BRIEF DEFINITIVE REPORT

The transcription factor Bhlhe40 is a switch of inflammatory versus antiinflammatory Th1 cell fate determination

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Type 1 T helper (Th1) cells play a critical role in host defense against intracellular pathogens and in autoimmune diseases by producing a key inflammatory cytokine interferon (IFN)-γ; some Th1 cells can also be antiinflammatory through producing IL-10. However, the molecular switch for regulating the differentiation of inflammatory and antiinflammatory Th1 cells is still elusive. Here, we show that Bhlhe40-deficient CD4 Th1 cells produced less IFN-γ but substantially more IL-10 than wildtype Th1 cells both in vitro and in vivo. Bhlhe40-mediated IFN-γ production was independent of transcription factor T-bet regulation. Mice with conditional deletion of Bhlhe40 in T cells succumbed to Toxoplasma gondii infection, and blockade of IL-10 signaling during infection rescued these mice from death. Thus, our results demonstrate that transcription factor Bhlhe40 is a molecular switch for determining the fate of inflammatory and antiinflammatory Th1 cells.

Introduction

Naïve CD4 T cells differentiate into distinct subsets of T helper (Th) cells during immune responses (Zhu et al., 2010). Th subsets play a critical role in protective immunity against a variety of infections and are involved in different forms of inflammatory diseases. Type 1 Th (Th1) cells are indispensable for fighting against infections with intracellular pathogens. Th1 cells are also responsible for the pathogenesis of many autoimmune diseases. The transcription factor T-bet is the master transcriptional regulator for the development and functions of Th1 cells (Szabo et al., 2000; Lazarevic et al., 2013). T-bet directly regulates the expression of Th1 effector cytokine IFN-γ (Yagi et al., 2010; Zhu et al., 2012). Besides T-bet, other Th1 lineage–specific transcription factors, such as Runx3 and Hlx, either directly or indirectly regulate IFN-γ expression (Mullen et al., 2002; Djuretic et al., 2007; Yagi et al., 2010). It is possible that other lineage-specific transcription factors are also involved in this process (Hu et al., 2013).

IL-10 is an antiinflammatory cytokine. IL-10–producing CD4 T cells that possess regulatory functions are designated as TR1 cells (Roncarolo et al., 2006). However, Foxp3-expressing regulatory T (T reg) cells and GATA3-expressing Th2 cells also express IL-10 (Maynard et al., 2007; Wei et al., 2011). Furthermore, some Th1 cells are capable of producing IL-10 during Leishmania major or Toxoplasma gondii infection, which elicits a very robust Th1 response (Anderson et al., 2007; Jankovic et al., 2007). The balance between the expression of inflammatory IFN-γ and anti-inflammatory IL-10 by Th1 cells is critical for host mounting an appropriate immune response in controlling parasites. IFN-γ– or IL-10–deficient mice succumb to T. gondii infection as a result of either ineffective or excessive immune response, respectively (Hunter et al., 1994; Gazzinelli et al., 1996; Neyer et al., 1997). However, the molecular mechanism of regulating the balance between IFN-γ and IL-10 production in T cells is still elusive.

The transcription factor Bhlhe40, also known as Bhlhb2, Dec1, and Stra13, is up-regulated during T cell activation (Sun et al., 2001). In fact, IRF4 and Bhlhe40 are the top two transcription factors whose expression is highly induced within 4 h of T cell activation (Hu et al., 2013). It has been reported that Bhlhe40 is critically important for inducing autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (Martinez-Llordella et al., 2013; Lin...
et al., 2014, 2016). However, the function of Bhlhe40 in type 1 immune response, particularly in vivo, has not been investigated.

Here, we report that transcription factor Bhlhe40 is required for optimal production of IFN-γ by Th1 cells both in vitro and in vivo, and this effect is independent of T-bet induction. However, Bhlhe40 suppresses IL-10 production by Th1 cells. Bhlhe40-deficient CD4 T cells, producing less IFN-γ but more IL-10, failed to induce colitis in mice in a transfer model. In addition, Bhlhe40 conditional knockout (cKO) mice are susceptible to T. gondii infection. Blockade of IL-10 signaling in Bhlhe40 cKO mice during T. gondii infection prevented these mice from death. Therefore, Bhlhe40 serves as an important molecular switch for the development of inflammatory and antiinflammatory Th1 cells.

**Results and discussion**

**Characterization of Bhlhe40 cKO mice in the context of previous studies**

Bhlhe40 is a transcription factor regulating circadian rhythms (Honma et al., 2002). Within the immune system, Bhlhe40 is not only expressed in activated T cells, but also expressed in eosinophils, macrophages, and dendritic cell subsets (Lin et al., 2016).

To investigate the role of Bhlhe40 in T cells, we generated a cKO mouse strain, Bhlhe40<sup>fl/fl</sup>-CD4-Cre, in which the Bhlhe40 gene is deleted only in T cells (Fig. S1 A). Bhlhe40 cKO mice were born at the expected Mendelian ratio and appeared to be as healthy as their Bhlhe40<sup>fl/wt</sup> WT littermates and C57BL/6 WT mice.

Because it has been reported that Bhlhe40 germline KO (Bhlhe40<sup>−/−</sup>) mice are resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were markedly resistant to EAE induction, we first examined our Bhlhe40 cKO mice using this model. Consistent with the previous studies (Miyazaki et al., 2010), Bhlhe40<sup>−/−</sup> mice were marked...
Figure 1. Bhlhe40 regulates IFN-γ production by in vitro differentiated Th1 cells without affecting T-bet induction. (A) In vitro differentiation of Th1 and Th17 cells from sorted naive CD4 T cells with plate-bound anti-CD3/CD28 for 4 d. Flow cytometric analysis of IFN-γ and IL-17A production by CD4+CD44hi Th1 and Th17 cells, respectively, from WT and cKO mice (left). Graphical representation of relative IFN-γ production of cKO mice compared with WT set as 1 (right). A representative of five independent experiments is shown. In each experiment at least two mice were used in each group (mean ± SEM, n = 5). Statistical significance was determined by a two-tailed unpaired Student’s t test.

Bhlhe40 cKO Th1 cells overproduce IL-10

Our RNA-Seq analysis revealed 245 genes that are regulated by Bhlhe40 in Th1 cells (Table S1; fold change > 2; false discovery rate < 0.01; reads per kilobase of exon model per million reads [RPKM] > 2). Strikingly, Il10 is the most up-regulated gene in cKO Th1 cells compared with WT Th1 cells (Fig. 2 A and Table S1). Interestingly, two IL-10–inducing transcription factors, c-Maf (Pot et al., 2009) and IKZF3 (Evans et al., 2014), were increased in Bhlhe40-deficient Th1 cells (Fig. 2 A and Table S1), suggesting that Bhlhe40’s effect on IL-10 expression may be indirect. Whether up-regulation of c-Maf and/or IKZF3 is responsible for increased IL-10 expression in Bhlhe40-deficient Th1 cells requires further investigation. The increased IL-10 production in cKO Th1 cells was confirmed at the protein level by intracellular staining (Fig. 2 B). Interestingly, although IL-10 was produced by WT Th1 cells that also expressed IFN-γ as previously reported (Jankovic et al., 2007), both IFN-γ–expressing and nonexpressing cKO Th1 cells were capable of producing IL-10.

To test whether increased IL-10 production is responsible for reduced IFN-γ by Bhlhe40-deficient Th1 cells, we used anti–IL-10R to block IL-10 signaling during the culture. Even in the presence of anti–IL-10R, Bhlhe40-deficient Th1 cells still produced less IFN-γ but more IL-10 compared with WT Th1 cells (Fig. 2 C), indicating that regulation of IFN-γ production by Bhlhe40 is largely independent of IL-10 up-regulation. The results from coculture of WT cells with Bhlhe40-deficient cells further confirm this conclusion (Fig. S2 A). Ifng mRNA was induced in both WT and Bhlhe40-deficient cells at 48 h of activation; however, Ifng transcription was transient in Bhlhe40-deficient cells (Fig. S2 B, upper panel). Il10 transcription kept rising over time in Bhlhe40-deficient Th1 cells, and WT Th1 cells expressed much less IL-10 (Fig. S2 B, lower panel). In addition, more IL-10 production was detected in Bhlhe40-deficient cells compared with WT cells when they were cultured in the presence of IL-27 or under Th2 conditions (Fig. 2 C). Overall, our data suggest that Bhlhe40 may serve as a molecular switch in determining Th1 cell fates for the expression of either inflammatory cytokine IFN-γ or antiinflammatory cytokine IL-10.

Bhlhe40-deficient CD4 T cells fail to induce colitis

To assess Bhlhe40 function in Th1 cells in vivo, we used the inflammatory bowel disease model induced by transferring naive CD4 T cells into Rag1−/− recipients. As expected, transferring WT naive CD4 T cells led to severe inflammation associated with OVA323–339 peptide alone, together with IFN-γ, or anti–IFN-γ antibody as indicated. Gray solid, OVA peptide alone; red line, OVA + IFN-γ; blue line, OVA+ anti–IFN-γ antibody. Data are representative of three independent experiments, and in each experiment at least three mice in each group were used (C and D). (E) Gene expression values (RPKM) of Ifng, Tbx21, and Runx3 from RNA-Seq analysis of C57BL/6 WT and Bhlhe40 cKO Th1 cells (n = 2). ns, not significant; *, P < 0.05; **, P < 0.01; ****, P < 0.0001; Student’s t test.
**Bhlhe40 promotes inflammatory Th1 cells**

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**Altered balance between IFN-γ and IL-10 in Bhlhe40 cKO mice that are susceptible to T. gondii infection**

Previous studies have shown that balance between the production of inflammatory IFN-γ and antiinflammatory IL-10 is critical for mounting an appropriate immune response against *T. gondii* infection (Gazzinelli et al., 1996; Neyer et al., 1997; Jankovic et al., 2007). To test whether altered expression of IFN-γ and IL-10 in the absence of Bhlhe40 has any physiological consequence, we infected WT and Bhlhe40 cKO mice with *T. gondii*. As a consequence of *T. gondii* exposure, serum levels of IL-12p40 were increased dramatically in both infected WT and cKO mice (Fig. S2 C). In contrast to WT mice, which survived for the entire period of the experiments, all the cKO mice infected with an avirulent strain of *T. gondii* succumbed to infection (Fig. 4 A). The mortality was observed in between acute and chronic phase of infection (between 2–4 wk after infection). Interestingly, treating cKO mice with anti–IL-10R–blocking mAb before and after infection rescued these mice from death (Fig. 4 B). As expected, Bhlhe40 cKO mice were less efficient than WT mice in clearing parasites; however, anti–IL-10R treatment rescued this defect (Fig. 4 C). Furthermore, the levels of a liver enzyme aspartate transaminase (AST) in response to infection were identical between WT and Bhlhe40 cKO mice, indicating that Bhlhe40 cKO mice do not die of excessive immune responses (Fig. S2 D). We also found no defect in the generation of antigen-specific cells in the absence of Bhlhe40 (Fig. 4 D, upper panel). However, IFN-γ production by cKO cells upon antigen stimulation was substantially lower than that detected in the similarly treated WT cells (Fig. 4 D, middle panel), whereas IL-10 production by cKO cells were higher than that of WT cells (Fig. 4 D, lower panel). IFN-γ from cKO mice injected with anti–IL-10R were dramatically increased compared with that in control mAb–treated cKO mice, indicating IL-10 blockade enhances IFN-γ production (Fig. 4 D, middle panel). However, such levels were still significantly lower than that found in the infected WT mice, indicating that Bhlhe40 is required for optimal IFN-γ production by Th1 cells in response to *T. gondii* infection. Whether Bhlhe40 also regulates IFN-γ production in antigen-specific CD8 T cells and, if so, how this defect may add to the defect of Bhlhe40-deficient CD4 T cells in host defense requires further investigation. Nevertheless, partial restoration of the balance between IFN-γ and IL-10 in cKO mice is sufficient to elicit protective immunity.

Collectively, our results indicate that Bhlhe40 serves as a molecular switch in determining the balance between the inflammatory cytokine IFN-γ and the antiinflammatory cytokine IL-10 in Th1 cells both in vitro and in vivo. The regulation of IFN-γ by Bhlhe40 is independent of T-bet regulation. Future understanding of pathways and/or molecules that regulate the expression of Bhlhe40 may offer new drug targets aiming to boost immune response or to limit inflammation.

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Figure 2. **Bhlhe40 suppresses IL-10 production by Th1 cells.** (A) Gene expression values (RPKM) of Il10, Maf, and Iκzf3 from RNA-Seq analysis of C57BL/6 WT and Bhlhe40 cKO Th1 cells (n = 2). (B) Sorted naive CD4 T cells cultured under Th1-polarizing conditions with plate-bound anti-CD3/CD28 for 4 d and restimulated with PMA-ionomycin for 4 h. Flow cytometric analysis of IFN-γ/IL-10 production (top) and IL-10 expression (bottom left) by CD4+CD44hi WT and cKO Th1 cells. Gray solid, WT; red line, cKO. Bottom right: IL-10 production from three independent experiments (≥2 mice per group). *, P < 0.05; **, P < 0.01; ***, P < 0.001; Student’s t test. (C) Sorted naive CD4 T cells cultured under indicated conditions with plate-bound anti-CD3/CD28 for 4 d and restimulated with PMA-ionomycin for 4 h. Flow cytometric analysis of IFN-γ/IL-10 production by CD4+CD44hi WT and Bhlhe40 cKO cells. Data are representative of two independent experiments (≥2 mice per group).

significant weight loss and colon thickening, whereas transferring cKO-naive CD4 T cells did not cause weight loss (Fig. 3 A). The failure of cKO CD4 T cells in inducing disease was not a result of a defect in cell expansion in vivo, because absolute cell number of CD4 T cells recovered from the recipients transferred with either WT or cKO naive cells was similar (Fig. 3 B).

Very few regulatory T cells were generated in this transfer model (<5%), and there was no difference between the WT and cKO groups (unpublished data). We also measured cytokine production by CD4 T cells recovered from spleen and lymph nodes of recipients at the end of experiments. Consistent with the data from in vitro studies, cKO CD4 T cells showed decreased IFN-γ but increased IL-17A production compared with WT CD4 T cells (Fig. 3 C). We also assessed IFN-γ and IL-10 expression 2 wk after transfer. Consistent with in vitro findings, IL-10 expression by cKO Th1 cells generated in vivo was increased, whereas IFN-γ expression was reduced compared with WT Th1 cells both at the mRNA and protein level (Fig. 3, D and E).

Although more experiments are needed to determine the role of IL-10 overproduction by cKO cells in this model, our data strongly indicate that Bhlhe40 plays an important role in inducing the expression of proinflammatory cytokine IFN-γ, but suppressing the expression of antiinflammatory cytokine IL-10 in Th1 cells in vivo.
Materials and methods

Mice

C57BL/6 mice and C57BL/6 Flpe (Taconic Line 7089) were purchased from Taconic Farms. C57BL/6-CD45.1 mice (Taconic Line 7), C57BL/6 Rag1−/− mice (Taconic Line 146), C57BL/6-CD44Rag1−/− OT-II (Taconic Line 187), and C57BL/6 CD4-Cre (Taconic Line 4196) were obtained from the National Institute of Allergy and Infectious Diseases (NIAID)-Taconic repository. To generate Bhlhe40fl/fl, CD4-Cre mice, mouse embryonic stem cells containing the Bhlhe40fl/fl(KOMP)Wtsi allele (clone EPD0208_6_A02) were obtained from the University of California, Davis, KOMP Repository. This allele has a trapping cassette, SA-β-neo-polA (splice acceptor-β-neo-polA), flanked by flipase recombinase (Flp) target FRT sites upstream of exon4, resulting in truncation of the endogenous transcript and thus creating a constitutive null mutation. The cassette also tags the gene with a lacZ reporter. The FRT flanked region can be removed by Flp, but exon4, a critical exon, remains intact. Exon4 can be further removed by the Cre recombinase to achieve a cKO. The embryonic stem cells were injected into C57BL/6 blastocysts. Chimeric mice were born and mice were genotyped by Southern blotting as per instructions of the IKMC project 24480. After successful germline transmission, mice were genotyped by Southern blotting as per instructions of the IKMC project 24480. Mice were weighed weekly. Statistical significance of body weight of Bhlhe40 cKO versus WT (mean ± SEM, n = 5) at different time points was determined by a two-tailed unpaired Student’s t test. Data are representative of three independent experiments. (B) Graphical representation of the absolute number of CD4 T cells harvested from spleen and mesenteric lymph node (MLN) of Rag1−/− mice, in which sorted naive CD4+CD45R0 T cells from each WT (red dots) or Bhlhe40 cKO (black squares) were i.v. transferred for 4 wk (n = 5 per group) are shown. Data are representative of two independent experiments. (C) Percentages of IFN-γ- and IL-17A+ cells among the CD4+CD44Rag1−/− OT-II cells in spleen and MLN from Rag1−/− mice received WT (dots) or Bhlhe40 cKO transfer (squares) for 8 wk (mean ± SEM, n = 5). Data are representative of three independent experiments. (D) Sorted naive CD4 T cells from WT or Bhlhe40 cKO were transferred into Rag1−/− mice. 2 wk after transfer, CD4 T cells were sorted from the spleens and RNAs were prepared from sorted cells. Real time PCR analysis of Ifng and Il10 mRNA was performed (mean ± SEM, n = 5). Data are representative of two independent experiments. (E) Sorted naive CD4 T cells from C57BL/6 or Bhlhe40 cKO were transferred into Rag1−/− mice. 2 wk after transfer, cells from MLN were restimulated with PMA and ionomycin, and then intracellular staining for IFN-γ and IL-10 was performed. Flow plots were gated on CD4+CD44Rag1−/− cells. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; Student’s t test. Data are representative of two independent experiments.
genotyped by PCR using primer pairs: 5’-AAAGGAGAAAAAGGCTTCC-3’ and 5’-ACACACGGGTTCTGGTTC-3’; 5’-GCTGCTGCA CAGACCTACT-3’ and 5’-CAGCTACCTACAACGAGGA-3’.

All the mice were bred and/or maintained in the NIAID-specific pathogen free animal facility, and the experiments were done when mice were 8- to 14-wk old under protocols approved by the NIAID Animal Care and Use Committee.

### Cell Preparation and cell sorting

Total CD4 T cells were purified by using mouse CD4 (L3T4) microbeads (Miltenyi Biotec Inc.) according to the manufacturer’s instructions. Naive CD4 T cells were isolated from lymph nodes. Lymph node cells were stained with FITC-anti-CD4, PE-anti-CD25, and APC-anti-CD45Rb and then sorted for CD4+CD25loCD25hi population using FACSAria (BD Biosciences). For some in vitro culture experiments, lymph node cells were stained with APC-anti-CD4, APC-Cy7-anti-CD44, PE-anti-CD25, and PB-anti-CD62L and then sorted for CD4+CD44loCD25hiCD62Llo naive CD4 population.

### Cell culture and proliferation assay

Cell culture medium and conditions were described previously (Yu et al., 2015). CFSE (Invitrogen Corporation) was used to detect the proliferation of naive CD4 T cells according to the manufacturer’s instructions. Cells were harvested on day 4, and the intensity of CFSE staining of CD4 T cells was determined by using a flow cytometer LSRII (BD Biosciences) and analysis was performed by FlowJo software (Tree Star). Equal numbers of sorted naive CD4 T cells were stimulated with plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) in the absence or presence of IL-2 (50 U/ml) containing RPMI 1640 media. The number of live cells was counted on day 4 after trypan blue staining.

### In vitro T cell differentiation and staining

Sorted naive CD4 T were stimulated with plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) or with APC prepared from T cell–depleted splenocytes (Zhu et al., 2012). All cytokines used in cell culture were purchased from PeproTech. T cell–depleted splenocytes were prepared by incubation with anti-Thy1.2 mAb supernatant and rabbit complement (Cedarlane Laboratories Limited) at 37°C for 45 min followed by irradiation at 30 Gy (3,000 rad). Naive CD4 T cells were cultured with irradiated T cell–depleted splenocytes at a ratio of 1:5 in the presence of 1 µg/ml of anti-CD3 and 3 µg/ml of anti-CD28 for 3–4 d with various combinations of antibodies and cytokines: for Th1 cell conditions, 50 IU/ml of hIL-2, 10 ng/ml of IL-12, and 10 µg/ml anti–IL-4 (11B11; some Th1 cell cultures include 10 ng/ml IFN-γ or IL-27 10ng/ml as specified); for Th17 cell conditions, 5 ng/ml TGF-β, 10 ng/ml IL-1β, 10 µg/ml anti-IL-4, 10 µg/ml anti-IL-12, and 10 µg/ml anti–IFN-γ; for Th2 cell conditions, 50 IU/ml of hIL-2, 10 ng/ml of IL-4, 10 µg/ml anti–IL-10, and 10 µg/ml anti–IFN-γ. For inducible T reg cell–polarizing condition, cells were cultured with plate-bound anti-CD3/CD28 in the presence of 100 IU/ml of hIL-2, 10 ng/ml of IL-12, and 10 µg/ml anti-IL-4 (11B11; some Th1 cell cultures include 10 ng/ml IFN-γ or IL-27 10ng/ml as specified); for Th17 cell conditions, 5 ng/ml TGF-β, 10 ng/ml IL-1β, 10 µg/ml anti-IL-4, 10 µg/ml anti–IL-12, and 10 µg/ml anti–IFN-γ. For cells cultured in neutral conditions (Thneu), 50 IU/ml of hIL-2. In some Thneu cell cultures, naive CD4 T cells from OTII mice with dendritic cells (DCs)
purified by CD11c microbeads (Miltenyi Biotec Inc.) were activated by OVA233−339 peptide (10 μM) in the absence or presence of either 10 ng/ml IFN-γ or 10 μg/ml anti–IFN-γ as indicated. All cytokines used in cell culture were purchased from PeproTech. All antibodies used in cell culture were purchased from Harlan. Cell surface molecules were stained in PBS with 2% FBS. Cells were stimulated with 10 ng/ml FMA and 500 nM ionomycin for 4 h or stimulated with plate-bound anti-CD3/anti-CD28 for 5 h in the presence of 2 μM monensin during the last 2 h. Cells were stained with a cocktail of fixable viability dye (eBioscience) and antibodies to cell surface markers and then fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized in PBS containing 0.5% Triton X-100 and 0.1% BSA. They were then stained intracellularly for cytokines. Staining for transcription factors was performed with the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. Flow cytometry data were collected with an LSR II (BD Biosciences) and the results were analyzed by using FlowJo’s software (Tree Star). Antibodies specific for mouse CD4 (RM4-5), CD25 (PC61.5), CD44 (IM7), CD45.1 (A20), CD45Rb (C363.16A), IL-4 (11B11), IFN-γ (XMG1.2), IL-17A (TC11-18H10), Foxp3 (FJK-16s), T-bet (4B10), and CD45RBhi T cells from either C57BL/6, Blhle40fl/fl, or Blhle40fl/fl-CD4-Cre mice were adoptively transferred to Rag1−/− recipient mice (Line 146). Each mouse received 2−3 × 105 naive CD4 T cells intravenously. In some experiments, CD4+CD25− YFP T reg cell population were sorted from lymph nodes of Blhle40fl/fl- Foxp3-Cre, Blhle40fl/fl, Foxp3-Cre mice stained with APC-anti-CD4 and PE-anti-CD25. They were cotransferred with sorted naive CD4+CD25−CD45RBhi T cells from CD45.1 congenic mice into Rag1−/− recipients at a 1:4 ratio. The body weight of mice was monitored weekly for all experiments.

**T cell transfer model of colitis**

Sorted naïve CD4+CD25−CD45RBhi T cells from either C57BL/6, Blhle40fl/fl, or Blhle40fl/fl-CD4-Cre mice were adoptively transferred to Rag1−/− recipient mice (Line 146). Each mouse received 2−3 × 105 naïve CD4 T cells intravenously. In some experiments, CD4+CD25− YFP T reg cell population were sorted from lymph nodes of Blhle40fl/fl- Foxp3-Cre, Blhle40fl/fl, Foxp3-Cre mice stained with APC-anti-CD4 and PE-anti-CD25. They were cotransferred with sorted naive CD4+CD25−CD45RBhi T cells from CD45.1 congenic mice into Rag1−/− recipients at a 1:4 ratio. The body weight of mice was monitored weekly for all experiments.

**Induction of EAE**

For induction of active EAE, 8- to 10-wk-old female Blhle40fl/fl WT or Blhle40fl/fl-CD4-Cre mice were immunized at two sites subcutaneously with 150 µg MOG 35–55 in complete Freund’s adjuvant (Sigma) containing Mycobacterium tuberculosis strain H37Ra (5 mg/ml; Difco). Mice were injected i.p. on days 0 and 2 with 200 ng pertussis toxin (Difco) in PBS. EAE was clinically assessed by daily assignment of scores on a scale of 0−5 as follows: partially limp tail, 0.5; completely limp tail, 1; limp tail and waddling gait, 1.5; paralysis of one hind limb, 2; paralysis of one hind limb and partial paralysis of the other hind limb, 2.5; paralysis of both hind limbs, 3; ascending paralysis, 3.5; paralysis of trunk, 4; moribund, 4.5; and death, 5.

**T. gondii infection**

Type II avirulent strain ME49 cysts were obtained from the brains of chronically infected C57BL/6 mice. To eliminate host cell contamination, the cyst preparations were treated with pepsin. Mice were inoculated i.p. with a mean of 15–20 cysts per animal. WT and Blhle40 CKO mice were injected i.p. with 1 mg per animal of the anti–IL-10R (1B1.3a) or the IgG1 isotype control antibody (BioXCell) on days −2 and 2 of T. gondii infection. Parasite burden was determined by counting infected cells in cytosin smears obtained from peritoneal exudate cells (PECs) on day 8 and 13. PECs harvested 13 d after infection were stained at 37°C for 1 h with Tetramer I-A^K−AIVEIHRPVGTAPPS-APC or -BV421 provided by the National Institutes of Health Tetramer Core Facility to assess AS15 peptide-specific CD4 T cells (Grover et al., 2012). Serum levels of AST were measured using a commercial kit (Roche Diagnostic) in an automatic analyzer (Cobas CS01; Roche).

**ELISA**

Total PEC samples were cultured with T. gondii–soluble tachyzoite antigen (STAg, 5 μg/ml) in complete RPMI for 3 d. The samples were centrifuged and the supernatant was used to measure the levels of IFN-γ and IL-10 using mouse Quantikine ELISA kits for IFN-γ and IL-10 (R&D systems). Experiments were performed following the manufacturer’s instructions.

**RNA Purification and Quantitative PCR**

Total RNAs were isolated using a combination of TRIzol (Invitrogen) and RNeasy kit (QIAGEN). cDNAs were prepared using SuperScript III Reverse transcription (Invitrogen). Quantitative PCR was performed on a 7900HT Sequence Detection System (Applied Biosystems) using the following predesigned primer/probe sets: Ifng (Mm99999071_m1), Il10 (Mm00439616_m1), and Cd4 (Mm00442754_m1; all purchased from Thermo Fisher).

**RNA-Seq**

Naïve CD4 T cells sorted from WT C57BL/6 or Blhle40fl/fl-CD4-Cre mice were cultured with plate-bound anti-CD3 and anti-CD28 under Th1-polarizing conditions for 3 d and then restimulated with PMA plus ionomycin for 2 h. Samples were prepared in duplicates. Total RNAs were isolated using QIagen’s miRNeasy micro kit (217084; QIagen). PolyA-tailed RNAs were purified from pure total RNA using Dynabeads mRNA DIRECT kit (61012; Ambion Life Technologies). Library constructions and RNA-Seq follow the protocols described previously (Chepelev et al., 2009). 50-bp reads were generated by the National Heart, Lung, and Blood Institute DNA Sequencing and Computational Biology Core. Sequence reads were mapped to mouse genome (mm9) by using bowtie 2 with default settings (Langmead and Salzberg, 2012). Reads mapped to multiple positions (MAPQ < 10) were discarded. The mRNA expression level of a gene was quantified by RPKM (Mortazavi et al., 2008) with in-house script. Differentially expressed genes were identified by edgeR 3 (Robinson et al., 2010) with the following criteria: false discovery rate < 0.01, fold change log2 ≥ 1, and RPKM ≥ 2.

**Statistics**

Groups were compared using the Prism 6 software (GraphPad) using a two-tailed unpaired Student’s t test or an ordinary one-way ANOVA. Data were presented as mean ± SEM. A p-value <0.05 was considered statistically significant and indicated as *; P < 0.01 was indicated as **; P < 0.001 was indicated as ***; and P < 0.0001 was indicated as ****. Not statistically significant was indicated as ns.
Evans, H.G., U. Roostal, C.J. Walter, N.J. Gullick, K.S. Frederiksen, C.A. Roberts, M. Sumner, D.L. Batten, J.G. Gerwin, A.P. Cope, et al. 2014. TNF-a blockade induces IL-10 expression in human CD4+ T cells. Nat. Commun. 5:3199. https://doi.org/10.1038/ncomms4199

Gazzinelli, R.T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kühn, W. Müller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J. Immunol. 157:798–805.

Grover, H.S., N. Blanchard, F. Gonzalez, S. Chan, E.A. Robey, and N. Shastri. 2012. The Toxoplasma gondii pep tide AS15 elicits CD4 T cells that can control parasite burden. Infect. Immun. 80:3279–3288. https://doi.org/10.1128/IAI.00425-12

Honna, S., T. Kawamoto, Y. Takagi, K. Fujimoto, F. Sato, M. Noshiro, Y. Kato, and K. Honna. 2002. Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature. 419:841–844. https://doi.org/10.1038/nature01123

Hu, G., Q. Tang, S. Sharma, F. Yu, T.M. Escobar, S.A. Muljo, J. Zhu, and K. Zhao. 2013. Expression and regulation of intergenic long noncoding RNAs during T cell development and differentiation. Nat. Immunol. 14:1990–1998. https://doi.org/10.1038/ni.7212

Hunter, C.A., C.S. Subauste, V.H. Van Cleave, and J.S. Remington. 1994. Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. Infect. Immun. 62:2818–2824.

Jankovic, D., M.C. Kullberg, C.F. Kuchroo, V.E. Arcehambault, J. Zhu, and G. Grunig. 2013. Expression and regulation of intergenic long noncoding RNAs in T-bet+Foxp3- Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. J. Exp. Med. 204:273–283. https://doi.org/10.1084/jem.20061715

Kanda, M., H. Yamanaka, S. Kojo, Y. Usui, H. Honda, Y. Sotomaru, M. Harada, M. Taniguchi, N. Suzuki, T. Atsumi, et al. 2016. Transcriptional regulator Bhlhe40 works as a cofactor of T-bet in the regulation of IFN-γ production in INKT cells. Proc. Natl. Acad. Sci. USA. 113:E3934–E4302. https://doi.org/10.1073/pnas.1604178113

Langmead, B., and S.L. Salzberg. 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods. 9:357–359. https://doi.org/10.1038/nmeth.1923

Lazarvec, V., L.H. Glumcher, and G.M. Lord. 2013. T-bet: a bridge between innate and adaptive immunity. Nat. Rev. Immunol. 13:777–789. https://doi.org/10.1038/nri3536

Lin, C.C., T.R. Bradstreet, E.A. Schwarzkopf, J. Sim, J.A. Carrero, C. Chou, L.E. Cook, T. Egawa, R. Taneja, T.L. Murphy, et al. 2014. Bhlhe40 controls cytokine production by T cells and is essential for pathogenicity in autoimmune neuroinflammation. Nat. Commun. 5:3551. https://doi.org/10.1038/ncomms4551

Lin, C.C., T.R. Bradstreet, E.A. Schwarzkopf, N.M. Jarjour, C. Chou, A.S. Archambault, J. Sim, B.H. Zinselmeyer, J.A. Carrero, G.F. Wu, et al. 2016. IL-1-induced Bhlhe40 identifies pathogenic T helper cells in a model of autoimmune neuroinflammation. J. Exp. Med. 213:251–271. https://doi.org/10.1084/jem.20150568

Martínez-Llordella, M., J.H. Esensten, S.L. Bailey-Bucktrout, R.H. Lipsky, A. Marini, J. Chen, M. Mughal, M.P. Matson, D.D. Taub, and J.A. Bluestone. 2013. CD28-inducible transcription factor DECI is required for efficient autoreactive CD4+ T-cell response. J. Exp. Med. 210:1803–1819. https://doi.org/10.1084/jem.20122387

Maynard, C.L., L.E. Harrington, K.M. Janowski, J.R. Oliver, C.L. Zindl, A.Y. Rudensky, and C.T. Weaver. 2007. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. Nat. Immunol. 8:931–941. https://doi.org/10.1038/nii1004

Miyazaki, M., M. Miyazaki, Y. Guo, M. Yamasaki, M. Kanno, Z. Honda, H. Oda, H. Kawamoto, and H. Honda. 2010. The role of the basic helix-loop-helix transcription factor Dec1 in the regulatory T cells. J. Immunol. 185:7330–7339. https://doi.org/10.1042/jimmunol.1001381

Mortazavi, A., B.A. Williams, K. McCue, L. Schaeffer, and B. Wold. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods. 5:621–628. https://doi.org/10.1038/nmeth.1226

Mullen, A.C., A.S. Hutchins, F.A. High, H.W. Lee, K.J. Sykes, L.A. Chodosh, and S.L. Reiner. 2002. Hlx is induced by and genetically interacts with T-bet to promote tolerable T(H)1 gene induction. Nat. Immunol. 3:652–658. https://doi.org/10.1038/ni807

Neyer, L.E., G. Grunig, M. Fort, J.S. Remington, D. Rennick, and C.A. Hunter. 1997. Role of interleukin-10 in regulation of T-cell-dependent and T-cell-independent mechanisms of resistance to Toxoplasma gondii. Infect. Immun. 65:1675–1682.
Pot, C., H. Jin, A. Awasthi, S.M. Liu, C.Y. Lai, R. Madan, A.H. Sharpe, C.L. Karp, S.C. Miaw, I.C. Ho, and V.K. Kuchroo. 2009. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J. Immunol. 183:797–801. https://doi.org/10.4049/jimmunol.0901233

Powrie, F., M.W. Leach, S. Mauze, S. Menon, L.B. Caddle, and R.L. Coffman. 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. Immunity. 1:553–562. https://doi.org/10.1016/1074-7613(94)90045-0

Robinson, M.D., D.J. McCarthy, and G.K. Smyth. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26:139–140. https://doi.org/10.1093/bioinformatics/btp616

Roncarolo, M.G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M.K. Levings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol. Rev. 212:28–50. https://doi.org/10.1111/j.0105-2896.2006.00420.x

Rubtsov, Y.P., J.P. Rasmussen, E.Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Slewe, A. Roers, W.R. Henderson Jr., et al. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity. 28:546–558. https://doi.org/10.1016/j.immuni.2008.02.017

Sun, H., B. Lu, R.Q. Li, R.A. Flavell, and R. Taneya. 2001. Defective T cell activation and autoimmune disorder in Stra13-deficient mice. Nat. Immunol. 2:1040–1047. https://doi.org/10.1038/ni2721

Szabo, S.J., S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, and L.H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 100:655–669. https://doi.org/10.1016/S0092-8674(00)80702-3

Wei, G., B.J. Abraham, R. Yagi, R. Jothi, K. Cui, S. Sharma, L. Narlikar, D.L. Northrup, Q. Tang, W.E. Paul, et al. 2011. Genome-wide analyses of transcription factor GATA3-mediated gene regulation in distinct T cell types. Immunity. 35:299–311. https://doi.org/10.1016/j.immuni.2011.08.007

Yagi, R., I.S. Junttila, G. Wei, J.F. Urban Jr., K. Zhao, W.E. Paul, and J. Zhu. 2010. The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-gamma. Immunity. 32:507–517. https://doi.org/10.1016/j.immuni.2010.04.004

Yu, F., S. Sharma, J. Edwards, L. Feigenbaum, and J. Zhu. 2015. Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. Nat. Immunol. 16:197–206. https://doi.org/10.1038/ni.3053

Zhu, J., H. Yamane, and W.E. Paul. 2010. Differentiation of effector CD4 T cell populations (*). Annu. Rev. Immunol. 28:445–489. https://doi.org/10.1146/annurev-immunol-030409-101212

Zhu, J., D. Jankovic, A.J. Oler, G. Wei, S. Sharma, G. Hu, L. Guo, R. Yagi, H. Yamane, G. Punkosdy, et al. 2012. The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. Immunity. 37:660–673. https://doi.org/10.1016/j.immuni.2012.09.007