The Transcription Factor Bhlhe40 Regulates Tissue-Resident Macrophages and Type 2 Immunity

Nicholas N. Jarjour
Washington University in St. Louis

Follow this and additional works at: https://openscholarship.wustl.edu/art_sci_etds

Part of the Allergy and Immunology Commons, Medical Immunology Commons, Microbiology Commons, and the Parasitology Commons

Recommended Citation

This Dissertation is brought to you for free and open access by the Arts & Sciences at Washington University Open Scholarship. It has been accepted for inclusion in Arts & Sciences Electronic Theses and Dissertations by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.
The Transcription Factor Bhlhe40 Regulates Tissue-Resident Macrophages and Type 2 Immunity

by

Nicholas Najib Jarjour

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

December, 2019
St. Louis, Missouri
### Table of Contents

List of Figures........................................................................................................................................iv
Acknowledgements....................................................................................................................................vi
Abstract ....................................................................................................................................................ix

**Chapter 1: Introduction** ..........................................................................................................................1
1.1 Transcription factors ..........................................................................................................................1
1.2 Bhlhe40..................................................................................................................................................2
1.3 Type 2 immunity ...................................................................................................................................4
1.4 Macrophages .........................................................................................................................................5
1.5 Transcriptional regulation of macrophages ........................................................................................7
1.6 Cell cycling of hematopoietic cells ......................................................................................................10
1.7 Resident macrophage self-renewal and expansion .............................................................................12
1.8 T cell responses in peripheral tissues ..............................................................................................14
1.9 Regulation of Th2 cell cytokine production .......................................................................................14

**Chapter 2: Bhlhe40 is a tissue-specific transcriptional regulator of the proliferation of peritoneal macrophages** .................................................................16
2.1 Abstract ..............................................................................................................................................16
2.2 Introduction .........................................................................................................................................17
2.3 Results ................................................................................................................................................19
   Loss of Bhlhe40 selectively reduces LPMs ...........................................................................................19
   Bhlhe40 is required in LPMs for self-renewal .....................................................................................20
   Bhlhe40 is required intrinsically in LPMs for cell cycling .................................................................21
   Loss of Bhlhe40 dysregulates a unique set of genes in LPMs ............................................................22
   Bhlhe40 is required for LPM responses during type 2 immunity .......................................................24
   Bhlhe40 regulates proliferation, but not polarization, of LPMs .......................................................25
   Monocytes can acquire a Bhlhe40-dependent proliferative program ..............................................26
   Bhlhe40 regulates LPM proliferation in response to *H. polygyrus* ................................................27
   Bhlhe40 controls cell cycle-related transcription .............................................................................28
   Bhlhe40 targets cell cycle-related loci directly ..................................................................................29
2.4 Discussion ..........................................................................................................................................31
2.5 Methods ............................................................................................................................................33

**Chapter 3: Bhlhe40 is required for protective Th2 cell responses to helminth infection** ..................76
Bhlhe40 is required for control of *H. polygyrus* rechallenge ........................................... 80
Bhlhe40 is required in T cells for normal immunity to *H. polygyrus* ..................................... 81
Bhlhe40 is required for a normal CD4\(^+\) T cell transcriptional response to *H. polygyrus* ........ 82
Loss of Bhlhe40 impairs T cell cytokine production in response to *H. polygyrus* .................. 83
Loss of the β\(_c\) chain impairs protective memory to *H. polygyrus* .................................. 84

3.4 Discussion ......................................................................................................................... 85
3.5 Methods .......................................................................................................................... 88

Chapter 4: Conclusions and Future Directions ........................................................................ 114
4.1 Introduction ...................................................................................................................... 114
4.2 Macrophages .................................................................................................................. 114
4.3 Helper T cells .................................................................................................................. 115
4.4 Type 2 immunity ............................................................................................................ 116
4.5 Common beta chain family cytokines ............................................................................. 118
4.6 A unifying role for Bhlhe40 .......................................................................................... 119
4.7 Concluding remarks ....................................................................................................... 120

References ............................................................................................................................. 122
List of Figures

Figure 2.1 Loss of Bhlhe40 dysregulates the cell cycle in LPMs........................................45
Figure 2.2 Bhlhe40 is specifically required in peritoneal and pleural macrophages............47
Figure 2.3 Bhlhe40 is cell-intrinsically required in LPMs to regulate the cell cycle.............49
Figure 2.4 Bhlhe40 is cell-intrinsically required in LPMs..................................................52
Figure 2.5 Bhlhe40 regulates a distinct set of genes related to alternative activation in LPMs....54
Figure 2.6 Bhlhe40 is required for normal accumulation of resident, but not recruited, macrophages in the peritoneum........................................................................56
Figure 2.7 Further analysis of responses to IL-4c in Bhlhe40-deficient mice.....................57
Figure 2.8 Bhlhe40 is required for normal cycling, but not polarization, of peritoneal macrophages during type 2 immunity.................................................................59
Figure 2.9 Loss of Bhlhe40 causes morphological changes in in vivo IL-4c-stimulated peritoneal macrophages .........................................................................................62
Figure 2.10 Bhlhe40 expression is tightly regulated in resident macrophages...................63
Figure 2.11 Bhlhe40 is required for normal proliferation of thioglycollate-elicited macrophages during type 2 immunity.................................................................65
Figure 2.12 Bhlhe40 is required for LPM proliferation in response to H. polygyrus..........66
Figure 2.13 Bhlhe40 regulates gene expression to modulate proliferation, but not alternative activation, in LPMs during type 2 immunity..................................................68
Figure 2.14 Bhlhe40 directly regulates gene transcription in LPMs.................................70
Figure 2.15 Bhlhe40 directly regulates gene expression in LPMs in an activation state-dependent manner........................................................................72
Figure 2.16 Bhlhe40 directly binds to cell cycle-related loci and is required to sustain normal gene expression .................................................................74
Figure 3.1 Bhlhe40 is required for a protective recall response to H. polygyrus.............96
Figure 3.2 Bhlhe40 is required for normal myeloid cell responses to H. polygyrus rechallenge...98
Figure 3.3 Loss of Bhlhe40 dysregulates myeloid cell responses to H. polygyrus rechallenge....99
Figure 3.4 Bhlhe40 is required in T cells for a normal memory response to H. polygyrus.......100
Figure 3.5 Loss of Bhlhe40 dysregulates the CD4+ T cell transcriptional response to *H. polygyrus* rechallenge ........................................................................................................................................102

Figure 3.6 Bhlhe40 regulates distinct gene sets in CD4+ T cells from naïve and *H. polygyrus*-rechallenged mice .......................................................................................................................................104

Figure 3.7 Bhlhe40 is required for normal CD4+ T cell production of βc chain-dependent cytokines .......................................................................................................................................106

Figure 3.8 Bhlhe40 is required in T cells for normal cytokine production during *H. polygyrus* rechallenge .......................................................................................................................................108

Figure 3.9 *In vitro*-polarized Th2 cells require Bhlhe40 for normal cytokine production........109

Figure 3.10 Genetic deletion of IL-10 in *Bhlhe40*+/− *Il10*+/− mice does not restore control of *H. polygyrus* rechallenge .......................................................................................................................................110

Figure 3.11 Combined deficiency in IL-5 and GM-CSF results in impaired control of *H. polygyrus* rechallenge ......................................................................................................................................111

Figure 3.12 The βc chain is necessary for control of *H. polygyrus* rechallenge .......................113
Acknowledgements

A moment like this is the culmination of a long journey, aided by many friends along the way. First and foremost, I would like to express my deepest gratitude to my mentor, Brian Edelson. Brian is one of the keenest intellectuals I’ve ever met and is deeply passionate about science. That enthusiasm for our work is a constant encouragement throughout the ups and downs that are inevitable in science. Brian is fiercely committed to the success of his trainees, achieving a rare balance between being supportive and also allowing us the space to flourish as independent scientists. I will always treasure the last 5 years and the discoveries we’ve made.

I would like to express a thousand thanks to the Edelson lab, especially Tara Bradstreet, Liz Schwarzkopf, Chih-Chung “Jerry” Lin, and Melissa Cook. Tara and Liz, thanks for putting up with all the crazy experiments and the literal shit. Absolutely none of this would have been possible without you, and I am so grateful! Jerry and Melissa, thank you for being a listening ear for new experiments or processing data. It has been my privilege to work with two scientists of such promise, and a joy to grow as intellectuals together. I can’t wait to see what the future holds for both of you!

To my classmates, Prachi Bagadia, Chin-Wen “RC” Lai, and Rachel Wong. It has been an honor to spend these years (mostly) together with you and to watch each of you grow as a scientist. When we came to Washington University, we thought we were gaining colleagues. In reality, we were gaining wonderful friends that would support us through all the trials of graduate school. We made it!

To my thesis committee, I have benefited immeasurably from your mentorship and advice along the way. Your generosity epitomizes the Washington University immunology
community, and your support of my career has been critical to my development into the scientist I am today. As I strike off on a new adventure, I hope to put those lessons to good use.

To the Washington University community. This is an extraordinary place because of each of you. Your tireless commitment, passion for science, intellectual brilliance, and generosity have made the last 6 years of my life a time I will treasure forever. This will forever be a home to me. To the next generation, uphold the tradition, but don’t be beholden to it.

To all those I’ve worked with over the years, including Teresa Ai, Paul Allen, Max Artyomov, Prachi Bagadia, Jennifer Bando, Wandy Beatty, Jacco Boon, Tara Bradstreet, Traci Bricker, Dorjan Brinja, Michael Buck, Javier Carrero, Marina Cella, Chih-Hao Chang, KC Choi, Marco Colonna, Melissa Cook, Alissa Cullen, Jonathan “JC” Curtis, Chris Farnsworth, Susan Gilfillan, Anshu Gounder, Matt Gubin, Paul Huang, Stan Huang, Jeremy Huynh, Stoyan Ivanov, Kiwook Kim, Jacqueline Kimmey, Chin-Wen Lai, Erica Lantelme, Jerry Lin, Ananya Mitra, Ashley Milam, David O’Sullivan, Santosh Panda, Bijal Parikh, Eugene Park, Swapneel Patel, Ed Pearce, Erika Pearce, Beatrice Plougastel, Jing Qiu, Gwen Randolph, Michelle Robinette, Brian Saunders, Elizabeth Schwarzkopf, Irina Shchukina, Dorothy Sojka, Christina Stallings, Thaddeus Stappenbeck, Sandeep Tripathy, Ashraf Ul Kabir, Emil Unanue, Joseph Urban, Jr., Steven Van Dyken, Gerritje Van Der Windt, Rachel Wong, Jesse Williams, Renee Wu, Wayne Yokoyama, Nan Zhang, and others unintentionally omitted. Thank you for the chance to do amazing science together! Also, a very special thank you to all of the DCM staff that makes our work possible.

To Amy Lovett-Racke, John Gunn, and the members of their labs at the Ohio State University. Thanks for putting up with a clueless undergraduate with endless patience. If not for your kindness and constant mentorship, I would not be here today.
I would like to thank my family for shaping me into the intellectual and man that I am today. Through your tireless efforts, I was given this opportunity, and I am so grateful for your support over the past six years and beyond. I would also like to thank my spiritual family in Missouri, Ohio, and beyond. Your support as I have pursued my dreams has meant the world to me.

Last, but certainly not least, I would like to thank my wife Cate for putting up with long hours, esoteric conversations, and late-night Skype conversations with Brian. You have been such a support and inspiration to me over the last three and a half years, and I have done my best work since I met you. You have taught me that there is so much more to life than science. You will forever be the greatest treasure from my time in St. Louis. Here’s to our new adventure together!

Nicholas Najib Jarjour

Washington University in St. Louis

December 2019
ABSTRACT OF THE DISSERTATION

The Transcription Factor Bhlhe40 Regulates Tissue-Resident Macrophages and Type 2 Immunity

by

Nicholas Najib Jarjour

Doctor of Philosophy in Biology and Biomedical Sciences

Immunology

Washington University in St. Louis, 2019

Assistant Professor Brian T. Edelson, Chair

Transcriptional control of gene expression is essential for life, tailoring protein production to development and environment to maintain organismal homeostasis. A limited set of proteins termed transcription factors are critical to this process. As our understanding of these central regulators has improved, new aspects of cell and organismal biology have been revealed. Herein, we demonstrate the importance of the transcription factor Bhlhe40 to tissue-resident macrophages, T helper type 2 cells, and type 2 immune responses, revealing novel transcriptional control of macrophages and unexpected cytokine regulation of helminth infection. We find that Bhlhe40 is cell-intrinsically required for normal proliferation of large serous cavity macrophages, but not other tissue-resident macrophage populations, revealing tissue-specific control of macrophage cycling active in homeostasis and type 2 immunity. Furthermore, we demonstrate that Bhlhe40 is critical for a normal transcription response of T helper cells to secondary infection with the helminth Heligmosomoides polygyrus bakeri (H. polygyrus). T cell-intrinsic loss of Bhlhe40 impairs protective memory to H. polygyrus and reveals novel
redundancy between the common beta chain-dependent cytokines GM-CSF and IL-5 in anti-helminth immunity.
Chapter 1: Introduction

1.1 Transcription factors

Transcriptional control is central to the structure and function of all life. Within the mammalian hematopoietic system, many transcription factors play indispensable roles in leukocyte lineages, including T-bet (T helper type 1 cells [T\textsubscript{H}1]) (Szabo et al., 2000), Gata binding protein 3 (Gata3, T\textsubscript{H}2 and type 2 innate lymphoid cells) (Zheng et al., 1997; Hoyler et al., 2012), retinoic acid receptor-related orphan receptor γt (RORγt, T\textsubscript{H}17 cells) (Ivanov et al., 2006)), Forkhead box P3 (FoxP3, regulatory T cells) (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003), Basic leucine zipper transcriptional factor ATF-like 3 (Batf3, CD8\textsuperscript{+} dendritic cells) (Hildner et al., 2008), PU.1 (macrophages and effects on other hematopoietic lineages) (Zhang et al., 1994), SpiC (red pulp macrophages) (Kohyama et al., 2009), Gata6 (large peritoneal macrophages [LPMs]) (Rosas et al., 2014; Gautier et al., 2014; Okabe and Medzhitov, 2014), Nuclear receptor subfamily 4 group A member 1 (Nr4a1, patrolling monocytes and thymic macrophages) (Hanna et al., 2011; Tacke et al., 2015), peroxisome proliferator-activated receptor γ (PPARγ, alveolar macrophages [AMs]) (Schneider et al., 2014), interferon response factor 4 (IRF4, small peritoneal macrophages [SPMs], CD4\textsuperscript{+} dendritic cells) (Kim et al., 2016; Tamura et al., 2005), IRF8 (CD8\textsuperscript{+} dendritic cells) (Tamura et al., 2005), liver X receptor α (LXRα, marginal zone macrophages) (A-Gonzalez et al., 2013), ThPOK (CD4\textsuperscript{+} T cells) (He et al., 2005; Sun et al., 2005), Runt-related transcription factors (Runx, CD8\textsuperscript{+} T cells) (Woolf et al., 2003; Setoguchi et al., 2008), Myb (hematopoietic stem cells) (Schulz et al., 2012), and many others. Transcription factors control every aspect of a cell’s biology and are regulated by environmental factors and cell-intrinsic cues to enforce proper genetic programs for each given cell type. To achieve the great diversity of organ systems and cell types found in mammalian systems, transcription
factors are thought to function in a combinatorial fashion to create complex outcomes from a limited number of regulatory proteins (Glass and Natoli, 2016). Transcriptional regulators are also constrained by the accessibility of gene loci within the three-dimensional structure of chromatin, which is itself regulated by transcription factors that recruit histone-modifying enzymes to remodel chromatin accessibility in response to various stimuli (Glass and Natoli, 2016). As a cell differentiates, transcription factors, in partnership with histone-modifying enzymes, begin to close chromatin, restricting possible gene expression. Chromatin accessibility is maintained or regained by the actions of pioneer transcription factors (or lineage-determining factors), which are often shared between closely related cell types (Glass and Natoli, 2016). The chromatin opened by pioneer transcription factors can then be accessed by signal-dependent transcription factors, creating the unique genetic program of each cell type (Glass and Natoli, 2016). While pioneer transcription factors determine the general differentiation state of a cell (e.g. macrophage, T cell, B cell), signal-dependent factors impart unique characteristics to the same cell type within different environments (e.g. a naïve T cell in the brachial lymph node versus a resident memory T cell in the skin). While we have a robust understanding of many pioneer transcription factors, their diversity pales in comparison to that of signal-dependent factors, which are much less understood. Therefore, it is critical to achieve a better understanding of this class of transcriptional regulators.

1.2 Bhlhe40

We had previously performed a screen to identify transcription factors with intriguing expression patterns in myeloid cells, and this led us to an interesting candidate gene expressed in resident macrophages. When looking for transcription factors selectively induced in monocytes by the
cytokines macrophage colony stimulating factor (M-CSF, or CSF-1) or granulocyte-macrophage colony stimulating factor (GM-CSF, or CSF-2), we discovered that Basic helix-loop-helix, member e40 (Bhlhe40) was selectively induced by GM-CSF, but not M-CSF. Bhlhe40 is a transcriptional regulator that can both activate and repress gene targets and has been identified as a retinoic acid responsive gene by multiple groups (Boudjelal et al., 1997; Kato et al., 2014; Ow et al., 2014; Gosselin et al., 2014) as well as a regulator of the circadian clock (Honma et al., 2002). Bhlhe40 is further known to modulate the cell cycle, transcriptionally regulating cyclin D1 and exhibiting transcriptional control by p53 (Wang et al., 2012; Wang et al., 2015). Intriguingly, a recent study demonstrated that normal cycling of B1a cells is dependent on Bhlhe40 and the related transcription factor Bhlhe41 (with a primary dependence on Bhlhe41) (Kreslavsky et al., 2017). Taken together, these data connect Bhlhe40 to the cell cycle, but there is little evidence establishing a major role for this transcription factor in regulating the proliferation of hematopoietic cells.

Within the immune system, Bhlhe40 is known to play an important role in various helper T cell subsets, including in T\textsubscript{H}1, T\textsubscript{H}17, and regulatory T cells (Yu et al., 2018; Lin et al., 2016; Miyazaki et al., 2010). While the related transcription factor Bhlhe41 has been shown to regulate T\textsubscript{H}2 cells (Yang et al., 2009; Liu et al., 2009), Bhlhe40 was not thought to regulate this population’s biology. However, a recent publication suggested that Bhlhe40 is a key regulator of \textit{in vitro}-polarized T\textsubscript{H}2 cells (Henriksson et al., 2019). We and others have previously shown that Bhlhe40 is a critical regulator of T cell cytokine production in the multiple sclerosis model experimental autoimmune encephalomyelitis (Martinez-Llordella et al., 2013; Lin et al., 2014; Lin et al., 2016). Furthermore, we have described a key role for Bhlhe40 in myeloid cells and T cells in repressing interleukin-10 (IL-10) production to allow control of \textit{Mycobacterium tuberculosis} (Huynh et al., 2018), while another group has shown a similar role in T cells in protective immune responses...
against *Toxoplasma gondii* (Yu et al., 2018). The best understood instance of cytokine regulation by Bhlhe40 is repression of the *Il10* locus, which occurs via direct repression by Bhlhe40 and by Bhlhe40-mediated suppression of c-Maf, which itself supports IL-10 production (Huynh et al., 2018; Yu et al., 2018; Gabrysova et al., 2018). Mechanistically, how Bhlhe40 antagonizes the Maf transcription factors is unclear. Taken together, these data collectively reveal important roles for Bhlhe40 in multiple T helper cell subsets, especially in the regulation of cytokine production, though it remains unclear whether Bhlhe40 regulates Th2 cells and type 2 immune responses. It is also uncertain whether Bhlhe40 regulates myeloid cells, despite marked expression in select macrophage populations (Lin et al., 2016).

### 1.3 Type 2 immunity

In contrast to many antiviral and antibacterial immune responses, the optimal type 2 response to helminths and allergens is often characterized by a state of tolerance, as these insults are generally present at delicate mucosal surfaces and in some cases are relatively innocuous. In the setting of many parasite infections, the sheer size of the organism renders phagocytosis an impossibility, requiring the secretion of anti-parasite effectors which are often stored in granules in eosinophils, basophils, and mast cells. These molecules, including histamine, leukotrienes, prostaglandins, and enzymes, are thought to either have directly toxic effects on parasites or to contribute to the so-called “weep and sweep” response to expel worms by mucus production and intestinal contractility (Motran et al., 2018). Tissue-resident and/or monocyte-derived macrophages are also thought to play a key role in directly targeting parasites, likely through resistin-like proteins, chitinases, and arginase enzymatic activity (Anthony et al., 2006; Allen and Sutherland, 2014; Maizels et al., 2018; Patel et al., 2009; Sorobetea et al., 2018).
Our understanding of the recruitment and activation of these effector populations has been significantly advanced by recent studies which have established an elegant system of cell-cell interactions and feedback loops prominently featuring innate lymphoid cells (ILCs) and epithelial tuft cells (Gerbe et al., 2016; Howitt et al., 2016; von Moltke et al., 2016). Activation of tuft cells increases their secretion of IL-25, which then acts on lymphocytes (initially type 2 ILCs (ILC2s)) to stimulate IL-13 production, which then acts back on epithelial progenitors to increase differentiation of tuft cells, thereby further enhancing IL-25 production and creating a positive feedback loop (Gerbe et al., 2016; Howitt et al., 2016; von Moltke et al., 2016). It is not yet entirely clear how this equation changes with the arrival of TH2 cells at the site of infection, but presumably these cells can replace or complement ILC2s in this circuit. While in some cases, helminths can be expelled without contributions from the adaptive immune system (Loser et al., 2019), in the majority of cases, TH2 cells will be recruited and a chronic infection will be established. In addition to production of IL-13, TH2 cells and/or ILC2s are stimulated to secrete other type 2 cytokines such as IL-5, which drives recruitment of eosinophils, and IL-4, which, along with IL-13, contributes to macrophage alternative activation (Patel et al., 2009; Jenkins et al., 2013; Allen and Sutherland, 2014; Maizels et al., 2018; Sorobetea et al., 2018). Key observations of the hyperproliferative behavior of macrophages during type 2 responses revolutionized our understanding of their biology and complemented a number of concurrent studies demonstrating that existing paradigms for their ontogeny were deeply flawed.

1.4 Macrophages
As a result of the work of many groups, we now understand that after conception, the yolk sac gives rise to the first wave of fetal macrophages, which colonize the developing embryo (Hoeffel
et al., 2015; Gomez-Perdiguero et al., 2015; Sheng et al., 2015). Later in development, these populations are largely replaced by a second wave of fetal liver-derived macrophages (Hoeffel et al., 2015; Gomez-Perdiguero et al., 2015; Sheng et al., 2015). Many of these embryonically-derived macrophages persist into adulthood; however, different macrophage populations do exhibit varying degrees of replacement by adult bone marrow-derived monocytes (Hoeffel et al., 2015; Gomez-Perdiguero et al., 2015; Sheng et al., 2015). Thus, throughout organismal life, macrophages colonize the body. In addition to phagocytosing dying cells during development and adult life (Cummings et al., 2016; A-Gonzalez et al., 2017), macrophages also possess a variety of tissue-specific accessory roles.

A simplified initial view of macrophage biology, termed the M1/M2 paradigm, stipulated that macrophages polarized one of two ways, either into an M1 inflammatory phenotype (characterized by production of cytokines like tumor necrosis factor α and expression of inducible nitric oxide synthase) or an M2 anti-inflammatory, reparative state (characterized by induction of arginase and production of cytokines like IL-10) (Ginhoux et al., 2016). While of some utility, this view is now seen as an oversimplification. One positive effect of the M1/M2 paradigm is that it countered the traditional view that defined macrophages as pathogen-focused phagocytes, leading to a more nuanced view emphasizing their roles as tissue stromal cells adapted to their environment.

Numerous examples of tissue-specific functions of macrophages have now been reported. AMs are required to handle surfactant and other material in the lung, as shown by the development of alveolar proteinosis in their absence (Shibata et al., 2001a; Shibata et al., 2001b; Schneider et al., 2014). Without red pulp macrophages of the spleen, clearance of red blood cells is impaired and an iron overload phenotype develops (Kohyama et al., 2009). Serous cavity macrophages have
been proposed to function as a reservoir of first responders to injuries of organs within these cavities or free bacteria (Wang et al., 2016; Zhang et al., 2019; Deniset et al., 2019). Splenic marginal zone macrophages are required for normal marginal zone development (A-Gonzalez et al., 2013). Furthermore, the field is just beginning to describe the signaling crosstalk connecting macrophages with non-hematopoietic cell types, for example with the nervous system in the gut or cardiomyocytes in the heart (Gabanyi et al., 2016; Hulsmans et al., 2017). Another example of such crosstalk is how adipose tissue macrophages can regulate thermogenesis (Nguyen et al., 2011). It is perhaps unsurprising that cells of such a ubiquitous nature as macrophages possess other critical roles, especially in organisms which rarely confront pathogens within the solid organs of the body. Thus, tissue-resident macrophages spend the majority of their lifespan fulfilling homeostatic roles, rather than responding to pathogens. Therefore, the identity of macrophages within different tissues must considerably differ to fulfill these unique accessory functions. At the transcriptional level, resident macrophages possess significant diversity, exceeding that of dendritic cells within the same tissues (Gautier et al., 2012). This point is logical because all dendritic cells possess a common functional program of antigen acquisition within tissues followed by migration through a network of lymphatics to one of many highly related lymph nodes. This is in stark contrast to resident macrophages’ different functions within each tissue, which are thought to be supported by distinct gene expression patterns created by tissue-specific transcription factors.

1.5 Transcriptional regulation of macrophages

One mechanism which enforces tissue adaptation on macrophages is tissue-specific transcription factors. The first of these factors to be identified, Spi-C, was shown to be required for development of red pulp macrophages (Kohyama et al., 2009). Three groups independently
demonstrated that Gata6 was required for the generation of LPMs (Rosas et al., 2014; Gautier et al., 2014; Okabe and Medzhitov, 2014). Other studies showed key roles for peroxisome proliferator-activated receptor (PPAR) γ in AMs, NR4A1 in thymic macrophages, Id2 in Langerhans cells, IRF4 in SPMs, and liver x receptor α in splenic marginal zone macrophages (Scheider et al., 2014; Tacke et al., 2015; Chopin et al., 2013; Hacker et al., 2003; Kim et al., 2016; A-Gonzalez et al., 2014). Additionally, C/EBPβ is required for normal populations of LPMs, SPMs, and AMs (Cain et al., 2013). The transcription factor Zeb2 was recently shown to act broadly to help maintain tissue-resident macrophage identity (Scott et al., 2018). Thus, the regulation of resident macrophages is complex, employing a combination of specific or shared transcriptional regulators that may also play a key role in monocytes, dendritic cells, or other macrophage populations (e.g. C/EBPβ, NR4A1, ID2, Zeb2) (Cain et al., 2013; Hanna et al., 2011; Hacker et al., 2003; Scott et al., 2018). While still controversial, there is also strong evidence that environment dominates over ontogeny in defining the transcriptional program of resident macrophages, though further epigenetic studies are needed (Gibbings et al., 2015; van de Laar et al, 2016). Taken together, it is apparent that our understanding of the specific transcriptional controls regulating resident macrophages and the stimuli which induce them remains limited. In light of this, it is perhaps unsurprising that attempts to use macrophages therapeutically remain far from the clinic, though a study correcting alveolar proteinosis by macrophage transplantation showed promise (Suzuki et al., 2014). Furthermore, much of the literature is founded on studies of in vitro-derived or cultured cells, such as bone marrow-derived macrophages. It is nearly impossible to develop a biologically relevant understanding of a cell like the macrophage in vitro because of the critical role of environment in enforcing cellular identity. To achieve an
understanding of macrophage biology and to develop therapeutic applications, it is critical to employ \textit{in vivo} macrophage populations amenable to manipulation.

A cell of historical importance, the LPM is one of the most accessible macrophage populations of mammals and is thus a useful experimental subject for the study of tissue-resident macrophages. From studies of macrophage epigenetics and transcriptional regulation heavily relying on LPMs, a paradigm has been proposed whereby PU.1, which is a pioneer transcription factor required for the macrophage developmental program, cooperates with lineage-determining transcription factors or tissue-specific signal-dependent to drive tissue-resident macrophage differentiation (Gosselin et al., 2014). This suggests a mechanism by which core macrophage genes driven by PU.1 could be activated in all macrophages, while still inducing subset specific gene expression. While as of yet there is little direct evidence of the biological importance of this unifying theory, this study also demonstrates the utility of LPMs as a model macrophage.

The development of LPMs is already known to require the LPM-specific transcription factor Gata6 (Rosas et al., 2014; Gautier et al., 2014; Okabe and Medzhitov et al., 2014), which may be induced by omentum-derived retinoic acid (Okabe and Medzhitov, 2014). As Bhlhe40 is also induced by retinoic acid in some cell types, this suggests that it may be of functional importance in LPMs. Until recently, the origins and regulation of the other population of peritoneal macrophages, SPMs, were unknown (Ghosn et al., 2010). We now know that SPMs are constantly replaced by monocytes and depend on IRF4 (Kim et al., 2017). In contrast to the maintenance of SPMs by monocyte replacement, studies on large serous cavity macrophages have revealed the remarkable capacity of macrophages to proliferate in homeostasis and type 2 immunity (Jenkins et al., 2011). Even in more inflammatory settings, LPMs retain a remarkable ability to self-renew after an enormous reduction in number within the peritoneal cavity, possibly from a subset of
LPMs expressing low levels of the renewal negative regulators MafB and c-Maf (Davies et al., 2011; Soucie et al., 2016). These findings helped to overturn aspects of “the mononuclear phagocyte system,” or the belief that resident macrophages were continuously connected to monocytes and bone marrow hematopoiesis by replacement (Hume, 2006). In many cases, we now know that self-renewal and population expansion are dependent on in situ proliferation of resident macrophages, which is remarkable in light of their terminally differentiated state.

1.6 Cell cycling of hematopoietic cells

The two most fundamental biological processes are proliferation and cell death. Proper regulation of these processes is essential to normal development, tissue repair, control of infection, and avoidance of cancer. Every organ system exists in a constant equilibrium between life and death, with stem cell populations giving rise to terminally differentiated cells with a finite lifespan. After exit from quiescence, or Gap 0, cells cycle through four phases, Gap 1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M) (Vermeulen et al., 2003; Satyanarayana and Kaldis, 2009). This process is tightly regulated by cyclins, cyclin-dependent kinases (CDKs), retinoblastoma (Rb), and other regulators, including tumor suppressors such as p53 and CDK inhibitors (i.e. p21, p27) (Vermeulen et al., 2003; Satyanarayana and Kaldis, 2009). Within G1, accumulation of D cyclins activates CDK4/6 to allow progression through the restriction point, or the point of irrevocable commitment to the cell cycle without further extrinsic factors (Vermeulen et al., 2003; Satyanarayana and Kaldis, 2009). CDK4/6 then activate transcription of E2F transcription factors and phosphorylates Rb, allowing E2Fs to activate cyclin E transcription (Vermeulen et al., 2003; Satyanarayana and Kaldis, 2009). Cyclin E accumulation then activates CDK2 to allow progression into S phase and genome replication (Vermeulen et al., 2003;
Satyanarayana and Kaldis, 2009). Cyclin A-activated CDK2 promotes progression through S phase, while accumulation of Cyclin B activates CDK1 to initiate mitosis (Vermeulen et al., 2003; Satyanarayana and Kaldis, 2009). However, further work has demonstrated that surprising deviations from this schema do occur and that loss of regulators deemed to be essential for cell proliferation can result in surprisingly mild phenotypes predominantly affecting specific cell types, as for genetic deletion of cyclin D2 or cyclin D3, which respectively affect B and T cells among other cell types (Barriere et al., 2007; Satyanarayana and Kaldis, 2009). These data indicate that there is nuance to the regulation of the cell cycle between different cell types (Barriere et al., 2007; Satyanarayana and Kaldis, 2009).

As for other organs, the hematopoietic system must maintain itself. However, this occurs in some unusual ways. One of the defining characteristics of adaptive immunity is clonal expansion, or the dramatic proliferative expansion of a rare antigen-specific lymphocyte clone in response to cognate antigen. In recent work, tissue-resident T cells have been shown to locally proliferate and to be competent to generate a tissue immune response independent of memory cells resident in the lymphoid system (Beura et al., 2018). While tissue macrophages were long thought to be constantly replaced by monocytes, recent work has clearly demonstrated that the majority of these populations self-maintain independent of the bone marrow (Hashimoto et al., 2013; Yona et al., 2013). This is also true of mast cells (Gentek et al., 2018). Furthermore, during certain immune responses, resident macrophages are capable of remarkable local proliferation, greatly expanding the population without contribution from monocytes (Jenkins et al., 2011). Thus, as for other biological systems, proliferation is essential to hematopoietic lineages. But it now appears that for many hematopoietic lineages, terminally differentiated cells are (surprisingly) capable of proliferation. As we have learned more about the role of proliferation
within the hematopoietic compartment, it has become clear that we have a very limited understanding of this process in many cell types. It is now known that the transcription factors c-Maf and MafB (multiple macrophage lineages); Bhlhe41 (B1a cells); and AP4 (T and B cells) are novel regulators of hematopoietic proliferation (Aziz et al., 2009; Soucie et al., 2016; Kreslavsky et al., 2018; Chou et al., 2014; Chou et al., 2016). However, this limited set of factors is likely only a tiny fraction of undiscovered regulatory proteins. A fascinating outstanding question is whether mechanisms that allow terminally differentiated hematopoietic cells to proliferate can be exploited to therapeutically stimulate stromal regeneration in injury and disease.

1.7 Resident macrophage self-renewal and expansion

Classically, tissue macrophages were considered to be constantly replaced by monocytes, as can occur during infection. In contrast to this, two seminal studies established the embryonic origin of tissue-resident macrophages by showing that microglia were derived from yolk sac macrophages and that development of many resident macrophage populations was independent of the transcription factor Myb, which is required for classical hematopoiesis (Ginhoux et al., 2010; Schulz et al., 2012). While both groups established that resident macrophage maintenance could occur independent of the adult bone marrow (Ginhoux et al., 2010; Schulz et al., 2012), the definitive proof that renewal in the steady state did occur independent of monocytes was provided several years later (Yona et al., 2013; Hashimoto et al, 2013). However, how resident macrophage self-renewal is regulated remains unclear. Recent studies have described the transcription factors Mafb and c-Maf (encoded by Mafb and Maf) as repressors of macrophage proliferation (including in LPMs), as well as the deacetylase Sirtuin1 as a positive regulator of proliferation (Aziz et al.,
2009; Soucie et al., 2016, Imperatore et al., 2017). In contrast, loss of Gata6 has been described to cause multinucleation of LPMs, but Gata6-deficient macrophages are strikingly different from normal LPMs, making it unclear whether this is indicative of a primary defect in proliferation (Rosas et al., 2014; Gautier et al., 2014; Okabe and Medzhitov, 2014). Therefore, whether there is a prominent role for tissue-specific regulators in this process is not well understood.

In addition to their self-renewal ability, resident macrophages are also capable of dramatic expansion in situ independent of the bone marrow (Jenkins et al., 2011). This process can be driven by the classic type 2 cytokines IL-4 and -13, as well as other type 2 cytokines (Jenkins et al., 2011, Jenkins et al., 2013, Jackson-Jones et al., 2016). Infection by helminths and other parasites drives T cell-mediated expansion and polarization of resident macrophages, and these populations are known to help control infection, both by cell-intrinsic and cell-cell mechanisms (Huang et al., 2014; Huang et al.; 2017, Minutti et al., 2017; Anthony et al., 2006; Satoh et al., 2010; Turner et al., 2018; Campbell et al., 2018). Until recently, such macrophage proliferation was assumed to be predominantly controlled by Janus kinase (JAK) and signal transducer and activation of transcription (STAT) transcription factors and to occur similarly in all macrophage lineages. However, a recent study has described lineage-specific regulation of macrophage alternative action and expansion by the secreted collagens surfactant protein A and C1q (Minutti et al., 2017). Furthermore, differences in alternative activation between monocyte-derived and resident macrophages also demonstrate that macrophage ontogeny can inform this process (Gundra et al., 2014, Gundra et al., 2017). Whether there are common regulators of both macrophage self-renewal and rapid proliferation during disease is unknown.
1.8 T cell responses in peripheral tissues

As our understanding of the biology of macrophages has evolved, so has our view of the mechanisms by which T helper cells support macrophage responses. In contrast to the traditional view of recruited T cells driving recruitment of monocytes to form macrophages at the site of infection, we now know that both the relevant T cell and macrophage populations can be resident in the tissue and imprinted with unique transcriptional programs by tissue residency (Masopust et al., 2001; Ginhoux et al., 2010; Schulz et al., 2012; T’Jonck et al., 2018). While it was formerly thought that T cells simply polarized macrophages via cytokine secretion, it is now understood that T cell-derived cytokines also can control their proliferative behavior and release the usual restrictions imposed by the availability of M-CSF on a tissue’s carrying capacity (Jenkins et al., 2011; Jenkins et al., 2013). Furthermore, while we are still at a very early stage, interactions between the tissue stroma and hematopoietic cells are beginning to be established, particularly in the case of epithelial and neural cells (Shibata et al., 2001a; Shibata et al., 2001b; Kohyama et al., 2009; Nguyen et al., 2011; A-Gonzalez et al., 2013; Schneider et al., 2014; Gabanyi et al., 2016; Hulsmans et al., 2017; Panduro et al., 2018; Cohen et al., 2018). In the case of T cells, much of our current understanding of this process is centered around their remarkable capacity for cytokine secretion in an antigen-driven fashion.

1.9 Regulation of Th2 cell cytokine production

Much effort has been spent on establishing the transcriptional regulation of Th2 cells, including the transcription factors BATF (Bao et al., 2016), Bhlhe41 (Yang et al., 2009; Liu et al., 2009), c-Maf (Gabrysova et al., 2018), Gfi-1 (Zhu et al., 2002), IRF4 (Lohoff et al., 2002), NFIL3 (Kashiwada et al., 2011), PPARγ (Nobs et al., 2017), TCF-1 (Yu et al., 2009), and YY1 (Hwang...
et al., 2013), often with a particular emphasis on differentiation. However, a new paradigm is emerging that transcription factors (such as BATF, Bhlhe40, c-Maf, and NFIL3) can regulate T cell effector functions downstream of differentiation (Bao et al., 2016; Martinez-Llordella et al., 2013; Lin et al., 2014; Lin et al., 2016; Huynh et al., 2018; Yu et al., 2018; Gabrysova et al., 2018; Kashiwada et al., 2011). c-Maf and Bhlhe40 are the two best characterized examples of this and likely overlap at the level of regulation of IL-10 (Gabrysova and O’Garra., 2018; Huynh et al., 2018; Yu et al., 2018). Bhlhe40 is now known to regulate GM-CSF, IL-10, and interferon γ production, suggesting that it is a central regulator of T cell cytokine production (Martinez-Llordella et al., 2013; Lin et al., 2014; Lin et al., 2016; Huynh et al., 2018; Yu et al., 2018). However, it is not clear whether Bhlhe40 regulates TH2 cell cytokine production. While unrelated to these studies, it is of great interest to further address the role of Bhlhe40 in regulating cytokine production in other hematopoietic lineages. There are some indications that Bhlhe40 may repress IL-10 production in myeloid lineages (Huynh et al., 2018)
Chapter 2: Bhlhe40 is a tissue-specific transcriptional regulator of the proliferation of peritoneal macrophages

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

Bhlhe40 mediates tissue-specific control of macrophage proliferation in homeostasis and type 2 immunity


*Nature Immunology*, 2019;20(6):687

2.1 Abstract

Most tissue-resident macrophage populations develop during embryogenesis, self-renew in the steady-state and expand during type 2 immunity. Whether shared mechanisms regulate the proliferation of macrophages in homeostasis and disease is unclear. Here we found that the transcription factor Bhlhe40 was required in a cell-intrinsic manner for the self-renewal and maintenance of large peritoneal macrophages (LPMs), but not that of other tissue-resident macrophages. Bhlhe40 was necessary for the proliferation, but not the polarization, of LPMs in response to the cytokine IL-4. During infection with the helminth *Heligmosomoides polygyrus bakeri*, Bhlhe40 was required for cell cycling of LPMs. Bhlhe40 repressed the expression of genes encoding the transcription factors c-Maf and Mafb and directly promoted expression of
transcripts encoding cell cycle-related proteins to enable the proliferation of LPMs. In LPMs, Bhlhe40 bound to genomic sites co-bound by the macrophage lineage-determining factor PU.1 and to unique sites, including Maf and loci encoding cell cycle-related proteins. Our findings demonstrate a tissue-specific control mechanism that regulates the proliferation of resident macrophages in homeostasis and type 2 immunity.

2.2 Introduction

Tissue-resident macrophages are established during embryogenesis (Ginhoux et al., 2010; Schulz et al., 2012; T’Jonck et al., 2018) and are largely maintained by local self-renewal within each organ (Hashimoto et al., 2013; Yona et al., 2013). While some transcription factors specifying distinct macrophage lineages have been described (T’Jonck et al., 2018), differences in the transcriptional basis for self-renewal in distinct macrophage populations are not well understood. Established regulators of self-renewal in multiple macrophage lineages include the anti-proliferative transcription factors c-Maf and MafB (Aziz et al., 2009; Soucie et al., 2016), as well as the pro-proliferative deacetylase Sirtuin1 (Imperatore et al., 2017). The transcription factor Gata6 may exercise tissue-specific control of macrophage self-renewal, as loss of Gata6 causes large peritoneal macrophages (LPMs) to become multinucleated and impairs their proliferation (Rosas et al., 2014). However, deletion of Gata6 also causes changes in the morphology, surface markers and gene expression profile of LPMs (Rosas et al., 2014; Gautier et al., 2014; Okabe and Medzhitov, 2014), illustrating that the study of tissue-specific control of resident macrophage self-renewal can be confounded by significant effects on macrophage identity. It remains unclear to what extent macrophage self-renewal is regulated in a tissue-specific manner and whether any tissue-specific regulation that does exist cooperates with broadly shared regulators.
In addition to their homeostatic self-renewal capacity, resident macrophages can become alternatively activated in response to type 2 cytokines produced in response to stimuli like helminth infection, resulting in dramatic proliferation concomitant with acquisition of a pro-repair or anti-helminth protein expression profile (Jenkins et al., 2011; Jenkins et al., 2013; Ruckerl and Allen, 2014; Minutti et al., 2017; Bosurgi et al., 2017). Until recently, proliferation of all macrophages elicited by type 2 immunity was assumed to be controlled by cytokine signaling through JAK kinases and STAT transcription factors. While it remains unclear whether tissue-specific, cell-intrinsic transcriptional regulation influences this process, the collagens SP-A and C1q act through the receptor Myo18a to mediate extrinsic, tissue-specific regulation of proliferation and alternative activation (Minutti et al., 2017). Signaling through receptors for apoptotic cells also influences these processes in tissue-resident macrophages (Bosurgi et al., 2017). Furthermore, differences in alternative activation between monocyte-derived and resident macrophages indicate that ontogeny influences responses during type 2 immunity (Gundra et al., 2014; Gundra et al., 2017). Whether there are common regulators of macrophage self-renewal at steady-state and proliferation during disease remains unknown.

The transcription factor Bhlhe40 is expressed in some hematopoietic and non-hematopoietic cell types (Kato et al., 2014; Ow et al., 2014), including select resident macrophage populations (Lin et al., 2016). Bhlhe40 binds to DNA at class B E-box motifs and functions primarily as a transcriptional repressor (Sun and Taneja, 2000; St.-Pierre et al., 2002; Park et al., 2012; Ow et al., 2014; Kato et al., 2014; Qian et al., 2014), although examples of transcriptional activation have been described (Li et al., 2006; Qian et al., 2014). Bhlhe40 is dysregulated in some cancers and may regulate cell cycling in specific contexts (Ow et al., 2014). A variety of hematopoietic cell types are regulated by Bhlhe40, including NKT cells and
B cells (Seimiya et al., 2004; Kanda et al., 2016; Kreslavsky et al., 2017; Camponeschi et al., 2018), and it controls cytokine production in T cells during infection and autoimmunity (Martinez-Llordella et al., 2013; Lin et al., 2014; Lin et al., 2016; Huynh et al., 2018; Yu et al., 2018). Bhlhe40 and c-Maf may be interconnected in the regulation of the cytokine IL-10, but how this would occur is unclear (Yu et al., 2018; Gabrysova and O’Garra, 2018). Despite an emerging view that Bhlhe40 is an important regulator of immunity, little is known regarding its role in myeloid cells. Bhlhe40 has been proposed as a tissue-specific binding partner of PU.1 in LPMs, but this has not been directly tested (Gosselin et al., 2014).

Here we found that Bhlhe40 had a unique and cell-intrinsic role in LPMs to regulate self-renewal, proliferation and accumulation during type 2 immunity. In LPMs, Bhlhe40 bound a subset of genomic sites bound by the macrophage lineage-specifying transcription factor PU.1, but also many unique sites, including loci encoding cell cycle-related proteins such as c-Maf. Loss of Bhlhe40 in LPMs led to higher expression of Maf and Mafb mRNA and lower expression of cell cycle-related transcripts. Our findings establish Bhlhe40 as a tissue-specific transcriptional regulator of LPM proliferation active in both homeostatic self-renewal and upon rapid cell cycling during type 2 immunity.

2.3 Results

Loss of Bhlhe40 selectively reduces LPMs

Because Bhlhe40 expression has been observed in select resident macrophage populations (Lin et al., 2016; Gautier et al., 2012; Lavin et al., 2014), we examined macrophages from mice transgenic for a Bhlhe40GFP bacterial artificial chromosome (Bhlhe40GFP+ mice hereafter) (Lin et al., 2016). We observed low or undetectable GFP expression in Ly6GCD115+ Ly6Chi and
Ly6C<sup>lo</sup> blood monocytes, F4/80<sup>hi</sup> splenic red pulp macrophages (hereafter red pulp macrophages), CD45<sup>int</sup>CD11b<sup>+</sup> central nervous system microglia, CD45<sup>+</sup>CD11b<sup>lo</sup>F4/80<sup>hi</sup> liver Kupffer cells (hereafter Kupffer cells), CD45<sup>+</sup>Ly6C<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>hi</sup> kidney macrophages (hereafter kidney macrophages) and CD45<sup>+</sup>Ly6C<sup>-</sup>F4/80<sup>+</sup>CD64<sup>+</sup>MHC-II<sup>+</sup> small intestinal lamina propria macrophages (hereafter SI macrophages), but found high expression of GFP in CD45<sup>+</sup>Siglec-F<sup>-</sup>CD11c<sup>+</sup> lung alveolar macrophages (hereafter AMs), CD115<sup>+</sup>CD11b<sup>+</sup>ICAM2<sup>+</sup>MHC-II<sup>+</sup> large peritoneal macrophages (hereafter LPMs), CD115<sup>+</sup>CD11b<sup>+</sup>MHC-II<sup>+</sup>ICAM2<sup>-</sup> small peritoneal macrophages (hereafter SPMs), CD115<sup>+</sup>CD11b<sup>+</sup>ICAM2<sup>+</sup>MHC-II<sup>+</sup> large pleural macrophages (hereafter large pleural macrophages) and CD115<sup>+</sup>CD11b<sup>+</sup>MHC-II<sup>+</sup>ICAM2<sup>-</sup> small pleural macrophages (hereafter small pleural macrophages) (Fig. 2.1A and Fig. 2.2A). Of the populations examined, only LPMs and SPMs were decreased in Bhlhe40<sup>−/−</sup> compared to Bhlhe40<sup>+/+</sup> mice (Fig. 2.1B, C and Fig. 2.2B-E). In some resident macrophage populations, including LPMs, Tim4 is a marker of embryonically-derived, long-lived resident macrophages (Scott et al., 2016; Bain et al., 2016; Shaw et al., 2018), while CD226 marks mature SPMs (Kim et al., 2016). Decreases in Tim4<sup>+</sup> LPMs and CD226<sup>+</sup> SPMs largely accounted for the reduced number of peritoneal macrophages in Bhlhe40<sup>−/−</sup> mice (Fig. 2.2F-I). The number of peritoneal CD115<sup>+</sup>MHC-II<sup>+</sup>CD19<sup>+</sup> B cells (hereafter B cells) was not reduced in Bhlhe40<sup>−/−</sup> compared to Bhlhe40<sup>+/+</sup> mice (Fig. 2.2J). Therefore, loss of Bhlhe40 selectively reduced the number of LPMs and SPMs.

**Bhlhe40 is required in LPMs for self-renewal**

To address whether the loss of LPMs in Bhlhe40<sup>−/−</sup> mice was due to impaired proliferation, we stained peritoneal cells from Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> mice for Ki67, a marker of cycling cells.
We observed a 4-fold increase in the frequency of Ki67+ LPMs (Fig. 2.1D, E), but little change in the frequency of Ki67+ SPMs and peritoneal B cells in Bhlhe40−/− compared to Bhlhe40+/+ mice (Fig. 2.1E). Despite normal numbers, we found an increase in the frequency of Ki67+ large pleural macrophages in Bhlhe40−/− compared to Bhlhe40+/+ mice (Fig. 2.2K, L). There was no difference in the uptake of bromodeoxyuridine (BrdU), which is incorporated during the S phase, in LPMs from Bhlhe40+/+ and Bhlhe40−/− mice 3 hours after injection with BrdU (Fig. 2.1F, G). Staining for the mitosis marker phosphohistone H3 (pHH3) was similar in Bhlhe40+/+ and Bhlhe40−/− LPMs (Fig. 2.1H, I). When using Ki67 and the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) to separate the phases of the cell cycle, we observed an increased number of LPMs in the G1 phase (Fig. 2.1J, K), but similar numbers of LPMs in the S, G2 and M phases in Bhlhe40−/− compared to Bhlhe40+/+ mice (Fig. 2.1J, K), suggesting that Bhlhe40−/− LPMs were impaired in progressing from G1, but proliferated sufficiently to maintain a stable, although reduced, population of LPMs. The proportion of LPMs staining for the viability dye 7-aminoactinomycin D (7-AAD) was similar in Bhlhe40+/+ and Bhlhe40−/− mice (Fig. 2.2M). Taken together, these data indicate that Bhlhe40 was required for normal proliferation of LPMs.

**Bhlhe40 is required intrinsically in LPMs for cell cycling**

To address whether the role of Bhlhe40 in LPMs was cell-intrinsic, we generated mixed bone marrow chimeras by co-transfer of equal numbers of Bhlhe40+/+ (CD45.1) plus either Bhlhe40+/+ (CD45.2) or Bhlhe40−/− (CD45.2) total bone marrow cells into irradiated Bhlhe40+/+ CD45.1/CD45.2 mice, which were allowed to reconstitute for >8 weeks. Out of peritoneal, blood, splenic, liver, kidney, SI lamina propria, lung and pleural hematopoietic populations examined, only LPMs and large pleural macrophages exhibited Bhlhe40-dependent
reconstitution (Bhlhe40+/+ outnumbering Bhlhe40−/− cells by more than 10:1) (Fig. 2.3A-D and Fig. 2.4A and data not shown). The small number of Bhlhe40−/− LPMs in mixed chimeras accumulated in the G1 phase (Fig. 2.3E, F), indicating that the alterations in cell cycling in Bhlhe40−/− LPMs were cell-intrinsic. Next, we bred LysM-Cre+ Bhlhe40fl/fl mice to delete Bhlhe40 in myeloid cells. Compared to LysM-Cre− Bhlhe40fl/fl mice, LysM-Cre+ Bhlhe40fl/fl mice had a nearly 2-fold reduction in the number of LPMs, with no change in SPMs or peritoneal B cells (Fig. 2.3G) and an increased proportion of LPMs in the G1 phase (Fig. 2.3H, I). Finally, we co-transferred Bhlhe40+/+ (CD45.1) plus either Bhlhe40+/+ (CD45.2) or Bhlhe40−/− (CD45.2) bulk peritoneal cells into resting Bhlhe40+/+ (CD45.1/CD45.2) mice at ratios calculated to result in the transfer of equal numbers of LPMs (200,000-300,000) from each donor. Over four weeks, the relative proportion of transferred Bhlhe40+/+ (CD45.1) to Bhlhe40−/− (CD45.2) LPMs was increased (Fig. 2.3J, K and Fig. 2.4B), while the relative proportion of transferred Bhlhe40+/+ (CD45.1) to transferred Bhlhe40−/− (CD45.2) B cells was maintained in the peritoneum (Fig. 2.3J, L and Fig. 2.4B), supporting a cell-intrinsic role for Bhlhe40 in mature LPMs. Thus, Bhlhe40 was cell-intrinsically required in LPMs for normal proliferation and maintenance.

Loss of Bhlhe40 dysregulates a unique set of genes in LPMs

Next, we performed gene expression microarrays to determine the transcriptional differences between LPMs from Bhlhe40+/+ and Bhlhe40−/− mice. 84 genes were dysregulated by 2-fold or more in Bhlhe40−/− compared to Bhlhe40+/+ LPMs (Fig. 2.5A), including expression changes in several genes related to macrophage alternative activation, such as Chil3, Clec10a, Mrcl and Arg1 (Fig. 2.5B). We validated these data by flow cytometry for several proteins encoded by differentially expressed genes (Emb, Clec10a, Lyve1) (Fig. 2.5C-E). When we assessed the
expression of gene ontology sets (Subramanian et al., 2005) in the absence of Bhlhe40 using the list of genes that were differentially expressed ≥1.5-fold between Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> LPMs, we found that the Regulation of cell proliferation gene set was enriched in Bhlhe40<sup>+/+</sup> LPMs, while some immune response-related gene sets were enriched in Bhlhe40<sup>−/−</sup> LPMs (Fig. 2.5F, G). We validated higher expression of Maf and Mafb mRNA in Bhlhe40<sup>−/−</sup> compared to Bhlhe40<sup>+/+</sup> LPMs in our microarrays (data not shown) by qRT-PCR (Fig. 2.5H), consistent with impaired proliferation of Bhlhe40<sup>−/−</sup> LPMs.

Because the transcription factor Gata6 is an important regulator of LPMs (Rosas et al., 2014; Gautier et al., 2014; Okabe and Medzhitov, 2014), we reanalyzed our microarray data and published microarray data (Gautier et al., 2014) from LysM-Cre<sup>−/−</sup>Gata6<sup>fl/fl</sup> and LysM-Cre<sup>+</sup> to look for differentially expressed genes regulated by both transcription factors. Expression of Gata6 mRNA was not substantially changed in Bhlhe40<sup>−/−</sup> LPMs (log<sub>2</sub> expression, Bhlhe40<sup>+/+</sup> 10.24, Bhlhe40<sup>−/−</sup> 10.10; data not shown), nor did loss of Gata6 cause substantial changes in the expression of Bhlhe40 in LPMs (log<sub>2</sub> expression, LysM-Cre<sup>−/−</sup>Gata6<sup>fl/fl</sup> 10.04, LysM-Cre<sup>+</sup> Gata6<sup>fl/fl</sup> 9.81; data not shown). Furthermore, the majority of Bhlhe40-dependent genes were not dependent on Gata6 and the converse was likewise true (Fig. 2.4C, D).

Next, we performed transcriptome analysis of AMs, which have high expression of Bhlhe40, from Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> mice. Compared to LPMs, Bhlhe40 controlled a largely distinct and smaller group of genes in AMs, mostly encoding proteins involved in antigen presentation by MHC class II (H2-Aa, H2-Ab1, H2-Eb1, Cd74; Fig. 2.5I). A set of genes selectively expressed in LPMs relative to AMs, splenic red pulp macrophages and microglia has been previously curated (Gautier et al., 2012). Bhlhe40 regulated the expression of only a small
subset of these genes (Lrg1, Stard13, Nedd4) (Fig. 2.4E). Therefore, Bhlhe40 regulated a cell type-specific set of genes in LPMs, but was dispensable for identity.

**Bhlhe40 is required for LPM responses during type 2 immunity**

We then asked whether Bhlhe40 was required for macrophage accumulation during peritoneal immune responses characterized either by the differentiation of monocyte-derived macrophages or the local proliferation of LPMs. Intraperitoneal (i.p.) injection of thioglycollate, which elicits the recruitment and differentiation of blood-derived monocytes to the peritoneum independent of proliferation (Gautier et al., 2013; Gundra et al., 2014), resulted in equivalent accumulation of CD115⁺CD11b⁺ICAM2⁺ thioglycollate-elicited macrophages (hereafter thioglycollate-elicited macrophages) in Bhlhe40⁺/⁻ and Bhlhe40⁻/⁻ mice after 4 days (Fig. 2.6A, B), while the i.p injection of IL-4+anti-IL-4 antibody complexes (hereafter IL-4c), which elicit the robust proliferation of resident macrophages (Jenkins et al., 2011), caused a 5-fold increase in the number of LPMs in Bhlhe40⁺/⁻ mice compared to Bhlhe40⁻/⁻ mice after 4 days (Fig. 2.6C, D). Similar findings were obtained in LysM-Cre⁻ Bhlhe40⁻/⁻ and LysM-Cre⁺ Bhlhe40⁻/⁻ mice (Fig. 2.6D), suggesting Bhlhe40 was required for the proliferation of resident LPMs in a cell-intrinsic manner. SPMs were not reduced in Bhlhe40⁻/⁻ and LysM-Cre⁺ Bhlhe40⁻/⁻ compared to Bhlhe40⁺/⁺ and LysM-Cre⁺ Bhlhe40⁻/⁻ mice in response to IL-4c (Fig. 2.6E). Because Bhlhe40 represses the production of IL-10 in T cells and myeloid cells (Lin et al., 2014; Lin et al., 2016; Huynh et al., 2018; Yu et al., 2018), we injected IL-4c i.p. into Bhlhe40⁻/⁻ Il10⁻/⁻ mice to test whether lack of IL-10 restored the IL-4c-driven accumulation of LPMs in the absence of Bhlhe40. Similar to Bhlhe40⁻/⁻ mice, Bhlhe40⁻/⁻ Il10⁻/⁻ mice had poor accumulation of LPMs after injection of IL-4c (Fig. 2.7A, B), indicating IL-10 did not contribute to the impaired response of Bhlhe40⁻/⁻ LPMs.
Taken together, these data indicate that Bhlhe40 was required for normal accumulation of LPMs in response to IL-4c.

**Bhlhe40 regulates proliferation, but not polarization, of LPMs**

We next assessed whether Bhlhe40 regulated induction of the alternative activation markers RELMα and Clec10a in LPMs in response to IL-4c. LPMs from Bhlhe40+/+, Bhlhe40−/−, LysM-Cre− Bhlhe40fl/fl and LysM-Cre+ Bhlhe40fl/fl mice all induced these proteins following i.p. injection with IL-4c (Fig. 2.8A, B). In contrast, IL-4c increased the proportions of BrdU+ LPMs and pH3+ LPMs by approximately 2-fold in Bhlhe40+/+ and LysM-Cre− Bhlhe40fl/fl mice compared to Bhlhe40−/− and LysM-Cre+ Bhlhe40fl/fl mice (Fig. 2.8C, D). IL-4c treatment also elicited a greater increase in the fraction of LPMs in the G1, S and G2M phases of the cell cycle in Bhlhe40+/+ compared to Bhlhe40−/− mice (Fig. 2.8E). Immunoblot analysis of cyclins D1-D3, cyclin dependent kinase (CDK) 2, CDK4, CDK6 and the transcription factor E2F2, which regulate the G1 and S phases of the cell cycle (Bertoli et al., 2013), showed increases in cyclin D3, CDK2 and CDK4 in LPMs from IL-4c-treated compared to naïve mice (Fig. 2.7C); however, their abundance was generally similar in Bhlhe40+/+ and Bhlhe40−/− LPMs (Fig. 2.7C). In contrast to cyclins and CDKs, E2F2 was similar in LPMs from mice injected or not with IL-4c (Fig. 2.7C). 7-AAD+ necrotic LPMs were somewhat increased in IL-4c-injected Bhlhe40−/− and LysM-Cre+ Bhlhe40fl/fl mice compared to Bhlhe40+/+ and LysM-Cre− Bhlhe40fl/fl mice (Fig. 2.8F). In mixed bone marrow chimeras (generated and reconstituted as in Fig. 2.3A-F) injected i.p. with IL-4c, a lower proportion of Bhlhe40−/− LPMs incorporated BrdU compared to Bhlhe40+/+ LPMs within the same recipient (Fig. 2.7D).
We used transmission electron microscopy of bulk peritoneal cells from naïve and IL-4c-treated Bhlhe40^{+/+} and Bhlhe40^{-/-} mice to assess cell morphology. Bhlhe40^{+/+} and Bhlhe40^{-/-} LPMs from IL-4c-treated mice showed increases in cell size and endoplasmic reticulum (ER) extent compared to naïve LPMs (Fig. 2.9). We observed no distinct morphology between naïve Bhlhe40^{-/-} and Bhlhe40^{+/+} LPMs, while LPMs from IL-4c-treated Bhlhe40^{-/-} mice were somewhat larger and more vacuolated than LPMs from IL-4c-treated Bhlhe40^{+/+} mice, without any severe morphologic defects (Fig. 2.9). Therefore, Bhlhe40 was required for LPMs to rapidly cycle in response to IL-4c, but was dispensable for normal morphology and induction of alternative activation markers.

**Monocytes can acquire a Bhlhe40-dependent proliferative program**

Next we asked whether Bhlhe40 was required for the IL-4c-induced proliferation of other macrophages. IL-4c injection i.p. into Bhlhe40^{GFP+} mice did not change the expression of GFP in LPMs, SPMs, AMs, kidney macrophages, red pulp macrophages and Kupffer cells compared to these populations in PBS-treated Bhlhe40^{GFP+} mice (Fig. 2.10A). IL-4c injection i.p. into Bhlhe40^{+/+} and Bhlhe40^{-/-} mice resulted in equivalent BrdU incorporation by, and numbers of, red pulp macrophages, Kupffer cells and AMs (Fig. 2.8G and Fig. 2.10B-E), in contrast to LPMs and large pleural macrophages, which required Bhlhe40 for a normal population of BrdU-incorporating cells in response to IL-4c (Fig. 2.8G).

Injection of thioglycollate and IL-4c i.p. causes monocyte-derived macrophages to proliferate and acquire alternative activation markers (Gundra et al., 2014; Gundra et al., 2017). When we asked whether Bhlhe40 was expressed in these monocyte-derived macrophages, we found that the combination of thioglycollate and IL-4c induced marked expression of GFP in the
thioglycollate-elicited macrophages in Bhlhe40^{GFP+} mice compared to a lower expression of GFP in macrophages elicited by thioglycollate alone (Fig. 2.8H). After treatment with thioglycollate and IL-4c, Bhlhe40^{−/−} mice had severely reduced proportions of BrdU^{+} and pHH3^{+} thioglycollate-elicited macrophages compared to Bhlhe40^{+/+} mice (Fig. 2.8I, J and Fig. 2.11A, B), while RELMα was acquired normally (Fig. 2.11C). Thus, these data indicate that Bhlhe40 is a specific regulator of large serous cavity macrophage proliferation in response to IL-4c and that monocyte-derived macrophages can acquire a Bhlhe40-dependent proliferative program similar to that of serous cavity resident macrophages.

**Bhlhe40 regulates LPM proliferation in response to H. polygyrus**

The intestinal helminth *Heligmosomoides polygyrus bakeri* (*H. polygyrus*) is a natural mouse pathogen that elicits robust proliferation of LPMs following oral infection (Ruckerl and Allen, 2014). Infection with *H. polygyrus* caused a 4-fold increase in the number of LPMs in infected Bhlhe40^{+/+} mice compared to Bhlhe40^{−/−} mice after 8 days (Fig. 2.12A, B). Similar findings were obtained in LysM-Cre^{−} Bhlhe40^{fl/fl} and LysM-Cre^{+} Bhlhe40^{fl/fl} mice (Fig. 2.12B). Furthermore, after *H. polygyrus* infection, the proportions of BrdU^{+} LPMs and pHH3^{+} LPMs were reduced in Bhlhe40^{−/−} and LysM-Cre^{+} Bhlhe40^{fl/fl} compared to Bhlhe40^{+/+} and LysM-Cre^{−} Bhlhe40^{fl/fl} mice (Fig. 2.12C, D). *H. polygyrus* infection elicited a greater increase in the fraction of LPMs in the G1, S and G2M phases of the cell cycle in Bhlhe40^{+/+} compared to Bhlhe40^{−/−} mice (Fig. 2.12E). The proportion of 7-AAD^{+} necrotic LPMs was also increased in Bhlhe40^{−/−} and LysM-Cre^{+} Bhlhe40^{fl/fl} compared to Bhlhe40^{+/+} and LysM-Cre^{−} Bhlhe40^{fl/fl} infected mice (Fig. 2.12F). These data suggested that Bhlhe40 is essential for the proliferation of LPMs during type 2 immunity.
**Bhlhe40 controls cell cycle-related transcription**

To determine the effects of Bhlhe40 on the expression profile of LPMs during type 2 immunity, we performed gene expression microarrays on sorted LPMs from IL-4c-treated Bhlhe40\(^{+/+}\), Bhlhe40\(^{+/+}\) and LysM-Cre\(^+\) Bhlhe40\(^{fl/fl}\) mice after 4 days. More genes (254; Fig. 2.13A, B) were differentially expressed by 2-fold or more between LPMs from IL-4c-treated Bhlhe40\(^{+/+}\) and Bhlhe40\(^{+/+}\) mice compared to LPMs from naive Bhlhe40\(^{+/+}\) and Bhlhe40\(^{+/+}\) mice (87 genes; Fig. 2.13A-C). To ask whether the transcriptional changes that occurred in alternatively activated LPMs were dependent on Bhlhe40, we selected the 55 genes most differentially expressed (≥10-fold different) between naive and IL-4c-treated Bhlhe40\(^{+/+}\) LPMs, including Mgl2, Chil3, Arg1 and Il1rl1 (Fig. 2.13D). These genes were generally normally expressed in Bhlhe40\(^{+/+}\), Bhlhe40\(^{+/+}\) and LysM-Cre\(^+\) Bhlhe40\(^{fl/fl}\) LPMs from IL-4c-treated mice (Fig. 2.13D), and we also found no defect in the expression of Myo18a, C1qa, C1qb and C1qc, which encode known regulators of LPM proliferation during type 2 immune responses (Minutti et al., 2017) (Fig. 2.14A).

Consistent with impaired proliferation, Maf (3.2-fold) and Mafb (3.8-fold) were more highly expressed in Bhlhe40\(^{-/-}\) compared to Bhlhe40\(^{+/+}\) LPMs by qRT-PCR (Fig. 2.13E). Gene set enrichment analysis (GSEA) for Hallmark gene sets (Liberzon et al., 2015) showed prominent enrichment of gene sets related to proliferation, including the E2F targets and Myc targets v1 gene sets in the gene expression data between LPMs from IL-4c-treated Bhlhe40\(^{+/+}\) and Bhlhe40\(^{-/-}\) mice (Fig. 2.13F). Further comparison of differentially expressed genes to the C5 gene ontology sets indicated that LPMs from IL-4c-treated Bhlhe40\(^{+/+}\) mice were substantially enriched for cell cycle and chromosome-related gene sets (Cell cycle, Cell cycle process, Mitotic cell cycle), while LPMs from IL-4c-treated Bhlhe40\(^{-/-}\) mice showed enrichment for the Vacuole gene set (Fig. 2.13G-I), consistent with increased vacuolar area by electron microscopy.
Therefore, Bhlhe40 was required in LPMs for normal regulation of cell cycle-related gene expression.

**Bhlhe40 targets cell cycle-related loci directly**

We next addressed whether Bhlhe40 regulated LPM gene expression via direct binding to gene loci by sorting LPMs from naïve and IL-4c-treated *Bhlhe40*+/+ mice after 4 days for Bhlhe40 chromatin immunoprecipitation sequencing (ChIP-seq). Motif analysis of the called peaks identified the expected CACGTG E-box sequence (Fig. 2.15A) and a majority of Bhlhe40 peaks (naïve 2,245 total peaks; IL-4c-treated 5,011 total peaks) were promoter-associated in both samples (Fig. 2.15B). In both conditions, Bhlhe40 bound sites in the *Bhlhe40* (single peak, within promoter) and *Il10* (single peak, 1 kilobase (kb) downstream of locus) loci previously described in T cells (Fig. 2.14B and Fig. 2.15C) (Huynh et al., 2018). We also identified a novel Bhlhe40 binding site 1.5kb downstream of the *Bhlhe40* locus that was occupied only in LPMs from IL-4c-treated mice (Fig. 2.15C). Many Bhlhe40 peaks were shared between LPMs from naïve and IL-4c-treated mice (1,684 sites, including peaks proximal to the *Klf4*, *Nr1d1*, *Plac8* and *Yy1* loci) (Fig. 2.15D), but the majority were unique to LPMs from IL-4c-treated mice (3,364 sites, including peaks proximal to the *Klf4* and *Nr1d1* loci) (Fig. 2.15D), often in association with shared peaks (as for the *Bhlhe40* locus).

Because Bhlhe40 and PU.1 may cooperate in LPMs (Gosselin et al., 2014), we compared our ChIP-seq data with previously published PU.1 ChIP-seq performed on LPMs from naïve C57BL/6 mice (Gosselin et al., 2014). PU.1 peaks overlapped with 22% or 24% of Bhlhe40 peaks in LPMs from naïve or IL-4c-treated mice, respectively, including the *Clec10a*, *Ccl2* and *Plac8* loci (Fig. 2.14C, D and Fig. 2.15E, F). However, the majority of Bhlhe40 peaks (naïve
1,754 peaks; IL-4c 3,822 peaks) were not associated with PU.1 binding (Fig. 2.15F), including at the Bhlhe40, Maf and Il10 loci. When we assessed whether Bhlhe40 bound directly to genes with Bhlhe40-dependent expression, we found that Bhlhe40 bound a small fraction of genes differentially expressed by two-fold or more between LPMs from naïve Bhlhe40+/+ and Bhlhe40−/− mice (11% of genes downregulated in Bhlhe40−/− LPMs, 17% of genes upregulated in Bhlhe40−/− LPMs, Fig. 2.14E and Fig. 2.15G) and bound a greater fraction of genes differentially expressed between LPMs from IL-4c-treated Bhlhe40+/+ and Bhlhe40−/− mice (15% of genes downregulated in Bhlhe40−/− LPMs, 48% of genes upregulated in Bhlhe40−/− LPMs, Fig. 2.14E and Fig. 2.15G), suggesting a direct role for Bhlhe40 in regulating gene expression in LPMs.

Further analysis of our ChIP-Seq data identified a Bhlhe40 peak within the Maf promoter in LPMs from naïve or IL-4c-treated mice (Fig. 2.15H), as well as two additional peaks closest to the Maf locus (200kb downstream (naïve and IL-4c) and 300kb downstream (IL-4c) of the locus) (Fig. 2.14F), suggesting that Bhlhe40 repressed the Maf locus. There was no clear Bhlhe40 peak uniquely associated with the Mafb locus (Fig. 2.14G, H). To address whether Bhlhe40 directly regulated other cell cycle-related loci, we performed GSEA analysis for the subset of genes directly bound by Bhlhe40 using the gene expression data from LPMs from IL-4c-treated Bhlhe40+/+ and Bhlhe40−/− mice. We found that differential expression of these genes between Bhlhe40+/+ and Bhlhe40−/− LPMs from IL-4c-treated mice largely recapitulated the enrichment of cell cycle-related modules observed when all gene expression data were analyzed (Fig. 2.15I and Fig. 2.16). Thus, Bhlhe40 functioned in LPMs as a direct transcriptional regulator of numerous genomic loci, including those encoding cell cycle-related proteins.
2.4 Discussion

Here we found that the transcription factor Bhlhe40 was an essential cell-intrinsic regulator of proliferation in LPMs. In the steady-state, Bhlhe40\(^{-/-}\) LPMs were reduced in number and a higher proportion accumulated in the G1 phase compared to Bhlhe40\(^{+/+}\) LPMs. During type 2 immunity, Bhlhe40 was essential for normal proliferation and accumulation, with a reduced proportion of Bhlhe40\(^{-/-}\) LPMs in the S and M phases compared to Bhlhe40\(^{+/+}\) LPMs, but Bhlhe40 was dispensable for acquisition of alternative activation markers. Bhlhe40 mediated repression of Maf and activation of multiple proliferation-related loci to allow LPM cell cycling. Bhlhe40 was a tissue-specific regulator of proliferation of LPMs, but could be acquired by peritoneal monocyte-derived macrophages to support a proliferative program.

How deletion of Bhlhe40 impairs cell cycle progression of LPMs remains unclear. We observed that a higher proportion of Bhlhe40\(^{-/-}\) LPMs were Ki67\(^{+}\) compared to Bhlhe40\(^{+/+}\) LPMs, suggesting that Bhlhe40\(^{-/-}\) LPMs might inappropriately enter the cell cycle. However, our data are not consistent with this notion, as we saw a selective increase in the proportion of Bhlhe40\(^{-/-}\) LPMs in the G1 phase, without a commensurate increase in LPMs in the S, G2, or M phases. Both Bhlhe40\(^{+/+}\) and Bhlhe40\(^{-/-}\) LPMs upregulated cyclins and CDKs when mice were treated with IL-4c. In contrast, Bhlhe40\(^{+/+}\) and Bhlhe40\(^{-/-}\) LPMs had low expression of cyclins and CDKs at steady-state. These data support the notion that impaired progression from the G1 phase rather than enhanced proliferation was the primary cause of accumulation of G1 phase LPMs in naïve and likely IL-4c-treated Bhlhe40\(^{-/-}\) mice. As expression of cyclin D and CDKs were comparable between Bhlhe40\(^{+/+}\) and Bhlhe40\(^{-/-}\) LPMs, alterations solely in the expression of these regulators likely do not explain the effect of Bhlhe40 deficiency on LPM proliferation. Instead, the phenotype of Bhlhe40\(^{-/-}\) LPMs was probably due to the impaired transcriptional regulation of a
broad set of cell cycle-related genes caused by loss of Bhlhe40 and upregulation of c-Maf and MafB.

Bhlhe40 and c-Maf may functionally interact in T cells (Huynh et al., 2018; Yu et al., 2018). Our data suggest that Bhlhe40 is a transcriptional repressor of Maf in LPMs. In contrast to T cells and other tissue-resident macrophages, LPMs require Bhlhe40 to support normal proliferation, suggesting that Bhlhe40-mediated repression of Maf has distinct effects in different cell types. Expression of both Bhlhe40 and c-Maf in LPMs results in a unique regulatory interaction not detected in macrophage subsets lacking expression of one of these transcription factors. Downregulation of Maf and Mafb expression is critical for proliferation in macrophages (Aziz et al., 2009; Soucie et al., 2016). Our data are most consistent with a role for Bhlhe40 in repressing Maf and Mafb to permit LPM cell cycling, along with Bhlhe40-mediated regulation of a wider set of target genes, some of them co-bound by PU.1, as previously proposed (Gosselin et al., 2014). It is likely that specific networks of integrated transcriptional regulators control the development and function of resident macrophages in each tissue. In LPMs, this network would include Bhlhe40, PU.1 (Gosselin et al., 2014), c-Maf and MafB (Soucie et al., 2016), as well as Gata6 (Rosas et al., 2014; Gautier et al., 2014; Okabe and Medzhitov, 2014) and C/EBPβ (Cain et al., 2013), two transcription factors whose loss results in impaired development of LPMs.

In addition to LPMs and large pleural macrophages, AMs and, in some contexts, monocyte-derived macrophages expressed Bhlhe40. Bhlhe40−/− AMs only showed minor transcriptional differences compared to Bhlhe40+/+ AMs and no evidence of a proliferative defect. c-Maf and MafB were poorly expressed in AMs (Soucie et al., 2016) and were not induced in Bhlhe40+/− AMs (data not shown), suggesting that Bhlhe40 was not required to repress these transcription factors in AMs. It is also possible that Bhlhe41, which is expressed highly in
AMs but not LPMs (data not shown) and can partially substitute for Bhlhe40 (Kreslavsky et al., 2017), may compensate for the absence of Bhlhe40 in AMs. In contrast to AMs, monocyte-derived macrophages can acquire a Bhlhe40-dependent proliferative program in response to thioglycollate and IL-4c. The similarities of this Bhlhe40-regulated transcriptional program in monocyte-derived macrophages to that of LPMs remain to be explored.

Whether common regulators can control macrophage cell cycling in response to different stimuli during homeostasis (e.g. CSF-1) or type 2 immunity (e.g. IL-4, IL-5, or IL-13) (Jenkins et al., 2013) is unclear. Our findings demonstrate the existence of shared regulation of macrophage proliferation in the steady-state and disease, as well as a crucial role for tissue-specific transcriptional regulation acting in concert with more broadly shared regulators like c-Maf. This suggests the possibility of therapeutically targeting the proliferation of select macrophage populations, including tumor-associated macrophages (TAMs), which are known to partly derive from tissue-resident macrophages and locally proliferate (Franklin et al., 2016; Zhu et al., 2017; Loyher et al., 2018; Mantovani et al., 2017).

Our results illustrate the complexity of tissue-specific control of macrophages, demonstrating that tissue-specific transcription factors are critical for the regulation of macrophage proliferation in health and disease. Our data provide direct evidence that resident macrophages are under constant control by a partnership of shared and tissue-specific transcription factors, with possible implications for therapies.

2.5 Methods

Mice
C57BL/6 (Taconic), B6.SJL (CD45.1, Taconic or Jackson), Il10<sup>−/−</sup> (B6.129P2-Il10tm1Cgn/J, Jackson) and LysM-Cre (B6N.129P2(B6)-Lyζ2<sup>tm1(cre)/IfJ</sup>, Jackson) mice were obtained from the vendors listed. Bhlhe40<sup>−/−</sup> (10 generations backcrossed to the C57BL/6 background) (Sun et al., 2001; Lin et al., 2014), Bhlhe40<sup>GFP+</sup> (10 generations backcrossed to the C57BL/6 background (Lin et al., 2016)) and Bhlhe40<sup>fl/fl</sup> (Huynh et al., 2018) mice have been previously reported. The Bhlhe40<sup>GFP+</sup> mouse strain, originally defined as STOCK Tg(Bhlhe40-EGFP)PX84Gsat/Mmucd, identification number 034730-UCD, was obtained from the Mutant Mouse Regional Resource Center (MMRRC), a NCRR-NIH funded strain repository, and was donated to the MMRRC by the NINDS funded GENSAT BAC transgenic project (The GENSAT Project, NINDS Contract #N01NS02331 to the Rockefeller University). All mice were maintained in our specific pathogen free animal facility. Sex-matched littermates were used for experiments whenever possible, although in some cases mice from multiple litters were used in a single experiment. All animal experiments were approved by the Animal Studies Committee of Washington University in St. Louis.

**Bone marrow chimeras**

Bhlhe40<sup>+/+</sup> CD45.1/CD45.2 mice were lethally irradiated with 1,000 rads from a gamma irradiator, followed by same-day i.v. transfer of 16 million total bone marrow cells (8 million Bhlhe40<sup>+/+</sup> (CD45.1) cells plus either 8 million Bhlhe40<sup>+/+</sup> (CD45.2) or Bhlhe40<sup>−/−</sup> (CD45.2) cells). Mice were given drinking water containing sulfamethoxazole (1.3 mg/ml) and trimethoprim (0.26 mg/ml) for 2 weeks after irradiation and were allowed to reconstitute for at least 8 weeks. In some experiments, chimeras were also made with CD45.1 recipients using CD45.1/CD45.2 and CD45.2 donor bone marrow cells.
Peritoneal cell transfers

Peritoneal cells were lavaged from the peritonea of \textit{Bhlhe40}^{+/+} (CD45.1), \textit{Bhlhe40}^{+/+} (CD45.2), and \textit{Bhlhe40}^{-/-} (CD45.2) donors, and aliquots of cells were analyzed by flow cytometry to determine the frequency of LPMs. Bulk peritoneal cells were then transferred i.p. into resting \textit{Bhlhe40}^{+/+} recipients (CD45.1/CD45.2) at ratios resulting in the transfer of equal numbers of LPMs from each donor (200,000-300,000 LPMs).

Treatment of mice with thioglycollate and interleukin-4 complexes (IL-4c)

A 3\% solution of thioglycollate was prepared in water, autoclaved and aged for three or more months (Gautier et al., 2013). Mice received 1 ml i.p. to induce peritonitis or a control injection of PBS (Gautier et al., 2013). IL-4c were prepared fresh as described (Finkelman et al., 1993; Jenkins et al., 2011). IL-4 (Shenandoah Biotechnology #200-18, resuspended in 0.1\% BSA in water) and anti-IL-4 antibody (clone 11B11; Leinco I-1071 or BioXCell BE0045) were combined in a 1:5 ratio by mass and a 1:1 ratio by volume, using ~1 mg/ml cytokine and ~5 mg/ml antibody. Complexes were incubated for ~2 minutes at room temperature (RT), diluted in 1x Dulbecco's PBS (DPBS) and injected i.p. Control injections were 0.1\% BSA diluted in 1x DPBS, while naïve mice were also used for assessment of cell cycling, due to acquisition of Ki67\(^{+}\) by LPMs 2 days after PBS injection as described (Jenkins et al., 2013). Mice received injections on day 0 and day 2, followed by sacrifice on day 4 as described (Jenkins et al., 2011). For treatment with thioglycollate and IL-4c complexes, mice were injected i.p. with thioglycollate on day 0 and IL-4c on days 0 and 2, as previously described (Gundra et al., 2014; Gundra et al., 2017).
**H. polygyrus infections**

*H. polygyrus bakeri* third-stage larvae (L3) were prepared as described (Camberis et al., 2003). Mice were orally gavaged with 200 L3 or water (mock) with a 20-gauge ball-tipped gavage needle. Mice were sacrificed on day 8 of infection for assessment of peritoneal cells.

**Leukocyte collection from tissues**

Peritoneal and pleural cells were collected from body cavities by lavage. Bone marrow was collected by flushing hind limb femurs and tibias. Blood was collected by submandibular bleeding into EDTA or lithium heparin tubes. Lungs, liver, spleen and kidney were excised, placed in Iscove's Modified Dulbecco's Medium (IMDM) containing 5% fetal bovine serum (FBS), minced finely, and digested at 37 °C for an hour with mechanical disruption with a stir bar and enzymatic digestion (lung and kidneys, 4 mg/ml collagenase D (Roche); spleen, 0.25 g/ml collagenase B (Roche) and 30U/ml DNase I (EMD); liver, 4 mg/ml collagenase D and 30U/ml DNase I). Microglia (Lin et al., 2016) and small intestinal lamina propria cells (Bando et al., 2018) were isolated as described. After digestion, enzymes were inactivated with 5 mM EDTA and samples were incubated on ice for 5 minutes.

All cells were passed through a 70 μm cell strainer before analysis. If necessary, tissues were treated with ACK lysis buffer to lyse red blood cells. Cells were counted with a hemocytometer using 3% acetic acid (naïve peritoneum and pleura) or trypan blue (all others).

**Flow cytometry**
Cell surface staining was conducted in sterile 1x PBS with 0.5% BSA and 2 mM EDTA (hereafter FACS buffer). In brief, cells were washed in FACS buffer, blocked with α-CD16/32 (clone 2.4G2, BioXCell) for 10 minutes at 4 °C, stained for 20 min at 4 °C and washed with FACS buffer before flow cytometry. In some experiments to assess cell death, 7-AAD (1:20 of a 50 μg/mL solution, BioLegend or BD) was added to cells for 15 minutes prior to flow cytometry. Flow cytometry was performed on FACSCanto II, LSRFortessa, LSRFortessa X20 and LSR II instruments (all BD). FlowJo software (Treestar) was used for analysis.

Gating of cell populations was as follows (all analysis pre-gated on FSC/SSC and a FSC-W/FSC-A singlet gate). Blood monocytes were gated as Ly6G^CD115^ and then divided by Ly6C expression. Peritoneal and pleural macrophages were gated as CD115^CD11b^+, then divided into ICAM2^MHC-II^int large macrophages and ICAM2^MHC-II^+ small macrophages. Thioglycollate-elicited macrophages were gated as CD115^CD11b^ICAM2lo. Liver Kupffer cells were gated as CD45^CD11b^loF4/80^hi, and in some contexts as Ly6C-. Kidney macrophages were gated as CD45^Ly6C^-CD11b^F4/80^hi. AMs were gated as CD45^Siglec-F^CD11c^+, and in some contexts as F4/80^CD11b-. Red pulp macrophages were gated as F4/80^hi and negative or low for other markers (CD11blo, MHC-II^lo, or CD11clo). Microglia were gated as CD45^intCD11b+. Small intestinal lamina propria macrophages were gated as CD45^-Ly6C^-F4/80^CD64^MHC-II^+. Peritoneal B cells were gated as CD115^-MHC-II^CD19+. Analysis of cells from the Bhlhe40^GFP^ reporter mouse used viability dyes (Po pro 1 or 7-AAD) when necessary to exclude dead cells.

**Intracellular staining for flow cytometry**

For Ki67, DAPI, RELMα, BrdU and pHH3 staining, the eBioscience FoxP3/Transcription Factor Staining Buffer set (00-5523-00) or the BioLegend True-Nuclear Transcription Factor Buffer set
(424401) was used. In brief, after surface staining, cells were fixed with 1x Fix Concentrate buffer in provided Fix Diluent for 30 minutes at 4 °C. Cells were then washed with FACS buffer and stored overnight. To permeabilize the cells, samples were washed with 1x Perm buffer diluted in water. Following blocking with 2% rat serum, samples were stained for 1 hour at RT, except for DAPI and secondary antibodies (20 minutes at RT), followed by washing in 1x Perm buffer and FACS buffer before flow cytometry.

For BrdU staining, mice were given 1 mg of BrdU i.p. (from BD kit, 552598, or Sigma, B5002) three hours before sacrifice as described (Jenkins et al., 2011). After sacrifice of mice and peritoneal lavage, samples were processed, fixed and stored overnight as for other intracellular antigens. BrdU-labelled cells were washed in 1x Perm buffer, incubated in DNase I (from BD kit, 552598 or Sigma, D4513) in 1x DPBS for 30 min at 37 °C, washed in 1x Perm buffer, blocked with 2% rat serum and stained for 1 hour at RT with α-BrdU antibody (BD, 552598), followed by washing in 1x Perm buffer and FACS buffer. Mice that did not receive BrdU were used as negative controls.

**Transmission electron microscopy**

For ultrastructural analyses, peritoneal cells were fixed in 2% PFA/2.5% glutaraldehyde (Polysciences Inc.) in 100 mM sodium cacodylate buffer, pH 7.2 for 1 hour at RT. Samples were washed in sodium cacodylate buffer at RT and postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 hour. Samples were rinsed in distilled water prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc.) for 1 hour. Following rinsing in distilled water, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome, stained with uranyl acetate and lead citrate and viewed on a JEOL 1200 EX transmission electron microscope (JEOL...
USA Inc.) equipped with an AMT 8 megapixel digital camera and AMT Image Capture Engine V602 software (Advanced Microscopy Techniques).

For morphological analysis, images were blinded and randomized. LPMs were identified as large cells with abundant cytoplasm and were distinguished from rare peritoneal mast cells by the absence of electron-dense granules. For measurement statistics, the ObjectJ plugin was used in ImageJ software (NIH). In brief, cell and vesicle cross-sectional area were calculated by tracing the outline of the cell or vesicles, respectively, and calculating the enclosed area. ER cross-sectional extent was calculated by tracing the ER with lines and adding these lengths together. For assessment of ER luminal width, a randomly placed grid was used to subdivide the cell into sections. Representative measurements were then taken across the lumen of the ER, and the measurements from each section were averaged.

Microarrays

The following cell populations were sorted on a FACSria II (BD) into FBS: for naïve LPM microarrays, B220\(^{-}\)F4/80\(^{+}\)CD11b\(^{+}\)ICAM2\(^{+}\) LPMs from untreated mice; for in vivo IL-4c-stimulated LPM microarrays, CD115\(^{+}\)CD11b\(^{+}\)ICAM2\(^{+}\)MHC-II\(^{int}\) LPMs from mice treated with IL-4c at days 0 and 2, with peritoneal cells collected at day 4; for naïve AM microarrays, CD45\(^{+}\)Ly6G\(^{-}\)Siglec-F\(^{+}\)CD11c\(^{+}\)CD11b\(^{lo}\) AMs from untreated mice. Cells were lysed and RNA was purified using the E.Z.N.A. MicroElute Total RNA kit (Omega Bio-Tek). Total RNA was submitted to the Genome Technology Access core at Washington University for cDNA synthesis (NuGen Pico SL) followed by microarray analysis on the Affymetrix Mouse Gene 1.0 ST platform. Data were analyzed using the DNASTAR ArrayStar program. Genes with an expression value of <5 (in log 2 scale) in all replicates were considered not expressed. For
analysis of naïve microarrays, which were conducted on three biologic replicates, the
differentially expressed gene list was also filtered on genes with a p-value significance of \( \leq 0.05 \)
by the moderated \( t \)-test. For analysis of \textit{in vivo} IL-4c-stimulated LPM microarrays, which were
conducted on two biologic replicates, no p-value filtering was applied. For comparison of naïve
to \textit{in vivo} IL-4c-stimulated LPMs, CEL files were normalized together to generate expression
data. Heatmaps were generated with Morpheus (software.broadinstitute.org/morpheus/). Venn
diagrams were generated with the Venn Diagram Plotter tool (Pacific Northwest National
Laboratories, omics.pnl.gov). Multiple differentially expressed probe sets representing a single
gene were presented in heat maps without exclusion, but only unique genes were counted in
Venn diagrams.

The macrophage alternative activation gene signature used to assess naïve macrophages
was generated from GSE69607 comparing M0, M1 and M2 bone marrow-derived macrophages
(BMDMs) (Jablonski et al., 2015). The 29 genes 20-fold upregulated in M2 vs. M1 BMDMs
were used to define a set of macrophage alternative activation-related genes. The LPM gene
signature was previously published (Gautier et al., 2012). For the LPM alternative activation
gene signature used to assess \textit{in vivo} IL-4c-stimulated LPMs, we compared our microarray data
from naïve and \textit{in vivo} IL-4c-stimulated \textit{Bhlhe40}^{+/+} LPMs and defined an alternative activation
signature for LPMs, composed of the 55 unique genes up- or down-regulated by ten-fold or
more. For comparison of the gene expression signature of \textit{Bhlhe40-} or \textit{Gata6}-deficient LPMs,
our data was analyzed in parallel with GSE37448 (Gautier et al., 2014) as above, as both data
sets were generated on the Affymetrix Mouse Gene 1.0 ST platform.

\textbf{Quantitative real-time polymerase chain reaction (qRT-PCR)}
LPMs were sorted as for microarrays. RNA was isolated with the E.Z.N.A. MicroElute Total RNA kit (Omega Bio-Tek), and cDNA was synthesized with 50 ng RNA using the High Capacity RNA to cDNA kit (Invitrogen). RNA concentration was assessed with a Nanodrop 2000 spectrophotometer (ThermoFisher). qRT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus Real-Time PCR machine (Applied Biosystems). Gene expression was determined relative to Hprt by the ΔC_T method. The following primers were used: Hprt, forward 5′-TCAGTCAACGGGGGACAT AAA-3′, reverse 5′-GGGGCTGTACTGCTTAACCAG-3′; Maf forward 5′-GGAGACCGACCCGATC-3′ reverse 5′-TCATCCAGTAGTAGCTCCAGG-3′; Mafb forward 5′-TTCGACCTTCTCAAGTTCGACG-3′, reverse 5′-TCGAGATGGGTCTTCGGTTCA-3′.

Immunoblotting

LPMs were sorted as for microarrays, pooled from multiple mice and lysed at a concentration of 25 million cells/mL using the RIPA lysis buffer system (Santa Cruz Biotechnology). Laemmli buffer (Bio-Rad, with added 2-mercaptoethanol) was added to the samples, which were then boiled for 10 minutes, and run on a Bio-Rad Miniprotein TGX gel with Precision Plus Dual Color molecular weight standards (Bio-Rad). Proteins were transferred to a BioBlot polyvinylidene fluoride membrane (Costar). Blots were blocked for 1-2 hours with 5% milk, followed by overnight staining with primary antibodies in 5% milk at 4 °C with shaking. After washing, blots were stained with horseradish peroxidase-conjugated secondary antibodies for 45-60 minutes at RT with shaking. After washing, blots were developed with either Super Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher) or Clarity Western ECL Substrate (Bio-Rad). Images were captured on a Chemidoc system (Bio-Rad) and inverted on Adobe
Illustrator for presentation. Blots were stripped with Restore PLUS Western Blot Stripping Buffer (ThermoFisher). Blots were then washed, reblocked and restained.

**Gene set enrichment analysis**

Lists of differentially expressed genes (≥1.5-fold up- or downregulated) were cross-referenced to the C5 gene sets in the MSigDB database. To further examine the enrichment of gene sets, the GSEA software from the Broad Institute was used to analyze all expressed genes or all Bhlhe40-bound expressed genes for gene set enrichment using the Hallmark and C5 databases (Subramanian et al., 2005; Liberzon et al., 2015).

**ChIP-Seq**

Anti-Bhlhe40 ChIP-seq was performed as previously published (Huynh et al., 2018). LPMs were sorted as for microarrays and pooled from multiple mice. Cells were fixed for 10 min at RT in 1% PFA with shaking. Cross-linking was stopped with glycine added to 0.125 M, cells were pelleted and dry pellets were stored at -80 °C. Cross-linked chromatin was sonicated and immunoprecipitated using rabbit anti-Dec1 (Bhlhe40) antibody (NB100-1800, Lot C1; Novus Biologicals). Following immunoprecipitation, the GenElute PCR cleanup kit (Sigma) was used to purify DNA. Library construction was followed by single-read sequencing on a HiSeq3000 (Illumina) at the Genome Technology Access Center at Washington University in St. Louis. Read length was 50 base pairs (bp). Quality control of FASTQ files used FastQC (0.11.3). Bowtie (1.1.1) was used to map reads onto the mm10 mouse reference genome. Input DNA samples were used for peak calling on Bhlhe40-immunoprecipitation samples using MACS v1.4.
with default settings (Zhang et al., 2008). Generated peaks were additionally required to have fold-enrichment $\geq 5$ and reads from unmapped regions (chrUn_xxxxx) were excluded.

Normalized tracks were generated with Deeptools (2.5.3), and tracks were visualized with the UCSC Genome Browser. Discriminative Regular Expression Motif Elicitation (DREME) (5.0.1) (Bailey, 2011) was used for motif enrichment analysis using 250 bp flanked summits of all acquired peaks. To annotate peaks, R package ChIPseeker (1.14.1) was used. The intersect function from the BEDtools suite (v2.25.0) was used to find shared peaks. The shared peak count was defined as the number of overlapping peaks in the naïve LPM Bhlhe40 ChIP-seq sample compared to the in vivo IL-4c-stimulated LPM Bhlhe40 ChIP-seq sample. Two group Venn diagrams were generated with the Venn Diagram Plotter tool.

ChIP-seq data for PU.1 performed on LPMs (GSM1533894) and the corresponding input sample (GSM1533895) (Gosselin et al., 2014) were downloaded in SRA format and converted to FASTQ format using the fastq-dump function (v2.8.1) from the SRA Toolkit. Subsequent processing and filtration was performed as described above. The shared peak count was defined as the number of overlapping peaks in the naïve LPM Bhlhe40 ChIP-seq sample compared to each of the other samples, except for the comparison of in vivo IL-4c-stimulated LPM Bhlhe40 ChIP-seq and LPM PU.1 ChIP-seq samples, which was defined as the number of overlapping peaks in the IL-4c Bhlhe40 sample. Three group Venn diagrams were generated with the eulerAP3 v3 tool (www.eulerdiagrams.org/eulerAPE/) (Micallef and Rodgers, 104).

**Statistical analysis**

All data are from at least two independent experiments, unless otherwise indicated. Data were analyzed by paired or unpaired two-tailed Student’s t-tests (Prism 7; GraphPad Software, Inc.) as
indicated in the figure legends, with $p \leq 0.05$ considered significant. For relevant comparisons where no p-value is shown, the p-value was $> 0.05$. For analysis of gene lists against the MSigDB database, the hypergeometric test performed by the Investigate Gene Sets tool was used to determine significance. For GSEA analysis, the NES score calculated by the GSEA software was used to account for set size effects when determining enrichment. The GSEA-calculated FWER p-value was used to determine significance, as this statistic is more conservative than the False Discovery Rate (FDR). Horizontal bars represent the mean and error bars represent the standard error of the mean (s.e.m.).
Figure 2.1. Loss of Bhlhe40 dysregulates the cell cycle in LPMs. (A) Flow cytometry of Bhlhe40<sup>GFP</sup> transgene reporter expression in blood monocytes (representative of 2
experiments, n=5 Bhlhe40^{GFP+}, 2 Bhlhe40^{GFP-}); red pulp macrophages, microglia, Kupffer
cells, kidney macrophages, SI macrophages, and peritoneal macrophages (representative of 2
experiments, n=4 Bhlhe40^{GFP+}, 2 Bhlhe40^{GFP-}); and AMs (representative of 3 experiments,
n=6 Bhlhe40^{GFP+}, 3 Bhlhe40^{GFP-}) from Bhlhe40^{GFP+} and Bhlhe40^{GFP-} mice. (B) Flow cytometry
of peritoneal macrophage subsets in Bhlhe40^{+/+} and Bhlhe40^{-/-} mice (representative of 6
experiments, n=22/group). (C) Numbers of LPMs as in (B), SPMs (pooled from 5
experiments, n=19/group), AMs (pooled from 4 experiments, n=13 Bhlhe40^{+/+}, 12 Bhlhe40^{-/-}),
red pulp macrophages (pooled from 3 experiments, n=9 Bhlhe40^{+/+}, 8 Bhlhe40^{-/-}), Kupffer
cells, and kidney macrophages (both pooled from 2 experiments, n=10/group) from Bhlhe40^{+/+}
and Bhlhe40^{-/-} mice. (D) Flow cytometry of Ki67 expression by Bhlhe40^{+/+} and Bhlhe40^{-/-}
LPMs (representative of 7 experiments, n=24 Bhlhe40^{+/+}, 22 Bhlhe40^{-/-}). (E) Frequency of
Ki67^{+} LPMs as in (D), SPMs, and B cells (both pooled from 8 experiments, n=30 Bhlhe40^{+/+},
29 Bhlhe40^{-/-}) from Bhlhe40^{+/+} and Bhlhe40^{-/-} mice. (F) Flow cytometry of BrdU incorporation
by Bhlhe40^{+/+} and Bhlhe40^{-/-} LPMs (representative of 5 experiments, n=18/group). (G)
Frequency of BrdU^{+} LPMs as in (F). (H) Flow cytometry of pH3 expression by Bhlhe40^{+/+}
and Bhlhe40^{-/-} LPMs (representative of 4 experiments, n=12 Bhlhe40^{+/+}, 11 Bhlhe40^{-/-}). (I)
Frequency of pH3^{+} LPMs as in (H). (J) Flow cytometry for discrimination of cell cycle
phases of Bhlhe40^{+/+} and Bhlhe40^{-/-} LPMs (representative of 4 experiments, n=12 Bhlhe40^{+/+},
11 Bhlhe40^{-/-}). (K) Numbers of LPMs in each phase of the cell cycle as in (J). Data are mean
± s.e.m. *P ≤ 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, significance calculated with
an unpaired two-sided Student’s t-test.
Figure 2.2. Bhlhe40 is specifically required in peritoneal and pleural macrophages. (A)

Flow cytometry of Bhlhe40GFP transgene reporter expression in pleural macrophages from Bhlhe40GFP+ and Bhlhe40GFP- mice (representative of 2 experiments, n=3 Bhlhe40GFP+, 2 Bhlhe40GFP-). (B-E) Flow cytometry for AMs (B), red pulp macrophages (C), kidney macrophages (D), and Kupfer cells (E).
macrophages (D), and Kupffer cells (E) from $Bhlhe40^{+/+}$ and $Bhlhe40^{-/-}$ mice as in Fig. 1C. (F) Flow cytometry for Tim4 expression on LPMs from $Bhlhe40^{+/+}$ and $Bhlhe40^{-/-}$ mice (representative of 11 experiments, n=40/group). (G) Numbers of Tim4$^+$ LPMs as in (F). (H) Flow cytometry for CD226 expression on SPMs from $Bhlhe40^{+/+}$ and $Bhlhe40^{-/-}$ mice (representative of 8 experiments, n=27/group). (I) Numbers of CD226$^+$ SPMs as in (H). (J) Numbers of peritoneal B cells from $Bhlhe40^{+/+}$ and $Bhlhe40^{-/-}$ mice (pooled from 12 experiments, n=35/group). (K) Numbers of large pleural macrophages from $Bhlhe40^{+/+}$ and $Bhlhe40^{-/-}$ mice (pooled from 6 experiments, n=16 $Bhlhe40^{+/+}$, 17 $Bhlhe40^{-/-}$). (L) Frequency of Ki67$^+$ large pleural macrophages from $Bhlhe40^{+/+}$ and $Bhlhe40^{-/-}$ mice (pooled from 3 experiments, n=7 $Bhlhe40^{+/+}$, 8 $Bhlhe40^{-/-}$). (M) frequency of 7-AAD$^+$ LPMs from $Bhlhe40^{+/+}$ and $Bhlhe40^{-/-}$ mice (pooled from 8 experiments, n=19 $Bhlhe40^{+/+}$, 18 $Bhlhe40^{-/-}$). Data are mean ± s.e.m. ***$P < 0.001$; ****$P < 0.0001$, significance calculated with an unpaired two-sided Student’s t-test.
Figure 2.3. Bhlhe40 is cell-intrinsically required in LPMs to regulate the cell cycle. (A)

Flow cytometry for the discrimination of donor and recipient LPMs (representative of 6
experiments, n=18 [Bhlhe40+/+ (CD45.1) +Bhlhe40+/+ (CD45.2)], 21 [Bhlhe40+/+ (CD45.1) +Bhlhe40−/− (CD45.2)] and peritoneal B cells (representative of 4 experiments, n=12 [Bhlhe40+/+ (CD45.1) +Bhlhe40+/+ (CD45.2)], 13 [Bhlhe40+/+ (CD45.1) +Bhlhe40−/− (CD45.2)]) from Bhlhe40+/+ (CD45.1) plus either Bhlhe40+/+ (CD45.2) or Bhlhe40−/− (CD45.2) mixed bone marrow chimeras. (B) Ratio of CD45.1 to CD45.2 LPMs as in (A). (C) Ratios of CD45.1 cells to CD45.2 cells for SPMs (pooled from 4 experiments, n=12 [Bhlhe40+/+ (CD45.1) +Bhlhe40+/+ (CD45.2)], 13 [Bhlhe40+/+ (CD45.1) +Bhlhe40−/− (CD45.2)]) and peritoneal B cells (representative of 4 experiments, n=12 [Bhlhe40+/+ (CD45.1) +Bhlhe40+/+ (CD45.2)], 13 [Bhlhe40+/+ (CD45.1) +Bhlhe40−/− (CD45.2)]) from Bhlhe40+/+ (CD45.1) plus either Bhlhe40+/+ (CD45.2) or Bhlhe40−/− (CD45.2) mixed bone marrow chimeras. (B) Ratio of CD45.1 to CD45.2 LPMs as in (A). (C) Ratios of CD45.1 cells to CD45.2 cells for SPMs (pooled from 4 experiments, n=12 [Bhlhe40+/+ (CD45.1) +Bhlhe40+/+ (CD45.2)], 13 [Bhlhe40+/+ (CD45.1) +Bhlhe40−/− (CD45.2)]) and peritoneal B cells (representative of 4 experiments, n=12 [Bhlhe40+/+ (CD45.1) +Bhlhe40+/+ (CD45.2)], 13 [Bhlhe40+/+ (CD45.1) +Bhlhe40−/− (CD45.2)]) from Bhlhe40+/+ (CD45.1) plus either Bhlhe40+/+ (CD45.2) or Bhlhe40−/− (CD45.2) mixed bone marrow chimeras. (D) Ratio of CD45.1 to CD45.2 large pleural macrophages (pooled from 2 experiments, n=6 [Bhlhe40+/+ (CD45.1) +Bhlhe40+/+ (CD45.2)], 8 [Bhlhe40+/+ (CD45.1) +Bhlhe40−/− (CD45.2)]) from mixed bone marrow chimeras. (D) Ratio of CD45.1 to CD45.2 large pleural macrophages (pooled from 2 experiments, n=6/group) from mixed bone marrow chimeras. (E) Flow cytometry for discrimination of cell cycle phases of Bhlhe40+/+ (CD45.1), Bhlhe40+/+ (CD45.2), or Bhlhe40+/+ (CD45.2) LPMs from mixed bone marrow chimeras (representative of 3 experiments, n=9 [Bhlhe40+/+ (CD45.1) +Bhlhe40+/+ (CD45.2)], 11 [Bhlhe40+/+ (CD45.1) +Bhlhe40−/− (CD45.2)]) from mixed bone marrow chimeras. (F) Frequency of G1 LPMs as in (E) with LPMs from each donor recovered from the same recipient connected by a line. (G) Numbers of LPMs, SPMs (both pooled from 6 experiments, n=14 LysM-Cre− Bhlhe40fl/fl, 15 LysM-Cre+ Bhlhe40fl/fl) and peritoneal B cells (5 experiments, n=12 LysM-Cre− Bhlhe40fl/fl, 13 LysM-Cre+ Bhlhe40fl/fl) from
LysM-Cre Bhlhe40^{fl/fl} and LysM-Cre^{+} Bhlhe40^{fl/fl} mice. (H) Flow cytometry for discrimination of cell cycle phases of LysM-Cre Bhlhe40^{fl/fl} and LysM-Cre^{+} Bhlhe40^{fl/fl} LPMs (representative of 3 experiments, n=8/group). (I) Numbers of LPMs in each phase of the cell cycle as in (H). (J) Flow cytometry for the discrimination of donor and recipient LPMs and peritoneal B cells from CD45.1/CD45.2 recipients of mixed peritoneal cells transferred from Bhlhe40^{+/+} (CD45.1) plus either Bhlhe40^{+/+} (CD45.2) or Bhlhe40^{−/−} mice (CD45.2) mice (representative of 2 experiments, n=3 [Bhlhe40^{+/+} (CD45.1) +Bhlhe40^{+/−} (CD45.2)], 5 [Bhlhe40^{+/+} (CD45.1) +Bhlhe40^{−/−} (CD45.2)]). (K, L) Ratio of CD45.1 LPMs to CD45.2 LPMs (K) and CD45.1 peritoneal B cells to CD45.2 peritoneal B cells (L) as in (J) (pooled from 2-3 experiments, n≥3 for all time points, except day 14 [Bhlhe40^{+/+} (CD45.1) +Bhlhe40^{−/−} (CD45.2)], n=2). Data are mean ± s.e.m. *P ≤ 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, significance calculated with an unpaired two-sided Student’s t-test, except in (F) which used an unpaired two-sided Student’s t-test.
Figure 2.4. Bhlhe40 is cell-intrinsically required in LPMs. (A) Ratio of CD45.1 to CD45.2 cells for small pleural macrophages and pleural B cells from mixed bone marrow chimeras as in Fig. 2D. (B) Flow cytometry for the discrimination of donor and recipient LPMs and peritoneal B cells from CD45.1/CD45.2 recipients of transferred peritoneal cells as in Fig. 2K, L. (C, D) Gene expression microarray data from Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>-/-</sup> LPMs (in this study) and LysM-Cre<sup>-</sup> Gata6<sup>fl/fl</sup>, and LysM-Cre<sup>+</sup> Gata6<sup>fl/fl</sup> LPMs (reanalyzed from Gautier et
al., 2014) were analyzed for shared and unique Bhlhe40 and/or Gata6-dependent genes (≥2-fold differentially expressed, depicted as a Venn diagram) (C) and differentially expressed genes dependent on both Bhlhe40 and Gata6 (D). (E) Gene expression microarray data were analyzed for expression of an LPM gene signature in LPMs and AMs from Bhlhe40+/+ and Bhlhe40+/- mice. Microarray data from LPMs (n=3/group) and AMs (n=2/group) are from a single experiment. Data are mean ± s.e.m. Significance calculated with an unpaired two-sided Student’s t-test.
Figure 2.5. Bhlhe40 regulates a distinct set of genes related to alternative activation in LPMs. (A, B) Gene expression microarray data were analyzed for genes differentially expressed by ≥2-fold (A) and expression of a macrophage alternative activation gene signature in Bhlhe40+/+ and Bhlhe40−/− LPMs (B). (C) Flow cytometry of Embigin-1 expression
and quantitation of geometric mean fluorescence intensity (gMFI) on LPMs (pooled from 4 experiments, n=8 Bhlhe40+/+, 7 Bhlhe40+/-; 1 experiment, n=3 LysM-Cre- Bhlhe40^{fl/fl}, 4 LysM-Cre+ Bhlhe40^{fl/fl}). (D) Flow cytometry of Clec10a expression and frequency of Clec10a+ LPMs (pooled from 6 experiments, n=19 Bhlhe40+/+ and 21 Bhlhe40-/-; 4 experiments, n=10 LysM-Cre- Bhlhe40^{fl/fl} and LysM-Cre+ Bhlhe40^{fl/fl}). (E) Flow cytometry of Lyve-1 expression and frequency of Lyve-1+ LPMs (pooled from 7 experiments, n=22 Bhlhe40+/+, 24 Bhlhe40-/-; 5 experiments, n=11 LysM-Cre- Bhlhe40^{fl/fl} and LysM-Cre+ Bhlhe40^{fl/fl}) from Bhlhe40+/+, Bhlhe40-/-, LysM-Cre- Bhlhe40^{fl/fl}, and LysM-Cre+ Bhlhe40^{fl/fl} LPMs. (F, G) MSigDB C5 gene set enrichment was analyzed using the lists of genes expressed at ≥1.5-fold in Bhlhe40+/+ vs. Bhlhe40-/- (G) or Bhlhe40-/- vs. Bhlhe40+/+ LPMs (G). (H) qRT-PCR of Maf and Mafb expression relative to Hprt in Bhlhe40+/+ and Bhlhe40-/- LPMs (pooled from 2 experiments, n=5 Bhlhe40+/+, 4 Bhlhe40-/-). (I) Gene expression microarray data were analyzed for shared and unique Bhlhe40-dependent genes in Bhlhe40+/+ and Bhlhe40-/- LPMs and AMs (≥2-fold differentially expressed, depicted as a Venn diagram). Heat map depicts all genes differentially expressed by ≥2-fold in Bhlhe40+/+ and Bhlhe40-/- AMs. Microarray data from LPMs (n=3/group) and AMs (n=2/group) are from a single experiment. Data are mean ± s.e.m. *P ≤ 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, significance calculated with an (C-E, H) unpaired two-sided Student’s t-test and a (F, G) one-sided hypergeometric test.
Figure 2.6. Bhlhe40 is required for normal accumulation of resident, but not recruited, macrophages in the peritoneum. (A) Flow cytometry of peritoneal macrophage subsets from Bhlhe40\textsuperscript{+/+} and Bhlhe40\textsuperscript{-/-} mice treated with thioglycollate (Thio) (representative of 3 experiments, n=6/group). (B) Numbers of CD115\textsuperscript{+}CD11b\textsuperscript{+} peritoneal macrophages from Bhlhe40\textsuperscript{+/+} and Bhlhe40\textsuperscript{-/-} mice treated with PBS or Thio as in (A) (pooled from 3 experiments, n=3/group for PBS, 6/group for Thio). (C) Flow cytometry of peritoneal macrophage subsets from Bhlhe40\textsuperscript{+/+} and Bhlhe40\textsuperscript{-/-} mice treated with IL-4c (representative of 6 experiments, 17 IL-4c-treated Bhlhe40\textsuperscript{+/+}, 19 IL-4c-treated Bhlhe40\textsuperscript{-/-}). (D, E) Numbers of LPMs (D) and SPMs (E) as in (C) from Bhlhe40\textsuperscript{+/+}, Bhlhe40\textsuperscript{-/-}, LysM-Cre\textsuperscript{-} Bhlhe40\textsuperscript{fl/fl}, and LysM-Cre\textsuperscript{+} Bhlhe40\textsuperscript{fl/fl} mice treated with PBS or IL-4c (pooled from 5 experiments, 6 PBS-treated Bhlhe40\textsuperscript{+/+} and Bhlhe40\textsuperscript{-/-}; IL-4c-treated as in c for Bhlhe40\textsuperscript{+/+} and Bhlhe40\textsuperscript{-/-}; 2 experiments, 2 PBS-treated LysM-Cre\textsuperscript{-} Bhlhe40\textsuperscript{fl/fl}, 3 PBS-treated LysM-Cre\textsuperscript{+} Bhlhe40\textsuperscript{fl/fl}, 9 IL-4c-treated LysM-Cre\textsuperscript{-} Bhlhe40\textsuperscript{fl/fl}; 7 IL-4c-treated LysM-Cre\textsuperscript{+} Bhlhe40\textsuperscript{fl/fl}). Data are mean ± s.e.m. **P < 0.01; ****P < 0.0001, significance calculated with an unpaired two-sided Student’s t-test.
Figure 2.7. Further analysis of responses to IL-4c in Bhlhe40-deficient mice. (A) Flow cytometry of peritoneal macrophage subsets from Bhlhe40<sup>+/+</sup>, Bhlhe40<sup>-/-</sup>, and Bhlhe40<sup>-/-</sup> Il10<sup>-/-</sup> mice treated with PBS or IL-4c (representative of 2 experiments, n=3 PBS-treated Bhlhe40<sup>+/+</sup>, 2 PBS-treated Bhlhe40<sup>-/-</sup>, 3 PBS-treated Bhlhe40<sup>-/-</sup> Il10<sup>-/-</sup>, 5 IL-4c-treated Bhlhe40<sup>+/+</sup>, 7 IL-4c-treated Bhlhe40<sup>-/-</sup>, 4 IL-4c-treated Bhlhe40<sup>-/-</sup> Il10<sup>-/-</sup>). (B) Numbers of LPMs as in (A). (C) Immunoblotting of cyclins D1-3, cyclin-dependent kinase (CDK) 2, CDK4, CDK6, E2F2, and beta actin in lysates of LPMs from Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>-/-</sup> mice unstimulated or treated with IL-4c (representative of 2 experiments, n=2/group). (D) Frequency of BrdU<sup>+</sup> Bhlhe40<sup>+/+</sup> (CD45.1), Bhlhe40<sup>+/+</sup> (CD45.2), or Bhlhe40<sup>-/-</sup> (CD45.2) LPMs from mixed bone marrow chimera mice (generated as in Fig. 2A-F) treated with PBS or IL-4c, with LPMs from each donor recovered from the same recipient connected by a line (pooled from 2 experiments, n=2 PBS-treated [Bhlhe40<sup>+/+</sup> (CD45.1) + Bhlhe40<sup>+/+</sup> (CD45.2)] and [Bhlhe40<sup>+/+</sup> (CD45.1) + Bhlhe40<sup>-/-</sup> (CD45.2)]; 4 IL-4c-treated [Bhlhe40<sup>+/+</sup> (CD45.1) + Bhlhe40<sup>+/+</sup> (CD45.2)]; 5 [Bhlhe40<sup>+/+</sup> (CD45.1) + Bhlhe40<sup>-/-</sup> (CD45.2)] + Bhlhe40<sup>-/-</sup> (CD45.2); 2 IL-4c-treated [Bhlhe40<sup>+/+</sup> (CD45.1) + Bhlhe40<sup>-/-</sup> (CD45.2)] + Bhlhe40<sup>-/-</sup> (CD45.2); 3 IL-4c-treated [Bhlhe40<sup>-/-</sup> (CD45.1) + Bhlhe40<sup>-/-</sup> (CD45.2)] + Bhlhe40<sup>-/-</sup> (CD45.2); 4 IL-4c-treated [Bhlhe40<sup>-/-</sup> (CD45.1) + Bhlhe40<sup>-/-</sup> (CD45.2)] + Bhlhe40<sup>-/-</sup> (CD45.2); 5 [Bhlhe40<sup>-/-</sup> (CD45.1) + Bhlhe40<sup>-/-</sup> (CD45.2)].
+$Bhlhe40^{+/−}$ (CD45.2)). Data are mean ± s.e.m. ***$P < 0.001$, significance calculated with an (B) unpaired or a (D) paired two-sided Student’s $t$-test.
Figure 2.8. Bhlhe40 is required for normal cycling, but not polarization, of peritoneal macrophages during type 2 immunity. (A-D) Frequency of RELMα+ LPMs (A) (pooled
from 3 experiments, n=4 PBS-treated Bhlhe40+/+ and Bhlhe40–/–; 15 IL-4c-treated Bhlhe40+/+ and Bhlhe40–/–; 2 experiments, 2 PBS-treated LysM-Cre– Bhlhe40fl/fl and LysM-Cre+
Bhlhe40fl/fl; 6 IL-4c-treated LysM-Cre– Bhlhe40fl/fl; 5 IL-4c-treated LysM-Cre+ Bhlhe40fl/fl), Clec10a+ LPMs (B) (pooled as in (A)), BrdU+ LPMs (C) (pooled from 3 experiments, n=4 PBS-treated Bhlhe40+/+; 5 PBS-treated Bhlhe40–/–; 13 IL-4c-treated Bhlhe40+/+; 17 IL-4c-treated Bhlhe40+/+; 3 PBS-treated LysM-Cre– Bhlhe40fl/fl and LysM-Cre+ Bhlhe40fl/fl; 8 IL-4c-treated LysM-Cre– Bhlhe40fl/fl; 7 IL-4c-treated LysM-Cre+ Bhlhe40fl/fl), and pHH3+ LPMs (D) (pooled from 3 experiments, n=6 PBS-treated Bhlhe40+/+; 7 PBS-treated Bhlhe40–/–; 16 IL-4c-treated Bhlhe40+/+; 18 IL-4c-treated Bhlhe40–/–; 3 PBS-treated LysM-Cre– Bhlhe40fl/fl and LysM-Cre+ Bhlhe40fl/fl; 8 IL-4c-treated LysM-Cre– Bhlhe40fl/fl, and LysM-Cre+ Bhlhe40fl/fl) from Bhlhe40+/+, Bhlhe40–/–, LysM-Cre– Bhlhe40fl/fl, and LysM-Cre+ Bhlhe40fl/fl mice treated with PBS or IL-4c. (E) Proportion of LPMs in each phase of the cell cycle from Bhlhe40+/+ and Bhlhe40–/– mice unstimulated or treated with IL-4c (pooled from 5 experiments, n=6/group for unstimulated; 16 IL-4c-treated Bhlhe40+/+; 15 IL-4c-treated Bhlhe40–/–). (F) Frequency of 7-AAD+ LPMs from Bhlhe40+/+, Bhlhe40–/–, LysM-Cre– Bhlhe40fl/fl, and LysM-Cre+ Bhlhe40fl/fl mice treated with PBS or IL-4c (pooled from 3 experiments, n=5 PBS-treated Bhlhe40+/+ and Bhlhe40–/–; 13 IL-4c-treated Bhlhe40+/+; 17 IL-4c-treated Bhlhe40–/–; 3 PBS-treated LysM-Cre– Bhlhe40fl/fl and LysM-Cre+ Bhlhe40fl/fl; 8 IL-4c-treated LysM-Cre– Bhlhe40fl/fl; 7 IL-4c-treated LysM-Cre+ Bhlhe40fl/fl). (G) Frequency of BrdU+ LPMs, large pleural macrophages, red pulp macrophages, Kupffer cells, and AMs from Bhlhe40+/+ and Bhlhe40–/– mice treated with PBS or IL-4c (pooled from 2 experiments, n=3/group for PBS; 6 IL-4c-treated Bhlhe40+/+; 7 IL-4c-treated Bhlhe40–/–; except for pleura, 3 experiments, n=4 PBS-treated Bhlhe40+/+; 3 PBS-treated Bhlhe40–/–; 7 IL-4c-treated Bhlhe40+/+; 6 IL-4c-treated
(H) Flow cytometry of Bhlhe40GFP transgene reporter expression in thioglycollate (Thio)-elicited macrophages from Bhlhe40GFP+ and Bhlhe40GFP- mice treated with Thio or Thio and IL-4c (1 experiment, n=2 Thio-treated Bhlhe40GFP+; 3 Thio and IL-4c-treated Bhlhe40GFP+; 1 Thio-treated Bhlhe40GFP-). (I) Flow cytometry of BrdU incorporation by Thio-elicited macrophages from Bhlhe40+/+ and Bhlhe40-/- mice treated with Thio and IL-4c (representative of 2 experiments, n=6/group). (J) Frequency of BrdU+ LPMs and Thio-elicited macrophages from Bhlhe40+/+ and Bhlhe40-/- mice treated with PBS, IL-4c, Thio, or Thio and IL-4c (pooled from 2 experiments, n=3 PBS-treated Bhlhe40+/+, 4 PBS-treated Bhlhe40-/-, 4 IL-4c-treated Bhlhe40+/+, 5 IL-4c-treated Bhlhe40-/-, 2 Thio-treated Bhlhe40+/+, 4 Thio-treated Bhlhe40-/-, Thio and IL-4c-treated as in (I)). Data are mean ± s.e.m. *P ≤ 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, significance calculated with an unpaired two-sided Student’s t-test.
Figure 2.9. Loss of Bhlhe40 causes morphological changes in in vivo IL-4c-stimulated peritoneal macrophages. (A, B) Transmission electron microscopy (TEM) images of LPMs from naïve (A) and IL-4c-treated (B) Bhlhe40+/+ and Bhlhe40−/− mice (representative of 2 experiments, n=2 mice (45-50 images)/group). Scale bar, 2 μm. (C-H) Cellular cross-sectional area (C), endoplasmic reticulum (ER) cross-sectional extent (D), ER luminal width (E), vesicle cross-sectional area (F), nucleoli/cross-section (G), mitochondria/cross-section (H) of LPMs as in (A, B) (pooled from 2 experiments, n=2 mice/group [17-60 cells analyzed]). Data are mean ± s.e.m. *P ≤ 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, significance calculated with an unpaired two-sided Student’s t-test.
Figure 2.10. *Bhlhe40* expression is tightly regulated in resident macrophages. (A) Flow cytometry of *Bhlhe40*<sup>GFP</sup> transgene reporter expression in LPMs, SPMs, red pulp macrophages, Kupffer cells, kidney macrophages, and AMs from *Bhlhe40*<sup>GFP<sup>+</sup></sup> and *Bhlhe40*<sup>GFP<sup>-</sup></sup> mice after PBS or IL-4c treatment (representative of 1-2 experiments, n=3 PBS-treated *Bhlhe40*<sup>GFP<sup>+</sup></sup>, 1 PBS-treated *Bhlhe40*<sup>GFP<sup>-</sup></sup>, 5 IL-4c-treated *Bhlhe40*<sup>GFP<sup>+</sup></sup>, 3 IL-4c-treated *Bhlhe40*<sup>GFP<sup>-</sup></sup>). (B) Numbers of LPMs, red pulp macrophages, Kupffer cells, and AMs from *Bhlhe40*<sup>+/+</sup> and *Bhlhe40*<sup>−/−</sup> mice treated with PBS or IL-4c (pooled from 2 experiments, n=4 PBS-treated *Bhlhe40*<sup>+/+</sup>, 3 PBS-treated *Bhlhe40*<sup>−/−</sup>, 8 IL-4c-treated *Bhlhe40*<sup>+/+</sup>, 7 IL-4c-treated *Bhlhe40*<sup>−/−</sup>). (C-E) Flow cytometry of BrdU incorporation by red pulp macrophages (C), Kupffer cells (D), and AMs (E) from *Bhlhe40*<sup>+/+</sup> and *Bhlhe40*<sup>−/−</sup> mice treated with PBS or IL-4c as in Fig. 5G. Data are mean ± s.e.m. *P ≤0.05, significance calculated with an unpaired two-sided Student’s *t*-test.
Figure 2.11. Bhlhe40 is required for normal proliferation of thioglycollate-elicited macrophages during type 2 immunity. (A) Flow cytometry of BrdU incorporation by LPMs and Thio-elicited macrophages from Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> mice treated with PBS, IL-4c, thioglycollate (Thio), or Thio and IL-4c as in Fig. 5J. (B, C) Frequency of pHH3<sup>+</sup> LPMs and Thio-elicited macrophages (B) (pooled from 2 experiments, n=3 PBS-treated Bhlhe40<sup>+/+</sup>; 4 PBS-treated Bhlhe40<sup>−/−</sup>; 4 IL-4c-treated and Thio-treated Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup>; 6 Thio and IL-4c-treated Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup>) and RELMα<sup>+</sup> LPMs and Thio-elicited macrophages (C) (pooled as in (B)) from Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> mice treated with PBS, IL-4c, thioglycollate (Thio), or Thio and IL-4c. Data are mean ± s.e.m. *P ≤ 0.05; ***P < 0.001, significance calculated with an unpaired two-sided Student’s t-test.
Figure 2.12. *Bhlhe40* is required for LPM proliferation in response to *H. polygyrus*. (A) Flow cytometry of peritoneal macrophage subsets from *Bhlhe40*+/+ and *Bhlhe40*−/− mice infected with *H. polygyrus* (representative of 4 experiments, n=15 *Bhlhe40*+/+, 14 *Bhlhe40*−/−). (B) Numbers of LPMs from *Bhlhe40*+/+, *Bhlhe40*−/−, LysM-Cre− *Bhlhe40*fl/fl, and LysM-Cre+ *Bhlhe40*fl/fl mice mock- or *H. polygyrus*-infected as in (A) (pooled from 3 experiments, n=6 mock-infected *Bhlhe40*+/+; 4 mock-infected *Bhlhe40*−/−; *H. polygyrus*-infected as in (A) for *Bhlhe40*+/+ and *Bhlhe40*−/−; 3 mock-infected LysM-Cre− *Bhlhe40*fl/fl and LysM-Cre+ *Bhlhe40*fl/fl; 12 *H. polygyrus*-infected LysM-Cre− *Bhlhe40*fl/fl and LysM-Cre+ *Bhlhe40*fl/fl). (C, D) Frequency of BrdU+ LPMs (C) (pooled from 3 experiments, n=4 mock-infected *Bhlhe40*+/+ and *Bhlhe40*−/−; 14 *H. polygyrus*-infected *Bhlhe40*+/+; 12 *H. polygyrus*-infected *Bhlhe40*−/−; 3 mock-infected LysM-Cre− *Bhlhe40*fl/fl and LysM-Cre+ *Bhlhe40*fl/fl; 12 *H. polygyrus*-infected LysM-Cre− *Bhlhe40*fl/fl and LysM-Cre+ *Bhlhe40*fl/fl) and pH3+ LPMs (D) (pooled from 3 experiments, n=6 mock-infected *Bhlhe40*+/+; 4 mock-infected *Bhlhe40*−/−; 15 *H. polygyrus*-infected *Bhlhe40*+/+; 14 *H. polygyrus*-infected *Bhlhe40*−/−; 3 mock-infected LysM-Cre− *Bhlhe40*fl/fl and LysM-Cre+ *Bhlhe40*fl/fl; 12 *H. polygyrus*-infected LysM-Cre− *Bhlhe40*fl/fl and LysM-Cre+ *Bhlhe40*fl/fl) from *Bhlhe40*+/+, *Bhlhe40*−/−, LysM-Cre− *Bhlhe40*fl/fl, and LysM-Cre+ *Bhlhe40*fl/fl.
mice mock- or *H. polygyrus*-infected. (E) Proportion of LPMs in each phase of the cell cycle from *Bhlhe40*+/+ and *Bhlhe40*−/− mice mock- or *H. polygyrus*-infected (pooled from 2 experiments, n=3 mock-infected *Bhlhe40*+/+, 2 mock-infected *Bhlhe40*−/−, 7 *H. polygyrus*-infected *Bhlhe40*+/+, 6 *H. polygyrus*-infected *Bhlhe40*−/−). (F) Frequency of 7-AAD+ LPMs from *Bhlhe40*+/+, *Bhlhe40*−/−, *LysM-Cre− Bhlhe40*fl/fl, and *LysM-Cre+ Bhlhe40*fl/fl mice mock- or *H. polygyrus*-infected (pooled from 3 experiments, n=4 mock-infected *Bhlhe40*+/+; 3 mock-infected *Bhlhe40*−/−; 12 *H. polygyrus*-infected *Bhlhe40*+/+ and *Bhlhe40*−/−; 3 mock-infected *LysM-Cre− Bhlhe40*fl/fl and *LysM-Cre+ Bhlhe40*fl/fl; 11 *H. polygyrus*-infected *LysM-Cre− Bhlhe40*fl/fl and *LysM-Cre+ Bhlhe40*fl/fl). Data are mean ± s.e.m. *P ≤ 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, significance calculated with an unpaired two-sided Student’s *t*-test.
Figure 2.13. Bhlhe40 regulates gene expression to modulate proliferation, but not alternative activation, in LPMs during type 2 immunity. (A) Gene expression microarray
data were analyzed for genes differentially expressed by ≥2-fold in LPMs from Bhlhe40<sup>+/+</sup>, Bhlhe40<sup>−/−</sup>, and LysM-Cre<sup>+</sup> Bhlhe40<sup>0/0</sup> mice treated with IL-4c. (B-D) Gene expression microarray data were analyzed for shared and unique Bhlhe40-dependent genes (≥2-fold differentially expressed, depicted as a Venn diagram) (B), shared Bhlhe40-dependent genes (C), and expression of the LPM alternative activation signature (D) in LPMs from Bhlhe40<sup>+/+</sup>, Bhlhe40<sup>−/−</sup>, and LysM-Cre<sup>+</sup> Bhlhe40<sup>0/0</sup> mice unstimulated or treated with IL-4c. (E) qRT-PCR of Maf and Mafb expression relative to Hprt in Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> LPMs from mice treated with IL-4c (pooled from 3 experiments, n=7 Bhlhe40<sup>+/+</sup>, 6 Bhlhe40<sup>−/−</sup>). (F, G) GSEA of gene expression microarray data for representative Hallmark (F) and C5 gene sets (G) enriched in Bhlhe40<sup>+/+</sup> vs. Bhlhe40<sup>−/−</sup> LPMs from IL-4c-treated mice. NES, normalized enrichment score. FWER, family-wise error rate. (H, I) MSigDB C5 gene set enrichment was analyzed using the lists of genes expressed at ≥1.5-fold in Bhlhe40<sup>+/+</sup> vs. Bhlhe40<sup>−/−</sup> (H) or Bhlhe40<sup>−/−</sup> vs. Bhlhe40<sup>+/+</sup> LPMs from IL-4c-treated mice (I). Microarray data from naïve LPMs (n=3/group, reanalyzed from Fig. 3) and in vivo IL-4c-stimulated LPMs (n=2/group) are from single separate experiments. Data are mean ± s.e.m. ***P < 0.001; ****P < 0.0001, significance calculated with an (E) unpaired two-sided Student’s t-test and a (H, I) one-sided hypergeometric test.
Figure 2.14. Bhlhe40 directly regulates gene transcription in LPMs. (A) Gene expression microarray data were analyzed for expression of genes encoding selective regulators of LPM proliferation (Myo18a and C1q) in LPMs from Bhlhe40<sup>+/+</sup>, Bhlhe40<sup>−/−</sup>, and LysM-Cre<sup>+</sup> Bhlhe40<sup>fl/fl</sup> mice unstimulated or treated with IL-4c. (B-D) Tracings of Bhlhe40 binding, PU.1 binding, and vertebrate conservation at the Il10 (B), Ccl2 (C), and Plac8 (D) loci. (E) Bhlhe40-bound, Bhlhe40-dependent genes (≥2-fold differentially expressed in Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> LPMs)
in LPMs from naïve mice and Bhlhe40-bound, Bhlhe40-dependent genes (≥2-fold differentially expressed in \textit{Bhlhe40}^{+/+} and \textit{Bhlhe40}^{-/-} LPMs) in LPMs from IL-4c-treated mice, as in Fig. 8I. Underlined genes are highlighted elsewhere in this study. (F-H) Tracings of Bhlhe40 binding, PU.1 binding, and vertebrate conservation at the \textit{Maf} (distal) (F), \textit{Mafb} (G), and \textit{Mafb} (distal) (H) loci. LPM Bhlhe40 ChIP-seq data (n=1/group), microarray data from naïve LPMs (n=3/group), and microarray data from IL-4c-stimulated LPMs (n=2/group) are from single separate experiments. LPM PU.1 ChIP-seq data reanalyzed from Gosselin et al., 2014.
Figure 2.15. Bhlhe40 directly regulates gene expression in LPMs in an activation state-dependent manner. (A) Bhlhe40 ChIP-seq data were analyzed for consensus binding motifs in LPMs from naive (left) and IL-4c-treated mice (right). (B) Bhlhe40 ChIP-seq data were analyzed for locations of Bhlhe40 peaks in the genome in LPMs from naive (left) and IL-4c-treated mice (right). UTR, untranslated region. (C) Tracings of Bhlhe40 binding, PU.1 binding, and vertebrate conservation at the Bhlhe40 locus. (D) Bhlhe40 ChIP-seq data were analyzed for shared and unique Bhlhe40 binding sites in LPMs from naive and IL-4c-treated mice (depicted as a Venn diagram). (E) Tracings of Bhlhe40 binding, PU.1 binding, and vertebrate conservation at the Clec10a locus. (F) Naïve LPM Bhlhe40 ChIP-seq data, in vivo IL-4c-stimulated LPM Bhlhe40 ChIP-seq data, and naïve LPM PU.1 ChIP-seq data were analyzed for shared and unique Bhlhe40 and PU.1-bound genes between the three samples (depicted as a Venn diagram). (G) The proportion of Bhlhe40-bound, Bhlhe40-dependent genes (≥2-fold differentially expressed in Bhlhe40+/+ and Bhlhe40−/− LPMs) in LPMs from naïve mice and Bhlhe40-bound, Bhlhe40-dependent genes (≥2-fold differentially expressed in Bhlhe40+/+ and Bhlhe40−/− LPMs) in LPMs from IL-4c-treated mice. (H) Tracings of Bhlhe40 binding, PU.1 binding, and vertebrate conservation at the Maf locus. (I) GSEA of gene expression microarray data for Bhlhe40-bound genes from LPMs from Bhlhe40+/+ and Bhlhe40−/− mice treated with IL-4c for the C5 Cell Cycle Process gene set. NES, normalized enrichment score. FWER, family-wise error rate. LPM Bhlhe40 ChIP-seq data (n=1/group) and microarray data from in vivo IL-4c-stimulated LPMs (n=2/group) are from single separate experiments. LPM PU.1 ChIP-seq data reanalyzed from Gosselin et al., 2014.
Figure 2.16. Bhlhe40 directly binds to cell cycle-related loci and is required to sustain normal gene expression. (A-D) The proportion of Bhlhe40-bound members of gene sets
enriched in Bhlhe40+/+ compared to Bhlhe40+/ LPMs from IL-4c-treated mice. Tracings of Bhlhe40 binding, PU.1 binding, and vertebrate conservation for a representative member of the core enrichment signature for each gene set is presented. Hallmark E2F Targets (A), Hallmark Myc Targets (v1) (B), C5 Chromosome Organization (C), and C5 Cell Cycle Process (D). (E-H) GSEA of expression of Bhlhe40-bound genes from gene expression microarray data from LPMs from Bhlhe40+/+ and Bhlhe40+/ mice treated with IL-4c for Hallmark E2F Targets (E), Hallmark Myc Targets v1 (F), C5 Chromosome Organization (G), and C5 Cell Cycle Process (H). C5 Cell Cycle Process is also presented in Fig. 8I. NES, normalized enrichment score. FWER, family-wise error rate. LPM Bhlhe40 ChIP-seq data (n=1/group) and microarray data (n=2/group) are from single separate experiments. LPM PU.1 ChIP-seq data reanalyzed from Gosselin et al., 2014.
Chapter 3: Bhlhe40 is required for protective Th2 cell responses to helminth infection

The contents of this chapter have been modified from the following preprint article (submitted):

Deficiency in Bhlhe40 impairs resistance to *H. polygyrus bakeri* and reveals novel Csf2rb-dependent regulation of anti-helminth immunity


3.1 Abstract

The cytokines GM-CSF and IL-5 are thought to possess largely divergent functions despite a shared dependence on the common beta (βC) chain to initiate signaling. Although IL-5 is part of the core type 2 cytokine signature and is required for protection against some helminths, it is dispensable for immunity to others, such as *Heligmosomoides polygyrus bakeri* (*H. polygyrus*). Whether this is due to compensatory mechanisms is unclear. The transcription factor Bhlhe40 has been shown to control GM-CSF production and is proposed to be a novel regulator of T helper type 2 cells. We have found that Bhlhe40 is required in T cells for a protective memory response to secondary *H. polygyrus* infection. *H. polygyrus* rechallenge elicited dramatic Bhlhe40-dependent changes in gene and cytokine expression by lamina propria CD4⁺ T cells and
in vitro-polarized T\textsubscript{H}2 cells, including induction of GM-CSF and maximal production of type 2 cytokines including IL-5. β\textsubscript{C} chain-deficient, but not GM-CSF-deficient, mice rechallenged with \textit{H. polygyrus} had severely impaired protective immunity. Our results demonstrate that Bhlhe40 is an essential regulator of T\textsubscript{H}2 cell immunity during helminth infection and reveal unexpected redundancy of β\textsubscript{C} chain-dependent cytokines.

3.2 Introduction

Helminthic worms are parasites of eukaryotic organisms which manipulate the immune system to establish chronic infections in diverse sites, often resulting in significant tissue damage (Allen and Sutherland, 2014; Maizels et al., 2018; Patel et al., 2009; Sorobetea et al., 2018). A stereotypical helminth infection results in production of alarmins including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which in turn stimulate the development of type 2 immunity characterized by production of IL-4, IL-5, and IL-13 (Allen and Sutherland, 2014; Maizels et al., 2018; Sorobetea et al., 2018). The helminth \textit{Heligmosomoides polygyrus bakeri} (hereafter \textit{H. polygyrus}) is a natural pathogen of mice and has been frequently employed as a model helminth infection (Camberis et al., 2003; Maizels et al., 2018; Patel et al., 2009; Sorobetea et al., 2018). Several cytokines contribute to protective immunity against \textit{H. polygyrus}, including IL-4 and IL-25, while IL-5, IL-33, and TSLP are considered dispensable for control of this infection (Massacand et al., 2009; Pei et al., 2016; Urban et al., 1991; Zaiss et al., 2013).

However, in many instances of type 2 immunity, IL-5 plays a crucial role (Bagnasco et al., 2017; Roufosse, 2018), including in some helminth infections (Allen and Sutherland, 2014;
Maizels et al., 2004; Sorobetea et al., 2018). 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 (Allen and Sutherland, 2014; Maizels et al., 2004). IL-5 binds to the unique IL-5Rα chain paired with the common beta (βc) chain, which is shared with the receptors for GM-CSF and IL-3 (Dougan et al., 2019; Robb et al., 1995). This raises the possibility that redundancy within this cytokine family could render individual members dispensable during helminth infection. While IL-3 signaling is maintained in mice in the absence of the βc chain (Csf2rb) through a unique beta chain (βIL3, Csf2rb2), GM-CSF and IL-5 signaling is abrogated in Csf2rb−/− mice (Dougan et al., 2019; Robb et al., 1995). Despite absolute dependence on this shared receptor chain, which is largely responsible for downstream signaling (Dougan et al., 2019), known roles for IL-5 and GM-CSF 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 (Becher et al., 2016; Croxford et al., 2015; Deepe et al., 1999; Gonzalez-Juarrero et al., 2005; Hirata et al., 2010; LeVine et al., 1999; Mandujano et al., 1995; Paine et al., 2000; Zhan et al., 1998). GM-CSF also contributes to pathological T_h2 responses to allergens (Cates et al., 2004; Sheih et al., 2017; Yamashita et al., 2002). 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* (Shim et al., 2012).

The transcription factor Bhlhe40 functions in B cells, NKT cells, T cells, and tissue-resident macrophages (Camponeschi et al., 2018; Huynh et al., 2018; Jarjour et al., 2019a; Kanda et al., 2016; Kreslavsky et al., 2017; Lin et al., 2016; Lin et al., 2014; Martínez-Llordella et al., 2013; Seimiya et al., 2004; Yu et al., 2018). We and others have described a crucial role for
Bhlhe40 as a modulator of Th1 and Th17 cell cytokine production in infection and autoimmunity, particularly by controlling GM-CSF and IL-10 production (Huynh et al., 2018; Lin et al., 2016; Lin et al., 2014; Martínez-Llordella et al., 2013; Yasuda et al., 2019; Yu et al., 2018). Recent studies of in vitro-polarized Th2 cells (Henriksson et al., 2019) and house dust mite (HDM)-elicited airway Th2 cells (Tibbit et al., 2019) have identified Bhlhe40 as a potential regulator of this cell type in vivo. We have also recently described a role for Bhlhe40 in large peritoneal macrophages (LPMs) during type 2 immunity (Jarjour et al., 2019a). Taken together, these data suggest that Bhlhe40 may regulate type 2 infections, possibly by controlling cytokine production from T cells or via a myeloid cell-intrinsic role.

Herein, we have found that Bhlhe40 and the βC chain are essential to protective memory to secondary *H. polygyrus* infection. Bhlhe40−/− mice exhibited severe defects during rechallenge infection with *H. polygyrus* and altered intestinal pathology, which were recapitulated in Cd4-Cre+Bhlhe40fl/fl, but not LysM-Cre+Bhlhe40fl/fl mice. We defined a helminth-induced gene signature in lamina propria CD4+ T cells, which was disrupted in the absence of Bhlhe40. In vitro-polarized Th2 cells and *H. polygyrus*-elicited CD4+ T cells from the small intestine lamina propria exhibited Bhlhe40-dependent production of GM-CSF, and Bhlhe40-deficient CD4+ T cells also exhibited reduced production of IL-5 and other cytokines. Secondary infection of Csf2rb−/−, but not Csf2−/−, mice resulted in severely impaired protective immunity and altered intestinal pathology. Overall, Bhlhe40 serves as a pivotal regulator of the Th2 cell transcriptional response to helminth infection, in part by modulating GM-CSF and IL-5 production, and reveals redundant roles for these cytokines not observed during deficiency of either factor alone.
3.3 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 (Filbey et al., 2014). 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. 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. 3.1A). While there was a trend towards increased adult worm burden in *Bhlhe40*−/− as compared to *Bhlhe40*+/+ mice after rechallenge (Fig. 3.1B), 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 (Filbey et al., 2014). Small intestines from *Bhlhe40*+/+ mice exhibited many granulomas, while those from *Bhlhe40*−/− mice resembled healthy tissue (Fig. 3.1C, D). 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. 3.1E, F).

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 (Bain et al., 2014; Scott et al., 2017) and another CD45+F4/80+CD64+MHC-IIloLy6Clo autofluorescent population, which we termed
granuloma-associated monocytes/macrophages (GMMs) (Fig. 3.2A, B). This latter population may correspond to previously described clodronate-sensitive alternatively activated macrophages seen by immunostaining during *H. polygyrus* infection (Anthony et al., 2006). SILP CD45^+^F4/80^+^CD64^+^CD11b^+^SSC-A^hi^ eosinophils were also significantly increased after secondary *H. polygyrus* infection (Fig. 3.2A, B and 3.3A). 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. 3.2A, B and 3.3B). Because *H. polygyrus* infection is known to change the cellular composition of the peritoneal cavity (Allen and Sutherland, 2014; Mohrs et al., 2005; Steinfelder et al., 2017), 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 RELMα staining (Fig. 3.2C-E and 3.3C-E). In contrast, serum *H. polygyrus*-specific IgG1 titers were not affected by loss of Bhlhe40 (Fig. 3.3F). These data indicated impaired responses by multiple myeloid cell lineages to *H. polygyrus* rechallenge in *Bhlhe40^-/-* mice.

**Bhlhe40 is required in T cells for normal immunity 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. 3.4A, B). When we assessed the response of myeloid cells to secondary infection, we found that loss of Bhlhe40 selectively in T cells was sufficient to perturb both the SILP and peritoneal responses (Fig. 3.4C-F). In contrast, *LysM-Cre^+^ Bhlhe40^fl/fl^ mice were able
to control infection and formed intestinal granulomas comparably to LysM-Cre Bhlhe40<sup>fl/fl</sup> mice (Fig. 3.4G, H). Therefore, Bhlhe40 expression was required in T cells to control secondary <i>H. polygyrus</i> infection, but was dispensable in LysM-expressing myeloid cells.

**Bhlhe40 is required for a normal CD<sup>4+</sup> T cell transcriptional response to <i>H. polygyrus**

We next asked whether loss of Bhlhe40 dysregulated CD<sup>4+</sup> T cell gene expression in response to <i>H. polygyrus</i> rechallenge. To address this, we sorted CD<sup>4+</sup> T cells from the SILP of naïve and rechallenged Bhlhe40<sup>+/+</sup> and Bhlhe40<sup>−/−</sup> mice for gene expression microarrays. By comparing CD<sup>4+</sup> T cells from naïve and <i>H. polygyrus</i>-rechallenged Bhlhe40<sup>+/+</sup> 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. 3.5A and 3.6A). When we assessed Bhlhe40-dependent genes after <i>H. polygyrus</i> rechallenge, we found that a significant majority were part of the helminth-induced signature and that Bhlhe40-dependent genes were distinct in CD<sup>4+</sup> T cells from naïve and <i>H. polygyrus</i>-rechallenged mice (Fig. 3.5B, C and 3.6B, C). 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<sup>+/+</sup> as compared to Bhlhe40<sup>−/−</sup> CD<sup>4+</sup> T cells after <i>H. polygyrus</i> 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. 3.5D, E). Furthermore, when we assessed differential expression of the lineage-specifying transcription factors of each T helper cell subset as well as a recently defined set of transcriptional regulators of in vitro-polarized Th2 cells (Henriksson et al., 2019), we observed reduced expression of Pparg in Bhlhe40<sup>−/−</sup> 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. 3.5E). Bhlhe40 expression was markedly induced in mice experiencing secondary infection as compared to naïve mice (Fig. 3.5E). Using Bhlhe40GFP bacterial artificial chromosome reporter mice (Lin et al., 2016), we observed a marked increase in SILP GFP+ CD4+ T cells after H. polygyrus rechallenge (Fig. 3.5F). 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 T cell cytokine production in response to H. polygyrus

Next, we restimulated SILP cells from naïve and rechallenged Cd4-Cre+ Bhlhe40fl/fl and Cd4-Cre+ Bhlhe40fl/fl mice ex vivo with phorbol 12-myristate 13-acetate (PMA) and ionomycin to assess cytokine production. Rechallenge with H. polygyrus induced production of IL-4, IL-5, and IL-13 from Cd4-Cre+ Bhlhe40fl/fl and Cd4-Cre+ Bhlhe40fl/fl CD4+ T cells; however, Bhlhe40 was required for normal frequencies of single- and multi-cytokine-producing cells, largely due to reductions in IL-5+ and IL-13+ CD4+ T cells (Fig. 3.7A-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+ Bhlhe40fl/fl CD4+ T cells) and that this required Bhlhe40 (~10% of Cd4-Cre+ Bhlhe40fl/fl CD4+ T cells) (Fig. 3.7D, E). TH2 cells disseminate widely in mice infected with H. polygyrus (Mohrs et al., 2005; Steinfelder et al., 2017). CD4+ T cell cytokine responses in the peritoneal cavity and mesenteric lymph nodes from H. polygyrus-rechallenged Cd4-Cre+ Bhlhe40fl/fl and Cd4-Cre+ Bhlhe40fl/fl mice were generally consistent with those in the SILP (Fig. 3.8). To assess whether Bhlhe40 was also required for normal cytokine production by a pure population of TH2 cells, we differentiated naïve splenic
CD4+ T cells into T\textsubscript{H}2 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 \textit{in vitro} production of type 2 cytokines (Fig. 3.9). Therefore, these data indicated that Bhlhe40 is required \textit{in vitro} and \textit{in vivo} for normal T\textsubscript{H}2 cell function.

As Bhlhe40 is a known repressor of IL-10 (Huynh et al., 2018; Lin et al., 2016; Lin et al., 2014; Yu et al., 2018), 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 \textit{H. polygyrus} rechallenge (Fig. 3.10A). Nevertheless, genetic deletion of IL-10 in Bhlhe40\textsuperscript{+/−} Il10\textsuperscript{−/−} mice was not sufficient to restore control of infection or normal intestinal granulomatous pathology (Fig. 3.10B, C). Collectively, these data demonstrated a key role for Bhlhe40 in CD4+ T cell cytokine responses to helminth infection, notably controlling production of the \textit{βC} chain family cytokines IL-5 and GM-CSF.

\textbf{Loss of the \textit{βC} chain impairs protective memory to \textit{H. polygyrus}}

As protective memory responses to \textit{H. polygyrus} rechallenge are unaffected by IL-5 blockade (Urban et al., 1991), we first asked whether loss of GM-CSF signaling was sufficient to impair control of a secondary infection with \textit{H. polygyrus}. Genetic deletion of GM-CSF (Csf2\textsuperscript{−/−} mice) did not result in severe defects in control of \textit{H. polygyrus} infection as compared to Csf2\textsuperscript{+/+} mice (Fig. 3.11A-F). As IL-5 and GM-CSF were individually dispensable, we then rechallenged Csf2rb\textsuperscript{+/+} and Csf2rb\textsuperscript{−/−} mice. Remarkably, Csf2rb\textsuperscript{−/−} mice had a severe defect in control of \textit{H. polygyrus} as compared to Csf2rb\textsuperscript{+/+} mice (Fig. 3.12A). Csf2rb\textsuperscript{−/−} mice did not form intestinal granulomas or develop a normal SILP myeloid cell response to \textit{H. polygyrus} rechallenge as compared to Csf2rb\textsuperscript{+/+} mice (Fig. 3.12B, C). Furthermore, loss of the \textit{βC} chain resulted in defects
in peritoneal myeloid cell responses similar to those seen in Bhlhe40−/− and Cd4-Cre+ Bhlhe40fl/fl mice (Fig. 3.12D-F). To exclude a role for βc chain-dependent IL-3 signaling, we singly blocked GM-CSF or IL-5 signaling with neutralizing antibodies or blocked both cytokines together. Double, but not single, blockade resulted in severely impaired protective immunity (Fig. 3.11G, H). Taken together, these data indicated that IL-5 and GM-CSF were collectively, but not individually, critical to control of H. polygyrus rechallenge.

3.4 Discussion

We have demonstrated that protective memory responses against H. polygyrus are critically dependent on Bhlhe40 and the βc chain. Bhlhe40 is specifically required in CD4+ T cells to promote a normal myeloid cell response to H. polygyrus by supporting expression of cytokine transcripts as well as other potential regulators of helminth infection, including Areg, Csf2, Il5, Il13, Nlrp3, and Pparg (Chen et al., 2017; Chenery et al., 2019; Zaiss et al., 2006). We have also described the CD4+ T cell transcriptome within the SILP during secondary H. polygyrus infection. These data provide unique insight into the CD4+ T cell global transcriptional response to helminths at the site of infection. We found that Bhlhe40 is a key regulator of T\textsubscript{H}2 cell cytokine production. Notably, GM-CSF and IL-5 were markedly stimulated by H. polygyrus rechallenge and this response was Bhlhe40-dependent. In light of these data, we assessed the importance of the βc chain during H. polygyrus rechallenge and found that it was critically required for protective immunity, despite control of secondary H. polygyrus infection during IL-5 blockade (this manuscript and Urban et al., 1991), during GM-CSF blockade, and in Csf2−/− mice. These data support redundant roles for Bhlhe40-dependent βc chain-dependent cytokines in protective immunity to H. polygyrus.
When we compared differentially expressed transcripts between *H. polygyrus*-elicited and naive SILP CD4$^+$ T cells, we found a remarkable similarity to the transcriptional profile of HDM-elicited airway T$_{H2}$ cells (*Bhlhe40, Cd200r1, Il6, Plac8, Igfbp7*) (Tibbit et al., 2019), demonstrating significant conservation of the T$_{H2}$ transcriptional program independent of tissue environment and stimulus. When we assessed whether Bhlhe40 was functionally required in T cells during type 2 immunity, we found that SILP CD4$^+$ T cells activated by *H. polygyrus* rechallenge required Bhlhe40 for normal expression of many helminth-induced genes and to control helminth rechallenge. We therefore demonstrate for the first time that Bhlhe40 regulates in vivo T$_{H2}$ cell responses, consistent with a recent screen for novel regulators of in vitro-polarized T$_{H2}$ cells that identified Bhlhe40 (Henriksson et al., 2019). Notably, *Pparg* expression was reduced in the absence of Bhlhe40, and as PPARγ is required for normal T$_{H2}$ cell responses and protective immunity to *H. polygyrus*, this may indicate that some of the effects of Bhlhe40 deficiency are indirect (Chen et al., 2017; Nobs et al., 2017). Our work and that of others has now established key roles for Bhlhe40 in T$_{H1}$, T$_{H2}$, and T$_{H17}$ cells as a pivotal regulator of GM-CSF, IL-10, and other cytokines (Huynh et al., 2018; Lin et al., 2016; Lin et al., 2014; Martínez-Llordella et al., 2013; Yasuda et al., 2019; Yu et al., 2018). These data and a recent study on c-Maf (Gabrysova et al., 2018) 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 factors regulating Bhlhe40 may possess significant therapeutic potential.

While GM-CSF and IL-5 have long been known to share common signaling through the $\beta_C$ 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 (Bagnasco et al., 2017;
Becher et al., 2016; Codarri et al., 2011; Roufosse, 2018). 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 βc 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 βc chain family cytokines is not well described, it is known that these cytokines can regulate eosinophils in a complementary manner (Esnault and Kelly, 2016). Future studies should identify the targets of βc family cytokine signaling and establish whether GM-CSF and IL-5 are collectively involved in protective immunity to other helminth infections. As βIL3 chain-dependent IL-3 signaling is preserved in Csf2rb−/− mice (Dougan et al., 2019; Robb et al., 1995), it is also of interest to establish whether additionally blocking IL-3 results in a more severe defect in immunity to *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 (Herbst et al., 2012; Schwartz et al., 2014), suggesting that IL-3 is likely important for control of *H. polygyrus* infection.

Our findings reveal novel transcriptional and cytokine regulation of the immune response to helminth infection, elucidating a critical function for Bhlhe40 during *in vivo* Th2 cell immunity and an unexpected role for βc chain-dependent signaling. Our data suggest that the importance of βc chain family cytokines may have been underestimated in type 2 diseases because of redundancy. Further studies are needed to elucidate whether compensation between βc chain family cytokines is a general theme of type 2 immunity and whether combinatorial targeting of these factors could yield improved clinical outcomes in diseases of pathological type 2 immunity.
3.5 Methods

Mice

C57BL/6 (Taconic and Jackson), Il10-/- (B6.129P2-Il10tm1Cgn/J, Jackson), Csf2-/- (B6.129S-Csf2tm1Mig/J, Jackson), Csf2rb-/- (B6.129S1-Csf2rbtm1Cgb/J, Jackson), Cd4-Cre (B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ, Jackson) and LysM-Cre (B6N.129P2(B6)-Lyz2tm1(cre)Ifo/J, Jackson) mice were obtained from the vendors listed. Bhlhe40-/- (10 generations backcrossed to the C57BL/6 background) (Lin et al., 2014; Sun et al., 2001), Bhlhe40GFP+ (10 generations backcrossed to the C57BL/6 background) (Lin et al., 2016), and Bhlhe40fl/fl (Huynh et al., 2018) mice have been previously reported. All mice were maintained in our specific pathogen free animal facility. Sex-matched littermates were used for experiments whenever possible. Experiments with Csf2-/- and Csf2rb-/- mice used cohoused, age-matched, sex-matched C57BL/6 controls bred in our animal facility or purchased from Jackson. All animal experiments were approved by the Animal Studies Committee of Washington University in St. Louis.

Heligmosomoides polygyrus bakeri infections

Infective Heligmosomoides polygyrus bakeri third-stage larvae (L3) were prepared as described (Camberis et al., 2003). For both primary and secondary infection, mice were orally gavaged with 200 L3 with a 20-gauge ball-tipped gavage needle. Female mice were used for experiments whenever possible. Fecal samples were collected at 8, 11, 14, and 21 days after primary and secondary infection and weighed. Eggs were counted by dissolution of feces in 5 ml water, followed by a 1:1 dilution with a saturated sodium chloride solution before loading into a McMaster counting chamber (Chalex LLC). For rechallenge experiments, mice were cleared of
infection by oral gavage with 2 mg of pyrantel pamoate (Columbia Laboratories) diluted in Dulbecco’s PBS on days 14 and 15 after primary infection and were rested for 3-4 weeks before reinfection. Blood was collected at day 21 post-rechallenge and allowed to clot for >1 hour before serum collection by centrifugation at 1,500 g for 15 minutes. Mice were sacrificed on day 17 or 22 of secondary infection for assessment of cellular responses. Macroscopic granulomas present at day 22 of secondary infection that were visible to the naked eye were counted along the length of the intact intestines. Assessment of the intestinal worm burden was performed at day 17 or 22 of primary or secondary infection. Adult worms were collected by cutting the intestine open and into ~4 in sections, placing the tissue and contents into a metal filter on top of a 50 ml tube of PBS, and setting the tube in a 37 °C water bath. Worms then actively migrated through the filter over several hours and sedimented. Worms were counted visually in petri dishes.

**In vivo antibody blockade**

Mice were infected, cleared, and rested as above. Immediately before reinfection, mice were injected i.p. with 300 µg of αGM-CSF (Leinco, G670, clone MP1-22E9), 300 ug of αIL-5 (BioXCell, BE0198, clone TRFK5), and/or control polyclonal rat IgG (Sigma Aldrich, I4131) and were given the same dose i.p. every other day until sacrifice (Codarri et al., 2011).

**In vitro Th2 cell culture**

The EasySep mouse naïve CD4+ T cell isolation kit (Stemcell Technologies, 19765A) was used to isolate T cells from the spleens of untreated mice. Cells were cultured in complete Iscove’s modified Dulbecco’s media (cIMDM, with added 10 % FBS, L-glutamine, sodium
pyruvate, non-essential amino acids, penicillin/streptomycin, and \( \beta \)-mercaptoethanol) in flat-bottom, tissue culture-treated 48 well plates with plate-bound anti-CD3 and anti-CD28. Plates were coated overnight with anti-CD3 antibody (BioXCell, 2 \( \mu \)g/mL, clone 145-2C11) and anti-CD28 antibody (BioLegend, 2 \( \mu \)g/ml, clone 37.51) before use. To polarize naïve T cells to T\(_{\text{H}2}\) cells, IL-4 (BioLegend, 10 ng/mL) and anti-IFN-\( \gamma \) (Leinco, 5 \( \mu \)g/mL, clone H22) were added at the start of culture. Cells were split at day 3, and on day 4 cells were stimulated for 24 hours with plate-bound anti-CD3 and anti-CD28 (coated as above) for assessment of secreted cytokines in the supernatant by ELISA.

**Enzyme-linked immunosorbent assay (ELISA)**

Nunc Maxisorp plates were coated with capture antibodies (for cytokine ELISAs) or \( H. \) *polygyrus* lysate (for \( H. \) *polygyrus*-specific IgG1 ELISA) overnight. Wells were washed with PBS-Tween 20 (0.05%), and 1% BSA in PBS was added for one hour to block. After washing, culture supernatants (cytokine ELISAs) or serum (IgG1 ELISA) were added for 1-2 hours, except for the GM-CSF ELISA (overnight). After washing, cytokine-detection biotinylated antibodies or horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 antibody were added for 1 hour, except for the GM-CSF ELISA (2 hours). For cytokine ELISAs, following another wash, streptavidin-conjugated horseradish peroxidase (HRP) was added for 1 hour. After washing, a 1:1 mixture of room temperature TMB A and B solutions (BD OptEIA) was added. The reaction was stopped with 1 M \( \text{H}_3\text{PO}_4 \). Samples were analyzed on an iMark microplate reader (BioRad). 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.
To generate \( H. \text{polygyrus} \) lysate, adult worms collected as above were washed repeatedly in PBS, and ground in a Dounce homogenizer in 1 mL of PBS. Debris was then 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. Lysate was titrated for an ELISA with the serum of \( H. \text{polygyrus} \)-rechallenged mice, and the greatest dilution of lysate (1:1000) which yielded maximal signal was used.

**Brightfield and epifluorescence microscopy**

The proximal 6 cm of the small intestine were formed into a swiss roll (Bialkowska et al., 2016) and were frozen in OCT media. 10 μm cryosections were cut using a Leica CM1950 cryostat. For hematoxylin and eosin staining, sections were fixed in 4% PFA, washed, and stained. For immunofluorescent staining, sections were fixed in acetone, washed, and blocked with CAS-Block (Invitrogen). Staining was performed with Cy3-anti-α-smooth muscle actin (clone 1A4, Sigma) and AlexaFluor647-anti-F4/80 (clone BM8, BioLegend) diluted in CAS block, followed by washing. Sections were mounted with Abcam Fluoroshield Mounting Medium with DAPI. All images were captured with a Nikon Eclipse E800 microscope equipped with a MicroPublisher 5.0 RTV camera (QImaging) for brightfield microscopy and an EXi Blue camera (QImaging) for fluorescence microscopy using QCapture software (QImaging). Fluorescence images were merged and levelled in Adobe Photoshop.

**Leukocyte collection from tissues**

Peritoneal cells were collected from body cavities by lavage. Mesenteric lymph nodes and spleens were harvested into cIMDM, homogenized through a 70 μm filter, and washed. Isolation
of small intestine lamina propria cells was performed as described (Bando et al., 2018). In brief, 4 cm of the proximal small intestine were excised, cleaned of fecal contents, and cut into 2-3 pieces. The tissue was shaken twice in 1x HBSS (with added HEPES, 10% FBS, EDTA, and DTT) for 20 minutes to remove the epithelial layer. The tissue was then shaken at 220 rpm at 37 °C for 1 hour in an Innova 4330 shaker (New Brunswick Scientific) with Collagenase IV (Sigma, C5138) in RPMI 1640 (with added 10% FBS, L-glutamine, penicillin/streptomycin, and 2-mercaptoethanol). Cells were then pelleted by centrifugation and washed.

All cells were passed through a 70 μm cell strainer before analysis. If necessary, tissues were treated with ACK lysis buffer to lyse red blood cells. Cells were counted with a hemocytometer using trypan blue.

Flow cytometry

Cell surface staining was conducted in PBS with 0.5% BSA and 2 mM EDTA (hereafter FACS buffer) with or without 0.02% sodium azide. In brief, cells were washed in FACS buffer, blocked with α-CD16/32 (clone 2.4G2, BioXCell) for 5-10 minutes at 4 °C, stained for 20 minutes at 4 °C, and washed with FACS buffer before flow cytometry. Flow cytometry was performed on LSRFortessa X20, FACSCanto II, and LSR II instruments (all BD). Analysis was performed with FlowJo software (Treestar).

Gating of cell populations was as follows (all analysis pre-gated on FSC/SSC followed by a FSC-W/FSC-A singlet gate). SILP macrophages were gated as CD45+F4/80+CD64+MHC-II+Ly6C−, while GMMs were gated as CD45+F4/80+CD64+MHC-IIloLy6Clo. SILP eosinophils were gated as CD45+F4/80+CD64−CD11b+SSC-Ahi, and this approach was validated in some experiments with Siglec-F staining. SILP CD3+ T cells were gated as CD45+F4/80+CD64+CD19−.
CD3+. SILP CD4+ T cells were gated as CD19-F4/80-TCRβ+CD4+CD8-. LPMs were gated as CD115+CD11b+ICAM2+MHC-IIlo. Peritoneal eosinophils were gated as Siglec-F+ICAM2-. Peritoneal CD4+ T cells were gated as ICAM2-TCRβ+CD4+CD8-. Mesenteric lymph node CD4+ T cells were gated as CD19+TCRβ+CD4+CD8-.

**Intracellular staining for flow cytometry**

For intracellular cytokine staining of T cells, total SILP, peritoneal, and mesenteric lymph node cells were cultured at 0.5-1 million cells/well in a 96 well V-bottom non-tissue culture-treated plate for 3-4 hours at 37 °C with 8% CO2 in the presence of PMA (50 ng/ml), ionomycin (1 μM), and brefeldin A (1 μg/ml) in cIMDM. Cells were then surface stained and fixed with 4% paraformaldehyde (PFA). Cells were then washed with FACS buffer and stored overnight. To permeabilize the cells, samples were washed with 1x Perm/Wash buffer (BD, 554714) and stained for 20 minutes at 4 °C, followed by washing in 1x Perm/Wash buffer and FACS buffer before flow cytometry.

For RELMα staining, the BioLegend True-Nuclear Transcription Factor Buffer set (424401) was used. After surface staining, cells were fixed with 1x Fix Concentrate buffer in Fix Diluent for 30 minutes at 4 °C. Cells were then washed with FACS buffer and stored overnight. Samples were washed with 1x Perm buffer diluted in water for permeabilization. After blocking with 2% rat serum, samples were stained for 1 hour at RT with anti-RELMα antibody followed by washing in 1x Perm buffer, staining with secondary antibody for 20 min at 4 °C, washing in 1x Perm buffer, and washing in FACS buffer before flow cytometry.

**Microarrays**
Singlet F4/80 TCRβ+ CD4+ CD8− SILP cells were sorted from naïve and *H. polygyrus*-rechallenged mice using a FACSria II (BD) into FBS, followed by lysis and RNA purification using the E.Z.N.A. MicroElute Total RNA kit (Omega Bio-Tek). RNA was submitted to the Genome Technology Access core at Washington University for cDNA synthesis (NuGen Pico SL) followed by analysis on the Affymetrix Mouse Gene 1.0 ST microarray platform. Data were analyzed with the DNASTAR ArrayStar program. All CEL files were normalized together. Genes with an expression value of >5 (in log 2 scale) in at least one replicate were considered expressed. For generation of lists of differentially expressed genes at a ≥2-fold differential expression cutoff between groups, p-value significance of ≤0.01 by the moderated t-test was also required. Morpheus was used to generate heatmaps (software.broadinstitute.org/morpheus/). The Venn Diagram Plotter tool (Pacific Northwest National Laboratories, omics.pnl.gov) was used to generate Venn diagrams. Multiple differentially expressed probe sets representing a single gene were presented in heat maps, but only unique genes were counted in Venn diagrams. To assess gene set enrichment, the GSEA software from the Broad Institute was used to analyze all expressed genes for enrichment of C5 database gene sets (Subramanian et al., 2005).

**Quantification and statistical analysis**

All data are from at least two independent experiments, unless otherwise indicated. Data were analyzed by unpaired two-tailed Student’s t-tests (Prism 7; GraphPad Software, Inc.) with p ≤ 0.05 considered significant. For relevant comparisons where no p-value is shown, the p-value was > 0.05. The NES score calculated by the GSEA software was used to account for set size effects when determining enrichment of gene sets. The GSEA-calculated FWER p value was
used as a conservative measure of significance. Horizontal bars represent the mean and error bars represent the standard error of the mean (s.e.m.).
**Figure 3.1.** *Bhlhe40* is required for a protective recall response to *H. polygyrus*. (A-C) *H. polygyrus*-infected *Bhlhe40<sup>+/+</sup>* and *Bhlhe40<sup>−/−</sup>* mice were analyzed for (A) quantitation of *H. polygyrus* eggs/gram feces over time during secondary infection, (B) quantitation of adult worms recovered from the intestines of mice experiencing primary or secondary infection, and (C) small intestine morphology after secondary infection. Arrows point to granulomas. Scale bar, 1 cm. (D) Quantitation of intestinal granulomas. (E, F) *H. polygyrus*-rechallenged *Bhlhe40<sup>+/+</sup>* and *Bhlhe40<sup>−/−</sup>* mice were analyzed histologically on adjacent sections by (E) hematoxylin and eosin staining and (F) immunostaining for F4/80, α-SMA, and DAPI performed 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. Data are representative of at least 3 independent experiments (C, E, F) or are pooled from at
least 3 independent experiments (A, B, D). Data are mean ± s.e.m. Significance calculated with an unpaired Student’s t-test.
Figure 3.2. Bhlhe40 is required for normal myeloid cell responses to *H. polygyrus* rechallenge. (A, B) Naïve and *H. polygyrus*-rechallenged *Bhlhe40*+/+ and *Bhlhe40*−/− mice were analyzed by flow cytometry for (A) SILP myeloid cells and (B) quantitation as in (A). (C-E) Naïve and *H. polygyrus*-rechallenged *Bhlhe40*+/+ and *Bhlhe40*−/− mice were analyzed by flow cytometry for quantitation of (C) peritoneal eosinophils, (D) LPMs, and (E) the frequency of RELMα+ LPMs. Data are representative of at least 3 independent experiments (A) or are pooled from at least 3 independent experiments (B-E). Data are mean ± s.e.m. Significance calculated with an unpaired Student’s *t*-test.
Figure 3.3. Loss of Bhlhe40 dysregulates myeloid cell responses to *H. polygyrus* rechallenge. (A, B) Naïve and *H. polygyrus*-rechallenged *Bhlhe40<sup>+/+</sup>* and *Bhlhe40<sup>−/−</sup>* mice were analyzed by flow cytometry for (A) SILP eosinophils and (B) quantitation of SILP CD3<sup>+</sup> T cells. (C-E) Naïve and *H. polygyrus*-rechallenged *Bhlhe40<sup>+/+</sup>* and *Bhlhe40<sup>−/−</sup>* mice were analyzed by flow cytometry for (C) peritoneal eosinophils, (D) LPMs, and (E) RELMα-expressing LPMs. (F) Naïve and *H. polygyrus*-rechallenged *Bhlhe40<sup>+/+</sup>* and *Bhlhe40<sup>−/−</sup>* mice were analyzed for serum anti-*H. polygyrus* IgG1 titers. Data are representative of at least three independent experiments (A, C-E) or are pooled from at least 3 independent experiments (B) or 2 independent experiments (F). Data are mean ± s.e.m. Significance calculated with an unpaired Student’s *t*-test.
Figure 3.4. Bhlhe40 is required in T cells for a normal memory response to *H. polygyrus*.

(A) *H. polygyrus*-rechallenged *Cd4-Cre* Bhlhe40^{flo/flo} and *Cd4-Cre* Bhlhe40^{f/f} mice were analyzed for quantitation of *H. polygyrus* eggs/gram feces over time. (B) Naïve and *H. polygyrus*-rechallenged *Cd4-Cre* Bhlhe40^{flo/flo} and *Cd4-Cre* Bhlhe40^{f/f} mice were analyzed for quantitation of intestinal granulomas. (C-F) Naïve and *H. polygyrus*-rechallenged *Cd4-Cre* Bhlhe40^{flo/flo} and *Cd4-Cre* Bhlhe40^{f/f} mice were analyzed by flow cytometry for quantitation of (C) SILP myeloid cells, (D) peritoneal eosinophils, (E) LPMs, and (F) the frequency of
RELMα+ LPMs. (G) *H. polygyrus*-rechallenged *LysM-Cre− Bhlhe40fl/fl* and *LysM-Cre+ Bhlhe40fl/fl* mice were analyzed for quantitation of *H. polygyrus* eggs/gram feces over time. (H) Naïve and *H. polygyrus*-rechallenged *LysM-Cre− Bhlhe40fl/fl* and *LysM-Cre+ Bhlhe40fl/fl* mice were analyzed for quantitation of intestinal granulomas. Data are pooled from 3 independent experiments (A-F) or 2 independent experiments (G, H). Data are mean ± s.e.m. Significance calculated with an unpaired Student’s *t*-test.
Figure 3.5. Loss of Bhlhe40 dysregulates the CD4+ T cell transcriptional response to *H. polygyrus* rechallenge. (A, B) Gene expression microarray data were analyzed for the 100 most differentially expressed probe sets in SILP CD4+ T cells from (A) naïve and *H. polygyrus*-rechallenged *Bhlhe40*+/+ mice and (B) *H. polygyrus*-rechallenged *Bhlhe40*+/+ and *Bhlhe40*−/− mice. (C) 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. (D) 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. (E) Gene expression microarray data were analyzed for (left) expression of cytokines induced by *H. polygyrus* rechallenge and (right) expression of Th2 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. (F) Flow cytometry of *Bhlhe40*GFP transgene reporter expression in SILP CD4+ T cells from *Bhlhe40*GFP+ mice. Data in (F) are representative of 2 independent experiments. Microarray data are from a single experiment.
Figure 3.6. Bhlhe40 regulates distinct gene sets in CD4+ T cells from naïve and *H. polygyrus*-rechallenged mice. (A) Gene expression microarray data were analyzed for genes induced by ≥2-fold in SILP CD4+ T cells from *H. polygyrus*-rechallenged as compared to naïve *Bhlhe40*+/+ mice. (B) Gene expression microarray data were analyzed for shared and unique *Bhlhe40*-dependent genes (≥2-fold differentially expressed) in SILP CD4+ T cells from naïve or *H. polygyrus*-rechallenged *Bhlhe40*+/+ and *Bhlhe40*+/- mice, depicted as a Venn diagram. (C) Gene expression microarray data were analyzed for genes differentially expressed by ≥2-fold in SILP CD4+ T cells from naïve *Bhlhe40*+/+ and *Bhlhe40*+/- mice. Microarray data are from a single experiment.
Figure 3.7. Bhlhe40 is required for normal CD4+ T cell production of βc 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, 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 2 independent experiments (A, D) or are pooled from 2 independent experiments (B, C, E). Data are mean ± s.e.m. Significance calculated with an unpaired Student’s t-test.
Figure 3.8. Bhlhe40 is required in T cells for normal cytokine production during *H. polygyrus* rechallenge. (A and B) Naive and *H. polygyrus*-rechallenged *Cd4-Cre* Bhlhe40<sup>fl/fl</sup> and *Cd4-Cre*<sup>+</sup> Bhlhe40<sup>fl/fl</sup> mice were analyzed by flow cytometry for quantitation of the frequency of IL-4<sup>+</sup>, IL-5<sup>+</sup>, IL-13<sup>+</sup>, and GM-CSF<sup>+</sup> CD4<sup>+</sup> T cells from the (A) peritoneal cavity and (B) mesenteric lymph nodes after *in vitro* PMA/ionomycin stimulation. Data are pooled from 2 independent experiments. Data are mean ± s.e.m. Significance calculated with an unpaired Student’s *t*-test.
Figure 3.9. In vitro-polarized T\textsubscript{H}2 cells require Bhlhe40 for normal cytokine production.

Naïve CD4\textsuperscript{+} T cells from Bhlhe40\textsuperscript{+/+} and Bhlhe40\textsuperscript{-/-} mice were differentiated in culture into T\textsubscript{H}2 cells and restimulated to assess production of GM-CSF, IL-4, IL-5, and IL-13 by ELISA. Data are pooled from 3 independent experiments. Data are mean ± s.e.m. Significance calculated with an unpaired Student’s $t$-test.
Figure 3.10. Genetic deletion of IL-10 in Bhlhe40+/− Il10−/− mice does not restore control of H. polygyrus rechallenge. (A) Naïve and H. polygyrus-rechallenged Cd4-Cre+ Bhlhe40fl/fl and Cd4-Cre+ Bhlhe40+/+ mice were analyzed by flow cytometry for quantitation of the frequency of IL-10+ CD4+ T cells after in vitro PMA/ionomycin stimulation of SILP cells. (B) H. polygyrus-rechallenged Bhlhe40+/+, Bhlhe40−/−, and Bhlhe40−/− Il10−/− mice were analyzed for quantitation of H. polygyrus eggs/gram feces over time. (C) Naïve and H. polygyrus-rechallenged Bhlhe40+/+, Bhlhe40−/−, and Bhlhe40−/− Il10−/− mice were analyzed for quantitation of intestinal granulomas. Data are pooled from 2 independent experiments. Data are mean ± s.e.m. Significance calculated with an unpaired Student’s t-test.
Figure 3.11. Combined deficiency in IL-5 and GM-CSF results in impaired control of *H. polygyrus* rechallenge. (A) *H. polygyrus*-rechallenged *Csf2*<sup>+/+</sup> and *Csf2*<sup>−/−</sup> mice were analyzed for quantitation of *H. polygyrus* eggs/gram feces over time. (B) Naïve and *H. polygyrus*-rechallenged *Csf2*<sup>+/+</sup> and *Csf2*<sup>−/−</sup> mice were analyzed for quantitation of intestinal granulomas. (C) Naïve and *H. polygyrus*-rechallenged *Csf2*<sup>+/+</sup> and *Csf2*<sup>−/−</sup> mice were analyzed by flow cytometry for quantitation of SILP myeloid cells. (D-F) Naïve and *H. polygyrus*-rechallenged *Csf2*<sup>+/+</sup> and *Csf2*<sup>−/−</sup> mice were analyzed by flow cytometry for quantitation of (D) peritoneal eosinophils, (E) LPMs, and (F) the frequency of RELMα<sup>+</sup> LPMs. (G) 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. (H) Naïve (open circles) and *H. polygyrus*-rechallenged mice (filled triangles) treated as in (G) were analyzed for quantitation of intestinal granulomas. Data are from single experiments. Data are mean ± s.e.m. Significance calculated with an unpaired Student’s *t*-test.
Figure 3.12. The βCy chain is necessary for control of *H. polygyrus* rechallenge. (A) *H. polygyrus*-rechallenged Csf2rb<sup>++</sup> and Csf2rb<sup>+</sup>- mice were analyzed for quantitation of *H. polygyrus* eggs/gram feces over time. (B) Naïve and *H. polygyrus*-rechallenged Csf2rb<sup>++</sup> and Csf2rb<sup>+</sup>- mice were analyzed for quantitation of intestinal granulomas. (C-F) Naïve and *H. polygyrus*-rechallenged Csf2rb<sup>++</sup> and Csf2rb<sup>+</sup>- mice were analyzed by flow cytometry for quantitation of (C) SILP myeloid cells, (D) peritoneal eosinophils, (E) LPMs, and (F) the frequency of RELMα<sup>+</sup> LPMs. Data are pooled from 2 independent experiments. Data are mean ± s.e.m. Significance calculated with an unpaired Student’s *t*-test.
Chapter 4: Conclusions and Future Directions

4.1 Introduction

As a result of our work and that of other groups, a remarkable variety of roles for Bhlhe40 within the hematopoietic system are now emerging. Bhlhe40 regulates T helper cell cytokine production (Martínez-Llordella et al., 2013; Lin et al., 2014; Lin et al., 2016; Yu et al., 2018; Jarjour et al., 2019b), macrophage proliferation (Jarjour et al., 2019a), and type 2 immunity (Jarjour et al., 2019a; Jarjour et al., 2019b). Herein, I will expand on these and related points.

4.2 Macrophages

While the literature on Bhlhe40’s role in the proliferation of lymphocytes is somewhat unclear and affected by redundancy with Bhlhe41 (Martínez-Llordella et al., 2013; Yu et al., 2018; Kreslavsky et al., 2018), we have established a crucial tissue-specific role for Bhlhe40 in the proliferation of peritoneal resident and monocyte-derived macrophages. The finding that macrophages have tissue-specific controls on their proliferation, even in an inflammatory context, has significant repercussions. Our data suggests that other proliferative macrophages, such as tumor-associated or pro-fibrotic populations (Franklin et al., 2016; Minutti et al., 2017; Mantovani et al., 2017; Zhu et al., 2017; Loyher et al., 2018), likely possess similar transcriptional regulators, which could be therapeutically manipulated. Furthermore, Bhlhe40 itself may be a promising target as peritoneal macrophages likely contribute to the tumor-associated macrophage pool in certain cancers, as these populations are known to incorporate resident macrophages (Zhu et al., 2017; Loyher et al., 2018). It is of great importance to identify these other regulators of macrophage proliferation; however, the case of Bhlhe40 suggests that
this may not be simple. Bhlhe40 is not exclusively expressed in LPMs, nor is its expression dramatically enhanced during proliferation. The most promising avenue to address this may be by focusing on regulators of Maf transcription factors, based on the premise that tissue-specific factors share an ability to regulate Mafs.

As our understanding of the relationship between monocytes and macrophages has advanced, it has become less clear why both populations are necessary. The most likely explanation is that tissue-resident macrophages are relatively restricted in their ability to respond to insults and may be more pro-tolerogenic, while monocytes are able to differentiate into every flavor of macrophage imaginable. Our data strongly supports this idea by showing that monocytes retain additional transcriptional plasticity as compared to tissue-resident macrophages, which could not acquire Bhlhe40 expression during type 2 immunity. Furthermore, our findings suggest that Bhlhe40 itself could be a therapeutic target in pathological monocyte-derived macrophages. Intriguingly, peritoneal monocyte-derived macrophages had a more profound proliferative defect in the absence of Bhlhe40 than LPMs, possibly due to compensation by Bhlhe41 in LPMs. It remains an open question whether double deficiency in Bhlhe40 and Bhlhe41 would impair the proliferation of more macrophage subsets, especially AMs (which highly express Bhlhe41).

### 4.3 Helper T cells

Our results clearly demonstrate that Bhlhe40 plays a crucial role in controlling T\(_H\)2 cell cytokine production, with marked similarities to its role in other helper T cell subsets. Bhlhe40 and c-Maf (Gabrysova et al., 2018) are emerging as potential master regulators of helper T cell cytokine production. The existence of such factors suggests that different helper T cell subsets
may share further regulation, despite their divergent transcriptional programs enforced by master lineage-specifying transcription factors. These findings suggest that T cell differentiation is less a series of irrevocable decisions (i.e. a golf ball in a lake) and more a limitation of the possible outcomes (i.e. a golf ball in a sand trap). While TH1 and TH2 cells likely are never able to exchange identities, they are capable of sharing secondary characteristics (i.e. a TH1-GM and TH2-GM). Conceptually, a corollary of this idea would be primary (Tbet, Gata3, RORγt, FoxP3, Bcl6) and secondary (Bhlhe40, c-Maf) helper T cell transcription factors.

Naturally, as in all things biological, there is likely a continuum between primary and secondary transcription factors. In light of the marked changes in the helminth-induced transcriptional signature in SILP CD4+ T cells lacking Bhlhe40 and the recent study by Henriksson et al describing Bhlhe40 as a TH2 regulator, it is of great interest to address whether Bhlhe40 also has unique functions in TH2 cells that contribute to the transcriptome of this cell type. Furthermore, because of the transcriptional similarities between ILC and helper T cell subsets, it is likely that Bhlhe40 also plays an important role in ILCs. If so, it would be fascinating to address the relative importance of Bhlhe40-mediated cytokine production in ILCs versus helper T cells.

4.4 Type 2 immunity

Because of the roles we have described for Bhlhe40 in peritoneal macrophages and TH2 cells, Bhlhe40 appears to be an important regulator of type 2 immune responses. Cooperative roles for a single transcription factor in multiple cell types during type 2 immunity is reminiscent of the role of IRF4 in dendritic cells and TH2 cells (Williams et al., 2013; Gao et al., 2013). Intriguingly, Bhlhe40 was connected to IRF4 by Henriksson et al, though our transcriptional data
did not reveal any significant difference in \textit{Irf4} expression between Bhlhe40-sufficient and deficient LPMs or T\textsubscript{H}2 cells. Bhlhe40 is highly expressed in other myeloid cell lineages which are important in type 2 immunity, suggesting that Bhlhe40 may be of even greater importance to this type of immune response. Furthermore, as type 2 immune responses prominently feature feedback loops (Ouyang et al., 2000; von Moltke et al., 2016), many of the indirect effects of Bhlhe40 deficiency in T cells remain to be explored, such as the effects on the epithelium (including tuft cells) and ILCs.

Bhlhe40 is highly expressed in a significant fraction of dendritic cells in the lung and spleen (Lin et al., 2016; unpublished data), and so it is a distinct possibility that Bhlhe40 could regulate \textit{Irf4} expression to modulate dendritic cells during type 2 immunity, in light of the role for IRF4 described above. As previously mentioned, our original interest in Bhlhe40 was piqued by its expression in monocytes in response to GM-CSF. As GM-CSF induces the differentiation of monocyte-derived dendritic cells (Briseno et al., 2016), it is possible that Bhlhe40 is important in both classical and monocyte-derived dendritic cells, which are potentially important in different type 2 models.

Bhlhe40 is expressed at a remarkably high level in lung, peritoneal, bone marrow, and intestinal eosinophils (Lin et al., 2016; unpublished data), suggesting that it also plays a role in this cell type. As eosinophils have the potential to secrete a variety of cytokines, including some more classically considered to be lymphocyte-derived, it is possible that Bhlhe40 also regulates their cytokine production (Davoine and Lacy, 2014).
4.5 Common beta chain family cytokines

While IL-5 and GM-CSF have been studied for decades, these factors have largely been interrogated in parallel. IL-5 has been a major focus of research on type 2 immunity, including parasitic, atopic, and other diseases, while GM-CSF has emerged as a proinflammatory mediator, particularly in TH1 and TH17 cell-driven diseases, such as autoimmunity, bacterial infections, and cancer. And yet, among all the known cytokines regulating the immune system, these two are uniquely linked as the only cytokines entirely dependent on the βchain for transducing downstream signaling (Dougan et al., 2019). The studies on these two factors present a conundrum of how essentially identical signaling can create such disparate effects. Of course, part of this is the availability of the relevant alpha chains, which are necessary to bind these cytokines. But this idea inevitably leads to the idea of redundancy of GM-CSF and IL-5 if both cytokines and both alpha chains are available.

We have found that either GM-CSF or IL-5 is sufficient to maintain protective immunity to *H. polygyrus*, but deficiency in both causes severely impaired immunity. This helps to address the long-standing confusion over the varying importance of IL-5 in anti-helminth immunity by revealing compensatory mechanisms (Maizels and Balic, 2004; Allen and Sutherland, 2014). It is now imperative to address whether GM-CSF and IL-5 broadly cooperate in other type 2 diseases, especially the various sorts of atopy. For example, trials of αIL-5 therapy in eosinophilic eosophagitis led to inconsistent clinical outcomes, and eosinophil levels were only partially suppressed by therapy (Roufosse et al., 2018). Would combined blockade of GM-CSF (using blocking antibodies already in clinical trials) and IL-5 yield an improved therapeutic modality?

While our work has clearly demonstrated that TH2 responses prominently feature GM-CSF production, thereby rendering this cytokine relevant during type 2 immunity, the question
remains of whether IL-5 is also important in a more proinflammatory context. GM-CSF is currently being therapeutically targeted in rheumatoid arthritis and multiple sclerosis (Dougan et al., 2019). Is it possible that combined blockade of IL-5 would eliminate a compensatory mechanism? There are two potential mechanisms by which IL-5 could compensate for GM-CSF in inflammatory diseases. One, IL-5 could play an undescribed role in these diseases (likely reparative or profibrotic), or two, IL-5 could be induced in the absence of GM-CSF signaling, resulting in compensation. While there is little-to-no literature on IL-5 during rheumatoid arthritis, it was previously shown that IL-5-deficient mice develop the murine model of multiple sclerosis normally (experimental autoimmune encephalomyelitis), suggesting that IL-5 is not normally involved in this disease, but not precluding a secondary, compensatory role vis a vis GM-CSF (Weir et al., 2003).

4.6 A unifying role for Bhlhe40

As of yet, the functions of Bhlhe40 appear to be quite distinct between different cell types. Bhlhe40 is induced by many different stimuli to play different roles in different cell types. One strong possibility is that this simply reflects the typical biology of transcription factors. As there are relatively few, they partner with each other in different ways in different cells to mediate different functions. However, it remains possible that there may be a unifying logic or mechanism behind these seemingly disparate functions. One noteworthy contender is the circadian clock. Bhlhe40 has previously been described to regulate circadian rhythms (Honma et al., 2001). Is it possible that Bhlhe40 couples cytokine production and proliferation to the time of day? In light of the circadian patterns of neutrophils (Adrover et al., 2019), Bhlhe40 might also
be relevant to the granulocyte clock, as it is expressed in multiple granulocyte subsets (Lin et al., 2016).

### 4.7 Concluding remarks

At the start of my thesis work, I proposed to unravel the role of Bhlhe40 in the biology of tissue-resident macrophages in both cellular and molecular detail. We have accomplished this by describing a tissue-specific role for Bhlhe40 in regulating the proliferation of peritoneal macrophages via repression of c-Maf and MafB, revealing a new paradigm of tissue-specific control of resident macrophage proliferation that is active in health and disease. This work was recently published in *Nature Immunology* (DOI 10.1038/s41590-019-0382-5). While we initially anticipated an important role for Bhlhe40 in alveolar macrophages, which highly express this factor, we found remarkably few differences in gene expression or changes in function in homeostasis. We suspect that this is in part due to high expression of Bhlhe41, leading to compensation, and the previously described lack of MafB and c-Maf in these cells (Soucie et al., 2016). It would be of great interest to assess whether Bhlhe40/41-deficient mice have significant changes in the behavior of alveolar macrophages.

In the course of these studies, I adopted a number of models of type 2 immunity, including the murine helminth *Heligmosomoides polygyrus*. We invested much effort in connecting peritoneal macrophages to the control of this infection, but were unable to do so. However, we did reveal a marked requirement for Bhlhe40 in T cells to control rechallenge infections with this pathogen. We found that Bhlhe40 was critical to the normal transcriptional response of small intestine lamina propria CD4$^+$ T cells to helminth infection, including expression of *Areg, Csf2, Il5, Il13, Nlrp3*, and *Pparg*. In light of the defects in production of the
common beta chain family cytokines GM-CSF and IL-5 in the absence of Bhlhe40, we assessed whether these cytokines were required for control of infection. Our work and previously published data on IL-5 neutralization in this model (Urban et al., 1991) demonstrate that single deficiency in either factor has little-to-no effect on protection against *H. polygyrus*, but loss of the common beta chain or double neutralization of both factors results in a profound defect. Therefore, our data may explain the inconsistent requirements for IL-5 in various helminth infections and suggest that GM-CSF and IL-5 may frequently cooperate in type 2 immunity and other diseases, suggesting the therapeutic potential of double blockade of these factors. This work has been published as a preprint on bioRxiv (DOI 10.1101/687541) and has been submitted for traditional publication.
References


