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BHLHE40 promotes T_H2 cell-mediated anti-helminth immunity and reveals cooperative CSF2RB family cytokines

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Abstract

The transcription factor BHLHE40 is an emerging regulator of the immune system. Recent studies suggest that BHLHE40 regulates type 2 immunity, but this has not been demonstrated *in vivo*. We found that BHLHE40 is required in T cells for a protective T_H2 cell response in mice infected with the helminth *Heligmosomoides polygyrus bakeri* (*H. polygyrus*). *H. polygyrus* elicited changes in gene and cytokine expression by lamina propria $CD4^+$ T cells, many of which were BHLHE40-dependent, including production of the common beta (β_C, CSF2RB) chain family cytokines GM-CSF and IL-5. In contrast to deficiency in GM-CSF or IL-5 alone, loss of both GM-CSF and IL-5 signaling impaired protection against *H. polygyrus*. Overall, we show that BHLHE40 regulates the T_H2 cell transcriptional program during helminth infection to support normal expression of *Csf2*, *Il5*, and other genes required for protection and reveal unexpected redundancy of β_C chain-dependent cytokines previously thought to possess substantially divergent functions.

Introduction

The transcription factor BHLHE40 has recently been characterized as a key regulator of the hematopoietic system (1–8). We and others have discovered that BHLHE40 modulates T_H1 and T_H17 cell cytokine production in infection and autoimmunity, particularly by controlling GM-CSF and IL-10 (1–3, 5, 6, 8). Several studies have also suggested a role for BHLHE40

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as a regulator of type 2 immunity, possibly by a T_H2 cell- (9, 10) or myeloid cell-intrinsic role (7).

Helminthic worms manipulate the immune system to establish chronic infections in diverse sites, often resulting in significant tissue damage (11, 12). Helminths elicit production of alarmins including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) and classic type 2 cytokines such as IL-4, IL-5, and IL-13 (11, 12). The helminth *Heligmosomoides polygyrus bakeri* (*H. polygyrus*) is a natural pathogen of mice and an oft-employed experimental model (12, 13). Several cytokines contribute to protective immunity against *H. polygyrus*, including IL-4 and IL-25, while IL-5, IL-33, and TSLP are considered dispensable for control of this infection (14–17).

In many other instances of type 2 immunity, IL-5 plays a crucial role (11, 18, 19). Controversy previously existed as to whether IL-5 was protective against helminths, but it is now appreciated that deficiency in IL-5 only affects immunity to select parasites and sometimes in a life cycle stage-dependent manner (11, 18). IL-5 binds to the unique IL-5Rα chain paired with the common beta (β_C) chain, which is shared with the receptors for GM-CSF and IL-3 (20). While IL-3 signaling is maintained in mice in the absence of the β_C chain (*Csf2rb*) through a unique beta chain (β_{IL3}, *Csf2rb2*), GM-CSF and IL-5 signaling is abrogated in *Csf2rb*$^{-/-}$ mice (20). Despite absolute dependence on this shared receptor chain, which is largely responsible for downstream signaling (20), known roles for GM-CSF and IL-5 are divergent. GM-CSF has been proposed to be a central regulator of inflammation driven by T_H1 and T_H17 cells and affects both autoimmunity and infection (21–26). GM-CSF also contributes to pathological T_H2 cell responses in allergy and asthma (27, 28). However, little is known regarding GM-CSF during type 2 infections, though it is thought to be dispensable during infection with the helminth *Nippostrongylus brasiliensis* (29).

We have found that BHLHE40 and β_C chain-dependent cytokine signaling are essential to protective memory to secondary *H. polygyrus* infection. We defined a T cell-intrinsic role for BHLHE40 to support the helminth-induced gene signature in lamina propria $CD4^+$ T cells, one of the first such analyses in helminth-infected mice. *In vitro* and *in vivo*, T_H2 cells exhibited BHLHE40-dependent production of GM-CSF, IL-5, and other cytokines. Secondary infection of mice deficient in both GM-CSF and IL-5 signaling resulted in severely impaired protective immunity. Overall, BHLHE40 serves as a pivotal regulator of the T_H2 cell transcriptional response to helminth infection, in part by modulating GM-CSF and IL-5, and reveals redundant roles for these cytokines not observed during deficiency of either factor alone.

Materials and Methods

Mice and infections

Il10$^{-/-}$, *Csf2*$^{-/-}$, *Csf2rb*$^{-/-}$, *Cd4-Cre*, and *LysM-Cre* mice were obtained from Jackson. C57BL/6 mice were obtained from Jackson or Taconic. *Bhlhe40*$^{-/-}$ (2, 30), *Bhlhe40*$^{GFP+}$ (3), and *Bhlhe40*$^{fl/fl}$ (5) mice have been reported. Mice were maintained in our specific pathogen free facility. Sex-matched littermates were used for experiments whenever

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possible. Animal experiments were approved by the Animal Studies Committee of Washington University.

Infective *Heligmosomoides polygyrus bakeri* third-stage larvae (L3) were prepared as described (13) and 200 L3 were given using a 20-gauge gavage needle. Fecal eggs were counted on a McMaster counting chamber (Chalex LLC) (13). For rechallenge experiments, infection was cleared with pyrantel pamoate p.o. (2 mg, Columbia Laboratories) on d14 and 15 after primary infection. Mice were rested for 3–4 weeks before reinfection. Blood was collected at d21 post-rechallenge to assess antibody titers. Mice were sacrificed on d17 or 22 of secondary infection for assessment of cellular responses, macroscopic intestinal granulomas, and intestinal worm burden.

For *in vivo* antibody blockade, mice were injected i.p. with 300 μg of αGM-CSF (Leinco, MP1–22E9), αIL-5 (BioXCell, TRFK5), or control polyclonal rat IgG (Sigma Aldrich I4131) every other day from the day of reinfection.

Cell culture

The EasySep mouse naïve CD4+ T cell isolation kit (Stemcell Technologies) was used to isolate splenic T cells. Cells were cultured in complete IMDM (cIMDM, with added 10% FBS, L-glutamine, sodium pyruvate, non-essential amino acids, penicillin/streptomycin, and 2-ME) with plate-bound anti-CD3 antibody (2 μg/mL) and anti-CD28 antibody (2 μg/ml). IL-4 (10 ng/mL) and anti-IFN-γ (5 μg/mL) were added at the start of culture to generate T_H2 cells. Cells were split at d3. On d4, cells were counted and stimulated for 24 hours with plate-bound anti-CD3 and anti-CD28 for assessment of secreted cytokines. In some experiments, naïve $CD4^+$ T cells were labelled with 40 μM CFSE (Tonbo) for 10 min at room temperature, followed by culture under T_H2 polarizing conditions as above and assessment of CFSE dilution by flow cytometry on d3.

ELISA

Nunc Maxisorp plates were coated with capture antibodies (for cytokine ELISAs) or *H. polygyrus* lysate (for *H. polygyrus*-specific IgG1 ELISA) overnight. Following blocking, culture supernatants (cytokine ELISAs) or serum (IgG1 ELISA) were added, followed by incubation with cytokine-detection biotinylated antibodies or horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 antibody. For cytokine ELISAs, streptavidin-conjugated horseradish peroxidase (HRP) was added. Substrate solution (BD OptEIA 555214) was added, reactions were stopped with 1 M H_3PO_4, and absorbance was read at OD_{450}. For cytokine ELISAs, standard curves were generated with purified cytokines. For the IgG1 ELISA, serum was diluted between 10^{-2} and 10^{-8} and the last well with an OD_{450} above 0.100 represented the titer. The Mouse IL-4 and IL-5 ELISA Max Standard Sets (BioLegend), Mouse IL-13 DuoSet ELISA (R&D), and C57BL/6 Mouse Immunoglobulin Panel (SouthernBiotech) were used. For detection of GM-CSF, biotinylated αGM-CSF (BioLegend, MP1–31G6), unconjugated αGM-CSF (BioLegend, MP1–22E9), and murine GM-CSF (Peprotech) were used.

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To generate *H. polygyrus* lysate, adult worms were washed in PBS and ground in 1 mL of PBS. Debris was pelleted by centrifugation at 16,000 g for 20 minutes at 4 °C. The supernatant was passed through a 0.2 μm filter and stored at −80 °C.

Microscopy

The proximal 6 cm of the small intestine were formed into a swiss roll, frozen in OCT media, and cryosectioned. For H&E staining, sections were fixed in 4% paraformaldehyde (PFA). For immunofluorescent staining, sections were fixed in acetone and blocked with CAS-Block (Invitrogen). Staining was performed with Cy3-anti-α-smooth muscle actin (Sigma, 1A4) and AlexaFluor647-anti-F4/80 (BioLegend, BM8) diluted in CAS-Block. Sections were mounted with Abcam Fluoroshield Mounting Medium with DAPI. Images were captured with a Nikon Eclipse E800 microscope and MicroPublisher 5.0 RTV or EXi Blue cameras (QImaging) using QCapture software. Fluorescence images were merged and levelled in Adobe Photoshop.

Cell isolation

Peritoneal cells were collected by lavage. Spleens were homogenized through a 70 μm filter. SILP cells were isolated from 4 cm of the proximal small intestine, cleaned of fecal contents and cut into 2–3 pieces. Tissues were shaken twice in 1x HBSS (with added 10% FBS, HEPES, EDTA, and DTT) for 20 minutes, vigorous vortexed, and then shaken at 220 rpm at 37 °C for 1 hour in an Innova 4330 shaker (New Brunswick Scientific) with Collagenase IV (0.8 mg/mL, Sigma C5138) in RPMI 1640 (with added 10% FBS, L-glutamine, penicillin/streptomycin, and 2-ME). RBC were lysed when needed.

Flow cytometry

Cells were blocked with αCD16/32 (BioXCell, 2.4G2) and stained with antibodies before flow cytometry on LSRFortessa X20 and FACSCanto II instruments (BD). Analysis was performed with FlowJo software (Treestar). The following antibodies were purchased from BioLegend: αCD3 APC/Cy7 (17A2), αCD4 PE/Cy7 or BV510 (RM4–5), αCD11b PE/Cy7 or APC/Cy7 (M1/70), αCD45.2 FITC (104), αCD64 APC (X54–5/7.1), αCD102 FITC or AF647 (3C4), αCD115 BV421 (AFS98), αI-A/I-E BV510 or PB (M5/114.15.2), αIL-4 APC (11B11), αIL-5 BV421 (TRFK5), αLy6C APC/Cy7 or BV510 (HK1.4), and αTCRβ PE (H57–597). The following antibodies were purchased from Tonbo: αCD8 PerCP/Cy5.5 or APC/Cy7 (53–6.7), αCD11b PE/Cy7 or APC/Cy7 (M1/70), αCD19 PerCP/Cy5.5 (1D3), αCD45 RF710 (30-F11), αCD115 PE (AFS98), αF4/80 PerCP/Cy5.5 (BM8.1), and αI-A/I-E RF710 (M5/114.15.2). The following antibodies were purchased from Becton Dickinson: αCD45 BV510 (30-F11), αGM-CSF BV421 (MP1–22E9), and αSiglec-F PE (E50–2440). αIL-5 FITC (TRFK5) was purchased from Leinco. Unconjugated αRELMα was purchased from Peprotech (500-P214).

SILP macrophages were gated as $CD45^+F4/80^+CD64^+MHC\text{-}II^+Ly6C^-$, while GMMs were gated as $CD45^+F4/80^+CD64^+MHC\text{-}II^{lo}Ly6C^{lo}$. SILP eosinophils were gated as $CD45^+F4/80^+CD64^-CD11b^+SSC\text{-}A^{hi}$ (validated by Siglec-F staining in some experiments). SILP $CD3^+$ T cells were gated as $CD45^+F4/80^-CD64^-CD19^-CD3^+$. SILP $CD4^+$ T cells were gated as $CD19^-F4/80^-TCR\beta^+CD4^+CD8^-$. LPMs were gated as $CD115^+CD11b$

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$^{+}$ICAM2^{+}MHC-II^{lo}. Peritoneal eosinophils were gated as Siglec-F^{+}ICAM2^{-}. Peritoneal CD4^{+} T cells were gated as ICAM2^{-}TCRβ^{+}CD4^{+}CD8^{-}.

For intracellular cytokine staining of T cells, total SILP and peritoneal cells were cultured in cIMDM for 3–4 hours at 37 °C in the presence of PMA (50 ng/ml), ionomycin (1 μM), and brefeldin A (1 μg/ml). Cells were then surface stained and fixed with 4% PFA. To permeabilize the cells, samples were washed with 1x Perm/Wash buffer (BD 554714) and stained for 20 minutes at 4 °C. For RELMα staining, the True-Nuclear Transcription Factor Buffer set (BioLegend 424401) was used.

Microarrays

CD4^{+} SILP T cells were sorted using a FACSAria II (BD). RNA was purified with E.Z.N.A. MicroElute Total RNA kits (Omega Bio-Tek). cDNA was synthesized from RNA (NuGen Pico SL) followed by analysis on Affymetrix Mouse Gene 1.0 ST microarrays. CEL files were normalized with the DNASTAR ArrayStar program. Genes with an expression value of >5 (in log 2 scale) in at least one replicate were considered expressed. Lists of differentially expressed genes required a p-value significance of ≤0.01 by the moderated *t*-test. Morpheus was used to generate heatmaps (Broad). GSEA software (Broad) was used to analyze enrichment of C5 database gene sets. Microarray data have been deposited in the Gene Expression Omnibus under accession number GSE135182 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135182).

Chromatin immunoprecipitation sequencing (ChIP-Seq)

Samples GSM3094815 and GSM3094816 from GSE113054 (5) were reanalyzed for BHLHE40 binding at the *Csf2* and *Il5* loci. Previously published peak calls were downloaded from the Gene Expression Omnibus.

Statistics

Statistical analysis was performed with Prism 7 (GraphPad) as described in the figure legends.

Results

BHLHE40 is required for control of *H. polygyrus* rechallenge

Primary oral challenge of C57BL/6 mice with *H. polygyrus* results in chronic infection, but rechallenge evokes a protective recall response which limits infection (31). To assess whether BHLHE40 was required for immunity to *H. polygyrus*, we challenged C57BL/6 *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice with infective *H. polygyrus* larvae (L3), cured them by treatment with pyrantel pamoate, and rechallenged them with infective L3. Mature *H. polygyrus* worms mate in the intestinal lumen, resulting in females laying eggs which are then shed in the feces (13). While *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice exhibited similar parasite fecal egg burdens during primary infection, *Bhlhe40*$^{-/-}$ mice had a much higher egg burden during secondary infection as compared to *Bhlhe40*$^{+/+}$ mice (Fig. 1A). Increased production of eggs could result from impaired expulsion of the parasites or enhanced parasite fitness. While there was a trend towards increased adult worm burden in *Bhlhe40*$^{-/-}$

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as compared to *Bhlhe40*$^{+/+}$ mice after rechallenge (Supplemental Fig. 1A), this did not reach statistical significance, indicating that their increased egg burden was primarily due to increased worm fecundity. *H. polygyrus*-rechallenged *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice had dramatically different pathology. Type 2 granulomas can form around developing parasites and have been correlated with protective immunity (31). Small intestines from *Bhlhe40*$^{+/+}$ mice exhibited many granulomas, while those from *Bhlhe40*$^{-/-}$ mice resembled healthy tissue (Fig. 1B, 1C). Histological analysis and immunostaining showed reduced immune infiltration and damage to the smooth muscle layer in *H. polygyrus*-rechallenged *Bhlhe40*$^{-/-}$ as compared to *Bhlhe40*$^{+/+}$ mice (Fig. 1D, 1E).

Flow cytometry of the small intestine from helminth-infected mice is challenging because of extensive tissue remodeling and enhanced mucus production. Therefore, we adapted a previously published protocol for intestinal flow cytometry for this purpose (32). When we explored the cellular composition of the small intestine lamina propria (SILP) following *H. polygyrus* rechallenge of *Bhlhe40*$^{+/+}$ mice by flow cytometry, we found both CD45^{+}CD64^{+}F4/80^{+}MHC-II^{+}Ly6C^{-} resident macrophages (33, 34) and another CD45^{+}F4/80^{+}CD64^{+}MHC-II^{lo}Ly6C^{lo} autofluorescent population, which we termed granuloma-associated monocytes/macrophages (GMMs) (Fig. 1F, 1G). This latter population may correspond to previously described clodronate-sensitive alternatively activated macrophages seen by immunostaining during *H. polygyrus* infection (35). SILP CD45^{+}F4/80^{+}CD64^{-}CD11b^{+}SSC-A^{hi} eosinophils were also significantly increased after secondary *H. polygyrus* infection (Fig. 1F, 1G, Supplemental Fig. 1B). However, GMMs and eosinophils were greatly reduced in *H. polygyrus*-rechallenged *Bhlhe40*$^{-/-}$ as compared to *Bhlhe40*$^{+/+}$ mice, in contrast to SILP CD3^{+} T cells (Fig. 1F, 1G, Supplemental Fig. 1C). Because *H. polygyrus* infection is known to change the cellular composition of the peritoneal cavity (11, 36), we assessed accumulation of LPMs and peritoneal eosinophils, and found severe reductions in both populations in *H. polygyrus*-rechallenged *Bhlhe40*$^{-/-}$ as compared to *Bhlhe40*$^{+/+}$ mice, as well as impaired LPM polarization as assessed by resistin-like molecule alpha (RELMα) staining (Supplemental Fig. 1D–H). In contrast, serum *H. polygyrus*-specific IgG1 titers were not affected by loss of BHLHE40 (Supplemental Fig. 1I). These data indicated impaired responses by multiple myeloid cell lineages to *H. polygyrus* rechallenge in *Bhlhe40*$^{-/-}$ mice.

BHLHE40 is required in T cells for a normal immune response to *H. polygyrus*

We next employed *Cd4-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ and *LysM-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice to address whether loss of BHLHE40 specifically in T cells or myeloid cells recapitulated the phenotype of *Bhlhe40*$^{-/-}$ mice during secondary *H. polygyrus* infection. *Cd4-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice were severely impaired in controlling *H. polygyrus* rechallenge and lacked intestinal granulomas as compared to *Cd4-Cre*$^{-}$ *Bhlhe40*$^{fl/fl}$ mice (Fig. 2A, 2B). When we assessed the cellular response to secondary infection, we found that loss of BHLHE40 selectively in T cells was sufficient to perturb both the SILP and peritoneal myeloid cell responses, as well as to reduce SILP T cells in some animals (Fig. 2C, Supplemental Fig. 1J–M). In contrast, *LysM-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice were able to control infection comparably to *LysM-Cre*$^{-}$ *Bhlhe40*$^{fl/fl}$ mice (Fig. 2D–F). Therefore, BHLHE40 expression was required

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in T cells to control secondary *H. polygyrus* infection, but was dispensable in LysM-expressing myeloid cells.

BHLHE40 is required for a normal CD4$^+$ T cell transcriptional response to *H. polygyrus*

We next asked whether loss of BHLHE40 dysregulated CD4$^+$ T cell gene expression in response to *H. polygyrus* rechallenge. To address this, we sorted CD4$^+$ T cells from the SILP of naïve and rechallenged *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice for gene expression microarrays. By comparing CD4$^+$ T cells from naïve and *H. polygyrus*-rechallenged *Bhlhe40*$^{+/+}$ mice, we defined a helminth-induced signature which included transcripts for cytokines (including *Areg*, *Il3*, *Il4*, *Il5*, *Il6*, *Il13*, *Csf1*, *Csf2*, *Lif*, *Tnf*, *Tnfsf11*), cytokine receptors (including *Il1rl1*, *Il1r2*, *Il17rb*), and transcription factors (including *Atf3*, *Bhlhe40*, *Gata3*, *Nfil3*, *Pparg*, *Rbpj*, *Vdr*, and *Zeb2*) (Fig. 3A, Supplemental Fig. 2A). When we compared differentially expressed transcripts between *H. polygyrus*-elicited and naive SILP CD4$^+$ T cells, we found a considerable overlap with the recently described transcriptional profile of HDM-elicited airway T$_H$2 cells (including *Bhlhe40*, *Cd200r1*, *Il6*, *Plac8*, and *Igfbp7*) (Fig. 3A, Supplemental Fig. 2A) (10), demonstrating significant conservation of the T$_H$2 cell transcriptional program independent of tissue environment and stimulus. When we assessed BHLHE40-dependent genes after *H. polygyrus* rechallenge, we found that a significant majority were part of the helminth-induced signature and that BHLHE40-dependent genes were distinct in CD4$^+$ T cells from naïve and *H. polygyrus*-rechallenged mice (Fig. 3A, 3B, Supplemental Fig. 2B, 2C).

When we used gene set enrichment analysis (GSEA) to look for BHLHE40-dependent gene modules, we noted that two of the most enriched sets in *Bhlhe40*$^{+/+}$ as compared to *Bhlhe40*$^{-/-}$ CD4$^+$ T cells after *H. polygyrus* rechallenge were “growth factor activity” and “cytokine activity,” reflecting altered expression of cytokine genes including *Areg*, *Il5*, *Il6*, *Il13*, *Csf1*, *Csf2*, and *Lif*, but not *Il3*, *Il4*, or *Tnf* (Fig. 3C, 3D). Furthermore, when we assessed differential expression of the lineage-specifying transcription factors of each T$_H$ cell subset as well as a recently defined set of transcriptional regulators of *in vitro*-polarized T$_H$2 cells (9), we observed reduced expression of *Pparg* in *Bhlhe40*$^{-/-}$ as compared to *Bhlhe40*$^{+/+}$ CD4$^+$ T cells from *H. polygyrus*-rechallenged mice (2.4-fold reduced), but only subtle changes in other factors including *Gata3* (1.4-fold reduced) (Fig. 3D). As PPARγ is required for normal T$_H$2 cell responses and protective immunity to *H. polygyrus*, this may indicate that some effects of BHLHE40 deficiency are indirect (37, 38). *Bhlhe40* expression was induced in mice experiencing secondary infection as compared to naïve mice (Fig. 3D). Using *Bhlhe40*^{GFP} bacterial artificial chromosome reporter mice (3), we observed a marked increase in SILP GFP$^+$ CD4$^+$ T cells after *H. polygyrus* rechallenge (Fig. 3E). Taken together, these data showed that BHLHE40 was induced by SILP CD4$^+$ T cells in response to *H. polygyrus* rechallenge and was critical for their normal transcriptional program.

Loss of BHLHE40 impairs CD4$^+$ T cell cytokine production in response to *H. polygyrus*

Next, we restimulated SILP cells from naïve and rechallenged *Cd4-Cre*$^-$ *Bhlhe40*$^{fl/fl}$ and *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice *ex vivo* with PMA and ionomycin to assess cytokine production. Rechallenge with *H. polygyrus* induced production of IL-4, IL-5, and IL-13 from *Cd4-Cre*$^-$ *Bhlhe40*$^{fl/fl}$ and *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ CD4$^+$ T cells; however, BHLHE40 was required for

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normal frequencies of single- and multi-cytokine-producing cells, largely due to reductions in IL-5$^+$ and IL-13$^+$ CD4$^+$ T cells, consistent with the results of our microarray gene expression analysis (Fig. 4A–C). Because *Csf2* was upregulated by *H. polygyrus* rechallenge in a BHLHE40-dependent fashion, we also assessed GM-CSF and found that it was markedly induced by rechallenge (~35% of *Cd4-Cre*$^-$ *Bhlhe40*$^{fl/fl}$ CD4$^+$ T cells) and that this required BHLHE40 (~10% of *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ CD4$^+$ T cells) (Fig. 4D, 4E). SILP CD4$^+$ T cells could coproduce GM-CSF and IL-5 (Supplemental Fig. 3A). T_H2 cells disseminate widely in mice infected with *H. polygyrus* (36). CD4$^+$ T cell cytokine responses in the peritoneal cavity and mesenteric lymph nodes from *H. polygyrus*-rechallenged *Cd4-Cre*$^-$ *Bhlhe40*$^{fl/fl}$ and *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice were generally consistent with those in the SILP (Supplemental Fig. 3B and data not shown). To assess whether BHLHE40 was also required for normal cytokine production by a more homogenous population of T_H2 cells, we differentiated naïve splenic CD4$^+$ T cells into T_H2 cells and restimulated them to assess cytokine production. We found that BHLHE40 was essential for production of GM-CSF and that loss of BHLHE40 also impaired *in vitro* production of IL-4, IL-5, and IL-13 (Supplemental Fig. 3C). As published literature is unclear as to whether Bhlhe40 controls T_H cell proliferation (1, 6), we assessed this *in vitro* with *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ T_H2 cells and found that BHLHE40 was dispensable for T_H2 cell proliferation (Supplemental Fig. 3D, 3E). As we have previously published BHLHE40 ChIP-Seq for *in vitro*-polarized T_H1 cells (5), we also assessed whether BHLHE40 was bound near the *Csf2* and *Il5* loci in this T_H subset. BHLHE40 was bound to the *Csf2* locus, while the nearest peaks to the *Il5* locus were more proximal to *Rad50* and *Irf1* (Supplemental Fig. 3F, 3G). Taken together, these data indicated that BHLHE40 was required *in vitro* and *in vivo* for normal T_H2 cell function.

As BHLHE40 is a known repressor of IL-10 (3, 5, 6), we also assessed IL-10 production from SILP CD4$^+$ T cells. Indeed, SILP CD4$^+$ T cells lacking BHLHE40 produced significantly higher levels of IL-10 after *H. polygyrus* rechallenge (Supplemental Fig. 4A). Nevertheless, genetic deletion of IL-10 in *Bhlhe40*$^{-/-}$ *Il10*$^{-/-}$ mice was not sufficient to restore control of infection or normal intestinal granulomatous pathology (Supplemental Fig. 4B, 4C). These data demonstrated a key role for BHLHE40 in CD4$^+$ T cell cytokine responses to helminth infection, notably controlling production of the β_C chain family cytokines GM-CSF and IL-5.

Loss of the β_C chain impairs protective memory to *H. polygyrus*

As protective memory responses to *H. polygyrus* rechallenge are unaffected by IL-5 blockade (14), we first asked whether loss of GM-CSF signaling was sufficient to impair control of a secondary infection with *H. polygyrus*. Genetic deletion of GM-CSF (*Csf2*$^{-/-}$ mice) did not substantially affect control of *H. polygyrus* infection as compared to *Csf2*$^{+/+}$ mice (Supplemental Fig. 4D–F). As GM-CSF and IL-5 were individually dispensable, we then rechallenged *Csf2rb*$^{+/+}$ and *Csf2rb*$^{-/-}$ mice. *Csf2rb*$^{-/-}$ mice had a severe defect in control of *H. polygyrus* as compared to *Csf2rb*$^{+/+}$ mice and did not form intestinal granulomas or develop normal SILP and peritoneal myeloid cell responses (Fig. 5A–C, Supplemental Fig. 4G–I). To exclude a role for β_C chain-dependent IL-3 signaling, we singly blocked GM-CSF or IL-5 signaling with neutralizing antibodies or blocked both

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cytokines together. Double, but not single, blockade resulted in impaired protective immunity (Fig. 5D, 5E). Taken together, these data indicated that BHLHE40 was required for normal production of GM-CSF and IL-5 from $CD4^+$ T cells and that these cytokines were collectively, but not individually, critical for control of *H. polygyrus* rechallenge.

Discussion

Our findings establish BHLHE40 as an essential, T cell-intrinsic regulator of type 2 immune responses. Mechanistically, we found that SILP $CD4^+$ T cells activated by *H. polygyrus* rechallenge required BHLHE40 for normal expression of many helminth-induced genes, most notably *Csf2* and *Il5*, and that these and potentially other factors were necessary for normal myeloid cell-mediated responses to *H. polygyrus*. In the absence of BHLHE40 in T cells, we observed a near-complete loss of the myeloid cell-mediated response, which encapsulates developing parasites in a type 2 granuloma structure and directly targets them through effector mechanisms including arginase-1 activity (35). These data also demonstrate the severity of type 2 immunopathology in the *H. polygyrus* model, as an impaired immune response in the absence of BHLHE40 protected mice from severe damage to the intestinal wall and smooth muscle layers. While our data suggest that BHLHE40 uniquely regulates some T_H2-specific genes, our work and that of others has now established a broader role for BHLHE40 in T_H1, T_H2, and T_H17 cells as a pivotal regulator of GM-CSF, IL-10, and other cytokines, acting as both a direct and indirect transcriptional regulator of these loci (1–3, 5, 6). These data and recent work on c-Maf (39) suggest that T cell production of many cytokines may be controlled by multi-lineage transcription factors in addition to lineage-restricted factors. In light of the critical role for BHLHE40 in multiple $CD4^+$ T cell subsets, clinical targeting of BHLHE40 may possess therapeutic potential.

A significant effect of BHLHE40 deficiency on $CD4^+$ T cells was reduced production of the β_C chain family members GM-CSF and IL-5. Using our previously published T_H1 cell ChIP-Seq data set (5), we found that BHLHE40 bound to the *Csf2* locus, while *Il5*-proximal peaks were more closely associated with other genes. As *Bhlhe40*$^{-/-}$ SILP $CD4^+$ T cells exhibited decreased expression of *Pparg*, a known regulator of type 2 cytokines (37, 38), BHLHE40 likely regulates *Csf2* and *Il5* via a combination of direct and indirect effects. While these cytokines have long been known to share common signaling through the β_C chain, their described functions are largely distinct, with GM-CSF contributing to T_H1 and T_H17 cell-driven inflammation and IL-5 contributing to type 2 immunity (19, 26). We have demonstrated that protective memory to a helminth infection unaffected by IL-5 blockade and also insensitive to GM-CSF deficiency is nonetheless dependent on the combination of GM-CSF and IL-5 signaling through the β_C chain. It remains to be determined how GM-CSF and IL-5 compensate for each other, whether by direct substitution or via effects on distinct arms of the type 2 response. While redundancy between β_C chain family cytokines is not well described, it is known that these cytokines can regulate eosinophils in a complementary manner (40). Future studies should establish whether GM-CSF and IL-5 are collectively involved in protective immunity to other helminth infections. As β_{IL3} chain-dependent IL-3 signaling is preserved in *Csf2rb*$^{-/-}$ mice (20), it is also of interest to establish whether additionally blocking IL-3 results in a more severe defect in immunity to

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H. polygyrus than is observed in *Csf2rb*$^{-/-}$ mice. IL-3 regulates basophilia in response to *H. polygyrus* and basophils help control infection with this helminth (41, 42).

Our findings reveal novel transcriptional and cytokine regulation of the immune response to a helminth infection, elucidating a critical function for BHLHE40 during *in vivo* T_H2 cell immunity and an unexpected role for β_C chain-dependent signaling. Our data indicate that the importance of β_C chain family cytokines may have been underestimated in type 2 diseases because of redundancy and suggest that combinatorial targeting of these factors could yield improved clinical outcomes in diseases of pathological type 2 immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations used in this article:

CSF2RB and β_C	beta common
BHLHE40	basic helix-loop-helix, member e40
GMM	granuloma-associated monocyte/macrophage
GSEA	gene set enrichment analysis
H. polygyrus	Heligmosomoides polygyrus bakeri
L3	infective stage *H. polygyrus* larvae
LPM	large peritoneal macrophage
RELMα	resistin-like molecule alpha
SILP	small intestine lamina propria

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Key points

- BHLHE40 is required for protective immunity to the helminth *H. polygyrus.*
- BHLHE40 is required for normal T_H2 gene transcription and cytokine production.
- CSF2RB-dependent cytokines can compensate for each other during type 2 immunity.

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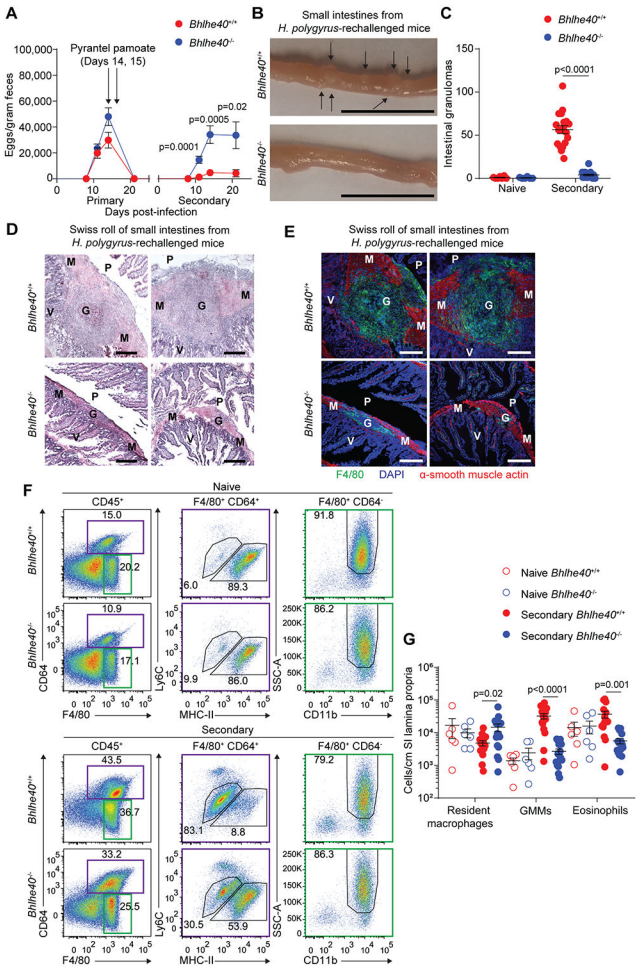

Figure 1. BHLHE40 is required for a protective recall response to *H. polygyrus*.

(A and B) *H. polygyrus*-infected *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice were analyzed for **(A)** quantitation of *H. polygyrus* eggs/gram feces and **(B)** small intestine morphology after secondary infection. Arrows point to granulomas. Scale bar, 1 cm. **(C)** Quantitation of intestinal granulomas. **(D and E)** *H. polygyrus*-rechallenged *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice were analyzed histologically on adjacent sections by **(D)** hematoxylin and eosin staining and **(E)** immunostaining for F4/80, α-SMA, and DAPI on swiss rolls of the proximal small intestine (2 representative lesions from each genotype). G, granuloma. M, muscle layers. P, peritoneal space. V, villi. Scale bar, 200 μm. **(F and G)** Naïve and *H. polygyrus*-rechallenged *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice were analyzed by flow cytometry for **(F)** SILP myeloid cells and **(G)** quantitation as in **(F)**. Data are representative of or pooled from at least 3 independent experiments. Data are mean ± s.e.m. Significance calculated with an unpaired Student's *t*-test.

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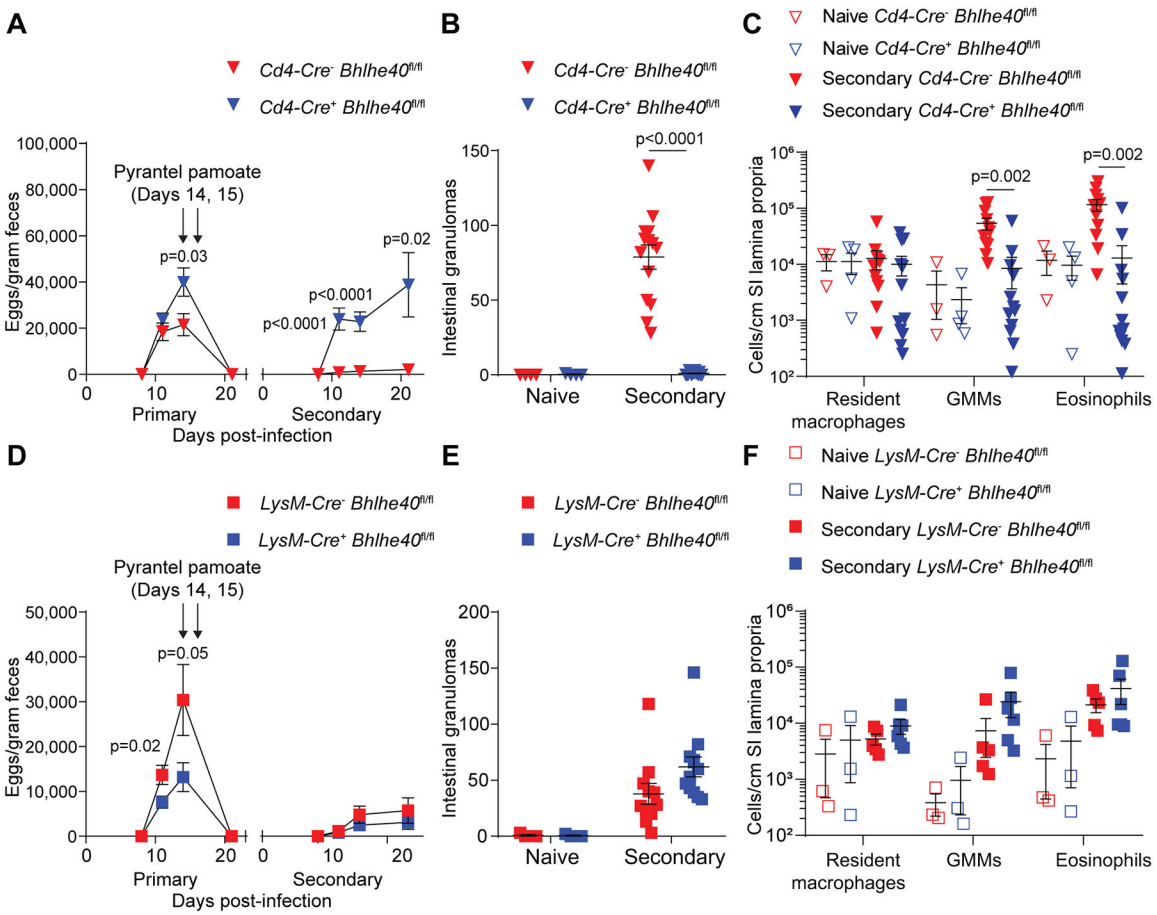

Figure 2. BHLHE40 is required in T cells for a normal memory response to *H. polygyrus*.

(A) *H. polygyrus*-rechallenged *Cd4-Cre*$^{-}$ *Bhlhe40*$^{fl/fl}$ and *Cd4-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice were analyzed for quantitation of *H. polygyrus* eggs/gram feces over time. **(B)** Naïve and *H. polygyrus*-rechallenged *Cd4-Cre*$^{-}$ *Bhlhe40*$^{fl/fl}$ and *Cd4-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice were analyzed for quantitation of intestinal granulomas. **(C)** Naïve and *H. polygyrus*-rechallenged *Cd4-Cre*$^{-}$ *Bhlhe40*$^{fl/fl}$ and *Cd4-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice were analyzed by flow cytometry for quantitation of SILP myeloid cells. **(D)** *H. polygyrus*-rechallenged *LysM-Cre*$^{-}$ *Bhlhe40*$^{fl/fl}$ and *LysM-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice were analyzed for quantitation of *H. polygyrus* eggs/gram feces over time. **(E)** Naïve and *H. polygyrus*-rechallenged *LysM-Cre*$^{-}$ *Bhlhe40*$^{fl/fl}$ and *LysM-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice were analyzed for quantitation of intestinal granulomas. **(F)** Naïve and *H. polygyrus*-rechallenged *LysM-Cre*$^{-}$ *Bhlhe40*$^{fl/fl}$ and *LysM-Cre*$^{+}$ *Bhlhe40*$^{fl/fl}$ mice were analyzed by flow cytometry for quantitation of SILP myeloid cells. Data are representative of or pooled from at least 2 independent experiments. Data are mean ± s.e.m. Significance calculated with an unpaired Student's *t*-test.

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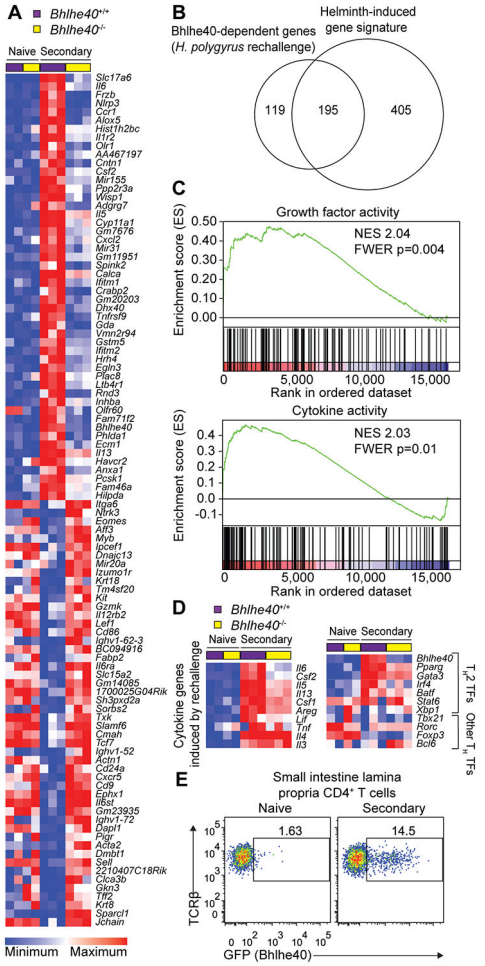

Figure 3. Loss of BHLHE40 dysregulates the CD4$^+$ T cell transcriptional response to *H. polygyrus* rechallenge.

(A) Gene expression microarray data were analyzed for the 100 most differentially expressed probe sets in SILP CD4$^+$ T cells from *H. polygyrus*-rechallenged *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice. **(B)** Gene expression microarray data were analyzed for shared and unique Bhlhe40-dependent genes (≥2-fold differentially expressed between SILP CD4$^+$ T cells from *H. polygyrus*-rechallenged *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice) with the helminth-induced signature (≥2-fold differentially expressed between SILP CD4$^+$ T cells from naïve and *H. polygyrus*-rechallenged *Bhlhe40*$^{+/+}$ mice), depicted as a Venn diagram. **(C)** GSEA of gene expression microarray data for selected C5 gene sets enriched in *Bhlhe40*$^{+/+}$ versus *Bhlhe40*$^{-/-}$ SILP CD4$^+$ T cells from *H. polygyrus*-rechallenged mice. NES, normalized enrichment score. FWER, family-wise error rate. **(D)** Gene expression microarray data were analyzed for (left) expression of cytokines induced by *H. polygyrus* rechallenge and (right) expression of T_H2 and T helper cell lineage-specifying transcription factors in SILP CD4$^+$ T cells from naïve and *H. polygyrus*-rechallenged *Bhlhe40*$^{+/+}$ and *Bhlhe40*$^{-/-}$ mice. TF, transcription factor. **(E)** Flow cytometry of *Bhlhe40*^{GFP} transgene reporter expression in SILP CD4$^+$ T cells from *Bhlhe40*$^{GFP+}$ mice. Data in **(E)** are representative of 2 independent experiments. Microarray data are from 1 experiment.

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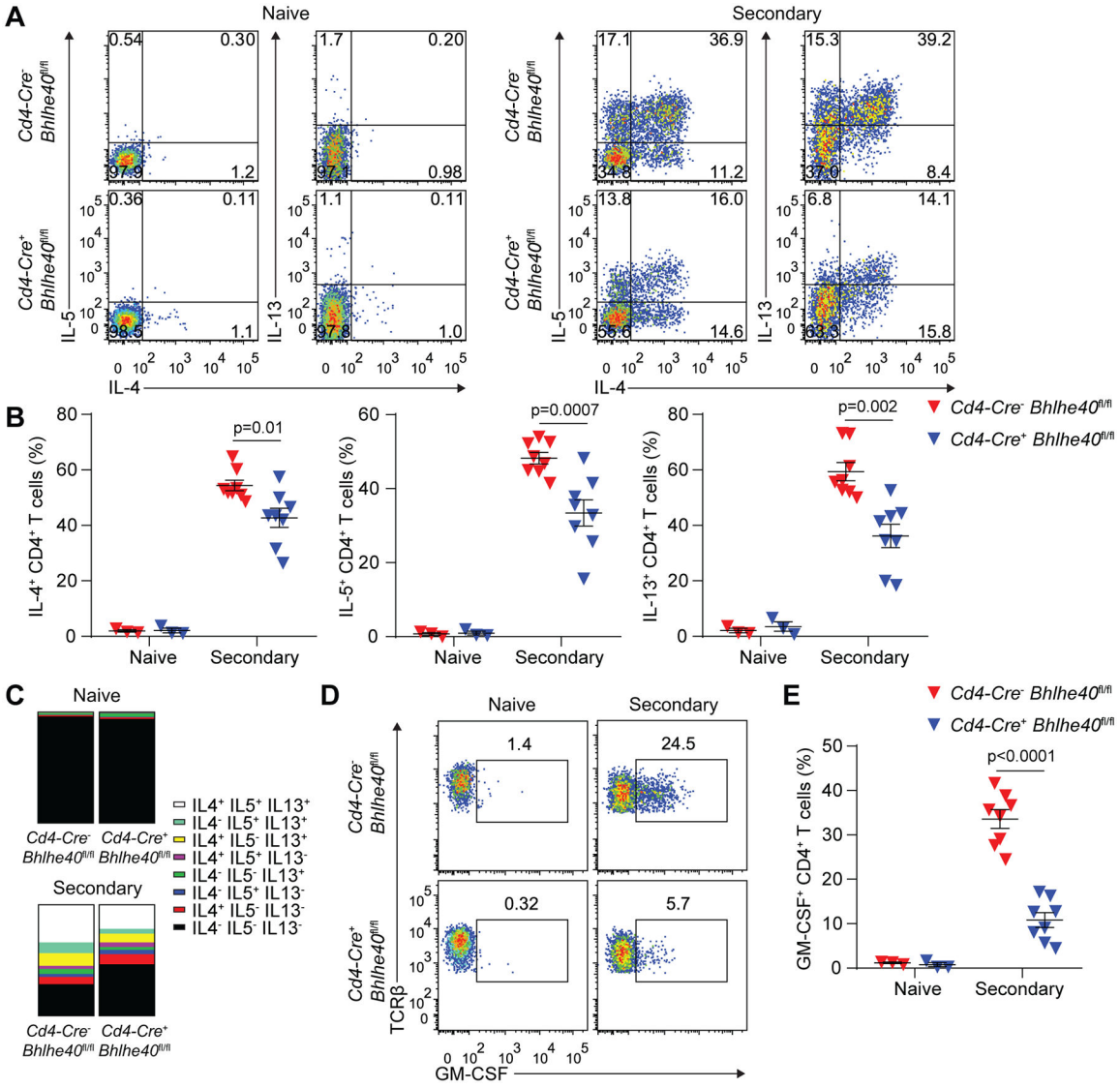

Figure 4. BHLHE40 is required for normal CD4$^+$ T cell production of β_C chain-dependent cytokines.

(A-C) Naïve and *H. polygyrus*-rechallenged *Cd4-Cre*$^-$ *Bhlhe40*$^{fl/fl}$ and *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice were analyzed by flow cytometry for **(A)** IL-4-, IL-5-, and IL-13-producing CD4$^+$ T cells, **(B)** quantitation of the frequency of IL-4$^+$, IL-5$^+$, and IL-13$^+$ CD4$^+$ T cells, and **(C)** quantitation of the frequency of CD4$^+$ T cells producing one or more cytokines after *in vitro* PMA/ionomycin stimulation of SILP cells. **(D and E)** Naïve and *H. polygyrus*-rechallenged *Cd4-Cre*$^-$ *Bhlhe40*$^{fl/fl}$ and *Cd4-Cre*$^+$ *Bhlhe40*$^{fl/fl}$ mice were analyzed by flow cytometry for **(D)** GM-CSF-producing CD4$^+$ T cells and **(E)** quantitation as in **(D)** after *in vitro* PMA/ionomycin stimulation of SILP cells. Data are representative of or pooled from 2 independent experiments. Data are mean ± s.e.m. Significance calculated with an unpaired Student's *t*-test.

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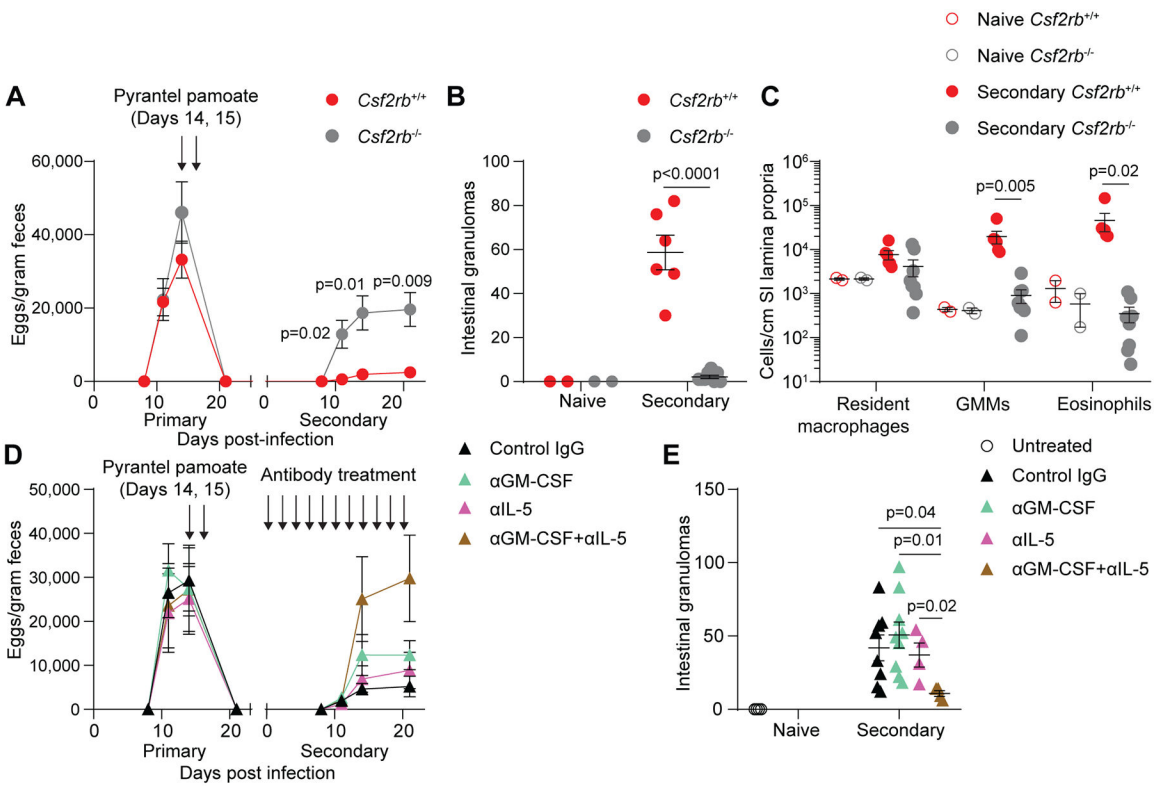

Figure 5. β_C chain signaling is required for control of *H. polygyrus* rechallenge.

(A) *H. polygyrus*-rechallenged *Csf2rb*$^{+/+}$ and *Csf2rb*$^{-/-}$ mice were analyzed for quantitation of *H. polygyrus* eggs/gram feces over time. **(B)** Naïve and *H. polygyrus*-rechallenged *Csf2rb*$^{+/+}$ and *Csf2rb*$^{-/-}$ mice were analyzed for quantitation of intestinal granulomas. **(C)** Naïve and *H. polygyrus*-rechallenged *Csf2rb*$^{+/+}$ and *Csf2rb*$^{-/-}$ mice were analyzed by flow cytometry for quantitation of SILP myeloid cells. **(D)** Wild type mice were rechallenged with *H. polygyrus* concurrent with control IgG, αGM-CSF, αIL-5, or αGM-CSF plus αIL-5 antibody treatment and were analyzed for quantitation of *H. polygyrus* eggs/gram feces over time. **(E)** Naïve (open circles) and *H. polygyrus*-rechallenged mice (filled triangles) treated as in **(D)** were analyzed for quantitation of intestinal granulomas. Data are pooled from 2 independent experiments **(A-C)** or are from 1 experiment **(D and E)**. Data are mean ± s.e.m. Significance calculated with an unpaired Student's *t*-test.