT and *Bhlhe40*^{-/-} mice. Data for IL-2, IFN- γ , IL-17A, and GM-CSF are combined from 3 independent experiments (n=9 mice per group). Data for IL-10 is from one representative experiment of two (n=4 mice per group). **c, e**, DLN cells from immunized WT and *Bhlhe40*^{-/-} mice (n=14 per group) were cultured with or without MOG(35-55) and with or without IL-1 β , IL-23, and/or IL-12 as indicated. **(c)** GM-CSF or **(e)** IL-10 was measured in

the supernatant at day 4. Data are combined from 5 independent experiments. Cells from all mice were not used in all conditions in each of the 4 experiments. **d**, DLN cells from immunized WT and *Bhlhe40*^{-/-} mice were cultured with MOG(35-55) with or without IL-1 β for 4 days, followed by ICS. Representative plots are gated on CD4⁺ T cells. **f**, 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. **g**, Frequencies of GM-CSF⁺ and IL-17A⁺ $\gamma\delta$ T cells in DLNs 7 days after immunization of WT and *Bhlhe40*^{-/-} mice (n=3 per group) as determined by ICS. **h**, DLN cells from immunized WT and *Bhlhe40*^{-/-} mice were cultured with or without MOG(35-55) and with or without IL-1 β and/or IL-23 as indicated for 4 days. Cells were stimulated with PMA/ionomycin in the presence of brefeldin A for 4 hours and then analyzed for IL-17A and GM-CSF by intracellular staining (i.e. our normal ICS protocol). Representative plots are gated on $\gamma\delta$ T cells.

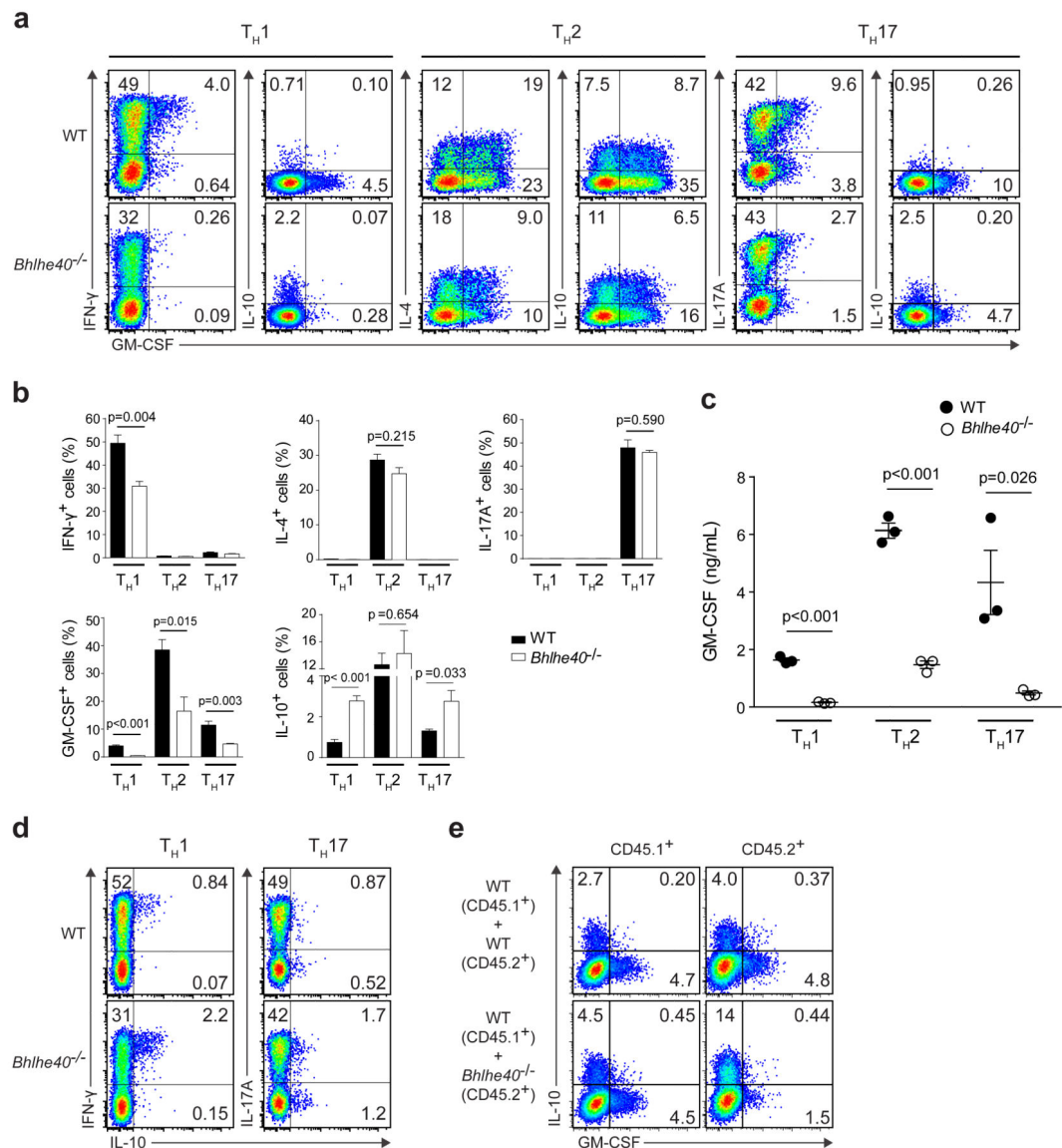


Figure 4. T_H cells require Bhlhe40 for normal cytokine production in vitro

a-d, CD4⁺ T cells from WT and *Bhlhe40*^{-/-} mice (n=3 per group) were polarized in T_{H1}, T_{H2}, or T_{H17} culture conditions for 4 days. **(a)** Representative ICS for the indicated cytokines. **(b)** Frequencies of IFN- γ ⁺, IL-4⁺, IL-17A⁺, GM-CSF⁺, and IL-10⁺ cells in T_{H1}, T_{H2}, and T_{H17} cultures. **(c)** T_{H1}, T_{H2}, and T_{H17} cells were stimulated for 24 hours with anti-CD3 and anti-CD28. GM-CSF was measured in the supernatant by ELISA. **(d)** Representative ICS for the indicated cytokines. **e**, CD4⁺ 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.

regions indicate the number of probes with greater than or equal to a 2-fold difference in expression (WT>KO or WT<KO). **d**, Quantitative RT-PCR analysis of *Ifng*, *Il4*, and *Il17a* expression in WT and *Bhlhe40*^{-/-} T_H1, T_H2, and T_H17 cells (n=3 per group). **e**, Quantitative RT-PCR analysis of the expression of the indicated 9 genes in WT and *Bhlhe40*^{-/-} T_H1, T_H2, and T_H17 cells (n=3 per group).

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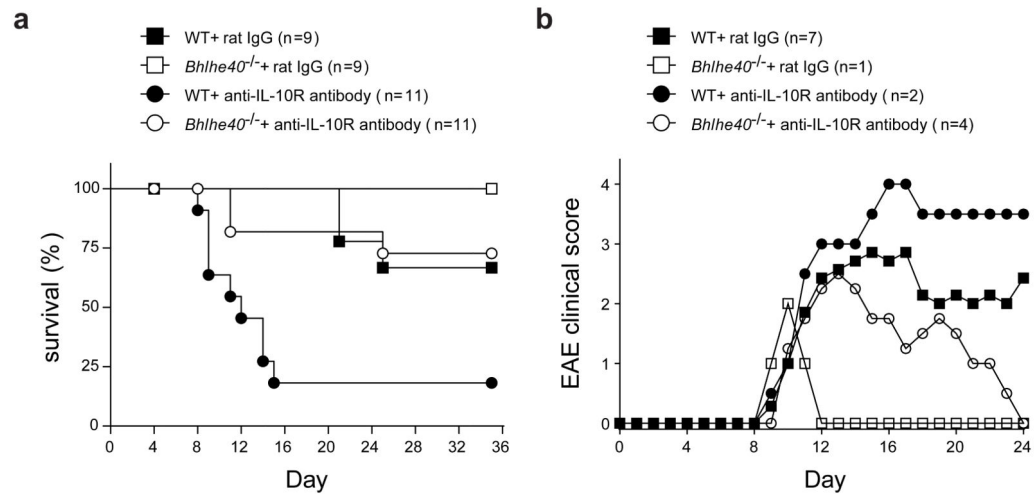


Figure 6. IL-10R blockade renders *Bhlhe40*^{-/-} mice susceptible to EAE

a, Survival of WT or *Bhlhe40*^{-/-} mice after EAE induction and biweekly treatment with control rat IgG or anti-IL-10R antibody. Data are combined from 3 independent experiments. **b**, Mean clinical scores of EAE in diseased WT or *Bhlhe40*^{-/-} mice treated biweekly with either control rat IgG or anti-IL-10R antibody. Data are combined from 3 independent experiments. Error bars are not shown for clarity. All s.e.m. values were ≤ 1 .

Table 1
Clinical EAE data in WT and *Bhlhe40*^{-/-} mice treated with rat IgG or anti-IL-10R antibody

	Day of onset*	Incidence	Maximum score*
WT + rat IgG	11 ± 0.4	100% (7/7)	3.43 ± 0.20
<i>Bhlhe40</i> ^{-/-} + rat IgG	9.0	11.1% (1/9)	2.00
WT + anti-IL-10R	10 ± 1.0	100% (2/2)	4.00 ± 0.00
<i>Bhlhe40</i> ^{-/-} + anti-IL-10R	12 ± 1.5	44.4% (4/9)	3.25 ± 0.25

* Mean ± s.e.m. of diseased mice.

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