CD4+ T cell differentiation and help to B cells

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Dedication

I dedicate this dissertation to my husband and newborn baby, Dmitri and Mishka Kotov, and my Mom, Shalin Chou. Thank you for supporting my scientific career.
Abstract

Vaccines save 2.5 million lives per year. Vaccine efficacy is largely dependent on the successful generation of antibodies by B cells for the elimination of pathogens like the influenza virus. T follicular helper (Tfh) cells are a type of CD4+ T cell that provides critical help to B cells for this process. My thesis research involves studying factors that promote Tfh cell formation and therefore B cell responses. This research is significant because it will provide better understanding of how Tfh cells are generated, which can be implemented during vaccine design. Using an approach to track both polyclonal antigen-specific CD4+ T and B cells within the same mouse after Complete Freund’s Adjuvant immunization, we found that the expression of BCOR protein in CD4+ T cells was critical for optimal Tfh formation. Reduced Tfh development as a result of BCOR absence also led to reduced germinal center B cell and antibody-secreting plasma cell formation. Thus, BCOR plays a critical role in promoting Tfh differentiation and B cell responses.

The role of BCOR in CD4+ T cell differentiation was also examined after infection of mice with the extracellular pathogen Streptococcus pyogenes (Group A Streptococcus). In this context, BCOR enhanced the development of another CD4+ T cell type called T helper 17 (Th17) cells. Th17 cells promote protection by secreting cytokines, like IL-17A, that drive trafficking of neutrophils to the site of infection to kill bacteria. Identifying the drivers of Th17 cells will inform the development of a Th17-focused Streptococcus pyogenes vaccine for humans, which is currently unavailable.
Because the efficacy of most vaccines is based on the process by which CD4\(^+\) T cells stimulate antibody responses, we wanted to better understand the role of different CD4\(^+\) T cell types on the B cell response. Tfh cells, in addition to Th1 and Th17 cells, were found to contribute to the production of early antibody-secreting B cells. This work provides insight into how non-Tfh cells also contribute to the B cell response, which is understudied in the field of B cell biology. Better understanding the process of CD4\(^+\) T cell help for generating antibody-secreting B cells is critical for producing efficacious vaccines.
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Chapter 1

Background and Introduction

1.1 CD4+ T and B cell development

T lymphocytes are a population of adaptive immune cells that play a critical role in immune protection. T lineage progenitor cells derive initially from the bone marrow and seed the thymus (1) where they undergo Variable-Diversity-Joining (VDJ) gene rearrangement of their T cell receptor (TCR). Thymocytes that express a functional TCRβ chain receive signals through the pre-TCR and go on to rearrange their TCRα loci (2). CD4+ CD8+ T cells scan cortical thymic epithelial cells presenting self-peptides loaded onto MHCI or MHCII. Cells with TCRs that bind at low affinity to self-peptide on MHCII will receive sufficient TCR signaling for their survival and development into CD4 single positive thymocytes (3, 4). This process is termed positive selection. The positively selected T cells then migrate to the thymic medulla for negative selection during which T cells scan medullary thymic epithelial cells, B cells, and dendritic cells presenting tissue-restricted self-peptides on MHCII. The binding strength of the TCR with self-peptides on MHCII plays an important role in the fate of the developing T cell. Strong binding, and therefore strong TCR signaling, leads to apoptosis or development into FOXP3-expressing thymic regulatory T (Treg) cells (5). Weak binding allows the T cells to obtain sufficient TCR signaling for survival and maturation into naïve CD4+ T cells that migrate to secondary lymphoid organs. The resulting population of naïve CD4+
T cells in the periphery contain specificity for various pathogen-derived peptides on MHCII.

Like T cells, B cells are adaptive immune cells that express clonally diverse surface antigen receptors. B cell development occurs in the bone marrow and derives from common lymphoid progenitors that receive B cell inducing signals such as that from the IL-7 cytokine (6). B cell progenitors then undergo a series of transformations during development in the following order: pre-pro-B, early pro-B, late pro-B, large pre-B, small pre-B, immature B, and finally mature B cells. These stages occur in the bone marrow except the transition from immature to mature B cells, which occurs in the spleen. Each B cell has a unique B cell receptor (BCR), which is a surface immunoglobulin (Ig) that recognizes a specific antigenic epitope. Igs contain two heavy and two light chains (7). Each heavy chain contains a VDJ segment and a constant (C) segment while each light chain contains VJ and C segments. During B cell development, the heavy chain is rearranged and the recombined heavy chain pairs with an invariant, surrogate light chain to form a pre-BCR (7). Cells that do not undergo pre-BCR signaling will die by apoptosis. Signaling through the pre-BCR indicates successful recombination and allows for progression to light chain rearrangement. These pre-B cells rearrange the light chain κ locus and then the λ locus, which pair with the μ heavy chain to generate a surface IgM antibody. B cells with a BCR composed of this IgM antibody are immature B cells. The BCR is then tested for reactivity to self-antigens in the bone marrow. B cells with BCRs that bind strongly to self-reactive epitopes undergo: receptor editing, clonal deletion, anergy, or ignorance. This process of negative selection is an important step for immune tolerance. Immature B cells do, however, need to bind to self-antigen with moderate
enough affinity to avoid death by apoptosis (8), which is the process of positive selection. The steps of recombination and selection during B cell development generate a vast repertoire of peripheral naïve B cells that are capable of recognizing a wide variety of foreign antigens.

### 1.2 Primary CD4⁺ T cell response

Naïve CD4⁺ T cells become activated in secondary lymphoid organs such as the spleen and lymph nodes upon recognition of their cognate antigen. Foreign antigen at an infection site drains into the regional lymph node or is brought to the lymph node by migrating dendritic cells (DCs) (9). DCs process antigen into peptides, load the peptides onto MHCII (p:MHCII), and present p:MHCII to naïve CD4⁺ T cells. CD4⁺ T cell recognition of p:MHCII via the TCR is the first signal required for CD4⁺ T cell activation. The second signal is offered by DCs in the form of costimulatory ligands (CD80 and CD86) that bind to the surface receptor CD28 on T cells (10). Finally, the third required signal is cytokines, which play a strong role in the fate decision of naïve CD4⁺ T cells. There are multiple options for activated CD4⁺ T cells to differentiate into including T helper 1 (Th1), T helper 17 (Th17), peripheral Treg, and T follicular helper (Tfh) cells. The cytokine IL-12 drives T helper 1 (Th1) differentiation (11), while TGF-β drives Treg (12), TGF-β and IL-6 drive Th17 (13, 14) and IL-6 drives Tfh cell differentiation (15).

The DC-derived priming signals induce a master transcription factor, which is a DNA-binding protein that is required for the differentiation of a given CD4⁺ T cell subset. TBET is required for Th1 cells (16), while RORγt, FOXP3, and BCL6 are
required for Th17 (17), Treg (18), and Tfh cells (19), respectively. Master transcription factors induce important functions of each subset while suppressing other subset fates. For example, RORγt binds to the promoters of Th17-specific genes such as Il17a and Ccr6 and stimulates their expression (20, 21). RORγt also inhibits expression of TBET and FOXP3 (20, 22), thereby preventing Th1 and Treg differentiation, respectively. Similarly, the transcription factor BCL6 promotes the Tfh fate by repressing TBET and RORγt to suppress the Th1 and Th17 fates, respectively (23). When transcription factors repress genes, they can recruit corepressors to assist in the task. BCL6 recruits corepressors such as BCL6-interacting corepressor (BCOR), nuclear receptor corepressor (NCOR), or nuclear receptor corepressor 2 (SMRT) that enhance the repressive function of BCL6 (24).

Each differentiated CD4+ T cell subset offers specialized functions during immune responses. Th1 cells express the chemokine receptor CXCR3, which induces Th1 cell migration to inflammatory sites (25) where Th1 cells offer help in the form of IFN-γ to macrophages. IFN-γ receptor signaling in macrophages is important for clearance of intracellular bacteria because it enhances the fusion of pathogen-containing phagosomes with lysosomes (26). In contrast, Th17 cells express the chemokine receptor CCR6, which induces migration toward mucosal sites containing extracellular bacteria (27, 28). Th17 cells promote protection by secreting cytokines, like IL-17A, that drive trafficking of neutrophils to the mucosal site to kill the bacteria (29, 30). Treg cells are unique in that they dampen immune responses, which is critical for immune tolerance. Treg cells express CTLA-4 and secrete IL-10, which inhibit DC activation of CD4+ T cells (31, 32). Tfh cells are considered specialized B cell helpers. Tfh cells express the
chemokine receptor CXCR5, allowing migration toward the T cell-B cell (T-B) border where Tfh cells interact with activated B cells who present cognate antigen (33). During these interactions, Tfh cells offer help in the form of IL-4 cytokine and CD40L (34). Tfh cells can also migrate from the T-B border to germinal centers (GCs) and are therefore termed GC-Tfh cells.

1.3 Primary B cell response

Within 24 hours of immunization, antigen arrives at lymphoid organs either through the blood or lymphatics, or is brought there by migrating dendritic cells (9). B cells containing a BCR specific for the antigen can capture antigen from DCs in the T cell zone during LN entry (35), directly from antigen that has diffused into the follicle (36), or from follicular DCs (37). Upon antigen binding, B cells become activated and upregulate CCR7 leading to B cell migration toward the CCL19 and CCL21 ligands that are rich in the T cell zone (38, 39). At the T-B border, B cells present p:MHCII complexes and interact with antigen-specific CD4\(^+\) T cells that were previously activated by conventional DCs in the T cell zone. These antigen-specific interactions lead to B cell proliferation and differentiation into three potential fates: 1) early short-lived plasmablasts (PBs) that migrate to the outer follicle where they produce antibodies, 2) GC B cells, and 3) early memory B cells (40). Each of these B cell subsets play an important role. Short-lived PBs only survive a matter of days, however, they arise early on in the immune response (three-four days) and secrete low affinity antibodies that contribute to neutralization of the pathogen.
About five-seven days after immunization, early GCs form in the follicle center where B cells proliferate and initiate somatic hypermutation (SHM) (41-43). SHM is a process by which B cells edit their BCR. Over the next two weeks, the GC matures and forms into a light zone (LZ) containing FDCs and Tfh cells, and a dark zone (DZ) containing rapidly proliferating B cells (40). GC B cells undergo cyclic migration between the two zones as they increase the antigen affinity of their BCR, a process referred to as affinity maturation. GC B cells that no longer contain a functional BCR that can bind to antigen presented by FDCs, and therefore cannot receive the necessary signals from Tfh cells for survival, die and are engulfed by tingible body macrophages (44). Additionally, B cells that have undergone SHM resulting in damaged Ig genes in the DZ undergo apoptosis (45). After 21 days of the immune response, GCs start to dissipate and can reignite upon antigen re-encounter.

Throughout the lifetime of the GC, long-lived plasma cells form and upregulate the chemokine receptor CXCR4, which allows their migration to the bone marrow. These long-lived plasma cells are retained in the bone marrow where they survive long-term and continue to secrete high-affinity antibodies against the antigen (46). The GC also gives rise to memory B cells that contain a higher affinity BCR as compared to their naïve precursors and are therefore a useful asset during antigen re-encounter. Thus, the primary B cell response is well-poised to neutralize pathogens at the time of invasion as well as fight off the pathogen during future encounters.

Overall, B cells play a protective role in the immune response and rely heavily on help from CD4$^+$ T cells. The ultimate product of activated B cells are plasma cells that produce antibody, which is the basis of vaccine efficacy.
1.4 CD4+ T cell help to B cells

Cytokine secretion is one source of CD4+ T cell help to B cells. BCR signaling in combination with IL-4, for example, promotes B cell proliferation (34). IL-4 is also known to drive IgE and IgG1 isotype switching (47, 48). IgE antibodies are critical for mediating protection against worms, while IgG1 antibodies provide protection against a variety of infections such as those caused by viruses like the influenza virus. IL-21 is also a B cell help factor that enhances B cell proliferation and stimulates plasma cell differentiation (49-52). The importance of IL-4 and IL-21 was demonstrated by severe defects in class switch recombination, germinal centers, and antibody production present in Il4−/−Il21r−/− mice (53).

Alongside cytokines, co-stimulatory molecules are critical for maximal B cell responses. During CD4+ T cell and B cell interactions, T cell expression of CD40L leads to CD40 signaling in B cells, which is another important factor for B cell proliferation (54). A study stimulating human GC B cells with CD40-specific antibody showed that CD40 signaling prevents apoptosis (55). Similarly, administration of CD40L blocking antibody in mice prevents GC formation (56). CD4+ T cell presence in GCs and CD40L expression by activated CD4+ T cells (57) suggests that CD4+ T cells provide the CD40 signaling required for GC maintenance. The importance of direct interactions between CD4+ T cells and B cells in the primary B cell response was tested by deleting Icos and Sh2d1a, genes important for T-B cell interactions. Deficiency in these genes was associated with defects in GCs and memory B cell formation, demonstrating the critical role of T-B cell interactions in B cell responses (58-61).
The CD4⁺ T cell subset that is primarily associated with providing B cell help are Tfh cells due to their ability to secrete B cell help factors including IL-4 and IL-21 (62-64), and their requirement for sustaining the GC response (19, 23, 65). While there is substantial evidence indicating that Tfh cells play a significant role in the B cell response (33), limited research has been done on the contribution of other CD4⁺ T cell subsets. Characteristics of other CD4⁺ T cell subsets indicate that they may also play a role in promoting B cell responses. For example, CD40L is not only expressed by Tfh cells but also other subsets including Th1 and Th17 cells (57). Furthermore, IFN-γ and IL-17, which are produced by Th1 and Th17 cells, respectively, are known drivers of IgG2a/c switching (66, 67), and IgG2a/c antibodies mediate enhanced protection against infections caused by the influenza and ebola viruses (68, 69). Therefore, it is possible that Th1 and Th17 cells may also promote protective B cell responses.

1.5 Statement of Thesis

My thesis is that BCOR plays a key role in Tfh and Th17 formation following *Listeria monocytogenes* or *Streptococcus pyogenes* infection, respectively, via repression of genes that inhibit the fate decision of each lineage; and that many Th subsets help B cells form early isotype switched plasmablasts in a manner that relies on CD40L expression by Th cells and cognate interactions.
Chapter 2

Bcl6-Interacting Corepressor Contributes to Germinal Center T Follicular Helper Cell Formation and B Cell Helper Function

2.1 Introduction

Germinal center (GC) T follicular helper (GC-Tfh) cells help B cells become long-lived plasma cells and memory B cells (33). The transcriptional repressor BCL6 promotes GC-Tfh formation by repressing the expression of transcription factors such as T-bet, GATA3, and RORγt required for the differentiation other effector cell lineages (23, 65). Repression by BCL6 depends on two domains, a middle repression domain 2 (RD2) domain and an N-terminal BTB domain (70, 71), which interact with corepressors. The RD2 domain can recruit the Metastasis-associated 3 (MTA3) corepressor (72), while the BTB domain can bind BCL6-interacting corepressor (BCOR), nuclear receptor corepressor (NCOR), or nuclear receptor corepressor 2 (SMRT) (24). BCOR potentiates transcriptional repression by BCL6 as part of a variant Polycomb complex, which may make epigenetic modifications that silence target genes (73).

The role, however, that these corepressors play in transcriptional repression by BCL6 in T cells is unclear. Mutation of the BCL6 RD2 domain leads to partial reduction in GC-Tfh differentiation (74). In contrast, it has been reported that GC-Tfh cell formation following sheep red blood cell immunization is normal in mice with a mutated BCL6 BTB domain (75), suggesting that none of the BTB-interacting corepressors are
involved in GC-Tfh differentiation. It remained possible, however, that a defect was not
detected in this experiment because relevant peptide:MHCII (p:MHCII)-specific T cells
were not monitored. Indeed, in the accompanying study (76), Crotty and colleagues
found that the BCL6 BTB domain contributes to GC-Tfh formation by viral p:MHCII-
specific CD4+ T cells during acute infection. Here, we evaluated BCOR for its role in
GC-Tfh formation. We found that BCOR deficiency in T cells led to a defect in
p:MHCII-specific GC-Tfh cell formation that correlated with reduced formation of
plasma cells and GC B cells. Therefore, BCOR was required for optimal GC-Tfh
formation by p:MHCII-specific CD4+ T cells, perhaps through its capacity to interact
with the BCL6 BTB domain.
2.2 Materials and Methods

Mice

The conditional Bcor allele (Bcor\textsuperscript{fl}), which contains LoxP sites flanking Bcor exons 9 and 10, was generated by homologous recombination (Wamstad et al, manuscript in preparation). Cre-mediated deletion results in a premature stop codon and a null allele.

Bcor\textsuperscript{fl/+} mice were backcrossed with C57BL/6NCr mice (NCI Frederick) for >6 (Fig. 1) or >10 generations (Fig. 3-4). B6.Cg-Tg(Lck-cre)3779Nik/J (The Jackson Laboratory) males were bred to Bcor\textsuperscript{fl/+} females to generate wild-type (WT; Bcor\textsuperscript{+/Y} or Bcor\textsuperscript{fl/Y} Lck-Cre\textsuperscript{-}) and T cell BCOR-deficient (Bcor\textsuperscript{fl/Y} Lck-Cre\textsuperscript{+}) males. C57BL/6 (B6 mice) (The Jackson Laboratory) used in Fig. 2 were housed in specific pathogen-free conditions while other mice were housed in a conventional facility at the University of Minnesota. All experimental protocols were performed in accordance with guidelines of the University of Minnesota Institutional Animal Care and Use Committee and National Institutes of Health.

Infections and Immunizations

Mice were infected intravenously with \textit{10}^{7} actA-deficient \textit{Listeria monocytogenes} (Lm) bacteria expressing FliC peptide RFNSAITNLGN (77) or 2W peptide EAWGALANWAVDSA fused to chicken ovalbumin (78), or immunized by i.p. injection of 200 µl of 0.6 ug of 2W peptide conjugated to 25 µg of PE (2W-PE)
emulsified in CFA (Sigma). The conjugate was formed by mixing biotinylated 2W peptide (Genscript) with streptavidin (SA)-PE (Prozyme) in a 4:1 ratio.

Cell Enrichment and Flow cytometry

Spleen and lymph node (LN) cells were divided for separate enrichments of CD4+ T cells and B cells. For p:MHCII-specific CD4+ T cell enrichment, cells were stained with CXCR5 (2G8; BD) and I-A\textsuperscript{b} tetramers containing listeriolysin O peptide NEKYAQAYPNVS (LLOp) or 2W peptide (79). Before PE-specific B cell enrichment, suspensions were incubated with dispase (Invitrogen), collagenase P (Roche), and DNase I (Roche) to release all B cell subsets. Enrichment was performed as previously described by mixing cell suspensions with 1 µg PE (Prozyme) (80). PE- or p:MHCII-bound cells were enriched using magnetic beads (Miltenyi) as previously described (79). Tetramer-enriched CD4+ T cells were stained with fluorochrome-labeled antibodies specific for B220 (RA3-6B2; all antibodies are from eBioscience unless otherwise indicated), CD11b (M1/70), CD11c (N418), CD44 (IM7), PD-1 (J43), CD90.2 (53-2.1), or CD4 (GK1.5; BD). For intracellular staining, cells were incubated in fixation/permeabilization buffer (eBioscience) and stained with fluorochrome-labeled anti-BCL6 (K112-91; BD) and anti-T-bet (4B10; Biolegend) antibodies. PE-enriched B cells were stained with fluorochrome-labeled antibodies against CD90.2 (53-2.1), CD11c (N418), F4/80 (BM8), and GR1 (RB6-8C5), CD38 (90), IgM (II/41), GL7 (GL-7), IgD (11-26c.2a; BD), and B220 (RA3-6B2; BD). Cells were fixed with 2% paraformaldehyde (Sigma) and stained
with anti-IgG [H+L] (Life Technologies). Cells were analyzed on a Fortessa (Becton Dickinson) flow cytometer and analyzed with FlowJo (TreeStar).

Statistical analysis

Statistical tests were performed using Prism (Graphpad) software and p values were obtained using two-tailed unpaired t tests with a 95% confidence interval.

2.3 Results and Discussion

BCOR deficiency in T cells causes a defect in GC-Tfh differentiation

Given that BCOR, NCOR, and SMRT potentiate BCL6 repression (81-83), it was possible that one or several of these corepressors promotes Tfh formation. We assessed the role of BCOR using Bcor0/0 Lck-Cre+ mice lacking BCOR specifically in CD4+ and CD8+ T cells. WT and Bcor0/0 Lck-Cre+ mice were infected intravenously with an attenuated strain of Lm that expresses listeriolysin O. The recipient mice contained about 80 LLOp:I-Ab-specific CD44low naive CD4+ T cells before infection (78, 84). Lm infection of WT mice generated a large population of CD44high LLOp:I-Ab-specific CD4+ effector cells by day 7 (Fig. 1A-B). As shown in previous studies (78, 84-86), this population contained CXCR5− PD-1− Th1 cells, CXCR5low PD-1− Tfh cells, and CXCR5high PD-1+ GC-Tfh cells (Fig. 1C-E). BCOR-deficient LLOp:I-Ab-specific CD4+ T cells produced an even larger effector cell population than that generated by WT cells
(Fig. 1B). The BCOR-deficient population had a higher fraction of Th1 cells, and a greater number of Th1 and Tfh cells (Fig. 1D-E) but a much lower fraction and number of GC-Tfh cells than the comparable WT population. Thus, BCOR expression in T cells is critical for GC-Tfh differentiation during Lm infection.

A caveat to these experiments was that BCOR-deficiency in CD8+ T cells, which play an important role in control of Lm bacteria (87), could have altered the infection in a way that indirectly affected GC-Tfh formation. We therefore examined the effects of BCOR deficiency on GC-Tfh differentiation during an immune response to a non-replicating Ag. WT and Bcorfl/Y Lck-Cre+ mice were immunized i.p. with a CFA emulsion containing PE or a 2W-PE conjugate. In the latter situation, PE-specific B cells that internalize 2W-PE through the BCR produce PE peptide:I-A\(^b\) and 2W:I-A\(^b\) complexes and could receive helper signals from either 2W:I-A\(^b\)- or PE peptide:I-A\(^b\)-specific T cells. PE-specific B cells underwent significantly more clonal expansion 7 days after immunization with 2W-PE/CFA than with PE/CFA (Fig. 2A-B). Thus, 2W:I-A\(^b\)-specific T cells were the main helpers of PE-specific B cells during the first week of the response when 2W-PE was the immunogen.

The effects of T cell-targeted BCOR deficiency on 2W:I-A\(^b\)-specific CD4+ T cells were then examined. Unlike after Lm infection, the total numbers of 2W:I-A\(^b\)-specific effector cells generated by 2W-PE immunization on day 7 were similar in WT and Bcorfl/Y Lck-Cre+ mice indicating that BCOR is not a regulator of T cell clonal expansion driven by a non-replicating Ag (Fig. 3A). BCOR-deficient 2W:I-A\(^b\)-specific effector cells, however, exhibited increased differentiation of CXCR5\(^{-}\) non-Tfh cells and decreased differentiation
of GC-Tfh cells relative to WT cells (Fig. 3B, D). Cells were also stained for intracellular T cell lineage-defining transcription factors to confirm a GC-Tfh defect. As shown in Fig. 3C, about 1% of the 2W:I-A<sup>b</sup>-specific WT effector cells were T-bet<sup>+</sup> BCL6<sup>-</sup> Th1 cells, while 30% were T-bet<sup>-</sup> BCL6<sup>+</sup> GC-Tfh cells indicating that i.p. peptide priming in CFA is a poor Th1 and good Tfh stimulus on day 7 (Fig. 3B-E). The BCOR-deficient 2W:I-A<sup>b</sup>-specific effector cell population contained about 3-fold more T-bet<sup>+</sup> BCL6<sup>-</sup> Th1 cells and 2-fold less T-bet<sup>-</sup> BCL6<sup>+</sup> GC-Tfh cells than the comparable population in WT mice. Therefore, BCOR was required for maximal GC-Tfh differentiation in response to a peptide Ag. The fact that BCOR was not essential indicates that some aspect of CFA priming, perhaps prolonged Ag presentation (88, 89), promotes BCOR-independent GC-Tfh formation.

*A reduction in GC-Tfh cells due to BCOR deficiency correlates with defects in B cell activation.*

GC-Tfh cells help GC B cells become long-lived plasma cells and memory cells (33). We therefore assessed B cell activation in B<sub>cor<sup>fl/Y</sup>Lck-Cre<sup>+</sup></sub> mice to see if the defect in GC-Tfh formation was associated with defects in B cell activation. As shown in Fig. 4A and B, PE-specific B cells in WT mice increased from about 50,000 cells on the day of immunization to about 400,000 cells on day 7 after injection of 2W-PE/CFA. The PE-specific B cell population consisted of plasma cells expressing large amounts of intracellular Ig heavy and light chains (IgG [H+L]) (Fig. 4A), B220<sup>high</sup> CD38<sup>-</sup> GL7<sup>-</sup> naïve or memory B cells, and CD38<sup>-</sup> GL7<sup>+</sup> GC B cells (Fig. 4A). PE-specific B cells in
*Bcor*<sup>Δ/Δ</sup>Lck-Cre<sup>+</sup> mice increased much less than WT cells after immunization and produced significantly fewer plasma cells and GC cells (Fig. 4B).

Our data show that BCOR deficiency hinders the differentiation of p:MHCII-specific GC-Tfh cells, which in turn reduces the formation of plasma cells and GC B cells. The recent report by Nance et al. (76) showing that the BCL6 BTB domain contributes to optimal GC-Tfh formation raises the likely possibility that the effects of BCOR on GC-Tfh differentiation depend on its interaction with the BCL6 BTB domain. It should be noted, however, that Huang et al. (75) found that GC-Tfh differentiation was induced normally by sheep red blood cells in mice with a mutated BCL6 BTB domain incapable of binding BCOR. It is therefore also possible that BCOR fosters GC-Tfh cell formation by a mechanism that depends on BCOR but not the BCL6 BTB domain. Such a mechanism could involve BCOR interaction with other known (90-94) or yet to be identified BCOR-binding transcription factors. Additional studies will be required to identify the molecular interactions that govern BCOR-dependent control of GC-Tfh formation during different immune responses.
FIGURE 1. A substantial defect in GC-Tfh differentiation occurs after Lm infection in BCOR-deficient CD4+ T cells. WT and Bcor^fl/^Lck-Cre^+ mice were infected with Lm bacteria. After 7 days, LLOp:I-A^b-specific CD4^+ T cells were enriched from spleen and LNs using LLOp:I-A^b tetramer. (A) B220^+ CD11b^+ CD11c^+ CD4^+ T cells from LLOp:I-A^b tetramer-enriched samples with gates on CD44^+ LLOp:I-A^b tetramer^+ cells. (B) Numbers of LLOp:I-A^b-specific cells in WT and Bcor^fl/^Lck-Cre^+ mice. (C) Identification of LLOp:I-A^b-specific (from gate in (A)) Th1, Tfh, and GC-Tfh cells based on PD-1 and CXCR5 expression. (D) Percentages and (E) numbers of LLOp:I-A^b-specific Th1, Tfh, or GC-Tfh cells in WT and Bcor^fl/^Lck-Cre^+ mice. Pooled data from two independent experiments are shown. ** p < 0.01, *** p < 0.001.
**FIGURE 2.** 2W:I-A^b^-specific CD4^+^ T cells provide help for PE-specific B cells after 2W-PE/CFA immunization. PE-specific B cells were enriched from spleen and LNs of naive B6 mice or mice that were immunized for 7 days with 2W mixed with PE (unlinked) or 2W-PE emulsified in CFA. (A) CD90.2^- CD11c^- F4/80^- Gr-1^- PE-specific cells (PE B) (top panels) gated for B220^{int} IgG [H+L]^+ plasma cells (middle panels) and B220^{int} IgG [H+L]^+ IgM^- plasma cells (bottom panels). (B) Numbers of PE-specific B cells (left panel) and IgM^- plasma cells (right panel) in naive, PE/CFA immunized, or
2W-PE/CFA immunized mice. Pooled data from two independent experiments are shown. ** $p < 0.01$, *** $p < 0.001$. 
FIGURE 3. A partial defect in GC-Tfh differentiation occurs in BCOR-deficient CD4+ T cells after CFA immunization. 2W:I-A\textsuperscript{b} T cells were enriched from spleen and LNs of WT or Bcor\textsuperscript{fl/Y} Lck-Cre\textsuperscript{+} mice using 2W:I-A\textsuperscript{b} tetramer 7 days after immunization with 2W-PE emulsified in CFA. (A) Numbers of 2W:I-A\textsuperscript{b} CD4+ T cells in naive and immunized WT or Bcor\textsuperscript{fl/Y} Lck-Cre\textsuperscript{+} mice. (B) Identification of 2W:I-A\textsuperscript{b}-specific Th1, Tfh, and GC-Tfh cells based on PD-1 and CXCR5 expression. (C) T-bet and BCL6 expression by 2W:I-A\textsuperscript{b}-specific CD4+ T cells. (D) Percentages of 2W:I-A\textsuperscript{b}-specific non-Tfh, Tfh, or GC-Tfh cells in WT and Bcor\textsuperscript{fl/Y} Lck-Cre\textsuperscript{+} mice. (E) Percentages of 2W:I-A\textsuperscript{b}-specific T-bet\textsuperscript{+} or BCL6\textsuperscript{+} cells in WT and Bcor\textsuperscript{fl/Y} Lck-Cre\textsuperscript{+} mice. Pooled data from two independent experiments are shown. ** p < 0.01, *** p < 0.001.
number of Th1 and Tfh cells (Fig. 1D, 1E) but a much lower fraction and number of GC-Tfh cells than did the comparable WT population. Thus, BCOR expression in T cells is critical for GC-Tfh cell differentiation during \textit{L. monocytogenes} infection.

A caveat to these experiments is that BCOR deficiency in CD8$^+$ T cells, which play an important role in the control of \textit{L. monocytogenes} bacteria (22), could have altered the infection in a way that indirectly affected GC-Tfh cell formation. Therefore, we examined the effects of BCOR deficiency on GC-Tfh cell differentiation during an immune response to a nonreplicating Ag. WT and \textit{Bcor$^{fl/Y}$} \textit{Lck-Cre$^+$} mice were immunized i.p. with a CFA emulsion containing 2W peptide mixed with PE (2W + PE) or a 2W-PE conjugate. In the latter situation, PE-specific B cells that internalize 2W-PE through the BCR produce PE peptide:I-A$^b$ and 2W:I-A$^b$ complexes and could receive helper signals from either 2W:I-A$^b$ – or PE peptide:I-A$^b$–specific T cells. PE-specific B cells underwent significantly more clonal expansion 7 d after immunization with 2W-PE/CFA than with 2W + PE/CFA (Fig. 2). Thus, 2W:I-A$^b$–specific T cells were the helper for PE-specific B cells during the first week of the response when 2W-PE was the immunogen.

Next, the effects of T cell–targeted BCOR deficiency on 2W:I-A$^b$–specific CD4$^+$ T cells were examined. Unlike after \textit{L. monocytogenes} infection, the total numbers of 2W:I-A$^b$–specific T cells were not different between WT and \textit{Bcor$^{fl/Y}$} \textit{Lck-Cre$^+$} mice (Fig. 3).

FIGURE 3. A partial defect in GC-Tfh cell differentiation occurs in BCOR-deficient CD4$^+$ T cells after CFA immunization. 2W:I-A$^b$ T cells were enriched from spleen and LNs of WT or \textit{Bcor$^{fl/Y}$} \textit{Lck-Cre$^+$} mice using 2W:I-A$^b$ tetramer 7 d after immunization with 2W-PE emulsified in CFA. (A) Numbers of 2W:I-A$^b$ CD4$^+$ T cells in naive and immunized WT or \textit{Bcor$^{fl/Y}$} \textit{Lck-Cre$^+$} mice. (B) Identification of 2W:I-A$^b$–specific non-Tfh, Tfh, and GC-Tfh cells based on PD-1 and CXCR5 expression. (C) T-bet and Bcl6 expression by 2W:I-A$^b$–specific CD4$^+$ T cells. (D) Percentages of 2W:I-A$^b$–specific non-Tfh, Tfh, or GC-Tfh cells in WT and \textit{Bcor$^{fl/Y}$} \textit{Lck-Cre$^+$} mice. (E) Percentages of 2W:I-A$^b$–specific T-bet$^+$ or Bcl6$^+$ cells in WT and \textit{Bcor$^{fl/Y}$} \textit{Lck-Cre$^+$} mice. Pooled data from two independent experiments are shown. ** $p$, 0.01, *** $p$, 0.001.

FIGURE 4. Plasmablast and GC B cell formation is reduced in mice with BCOR-deficient T cells. PE-specific B cells were enriched from spleen and LNs of WT or \textit{Bcor$^{fl/Y}$} \textit{Lck-Cre$^+$} mice 7 d after immunization with 2W-PE emulsified in CFA. (A) CD90.2$^+$CD11c$^+$F4/80$^+$PE-specific cells (top panels) gated for B220$^+$IgG [H+L]$^+$ plasma cells (middle panels) or B220$^+$CD38$^+$GL7$^+$ naive/memory or CD38$^+$GL7$^+$ GC cells (bottom panels). (B) Number of PE-specific B cells (upper left panel), plasma cells (upper right panel), GC B cells (lower left panel), and naive or memory B cells (lower right panel) in individual mice. Results are from a single experiment. Similar results were obtained in a second experiment, although the overall magnitude of the PE-specific B cell response was lower than in the first experiment. * $p$, 0.05, ** $p$, 0.01.
FIGURE 4. Plasmablast and germinal center B cell formation is reduced in mice with BCOR-deficient T cells. PE-specific B cells were enriched from spleen and LNs of WT or Bcor<sup>fl/Y</sup> Lck-Cre<sup>+</sup> mice 7 days after immunization with 2W-PE emulsified in CFA. (A) CD90.2<sup>-</sup> CD11c<sup>-</sup> F4/80<sup>-</sup> Gr-1<sup>-</sup> PE-specific cells (PE B) (top panels) gated for B220<sup>int</sup> IgG [H+L]<sup>+</sup> plasma cells (middle panels) or B220<sup>+</sup> CD38<sup>+</sup> GL7<sup>-</sup> naive/memory or CD38<sup>-</sup> GL7<sup>+</sup> GC cells (bottom panels). (B) Numbers of PE-specific B cells (top, left panel), plasma cells (top, right panel), GC B cells (bottom, left panel), and naive or memory B cells (bottom, right panel) in individual mice. Results are from a single experiment. Similar results were obtained in a second experiment although the overall magnitude of the PE-specific B cell response was lower than in the first. *<i>p</i> < 0.05, **<i>p</i> < 0.01.
Chapter 3

Bcl6-Interacting Corepressor Contributes to Th17 Cell Formation by Inhibiting Th17 Fate Suppressors

3.1 Introduction

CD4+ T cells participate in host immunity by using T cell antigen receptors (TCRs) to recognize major histocompatibility complex II (MHCII)-bound microbial peptides on host cells. TCR signaling causes naïve CD4+ T cells to proliferate and differentiate into specialized Th1, Th2, Th9, Th17, T follicular (Tfh), or induced regulatory T (Treg) cells depending on signals from cytokine receptors. Th17 cells are generated from naïve precursors that proliferate in the context of signals from receptors for innate immune system cytokines IL-6 and TGF-β (13, 14). These receptors activate the transcription factors STAT3 and SMAD3 (95, 96), which activate transcription of Rorc, the gene encoding RORγt, the master transcription factor for Th17 cells (17). RORγt binds to the promoters of Th17-specific genes such as Il17a, Il17f, Il22, and Ccr6 and stimulates their expression (20, 21). CCR6 directs Th17 cells to effector mucosal sites (27, 28) where they secrete cytokines including IL-17A, IL-17F, and IL-22. These cytokines act on other immune cells including neutrophils to promote clearance of extracellular bacteria, such as Streptococcus pyogenes (Sp), Klebsiella pneumoniae, and Bordetella pertussis (29, 30, 97-100).
Repression of genes that dictate other fates is another important component of Th differentiation. For example, RORγt promotes Th17 differentiation not only by gene activation but also by inhibiting expression of *Tbx21* and *Foxp3* (20, 22), which encode proteins that promote Th1 or Treg cell formation, respectively (16, 18). Similarly, the transcription factor BCL6 promotes the Tfh fate by repressing *Tbx21* and *Rorc* to suppress the Th1 and Th17 fates (23). Previous work from our lab and others suggests that BCL6 represses genes during T cell differentiation by recruiting the BCL6 co-repressor (BCOR), a component of a variant Polycomb repressive complex, PRC1.1 (76, 101). BCOR-mediated repression is required for orchestrating many aspects of cellular differentiation (102, 103) and although originally named for its interaction with BCL6 (104), BCOR can be recruited independently of BCL6 by other components of PRC1.1 such as KDM2B (105, 106).

Here, we show that BCOR-mediated repression also facilitates the formation of Th17 cells. We found that the loss of BCOR or KDM2B, but not BCL6, led to a reduction in the formation of Th17 cells. Chromatin immunoprecipitation sequencing (ChIP-seq) and RNA expression analysis revealed that BCOR was bound to and repressed the *Lef1*, *Runx2*, and *Dusp4* genes, which encoded proteins that suppress the Th17 cell fate thereby enhancing Th17 development.

### 3.2 Materials and Methods

*Mice*
Mice with a conditional Bcor allele (Bcor\(^{fl}\)) were generated by homologous recombination (M.Y. Hamline, J.A. Wamstad, C.M. Corcoran, I. Miletiche, J. Fenge, P.T. Sharpe, M.D. Gearhart, V.J. Bardwell, manuscript in preparation). Bcor\(^{fl}\) mice contain LoxP sites flanking Bcor exons 9 and 10 on the X chromosome, and Cre-mediated deletion causes a premature stop codon and a null allele. Bcor\(^{fl/+}\) mice were fully backcrossed with B6 mice (NCI Frederick) for over 10 generations. Bcor\(^{fl/+}\) mice were bred with Lck-cre mice (B6.Cg-Tg(Lck-cre)3779Nik/J; The Jackson Laboratory stock #012837) (107) to generate WT (Bcor\(^{fl/}\);Lck-Cre\(^{-}\) or Bcor\(^{fl/Y}\);Lck-Cre\(^{-}\)) and BCOR T cell-deficient (Bcor\(^{fl/}\);Lck-Cre\(^{+}\) or Bcor\(^{fl/Y}\);Lck-Cre\(^{+}\)) mice. Bcor\(^{fl/+}\);Lck-Cre\(^{+/-}\) mice were bred with Rorc\(^{GFP}\) reporter mice (B6.129P2(Cg)-Rorc\(^{tm2Litt}\)/J; The Jackson Laboratory stock #007572) (108) to generate Bcor\(^{fl/}\);Lck-cre\(^{-}\);Rorc\(^{GFP}\) or Bcor\(^{fl/}\);Lck-cre\(^{+}\);Rorc\(^{GFP}\) reporter mice for cell sorting and RNA sequencing analysis. Bcl6\(^{fl}\) mice (B6.129S(FVB)-Bcl6\(^{tm1.1Dent}\)/J; JAX stock #023727) (109) were bred with Lck-cre mice described above to generate Bcl6\(^{fl/+}\);Lck-Cre\(^{-}\) mice. Cas9 eGFP\(^{+}\) mice (B6(C)-Gt(ROSA)26Sor\(^{em1.1(CAG-cas9*,-EGFP)Rsky}\)/J; The Jackson Laboratory stock #028555) (110) were crossed with CD90.1\(^{+}\) ovalbumin peptide 323-339:I-A\(^{b}\)-specific (OT-II) Rag1\(^{-/-}\) TCR transgenic mice (111) to generate CD90.1\(^{+}\) Cas9 eGFP\(^{+}\) OT-II Rag1\(^{-/-}\) mice for CRISPR experiments. CD45.1 (B6.SJL-PtprcaPepe\(^{b}/\)BoyJ; The Jackson Laboratory stock #002014), CD90.1 (B6.PL-Thy1\(^{a}\)/ CyJ; The Jackson Laboratory stock #000406), and Cd25\(^{-/-}\) mice (B6;129S4-I1r2a\(^{tm1Dw}\)/J; The Jackson Laboratory stock #002462) (112) were used for bone marrow chimera experiments. All mice were housed in specific pathogen-free conditions at the University of Minnesota. All experimental protocols were
performed in accordance with guidelines of the University of Minnesota Institutional Animal Care and Use Committee and National Institutes of Health.

**Bone Marrow Chimera**

Bones from CD45.1\(^+\) WT and CD45.2\(^+\) \(Cd25^{-/-}\) mice were crushed separately with a mortar and pestle in PBS to prepare bone marrow cells. Bone marrow cells were resuspended in 10% DMSO in FBS (Omega), aliquoted and frozen. Frozen aliquots were thawed, spun down in DMEM containing 10% FBS, Pen Strep (Gibco), and Glutamax (Gibco), and resuspended in PBS. Recipient CD90.1\(^+\) mice at least 8 weeks of age were irradiated with 2 x 500 rad with 6 hours between doses. After the second irradiation, each CD90.1\(^+\) recipient was retro-orbitally injected with 4 x 10\(^6\) CD45.1\(^+\) WT and 1 x 10\(^6\) \(Cd25^{-/-}\) CD45.2\(^+\) bone marrow cells (80:20). After 8-10 weeks, the recipient mice were immunized with heat-killed \(Sp\)-2W.

**Infections and Immunizations**

Male or female mice 6-12 weeks of age were infected intranasally with 10 ul of 2 x 10\(^8\) CFU per mouse of live *Streptococcus pyogenes* expressing 2W (\(Sp\)-2W) peptide in phosphate buffered saline (PBS) (97). Alternatively, mice were immunized intranasally with 15 ul of 3 x 10\(^8\) CFU heat-killed \(Sp\)-2W (97) or *Streptococcus pyogenes* expressing Ovalbumin\(_{323-339}\) peptide (\(Sp\)-OVA) in PBS (113).

**CRISPR/Cas9 and Retroviral Transductions**
Interpro was used to identify functional motifs within target genes and two-three guide RNAs (gRNAs) targeting the functional motifs for each gene were designed using Benchling (114). Primers encoding the gRNA were generated using the Voytas laboratory toolkit and the cloning of the tRNA-gRNA array was performed as described previously (115) with modifications as follows. An MSCV-based gamma retroviral vector was created for generating gRNA delivery. This vector involves modification of the LMP-Amt vector (116), a gift from S. Crotty (La Jolla Institute). The SapI cut site in the vector was disrupted by mutation. Then, the shRNA-encoding segment was replaced with CCDB bacterial gene flanked by SapI cut sites using In-Fusion cloning (Takara Bio USA). The CCDB gene derived from the pMOD_B2303 plasmid from D. Voytas (University of Minnesota). The PGK promoter present in the LMP-Amt vector was replaced with an internal ribosome entry site (Addgene Plasmid #52109) to generate the pMCIA vector. A golden gate reaction was performed to generate the tRNA-gRNA arrays (117-119) using AarI (ThermoFisher Scientific) and T4 DNA ligase (New England BioLabs). The product was purified with a MinElute PCR Purification Kit (Qiagen) and a second golden gate reaction was performed using SapI (New England Biolabs) and T4 DNA ligase (New England Biolabs) to replace the CCDB gene in the pMCIA vector with the tRNA-gRNA arrays made from the first golden gate reaction.

Retroviral supernatant was prepared as previously described (120) with some alterations. Briefly, Platinum-E cells (Cell Biolabs) were plated in 6 well plates the evening prior to transfection. Platinum-E cells were grown in D10 media: DMEM media (Gibco) supplemented with 10% Fetal Bovine Serum (Omega), Pen/Strep (Life Technologies),
Glutamax (Life Technologies), β-mercaptoethanol (Sigma), HEPES (Life Technologies), Sodium Pyruvate (Life Technologies), MEM Non-Essential Amino Acids (Life Technologies), and plasmocin prophylactic (Invivogen). The following morning, media was replaced and after one-two hours, a transfection solution was added dropwise to the cells. The transfection solution contains DMEM media (Gibco) with Polyethylenimine, Linear, MW 25,000 for transfection (Polysciences), pCL-Eco and plasmid DNA containing guide RNA. After five-six hours, the media was aspirated and D10 media supplemented with ViralBoost reagent (Alstem Cell Advancements) and 30 uM water soluble cholesterol (Sigma) was added to the cells. After 24 and 48 hours, viral sup was collected, combined, filtered through a 0.45 um Nylon 25 mm Syringe Filter (Fisher), and frozen at -80°C for retroviral transductions.

Retroviral transduction of CD4⁺ T cells were performed as previously described (120) with some alterations. Cas9 eGFP⁺ CD90.1⁺ OT-II Rag1⁻/⁻ CD4⁺ T cells were isolated using a negative CD4⁺ T cell isolation kit (Stemcell) and incubated in a 96 well plate coated with anti-CD3 (8 µg/ml; 2C11; BioXcell), anti-CD28 (8 µg/ml; 37.51; BioXcell), and IL-7 (2 ng/ml, Tonbo biosciences). Cells were grown in I10 media: IMDM media (Sigma) supplemented with 10% fetal bovine serum (Omega), Pen/Strep (Life Technologies), Glutamax (Life Technologies), β-mercaptoethanol (Sigma), HEPES (Life Technologies), Sodium Pyruvate (Life Technologies), MEM Non-Essential Amino Acids (Life Technologies), and plasmocin prophylactic (Invivogen). At approximately 24 and 40 hours post-stimulation, two spin transductions were conducted by removing the media, adding retroviral supernatant, and spinning the cells at 1500 rpm for two hours at
37°C. After transductions, retroviral supernatants were replaced with I10 media containing IL-2 (10 ng/ml, Peprotech). About 24 hours after the second transduction, cells were removed from the anti-CD3 and anti-CD28 stimulating conditions and split into a 24 well plate. The next day, additional IL-2-containing media was added and the day after that, the cells are washed out of the IL-2 containing media and resuspended in I10 media containing IL-7 (2 ng/ml, Tonbo biosciences). After 24 hours, OT-II cells were cell sorted for guide-mAmetrine expression using a FACS Aria (BD). Then 10,000 mAmetrine+ OT-II cells were transferred into B6 mice. After 4 days of in vivo rest, the mice were immunized with heat-killed Sp-2W and organs were harvested 6 days later for processing as described below.

**Cell Enrichment and Flow cytometry**

Spleen and cervical lymph node cells were stained with CXCR5 antibody (L138D7; Biolegend) and fluorochrome-labelled I-A\(^b\) tetramers containing 2W (2W:I-A\(^b\)) for 1 hour at room temperature (79). For the detection of CD90.1\(^+\) OT-II CD4\(^+\) T cells, suspensions were incubated with CD90.1 antibody (OX-7; Biolegend) for 8 minutes at room temperature and then CXCR5 antibody for 1 hour at room temperature. Cell suspensions were enriched for 2W:I-A\(^b\)-specific or CD90.1\(^+\) CD4\(^+\) T cells using the EasySep\(^\text{TM}\) Mouse fluorochrome positive selection kit (Stemcell). Briefly, cells were incubated with 12.5 ul of EasySep\(^\text{TM}\) fluorochrome selection cocktail per sample for 15 minutes at room temperature and 12.5 ul of EasySep\(^\text{TM}\) magnetic particles per sample for 10 minutes at room temperature. The magnetically bound cells were isolated using magnets (Stemcell) per the manufacturer’s protocol. The positively enriched CD4\(^+\) T
cells were surface stained with fluorochrome-labeled antibodies specific for B220 (RA3-6B2; all antibodies are from eBioscience unless otherwise indicated), CD11b (M1/70), CD11c (N418), CD44 (IM7), CD27 (LG.3A10; BD), and CD4 (GK1.5; BD). For intracellular staining, cells were incubated in intracellular fixation & permeabilization buffer (eBioscience) for 1 hour at room temperature and stained with fluorochrome-labeled anti-RORγt (Q31-378; BD), anti-BCL6 (K112-91; BD), anti-TBET (4B10; Biolegend), and anti-FOXP3 (FJK-16s) antibodies in permeabilization buffer (Tonbo Biosciences) overnight.

For in vivo cytokine secretion analysis, mice were injected with 100 ug of 2W peptide in 200 ul PBS intravenously two hours prior to harvesting the spleen and cLN. After harvesting, samples were processed in Brefeldin A (eBioscience)-containing FACS media for all steps prior to fixation with Cytofix/Cytoperm (BD) for 15 minutes at 4°C and staining with fluorochrome-labeled IL-17A antibody (TC11-18H10; BD) overnight. Cells were analyzed on a Fortessa (Becton Dickinson) flow cytometer and analyzed with FlowJo (TreeStar).

RNA sequencing

*Bcor* fl/fl;Lck-cre;RorcGFP or *Bcor* fl/fl;Lck-cre+;RorcGFP reporter mice were intranasally infected with *Sp*-2W and 2W:I-A^b^-specific CD4^+^ T cells were isolated seven days later and surface stained as described above. Th17 (RORγt^+^ CXCR5^+^), Tfh (RORγt^+^ CXCR5^+^) and RORγt^-^ CXCR5^-^ 2W:I-A^b^-specific CD4^+^ T cells were individually sorted with a FACS Aria (BD) into tubes containing Trizol (Invitrogen). Total RNA was isolated from
the Trizol samples and treated with DNase using an RNA isolation kit (Qiagen). Sequencing libraries were made from PolyA+ mRNA using the KAPA mRNA Hyper Prep kit per manufacturer’s instructions (KAPA Biosystems). The libraries were submitted to the University of Minnesota Genomics Center for 2 x 50 base pair sequencing using the High-Output HiSeq 2500 (Illumina). Demultiplexed reads were trimmed with Trimomatic (v0.32, parameters: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:5 MINLEN:25). Trimmed reads were mapped to the GRCm38 version of the mouse genome with STAR (v2.4.2a_modified), indexed and sorted with samtools (v1.0). PCR Duplicates were removed using Picard MarkDuplicates (2.17.10-SNAPSHOT). Reads that overlap features in the Gencode M16 annotation were enumerated with Rsubread (1.22.3). Differential expression analysis was performed with R (v3.5.1) and DESeq2 (v1.20.0) using custom scripts available at https://github.com/micahgearhart/th17. Raw sequence data, count tables, and normalized FPKM values are available in the Gene Expression Omnibus under accession number GSE121766.

**Chromatin Immunoprecipitation (ChIP) sequencing of in vitro Th17 cells**

CD4+ T cells were isolated from B6 mice using a CD4 T cell positive isolation kit (Stemcell) and stimulated with Th17 polarizing conditions: plate-coated CD3 (4 µg/ml; 2C11; BioXcell) and CD28 (4 µg/ml; 37.51; BioXcell) antibodies, IL-6 (60 ng/ml, Tonbo biosciences), TGF-β1 (0.8 ng/ml, Tonbo biosciences), IL-23 (20 ng/ml, Miltenyi), IL-1β (20 ng/ml; Tonbo biosciences), and IFN-γ (10 µg/ml; XMG1.2; ), IL-12 (10 µg/ml; C17.8; Tonbo biosciences), and IL-4 (10 µg/ml; 11B11; Tonbo biosciences) antibodies.
Cells were grown in IMDM media (Sigma) supplemented with 10% fetal bovine serum (Omega), penicillin and streptomycin (Life Technologies), Glutamax (Life Technologies), β-mercaptoethanol (Sigma), HEPES (Life Technologies), sodium pyruvate (Life Technologies), and MEM non-essential amino acids (Life Technologies). After 3 days, the cells were washed with PBS two times and resuspended in PBS at a concentration of 10 million cells per mL. DNA and proteins were cross-linked by incubating cells with 1.5 mM ethylene glycol bis[succinimidyl succinate] (EGS; Thermo Fisher) for 30 minutes at room temperature on a rotator. Paraformaldehyde was added at a final concentration of 1% during the last 10 minutes. The cross-linking reaction was quenched by adding 2.5M glycine at a 1:20 dilution and washed two times with PBS. After the last spin, media was completely removed and the cell pellet was frozen using dry ice and isopropanol, and kept at -80°C until shearing. The truChIP Chromatin Shearing Reagent kit (Covaris) was applied to lyse the cells, isolate nuclei, and shear chromatin with the AFA™ Focused-ultrasonicator per manufacturer’s instructions. An aliquot was taken for the input. The rest of the sample was enriched for BCOR-bound DNA by incubating sheared chromatin overnight with Protein A Dynabeads (Invitrogen) bound to rabbit polyclonal anti-mouse BCOR antibody (RRID: AB_2750631). The sample was washed using the DynaMag-2 Magnet (Invitrogen) and buffers in the following order: low salt buffer (0.1% SDS, 1.0% Triton X-100, 2mM EDTA, 20 mM Tris-HCL pH 8.0, 150 mM NaCl), high salt buffer (0.1% SDS, 1.0% Triton x-100, 2mM EDTA, 20 mM Tris-HCL pH 8.0, 500 mM NaCl), lithium chloride buffer (0.25 M lithium chloride, 1% NP-40 or IGEPAL-CA630 1% DOC, 1mM EDTA, 10 mM Tris pH 8.0), Morohashi RIPA buffer (50mM Tris pH 7.5, 150 mM NaCl, 5mM EDTA, 0.5%
NP40, 0.1% SDS), DOC/Triton buffer (25mM Tris pH 7.5, 150 mM NaCl, 5mM EDTA, 1% Triton-X-100, 0.5% DOC), and TE buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA). DNA was eluted off the magnetic beads using elution buffer (1% SDS, 100 mM NaHCO₃). Cross-links were reversed for the BCOR-enriched DNA and input samples by incubating with 200 mM NaCl at 55°C overnight. DNA was purified using a PCR purification kit (Qiagen) and cDNA sequencing libraries were made using the KAPA Hyper Prep kit per manufacturer’s instructions (KAPA Biosystems). The libraries were submitted to the University of Minnesota Genomics Center for 1 x 50 base pair sequencing using the High-Output HiSeq 2500 (Illumina). Reads were trimmed (Trimmomatic 0.32), mapped to the GRCh38 reference genome (BWA mem 0.7.12-r1039), sorted and indexed (Samtools 1.0). Peaks were identified using MACS version 2.1.1.20160309 using the --broad --broad-cutoff 0.1 options. BCOR peak locations were analyzed in relationship to the Gencode M16 reference annotation and the UCSC defined CpG islands using custom R scripts which are available at https://github.com/micahgearhart/th17. Raw sequence data, coverage tracks and peak lists are available in the Gene Expression Omnibus under accession number GSE121766.

Statistical analysis

Statistical tests were performed using Prism (Graphpad) software and $p$ values were obtained using two-tailed unpaired $t$ tests with a 95% confidence interval. Statistical significance testing for RNA-seq data was performed using the Wald Test in DESeq2 and $p$-values were calculated using the Benjamini-Hochberg correction for multiple testing. For the ChIP-seq data, $q$-values for each peak were determined using MACS software.
3.3 Results

**BCOR is required for optimal Th17 differentiation after *Streptococcus pyogenes* infection**

We previously found that BCOR-mutant T cells produce fewer Tfh cells of the germinal center subtype, and more Th1 cells than wild-type (WT) T cells during an immune response to *Listeria monocytogenes* (101). We compared T cell responses of WT and BCOR-mutant T cells to a Th17-inducing pathogen to determine whether BCOR also influences Th17 differentiation. As in our previous study (101), we used a *Bcor* conditional allele, *Bcor*\textsuperscript{fl}, with Lck-cre to mutate *Bcor* in T cells. Cre mediated deletion of this allele removes exons 9 and 10 and results in a premature stop codon. The resulting truncated protein product, if stable, is incapable of incorporation into PRC1.1. We refer to *Bcor*\textsuperscript{fl/fl};Lck-cre mice as “BCOR-deficient” throughout the paper.

We used *Streptococcus pyogenes* (*Sp*) infection to generate a robust Th17 response (97, 121). Intranasal infection of an engineered *Sp* strain expressing a model antigenic peptide called 2W (*Sp*-2W) (97, 122) allowed us to follow the T cell response to this specific antigen. Both WT and BCOR-deficient mice are on a C57BL/6 (B6) background and express I-A\textsuperscript{b} MHCII molecules. The *Sp*-2W strain was used so that a fluorochrome-labeled 2W:I-A\textsuperscript{b} tetramer and flow cytometry could be used to monitor the fate of a Th population specific for an epitope from the microbe because no natural I-A\textsuperscript{b}-binding *Sp* epitopes have been discovered.

We first determined whether BCOR-deficiency affected the clonal expansion of 2W:I-A\textsuperscript{b}-specific CD4\textsuperscript{+} T cells. 2W:I-A\textsuperscript{b} tetramer-based cell enrichment (79) was
performed to identify 2W:I-A\textsuperscript{b}-specific CD4\textsuperscript{+} T cells in spleen and lymph node samples on day 7 after \textit{Sp}-2W infection. The approximately 300 2W:I-A\textsuperscript{b}-specific CD4\textsuperscript{+} T cells in uninfected mice (79) underwent extensive and similar clonal expansion in WT and T cell BCOR-deficient mice (Fig. 5A-B).

We then examined CD4\textsuperscript{+} T cell subsets within the 2W:I-A\textsuperscript{b}-specific populations by staining for the lineage defining markers ROR\gamma (Th17), CXCR5 (Tfh), TBET (Th1), and FOXP3 (Treg) (16-18, 33). The population in WT mice contained ROR\gamma\textsuperscript{+} CXCR5\textsuperscript{-} Th17 cells, CXCR5\textsuperscript{+} Tfh cells, and ROR\gamma\textsuperscript{-} CXCR5\textsuperscript{-} cells. (Fig. 5C). The ROR\gamma\textsuperscript{-} CXCR5\textsuperscript{-} population contained a subset of TBET\textsuperscript{+} Th1 cells, very few FOXP3\textsuperscript{+} Treg cells and other cells, which will be referred to hereafter as uncommitted cells. In total, the 2W:I-A\textsuperscript{b}-specific population in WT mice was composed of 35% Th17, 40% Tfh, 5% Th1, 0.4% Treg, and 16% uncommitted cells. The population in T cell BCOR-deficient mice contained significantly smaller fractions of Th17 and significantly larger fractions of uncommitted cells than WT mice. Although the Tfh populations were about the same size, the one in WT mice contained a small ROR\gamma\textsuperscript{low} subset that was absent in T cell BCOR-deficient population (Fig. 5C). Thus, BCOR was required for maximal formation of Th17 cells and a Tfh subset that expressed ROR\gamma (Fig. 5D).

We also determined whether BCOR was required for expression of CCR6 and IL-17, two proteins that provide canonical functions of Th17 cells. Most of the 2W:I-A\textsuperscript{b}-specific Th17 cells in WT mice on day 7 after \textit{Sp}-2W infection expressed CCR6, while most Tfh cells did not (Fig. 6A-B). In addition, intravenous injection of 2W peptide caused most of Th17 cells but few of Tfh cells to produce IL-17A (Fig. 6C-D). BCOR-deficiency led to significant reductions in CCR6 expression and blunted peptide-induced
IL-17A expression by the Th17 cells. In summary, these data suggest that BCOR is not only important for Th17 cell formation, but also for expression of Th17 functions in cells that manage to develop without intact BCOR.

KDM2B but not BCL6 is required for optimal Th17 differentiation

We asked whether BCL6, which is known to directly interact with BCOR, promotes Th17 cell differentiation as it does to promote Tfh differentiation (76, 101). This possibility was tested by immunizing WT and Bcl6\textsuperscript{fl/fl};Lck-cre mice, which have a Bcl6 mutation in T cells, with a 2W peptide-phycoerythrin (2W-PE) conjugate in complete Freund’s adjuvant (CFA). In WT cells, the 2W-PE immunogen induced 2W:I\textsuperscript{a}b-specific Th17 and Tfh cells (Fig. 7A). As expected, the mutation of Bcl6 in T cells led to reduced expansion of 2W:I\textsuperscript{a}b-specific CD4\textsuperscript{+} T cells (Fig. 7B), and significant defects in the formation of Tfh (Fig. 7A-B) and PE-specific germinal center B cells (data not shown). BCL6 did not play a role in Th17 differentiation as the Th17 cell numbers were equivalent between WT and Bcl6\textsuperscript{fl/fl};Lck-cre mice (Fig. 7B). These results indicate that BCOR does not cooperate with BCL6 to promote Th17 differentiation.

To confirm the importance of the PRC1.1 complex in Th17 differentiation, we investigated the role of KDM2B, a second unique component of PRC1.1. KDM2B is a histone demethylase and has a CXXC motif that can recruit BCOR/PRC1.1 to non-methylated CpG islands in other cell types (105, 106). We adopted an in vitro CRISPR/Cas9-based gene targeting approach to test the role of KDM2B in Th17 differentiation in vivo. OT-II TCR transgenic CD4\textsuperscript{+} T cells expressing Cas9 and a chicken ovalbumin peptide:I\textsuperscript{a}b-specific TCR (123) were transduced in vitro with
retroviruses encoding the mAmetrine fluorescent protein and guide RNAs (gRNAs) targeting genes of interest. The efficiency of this approach was first tested by targeting the \textit{Il2ra} (encoding CD25) and \textit{Cd27} genes, which are highly expressed during the \textit{in vitro} stimulation protocol. mAmetrine$^+$ OT-II cells transduced with \textit{Il2ra} or \textit{Cd27} gRNAs had 93% and 83% fewer CD25$^+$ or CD27$^+$ cells, respectively, than mAmetrine$^+$ OT-II cells transduced with irrelevant \textit{LacZ} gRNAs (data not shown) indicating that this targeting system was very efficient.

This approach was then used to determine the role of KDM2B in Th17 differentiation \textit{in vivo}. mAmetrine$^+$ Cas9-expressing CD90.1$^+$ OT-II cells were sorted after transduction with retroviruses encoding gRNAs targeting \textit{LacZ}, \textit{Bcor}, or \textit{Kdm2b} and then transferred into naive CD90.2$^+$ B6 mice (Fig. 7C). The recipients were immunized intranasally with heat-killed \textit{Sp} expressing the chicken ovalbumin peptide (\textit{Sp-OVA}) (113) four days after transfer. Six days later, OT-II cells were enriched from spleen and cervical lymph nodes cells after staining with CD90.1 antibody. The \textit{LacZ}-targeted OT-II cells maintained mAmetrine expression (Fig. 7D) and proliferated and differentiated into ROR$\gamma$t$^+$ Th17 cells, CXCR5$^+$ Tfh cells, or uncommitted cells (Fig. 7E) after administration of heat-killed \textit{Sp-OVA} bacteria. Deletion of \textit{Kdm2b} or \textit{Bcor} did not affect OT-II cell proliferation (Fig. 7F). The \textit{Bcor}-targeted OT-II cell population, however, contained a smaller fraction of Th17 cells than the \textit{LacZ}-targeted population (Fig. 7G). \textit{Bcor} deletion also led to a reduction in ROR$\gamma$t$^-$ CXCR5$^+$ Tfh cells, which was only observed for the ROR$\gamma$t$^{low}$ CXCR5$^+$ subset during live \textit{Sp}-2W infection (Fig. 5D). The \textit{Kdm2b}-targeted OT-II cell population exhibited reduced Th17 formation with no significant alteration in Tfh differentiation as compared to the \textit{LacZ} control (Fig. 7E-G),
confirming the importance of the PRC1.1 complex in Th17 differentiation and suggesting that KDM2B may contribute to the recruitment of BCOR to DNA in Th17 but not Tfh cells. Both Bcor- and Kdm2b-targeted OT-II cell populations had increased fractions of uncommitted cells (Fig. 5D, 7G), indicating BCOR and KDM2B drive uncommitted cells to become Th17 cells.

Expression profiling discriminates T cell subsets in WT and BCOR-deficient CD4⁺ T cells

We used genome-wide expression profiling to further characterize the T cell subsets generated upon Sp infection in both WT and BCOR-deficient populations. WT Rorc$^{GFP}$ or Bcor$^{fl/fl}$;Lck-cre⁺;Rorc$^{GFP}$ mice were infected with the Sp-2W bacterial strain and 7 days later, cells were harvested from the spleen and cervical lymph nodes. 2W:1-A$^b$ tetramer-binding Th17, Tfh, and RORγ−CXCR5− cells were sorted based on RORγt and CXCR5 expression (Fig. 8A). The Rorc$^{GFP}$ reporter-based strategy led to better resolution of the RORγ$^{t^{low}}$CXCR5$^+$ cells than RORγt antibody-based intracellular staining used in other experiments. As noted in Figure 5, however, very few of these cells were present in BCOR-deficient mice, precluding their inclusion in this analysis. Libraries for RNA sequencing were generated and gene expression values were used for principal component analysis. This analysis showed that the WT Th17, Tfh, and uncommitted subsets formed distinct clusters (Fig. 8B, ovals) and that BCOR-deficient Th17, Tfh, and RORγ−CXCR5− populations formed clusters that were related to but distinct from those formed by the WT populations. The first principle component along the horizontal axis separated the Tfh and Th17 subsets and was driven by variation in
expression of Ccr2, Adam8, Cxcr5, Nhsl2, and Il17re. The second principle component separated the WT and BCOR-deficient samples within each subset and was driven by variation in Spock2, Ccr6, Il17re, Nkg7, and Rorc (encoding RORγt). The analysis revealed that Th17 cells highly expressed Th17-related genes such as Rorc, Ccr6, and Maf, while Tfh cells highly expressed Tfh-related genes such as Bcl6, Cxcr5, and Pdcd1 (encoding PD-1). The RORγt−CXCR5− population expressed higher levels of Tbx21 and Ifng than the other populations (Fig. 8C), consistent with the observation that it contains a Th1 subset (Fig. 5C-D). Taken together, the expression profiles recapitulate the known characteristics of the T cell subsets.

**Identification of genes directly regulated by BCOR**

We sought to identify BCOR regulated genes given the distinct nature of the BCOR-deficient cells in the principal component analysis. We found only 16 differentially expressed genes for the Th17 subset, while 478 and 468 genes were identified in the Tfh and RORγt−CXCR5− populations, respectively (2 fold, up or down, and Benjamini-Hochberg adjusted p-value < 0.05) (Fig. 9A). Because of the possibility that BCOR enhances Th17 cell formation by repressing genes that oppose the Th17 cell fate, we focused on the 157 genes that were expressed more highly in BCOR-deficient than WT cells for one or more subsets (2 fold up and Benjamini-Hochberg adjusted p-value < 0.05). We then used BCOR-specific ChIP-seq to determine which of these genes were direct BCOR targets. WT CD4+ T cells were cultured in vitro for 3 days under Th17 inducing conditions to mimic a situation whereby BCOR represses gene targets in the context of Th17 differentiation. We identified 10,761 BCOR peaks above threshold.
levels (q-value < 0.05), 82% of which were found at promoters. CpG islands were observed at 83% of the peaks consistent with the CXXC domain of KDM2B contributing to the recruitment of BCOR. Of the 157 genes that were more highly expressed in BCOR-deficient cells, 123 were directly bound by BCOR in Th17 cells.

Eleven of the 123 BCOR-repressed direct target genes (Tsc22d3, Runx2, Id3, Bach2, Scml4, Foxo3, Hipk2, Dusp6, Dusp4, Ptc1, and Lef1) were chosen for further study based on expression level (mean counts of BCOR-deficient cells > 150) and because they encoded proteins with the potential to suppress differentiation by regulating transcription or phosphatase activity. BCOR was found at promoters and CpG islands for 10 of the 11 target genes (examples for Lef1, Dusp4, and Hipk2 are shown in Fig. 9B). BCOR-deficient Tfh and RORγt−CXCR5− cells expressed these genes to a higher level than the comparable WT subsets (Fig. 9C). BCOR-deficiency led to more modest increases in the expression of these genes in the Th17 cells that formed in the absence of BCOR (Fig. 9C). This observation led us to investigate the efficiency of Cre mediated deletion of exons 9 and 10 of Bcor in each subset, since inefficient deletion would result in expression of intact BCOR protein. Indeed, quantification of RNA-seq reads mapping to exons 9 and 10 indicated that the residual Th17 cells in Bcorfl/fl;Lck-cre+ mice retained these exons more often than Tfh or RORγt−CXCR5− cells (Fig. 9D). The presence of intact Bcor alleles in some Th17 cells is consistent with the small numbers of significant gene expression changes observed for this subset (Fig. 9A). This result suggests that many of the Th17 cells that developed in Bcorfl/fl;Lck-cre+ mice arose from precursors that expressed intact BCOR. The enrichment of cells that failed to delete Bcor in the Th17 subset is strong evidence that BCOR is beneficial to their development.
In vivo CRISPR/Cas9 screen reveals novel Th17 regulators

As described above, we found genes that were significantly upregulated in the Tfh and ROR\(\gamma\)^{+} CXCR5\(^{-}\) subtypes upon BCOR depletion (Fig. 9C). We tested the hypothesis that products of these genes suppress the Th17 fate during normal Th cell differentiation. Cas9\(^{+}\) OT-II cells were transduced in vitro with retroviruses expressing mAmetrine and gRNAs targeting each of the 11 genes. mAmetrine\(^{+}\) cells were transferred into recipient mice, which were immunized intranasally with heat-killed Sp-OVA bacteria four days after transfer. The transferred OT-II cells were enriched from spleen and cervical lymph node cells after staining with CD90.1 fluorochrome-conjugated antibody and fluorochrome antibody-conjugated magnetic beads. Targeting the BCOR-repressed genes did not result in significant alterations to OT-II cell proliferation when compared to a LacZ control (Fig. 10B). Ablation of Id3, Foxo3, Bach2, Scml4, and Ptch1 had little to no effect on the differentiation of the OT-II cells. In contrast, disruption of Lef1, Runx2, and Dusp4 enhanced Th17 differentiation, reduced Tfh differentiation, and had no effect on uncommitted cells (Fig. 10A-B). These results indicate that the products of these genes suppress the Th17 fate. BCOR repression of these genes in WT cells would therefore be predicted to enhance Th17 differentiation. Surprisingly, disruption of Dusp6, Hipk2, and Tsc22d3 reduced Th17 and increased the differentiation of uncommitted cells (Fig. 10A-B), indicating that the products of these genes enhance Th17 differentiation in WT cells. These results suggest that BCOR is targeted to some genes that enhance Th17 differentiation and others that suppress it.
Enhancement must dominate suppression, however, since the net effect of BCOR
deficiency is a reduction in Th17 formation.

The CD25-STAT5-BLIMP1 pathway promotes Th17 differentiation

The mechanism of action of dual specificity phosphatase 4 (DUSP4), the protein
product of Dusp4 gene during Th17 formation, was explored in more detail. DUSP4 is a
phosphatase that inhibits the phosphorylation and action of the STAT5 transcription
factor (124, 125). During Th1 priming conditions, STAT5 phosphorylation is triggered
by TCR signaling and induces Il2ra (encodes CD25), a component of the IL-2 receptor,
which promotes the Th1 fate (126-129). It was therefore possible that STAT5 signaling is
also required to promote the Th17 fate during Th17 priming conditions, and that BCOR
promotes this process by preventing DUSP4 from dephosphorylating STAT5. We first
tested the importance of the CD25-STAT5 pathway for Th17 differentiation using an
Il2ra loss-of-function allele in a radiation chimera-based approach. A mixture of 80%
WT CD45.1+ bone marrow and 20% Il2ra−/− CD45.2+ bone marrow (Fig. 11A) was
transplanted into lethally-irradiated CD90.1+ B6 mice. This strategy was used to prevent
the autoimmunity that occurs in intact Il2ra−/− mice due to Treg cell insufficiency (130).
After reconstitution, these mice were immunized with heat-killed Sp-2W bacteria and
CD44high 2W:I-Ab+ CD4+ T cells were identified six days later (Fig. 11B). WT or Il2ra−/−
2W:I-Ab-specific CD4+ T cells were identified based on congenic markers and classified
as Th17, Tfh, or uncommitted cells based on the expression of RORγt, CXCR5, or
neither (Fig. 11B). CD25 deficiency reduced Th17 differentiation, enhanced Tfh
differentiation, and had no effect on the uncommitted population (Fig. 11C). These
results demonstrate that CD25 expression is beneficial for Th17 differentiation and harmful for Tfh differentiation.

During Th1 priming conditions, CD25 and STAT5 enhance Th1 formation in part by preventing the Tfh fate via activation of Prdm1, which encodes BLIMP1 (129, 131). Stat5 and Prdm1 were targeted in OT-II cells using the CRISPR/Cas9-based approach described above (Fig. 7C-G) to determine whether a similar pathway favors Th17 cell formation under Th17 priming conditions. Rorc, which encodes RORγt, the master transcription factor for Th17 cells (17), was also targeted to serve as a control. As expected, Rorc-targeted OT-II cells exhibited reduced Th17 differentiation and enhanced differentiation of Tfh and uncommitted cells (Fig. 11D-F). Ablation of Stat5 or Prdm1 had similar effects (Fig. 11D-F). These results demonstrate that STAT5 and BLIMP1 are required for optimal Th17 cell differentiation and raise the possibility that DUSP4 acts as a Th17 fate suppressor by inhibiting the CD25-STAT5-BLIMP1 signaling pathway.

3.4 Discussion

We found that BCOR and KDM2B are essential for the optimal differentiation of the Th17, but not most Tfh cells that form after Sp infection. It is likely that the role of BCOR in this process is based on its interaction with KDM2B, which contains a CXXC DNA-binding domain that specifically targets unmethylated CpG nucleotides (105). Thus, KDM2B may contribute to targeting the repressive activity of BCOR to unmethylated CpG islands in genes encoding proteins that suppress the Th17 cell fate. Th cells that induce RORγt via the action of IL-6 and TGF-β (132) and experience KDM2B-BCOR-mediated repression of Th17 inhibitors would therefore become Th17 cells.
Although all Tfh cells induced during Sp infection are dependent on BCL6, most are not dependent on BCOR as was observed for PD-1- Tfh cells that form during Listeria monocytogenes infection (101). Thus, many Th cells may achieve the BCL6-mediated repressive effects needed to become Tfh cells via BCL6 interactions with other corepressors such as SMRT or NCOR (24). However, the RORγtdlow Tfh cells and PD-1+ germinal center Tfh cells that form after Sp and L. monocytogenes infections, respectively, are both BCOR-dependent. Notably, these two Tfh subsets express more BCL6 than other Tfh cells and thus may contain sufficient BCL6-BCOR complexes to influence their differentiation.

Unlike RORγt, which primarily binds to and activates the genes that define the Th17 lineage, BCOR promotes the Th17 fate by repressing genes that encode proteins that suppress this fate. ChIP sequencing experiments demonstrated that BCOR is directly targeted to the Lef1, Runx2, and Dusp4 genes, while CRISPR/Cas9 targeting studies showed that ablation of these genes enhanced Th17 cell formation. Therefore, BCOR may enhance Th17 cell generation by repressing Lef1, Runx2, and Dusp4 expression. The fact that the products of these genes have different functions indicates that BCOR-mediated repression facilitates Th17 formation via multiple pathways. For example the runt-related transcription factor 2 (RUNX2), which promotes the Tfh fate in the context of LCMV infection (133), could activate transcription of genes encoding proteins that repress Th17 cell differentiation. In contrast, BCOR-mediated repression of DUSP4 likely promotes Th17 cell formation by enhancing the STAT5 signaling pathway. During infections that stimulate IL-12 production, relevant epitope-specific naive T cells experience IL-2 receptor-induced phosphorylation of STAT5, which ultimately results in
expression of BLIMP1. BLIMP1 is a repressor of the Tfh pathway (19) and in the presence of STAT4, STAT5, and BLIMP1 signaling, T cells are more likely to become Th1 cells. Our results show that the IL-2 receptor CD25, STAT5, and BLIMP1 also favor Th17 cell formation during Sp infection. Since DUSP4 is a barrier to STAT5 signaling, it is possible that BCOR-mediated repression of Dusp4 promotes Th17 formation by removing this barrier. It should be noted that in vitro experiments suggest that STAT5 and DUSP4 promote Th17 cell formation (125) (134). The difference between these results and ours emphasizes the importance of analyzing Th cell differentiation in vivo during relevant infections.

Although we found evidence that BCOR enhances Th17 cell formation by repressing genes that suppress the Th17 cell fate, we also identified BCOR target genes such as Dusp6, Hipk2, and Tsc22d3, which encoded proteins that enhanced Th17 formation. The role of DUSP6 in promoting the Th17 fate is consistent with previous findings showing that Dusp6−/− CD4+ T cells have reduced production of the Th17 cytokine IL-17A and enhanced production of the Th1 cytokine IFN-γ after in vitro stimulation (135). The roles of homeodomain-interacting protein kinase 2 (HIPK2) and TSC22 domain family member 3 (TSC22D3) will require additional studies to understand their importance in the context of Th17 differentiation. In any case, although BCOR represses genes encoding proteins that promote and suppress the Th17 cell fate, the fact that BCOR is required for optimal Th17 cell formation suggests that its dominant activity in Th cell differentiation is to repress genes that inhibit the Th17 cell fate.
FIGURE 5. BCOR is required for optimal Th17 differentiation after *Streptococcus pyogenes* (*Sp*) infection. WT and *Bcor*^fl/fl^;Lck-cre^+^ (BCOR-deficient) mice were infected with *Sp*-2W. After 7 days, 2W:I-A^b^-specific CD4^+^ T cells were enriched from spleen and cervical lymph nodes using 2W:I-A^b^-tetramer and a magnetic based bead enrichment strategy. (A) B220^-^CD11b^-^CD11c^-^CD4^+^ T cells from 2W:I-A^b^-tetramer-enriched samples with gates on CD44^+^ 2W:I-A^b^-tetramer^+^ cells. (B) Numbers of 2W:I-A^b^-specific cells in WT and *Bcor*^fl/fl^;Lck-cre^+^ mice. (C) Identification of 2W:I-A^b^-specific (from gate in (A)) Th17, Tfh, Th1, Treg, and uncommitted subsets based on RORγt, CXCR5, BCL6, TBET, and FOXP3 expression. (D) Percentages of each subset among 2W:I-A^b^-specific cells. Subsets were identified in (C). Data are representative of two independent experiments (*n* = 6-11 mice/group). * p < 0.05, *** *p < 0.001.
FIGURE 6. BCOR is important for the induction of canonical Th17-associated chemokine receptor and cytokine expression. WT and BCOR-deficient mice were infected with Sp-2W and 2W:I-A^b-specific CD4^+ T cells from spleen and cervical lymph nodes were analyzed 7 days later. Cytokine expression was assessed following 2W peptide injection 2 hours prior to harvest. (A) CCR6 expression of 2W:I-A^b-specific Th17 (RORγt\(^+\) CXCR5\(^-\)) and Tfh (RORγt\(^-\) CXCR5\(^+\)) cells. (B) CCR6 percentage of each subset from (A). (C) IL-17A secretion of 2W:I-A^b-specific subsets after 2 hours of \textit{in vivo} 2W peptide stimulation. (D) IL-17A percentage of each subset from (C). Data are representative of two independent experiments (n = 2-9 mice/group). *** p < 0.001.
FIGURE 7. KDM2B but not BCL6 is required for optimal Th17 differentiation. WT and Bcl6\textsuperscript{fl/fl};Lck-cre\textsuperscript{+} mice were immunized with 2W-PE in CFA. After nine days, 2W:I-A\textsuperscript{b}-specific CD4\textsuperscript{+} T cells were enriched from spleen and cervical lymph nodes using 2W:I-A\textsuperscript{b} tetramer and a magnetic based bead enrichment strategy. (A) B220\textsuperscript{-}CD11c\textsuperscript{-}CD11c\textsuperscript{-}CD44\textsuperscript{+}2W:I-A\textsuperscript{b}-specific T cells from tetramer-enriched samples with gates on ROR\textgammat\textsuperscript{+} Th17 and CXCR5\textsuperscript{+} Tfh cells. (B) Numbers of total 2W:I-A\textsuperscript{b}-specific cells, Tfh, and Th17 cells in WT and Bcl6\textsuperscript{fl/fl};Lck-cre\textsuperscript{+} mice based on gates in (A). Cas9-expressing OT-II transgenic CD4\textsuperscript{+} T cells were \textit{in vitro} stimulated and retrovirally transduced prior to adoptive transfer of 10,000 mAmetrine\textsuperscript{+} OT-II cells into naive C57BL/6 mice. After four days of \textit{in vivo} rest, mice were immunized with heat-killed \textit{Sp} expressing OVA, and spleen and cervical lymph nodes were taken 6 days later to assess
OT-II differentiation. (C) Example sort strategy for Cas9+ OT-II CD4+ T cells pre-gated on singlets. Cells were transduced with control guide RNA targeting LacZ. (D) After six days of immunization with heat-killed Sp-OVA, OT-II cells were enriched from spleen and cervical lymph nodes by staining with Thy1.1-APC and a magnetic based bead enrichment strategy. Flow cytometry plots show mAmetrine expression of Thy1.1-enriched B220- CD11b- CD11c- cells with gates on Thy1.1+ OT-II cells and endogenous CD4+ T cells. (E) Identification of Th17, Tfh, and uncommitted (Uncom) OT-II cells based on RORγt and CXCR5 expression for LacZ, Bcor, and Kdm2b targeted OT-II cells. (F) Numbers of Thy1.1+ OT-II CD4+ T cells. (G) Percentages of each subset among OT-II cells based on gates in (E). Data are representative of two independent experiments (n = 2-6 mice/group) for (A-B) and three independent experiments (n = 2-5 mice/group) for (C-H). ** p < 0.01, *** p < 0.001.
FIGURE 8. RNA sequencing of WT and BCOR-deficient CD4+ T cells following Sp infection. WT (RorcGFP) or BCOR-deficient (Bcorfl/fl;Lck-cre+;RorcGFP) mice were infected with Sp-2W. After 7 days, 2W:I-Ab-specific CD4+ T cell subsets from spleen and cervical lymph nodes were sorted for RNA sequencing analysis. (A) Flow cytometry plots displaying B220−CD11b−CD11c−CD4+CD44+2W:I-Ab-specific T cells from tetramer-enriched samples. 2W:I-Ab-specific Th17, Tfh, and RORγt+CXCR5− cells were identified based on RORγt-GFP and CXCR5 expression. (B) Principal component analysis of WT (open circles) and BCOR-deficient (closed circles) CD4+ T cell subsets.
(C) Centered and scaled Log2 FPKM expression values of canonical markers used to identify Th subsets.
FIGURE 9. RNA expression analysis and BCOR ChIP-seq reveal genes directly regulated by BCOR. (A) Venn diagram displaying the number of BCOR regulated genes within each Th subset based on the RNA sequencing in Fig. 8 (2 fold, up or down, and Benjamini-Hochberg adjusted p-value < 0.05). (B) Input and BCOR-enriched ChIP tracks for select BCOR bound genes in WT CD4+ T cells cultured in vitro with Th17-polarizing conditions for 3 days. (C) BCOR repressed genes from RNA sequencing data were cross-referenced with BCOR-specific binding peaks from ChIP sequencing data and 11 genes to examine further were chosen based on expression level (mean counts of
BCOR-deficient cells > 150) and because they encoded proteins with the potential to suppress differentiation by regulating transcription or phosphatase activity. ChIP peak score ($10^*\log10qvalue$), scaled Log2 FPKM values, and gene ontology terms are displayed for each sample. (D) Representative RNA sequencing tracks for BCOR exons 7-10 (left panel) and splicing efficiency of exons 9-10 (right panel).
**FIGURE 10. In vivo CRISPR/Cas9 screen reveals novel Th17 regulators.** Cas9-expressing OT-II transgenic CD4+ T cells were *in vitro* stimulated and retrovirally transduced with guides targeting *LacZ* or genes listed in Fig 5C. 10,000 mAmeterine+ OT-II cells were adoptively transferred into naive B6 mice and immunized with heat-killed *Sp*-OVA four days later. Spleen and cervical lymph nodes were taken six days after immunization to assess OT-II differentiation. (A) Flow cytometry plots displaying B220-CD11b-CD11c-CD4+CD44+Thy1.1+ OT-II T cells from Thy1.1-enriched samples. Th17, Tfh, and uncommitted (Uncom) cells were identified based on RORγt and CXCR5 expression for select guide RNA. (B) Numbers of OT-II CD4+ T cells and percentages of each subset among OT-II cells as gated in (A). Data are representative of two to four independent experiments (*n* = 2-5 mice/group). * p < 0.05, ** p < 0.01, *** p < 0.001.
FIGURE 11. The CD25-STAT5-BLIMP1 pathway promotes Th17 differentiation.

(A) Model for WT and CD25-/- mixed bone marrow chimera experiment. (B) Representative flow cytometric identification of 2W:I-A\(^b\)-specific WT or CD25-/- CD4\(^+\) T cells and their differentiation into Th17, Tfh, and uncommitted cells six days after heat-killed Sp-2W immunization. (C) Percentage of subsets among 2W:I-A\(^b\)-specific WT or CD25-/- CD4\(^+\) T cells. Cas9-expressing OT-II transgenic CD4\(^+\) T cells were in vitro transduced with guide RNA targeting LacZ, Rorc, Stat5, or Prdm1 prior to in vivo transfer to assess differentiation, as described in Fig. 7C. (D) Flow cytometry plots
displaying B220<sup>-</sup> CD11b<sup>-</sup> CD11c<sup>-</sup> CD4<sup>+</sup> CD44<sup>+</sup> Thy1.1<sup>+</sup> OT-II T cells from Thy1.1-enriched samples. Th17, Tfh, and uncommitted (Uncom) cells were identified based on RORγt and CXCR5 expression. (E) Numbers of OT-II CD4<sup>+</sup> T cells. (F) Percentages of each subset among OT-II cells as gated in (D). Data are representative of two independent experiments (n = 2-8 mice/group). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Maximal production of high affinity isotype-switched Abs by B cells depends on signals from CD4⁺ helper T (Th) cells. Antigen binding to surface Ig molecules (BCRs) causes naïve B cells to migrate to the border between the follicle and T cell area and present MHCII-bound antigen-derived peptides (p:MHCII) to Th cells (136-138) that were previously activated by the same p:MHCII complex on dendritic cells. The antigen-specific B cells then receive signals from the Th cells, proliferate, and undergo isotype switching (42, 139, 140). Some of the activated B cell progeny become extrafollicular Ab-secreting plasmablasts (PBs) while others enter germinal centers (GCs) along with specialized follicular helper (Tfh) cells that express the Bcl-6 transcription factor and the follicle-homing chemokine receptor CXCR5 (33). Tfh cells then engage in p:MHCII-dependent interactions with the GC B cells and drive somatic mutations and formation of high-affinity memory B cells and long-lived plasma cells (141, 142).

Early PB formation also depends on CD4⁺ T cells, (80, 143), but it is uncertain whether Tfh cells are required (144-146). Although isotype switched Abs were severely impaired in the absence of Tfh cells after NP-OVA in alum immunization (19) and Salmonella infection (147), another study showed that Th1 cells played a critical role in generating influenza-specific IgG2 Abs independently of Tfh cells (148). Furthermore,
little is known regarding the contribution of other Th subsets such as Th17 cells to the B cell response in vivo. We therefore examined the contribution of Tfh, Th1, and Th17 cells to PB formation and isotype switching in response to a stimulus that primes all three Th subsets. The results show that although GC B cell formation was defective in mice lacking Tfh cells, formation of isotype-switched PBs occurred normally in mice lacking Tfh, Th1, or Th17 cells. Isotype-switched PB formation was defective when CD154-CD40 interactions were absent or B cells could not present p:MHCII complexes. These results indicate that isotype-switched PB production requires a CD40-dependent form of cognate T cell help that does not depend on a highly differentiated Th cell subset.

4.2 Materials and methods

Mice. Six-12 week old male and female mice were used. C57BL/6 (B6) and CD45.1+ (B6.SJL-Ptprca Pep3b/BoyJ) mice were purchased from the National Cancer Institute (Frederick, MD). Bcl6fl/fl (B6.129S(FVB)-Bcl6tm1.Dent/J) (109), Tbx21fl/fl (B6.129-Tbx21tm2Sn/J) (149), Rorcfl/fl (B6(Cg)-Rorc tm3Lin/J), Lck-cre (B6.Cg-Tg(Lck-icre)3779Nik/J), Tcratm1 (B6.129S2-Tcratm1Mom/J), CD154−/− (B6.129S2-Cd40lgtm1Imx/J) (150), H2d/LiAb1-Ea (MHCII-deficient; B6.129S2-H2d/LiAb1-Ea/J) (151), and B cell-deficient (µMT; B6.129S2-Ighm tm1Cgn/J) (152) mice were purchased from The Jackson Laboratory. Mice with floxed alleles were crossed to Lck-cre mice to obtain mice with two floxed alleles and one Lckcre allele. Mice with floxed alleles but lacking the Lckcre allele served as controls. A.L. Dent (Indiana University) provided WT and Bcl6−/− mice (153). All mice were housed in specific pathogen–free conditions at the
University of Minnesota. Experimental protocols were performed in accordance with guidelines of the University of Minnesota Institutional Animal Care and Use Committee and National Institutes of Health.

**Immunizations.** Biotinylated 2W peptide (GenScript) was mixed with streptavidin-PE (ProZyme) in PBS at a 4:1 molar ratio to form 2W-PE. Each mouse was injected i.p. with 100 µl of 0.6 µg 2W peptide conjugated to 25 µg PE emulsified in 100 µl CFA (Sigma-Aldrich).

**Bone marrow chimeras and cell transfer experiments.** Recipient CD45.1+ mice were irradiated twice with 500 rad with 6 hours between doses. After the second irradiation, 1 x 10^6 CD45.2+ Bcl6−/− or WT bone marrow cells were injected into recipients. After 8-10 weeks, CD45.2+ Bcl6−/− or WT T cells T cells were isolated by negative selection using the EasySep™ Mouse T Cell Isolation Kit (Stemcell) with added biotin-conjugated CD45.1 Ab (eBioscience. Thirteen-16 x 10^6 CD45.2+ Bcl6−/− or WT CD4+ T cells were injected into Tcra−/− T cell-deficient mice before immunization with 2W-PE in CFA the next day. B cells were isolated from WT and MHCII-deficient mice using a negative selection kit (Miltenyi Biotec) and 88 x 10^6 B cells were injected separately into µMT mice before immunization with 2W-PE in CFA.

**Cell enrichment and flow cytometry.** Single cell suspensions of spleens and lymph nodes were split equally for Th and B cell analyses. For 2W:1-A^b-specific T cell analysis, cells were stained with fluorochrome-conjugated CXCR5 (2G8; BD) Ab and
allophycocyanin-conjugated I-A<sup>b</sup> tetramer containing 2W peptide (EAWGALANWAVDSA) for one hour at room temperature. Tetramer-bound cells were positively enriched using allophycocyanin-specific magnetic isolation (Stemcell). Tetramer-enriched cells were stained with fluorochrome-labeled Abs specific for B220 (RA3-6B2; all Abs from eBioscience unless otherwise indicated), CD11b (M1/70), CD11c (N418), CD44 (IM7), PD-1 (J43), CD90.2 (53-2.1), or CD4 (GK1.5; BD). Cells were incubated in fixation/permeabilization buffer (eBioscience) and the fluorochrome-labeled Bcl-6 (K112-91; BD), T-bet (4B10; BioLegend), and RORγt (Q31-378; BD) Abs in permeabilization buffer (eBioscience).

For PE-specific B cell analysis, spleens and lymph node fragments were incubated with Dispase (Invitrogen), collagenase P (Roche), and DNase I (Roche) at 37°C for 20 min. The released cells were mixed with unlabeled CD16/CD32 (2.4G2) Ab (Tonbo) and fluorochrome-labeled Abs specific for IgG1 (A85-1; BD), IgG2b (polyclonal; Life Technologies), IgG3 (polyclonal; Life Technologies), or IgA (C10-3; BD). Cells were then incubated with 1 µg of PE (ProZyme) for 30 minutes at 4°C (15). PE-bound B cells were positively enriched using PE-specific magnetic isolation (Stemcell). PE-enriched B cells were stained with fluorochrome-labeled Abs against CD90.2 (53-2.1), CD11c (N418), F4/80 (BM8), GR1 (RB6-8C5), CD38 (90), IgM (II/41), GL7 (GL-7), IgD (11-26c.2a; BD), and B220 (RA3-6B2; BD). Cells were fixed with Fixation/Permeabilization buffer (BD) and stained with fluorochrome-labeled Abs against IgG1, IgG2b, IgG3, and IgA followed by biotin-labeled IgG2c Ab (IgG2a [b]; 5.7; BD) and then IgG [H+L] Ab (Life Technologies). Cells were analyzed on a Fortessa (Becton Dickinson) flow cytometer and analyzed with FlowJo (TreeStar).
**Statistical analysis.** Statistical tests were performed using Prism (Graphpad) software. Data were log transformed and $p$ values were obtained from a one-way ANOVA and Dunnett post-test comparing all groups to the WT control group with a 95% confidence interval. In cases where only two groups are compared to one another, $p$ values were obtained from two-tailed unpaired $t$ tests were performed with a 95% confidence interval.

4.3 Results and Discussion

**Analysis of mice deficient in Tfh, Th1, and Th17 cells**

We used mice lacking functional exons of Th lineage-defining transcription factors (16, 17, 19) to study the contribution of Th cell subsets to B cell responses. WT, Lck-cre$^+\text{Bcl6}^{fl/fl}$ (Tfh-deficient), Lck-cre$^+\text{Tbx21}^{fl/fl}$ (Th1-deficient), Lck-cre$^+\text{Rorc}^{fl/fl}$ (Th17-deficient), and Tcra$^-$ (T cell-deficient) C57BL/6 (B6) mice, which express the I-A$^b$ MHCII molecule, were immunized with a CFA emulsion containing an immunogenic I-A$^b$ binding peptide called 2W (122) linked to phycoerythrin (PE) (2W-PE). This immunization strategy allowed for analysis of 2W:I-A$^b$-specific CD4$^+$ T cell and PE-specific B cell responses in the same host (117). 2W:I-A$^b$-specific CD4$^+$ T cells in secondary lymphoid organs were identified by flow cytometry after 2W:I-A$^b$ tetramer staining and magnetic bead enrichment (79) (Fig. 12A). WT mice, which have about 300 2W:I-A$^b$-specific CD4$^+$ naïve T cells (79), contained about 6,000 2W:I-A$^b$-specific CD4$^+$ CD44$^{high}$ effector T cells in secondary lymphoid organs on day 11 after immunization with 2W-PE in CFA (Fig. 12B). The expanded effector cell population in WT mice
contained on average 1,300 CXCR5+ PD-1− Tfh and 1,000 CXCR5+ PD-1+ GC-Tfh, 600 CXCR5− T-bet+ Th1, and 500 RORγt+ Th17 cells (Fig. 12C-D). The Bcl-6-deficient effector T cell population contained 10-30-fold fewer Tfh and GC-Tfh cells than the WT population but had normal numbers of Th1 and Th17 cells (Fig. 12D). Mice with T-bet-deficiency generated 10-folder fewer 2W:I-Ab-specific Th1 cells than WT mice but had normal numbers of Tfh, GC-Tfh, and Th17 cells, whereas RORγt-deficient mice generated 10-fold fewer Th17 and 4-fold fewer Tfh and GC-Tfh cells than WT mice but had normal numbers of Th1 cells. These results demonstrate that the expected T cell subsets were missing in mice lacking Bcl-6, T-bet, or RORγt with exception that RORγt-deficiency created a small reduction in Tfh and GC-Tfh cells in addition to Th17 cells.

Antigen-specific B cell analysis in mice deficient in Tfh, Th1, and Th17 cells

We then assessed the roles of individual CD4+ T cell subsets in the PE-specific B cell response. PE-specific B cells, PBs, GC B cells (Fig. 13A) and isotype-switched PBs cells (Fig. 13C) were detected in secondary lymphoid organs by flow cytometry after PE staining and magnetic bead enrichment (80). The ~20,000 naive PE-specific B cells in WT mice (80) produced about 300,000 activated B cells by day 11 after immunization with 2W-PE in CFA (Fig. 13B). The activated B cell population contained 70,000 CD38− GL7+ GC B cells (Fig. 13B) and 40,000 intracellular Ighigh isotype-switched (IgM− IgD−) PBs (Fig. 13C-D) including cells with the IgG1, IgG2b, IgG2c, IgG3, or IgA isotypes (Fig. 16). In contrast, only 40,000 activated PE-specific B cells, including less than 100 GC B cells and 400 isotype-switched PBs were generated in T cell-deficient Tcra−/− mice (Fig. 13B,D), confirming the T cell dependence of the PE-specific response (80). Mice
with T cell-specific deficiency in Bcl-6 and lacking Tfh cells produced 2-fold fewer PE-specific activated B cells than WT mice. This reduction was due to a 15-fold drop in the number of PE-specific GC B cells, confirming that GC B cell formation depends on Tfh cells (33). Surprisingly, however, mice lacking Tfh cells produced on average 22,000 isotype-switched PBs, a number that was not significantly different from the 37,000 produced in WT mice. The isotype switched PE-specific PB population that formed in mice with T cell-specific Bcl-6 deficiency also had the same composition of isotypes as WT mice (Fig. 16). Similarly, mice with T cell-specific deficiencies in T-bet or RORγt and lacking Th1 or Th17 cells, respectively, produced the same number of PE-specific total and GC B cells, and isotype-switched PBs with the same isotypes as WT mice. These results indicate that although formation of isotype-switched PBs is T cell-dependent, Tfh, Th1 or Th17 cells are not uniquely required for this function.

**Confirmation that early switched PBs form in the absence of Tfh cells**

Recent work in an influenza infection model showed that production of IgG1 Abs was reduced in mice without Tfh cells (148). Thus, it was surprising to find that PE-specific IgG1+ PBs formed normally in Lck-cre⁺\(^{Bcl6Δ/Δ}\) Tfh-deficient mice (Fig. 16). It was concerning, however, that these mice still generated some PE-specific GC B cells (Fig. 13B). Lck-cre⁺\(^{Bcl6Δ/Δ}\) mice have a deletion in exons 7-9 (109) of the Bcl6 gene and therefore could express a truncated but partially functional version of the Bcl-6 protein that could have supported weak Tfh formation in the experiment shown in Fig. 12. Residual Tfh cells may have been sufficient for the weak GC B cell and normal isotype-switched PB formation observed in these mice (Fig. 13). This concern was ameliorated
by transferring $Bcl6^{-/-}$ T cells, which completely lack the $Bcl6$ gene, into T cell-deficient mice prior to immunization with 2W-PE in CFA. Recipients of $Bcl6^{-/-}$ T cells made no more PE-specific GC B cells than mice that did not contain T cells, and many fewer than T cell-deficient mice that received WT T cells (Fig. 14A,C). T cell-deficient mice that received $Bcl6^{-/-}$ T cells, however, generated the same number of isotype-switched PBs that had the same distribution of isotypes as T cell-deficient mice that received WT T cells (Fig. 13B,C). Thus, T cell-dependent isotype-switched PB formation does not require Tfh cells in this system.

**Early switched PBs require interactions with CD4$^+$ T cells and CD40 signaling**

Previous work showed that T cells other than CD4$^+$ T cells can contribute to humoral responses. For example, $\gamma\delta$ and NK T cells can influence isotype switching (154, 155). We therefore explored the nature of the T cell-dependence of isotype-switched PB formation by determining whether it depended on p:MHCII presentation by B cells as expected if cognate interaction between Th cells and B cells is required. B cells from WT or $H2^{dIab1-Ea}$ (MHCII-deficient) mice (151) were transferred into B cell-deficient $\mu$MT mice (152) before immunization with 2W-PE to test this possibility. $\mu$MT recipients of MHCII-deficient B cells formed 200-fold fewer PE-specific isotype-switched PBs than recipients of WT B cells (Fig. 15A-B) indicating that cognate interactions with Th cells were required. We then determined whether CD40 signaling in the B cells, which occurs during cognate interactions with CD154$^+$ Th cells (133), was also required for PE-specific isotype-switched PB formation. Indeed, PE-specific isotype-switched PB
formation was as defective in *Cd154*−/− mice immunized with 2W-PE in CFA as in mice lacking all Th cells (Fig. 15C-D).

Our results suggest a model in which early cognate interactions between undifferentiated Th cells and B cells are sufficient for the generation of isotype-switched PBs. The independence of this response from Tfh cells, which use CXCR5 to exert their actions in follicles (62, 156, 157) indicates that it could occur outside of this location. These results are in line with previous work showing that isotype switching can be achieved by extrafollicular PBs, outside of the Tfh-rich GC environment (158, 159). Our work provides the additional insight that this process does not rely on other Th subsets like Th1 and Th17 cells.

Recently, it has been shown that antigen-stimulated Th cells rapidly induce the G-protein-coupled receptor EBI2 (GPR183) (160) and migrate to the outer T cell zone to meet antigen-stimulated B cells (136, 137). Because this migration does not require CXCR5 it could be achievable by as yet undifferentiated Th cells. Once in the outer T cell zone, these Th cells could deliver CD40 signals to their B cell partners, driving them to proliferate and become extrafollicular PBs.
FIGURE 12. Analysis of 2W:I-A\textsuperscript{b}-specific T cells in Tfh-, Th1- and Th17-deficient mice. (A) CD4\textsuperscript{+} 2W:I-A\textsuperscript{b} tetramer\textsuperscript{+} cells (shown in the rectangular gates) were identified amongst B220\textsuperscript{−}CD11b\textsuperscript{−} CD11c\textsuperscript{−} CD4\textsuperscript{+} T cells from 2W:I-A\textsuperscript{b} tetramer-enriched spleen and lymph node samples from the indicated mice immunized with 2W-PE/CFA 11 days earlier. (B) Numbers of 2W:I-A\textsuperscript{b}-specific cells from the groups shown in (A). (C) Identification and (D) percentages of 2W:I-A\textsuperscript{b}-specific Tfh, GC-Tfh, Th1, and Th17 cells (from gate in (A)), based on CXCR5, PD-1, T-bet, and ROR\gamma\textsuperscript{t} expression. Data are expressed as mean values and are representative of two independent experiments (n = 3-6 mice/group). p values were obtained from a one-way ANOVA and Dunnett post-test that compares all groups to the WT control group (* p < 0.05, ** p < 0.01, *** p < 0.001).
Early T cell–dependent PBs form in the absence of individual CD4+ T cell subsets. (A) CD90.2− CD11c− F4/80− Gr-1− cells from PE-enriched spleen and lymph node samples from the indicated mice immunized with 2W-PE/CFA 11 days earlier with a gate on PE-binding B cells. (B) PE-binding B cells from (A) with gates on B220low IgG [H+L]hi plasmablasts (PB) or B220hi IgG [H+L]int B cells. (C) B220hi IgG [H+L]int B cells from (B) with a gate on CD38− GL7+ GC B cells. (D) PB from (B) with a gate on IgM− isotype-switched cells. (E) Numbers of PE-specific total B cells (left panel), GC B cells (middle panel), and IgM− IgD− PBs (right panel). (F) Numbers of...
isotype switched PB from gate in (B) that are IgA⁺, IgG1⁺, IgG2b⁺, IgG2c⁺, or IgG3⁺.

Data are expressed as the mean value and are representative of two independent experiments (n = 2-6 mice/group). *p* values were obtained from a one-way ANOVA and Dunnett post-test that compares all groups to the WT control group (* p < 0.05, *** p < 0.001).
FIGURE 14. Transfer of Bcl6<sup>−/−</sup> CD4<sup>+</sup> T cells into TCRα knockout mice confirms that plasmablasts form in the absence of Tfh cells. CD45.2<sup>+</sup> WT or Bcl6<sup>−/−</sup> T cells were isolated and transferred into T cell-deficient mice, which were subsequently immunized with 2W-PE/CFA. B6 and T cell-deficient mice with no T cells transferred into were also immunized as controls. After 11 days, PE-specific B cells were enriched from the spleen and lymph nodes. (A) CD90.2<sup>−</sup> CD11c<sup>−</sup> F4/80<sup>−</sup> Gr-1<sup>−</sup> PE-specific B cells from the indicated groups with a gate on B220<sup>hi</sup> IgG [H+L]<sup>int</sup> CD38<sup>−</sup> GL7<sup>+</sup> GC B cells or (B) on B220<sup>int</sup> IgG [H+L]<sup>hi</sup> plasmablasts. (C) Numbers of PE-specific GC B cells (left panel) and IgM<sup>−</sup> IgD<sup>−</sup> plasmablasts (right panel). Data are expressed as mean values and are representative of two independent experiments (n = 2-5 mice/group). p values were obtained from a one-way ANOVA and Dunnett post-test that compares all groups to the control group (WT T cell transfer into T cell-deficient group) (* p < 0.05, *** p < 0.001).
CD40 signaling

Previous work showed that T cells other than CD4

... PBs formed normally in Lck-cre (17). Thus, it was surprising to find that PE-specific IgG1

Confirmation that early switched PBs form in the absence of Tfh cells

... Th cells were required. We then determined whether CD40

B cells from WT or CD154-deficient mice formed 200-fold fewer

... Tfh cells in this system.

Our results suggest a model in which early cognate inter-

FIGURE 15. Early plasmablasts require interactions with CD4+ T cells and CD40-

CD40L signaling. (A) CD90.2− CD11c− F4/80− Gr-1− PE-specific WT or MHCII-

deficient B cells from μMT recipient mice on day 11 after 2W-PE/CFA immunization

with a gate on on B220int IgG [H+L]hi plasmablasts. (B) Numbers of PE-specific total B

cells (left panel) or IgM− IgD+ plasmablasts of the indicated types (right panel). (C)

CD90.2− CD11c− F4/80− Gr-1− PE-specific B cells from T cell-deficient, WT, or CD154-

deficient mice on day 11 after 2W-PE/CFA immunization with a gate on on B220int IgG
[H+L]^{hi} plasmablasts. (D) Numbers of PE-specific total B cells (left panel) or IgM⁻ IgD⁻ plasmablasts of the indicated types (right panel). Data are expressed as mean values and are representative of two independent experiments ($n = 2-5$ mice/group). In (B), the $p$ value was obtained from a two-tailed unpaired $t$ test ($*** p < 0.001$). In (D), $p$ values were obtained from a one-way ANOVA and Dunnett post-test that compares all groups to the WT control group ($** p < 0.01, *** p < 0.001$).
FIGURE 16. PE-specific isotype switched plasmablasts after 2W-PE/CFA immunization. (A) Representative isotype gating strategy for IgM− IgD− PBs gated in Fig. 13C. (B) Numbers of isotype switched PBs that are IgA+, IgG1+, IgG2b+, IgG2c+, or IgG3+. Data are expressed as the mean value and are representative of two independent experiments (n = 2-6 mice/group). P values were obtained from a one-way ANOVA and Dunnett post-test that compares all groups to the WT control group (**p < 0.001).
Chapter 5

Conclusions

This work focuses heavily on the importance of repression for establishing CD4⁺ T cell fates. BCL6 is the master transcription factor required for Tfh differentiation and solidifies this fate decision by inhibiting the expression of genes that promote differentiation into other lineages. For example, BCL6 enhances the Tfh fate by repressing TBET and RORγt to suppress the Th1 and Th17 fates, respectively (23). DNA binding proteins enlist the help of corepressors to enhance repressive function at genetic target sites. Therefore, we hypothesized that recruitment of corepressors by BCL6 plays an important role in establishing Tfh differentiation. BCOR is a corepressor that potentiates transcriptional repression by BCL6 as part of a variant Polycomb complex, which make epigenetic modifications that silence target genes (73). We demonstrate that mice with a T cell-targeted BCOR deficiency exhibit a substantial loss in peptide:MHCII-specific Tfh cells following *Listeria monocytogenes* infection and immunization with peptide in CFA. The loss in Tfh cells correlated with a defect in GC B cell formation, as expected. These results suggest that BCL6-mediated repression via corepressor recruitment is critical for optimal Tfh differentiation and humoral immunity.

During our initial studies in discovering the importance of BCOR-mediated repression for Tfh differentiation, we also found that BCOR plays a positive role in Th17 differentiation after peptide in CFA immunization. This result drove our examination of the CD4⁺ T cell response in T cell-targeted BCOR deficient mice after a Th17-inducing pathogen like *Streptococcus pyogenes*. We characterized a mechanism by which BCOR
promotes Th17 formation by repressing genes that inhibit the Th17 lineage. Because the absence of BCL6 did not negatively impact Th17 formation, BCL6 is unlikely to recruit BCOR to enhance Th17 differentiation. Instead, a different BCOR-recruiting DNA binding protein, KDM2B, was important for Th17 differentiation, suggesting that KDM2B recruits BCOR for enhancing Th17 differentiation. We applied genome-wide expression and BCOR chromatin immunoprecipitation studies to show that BCOR directly represses Lef1, Runx2, and Dusp4, whose products inhibit Th17 differentiation. Together, the results suggest that PRC1.1 components, BCOR and KDM2B, work together to enhance Th17 cell formation by repressing Th17 fate suppressors.

While there are genes that need to be turned on for each CD4+ T cell subset to function, it is also important to turn off genes that would re-direct the subset to another lineage. For example, RORγt promotes Th17-specific genes such as Il17a and Ccr6 (20, 21). RORγt also inhibits expression of Tbx21 and Foxp3 (20, 22), which encode the master transcription factors for Th1 and Treg cells, respectively (16, 18). The BCOR studies in this body of work provide additional evidence for the importance of repressive mechanisms to establish a given CD4+ T cell fate.

Differentiation of Th17 cells is an important area of research because of the role of this process in protective immunity. Expression of CCR6 directs Th17 cells to effector mucosal sites (27, 28) where they secrete cytokines. These cytokines act on other immune cells including neutrophils to promote clearance of extracellular bacteria, such as Streptococcus pyogenes, Klebsiella pneumoniae, and Bordetella pertussis (29, 30, 97-100). Identifying the drivers of Th17 cells will inform the development of a Th17-focused Streptococcus pyogenes vaccine for humans, which is currently unavailable.
Another major component of this thesis is the study of CD4\(^+\) T cell help to B cells. There is a large body of evidence describing the contribution of Tfh cells for maintaining the GC reaction (33) and driving the production of high affinity antibodies against pathogens. The contribution of other CD4\(^+\) T cell subsets to B cell responses has not been fully explored \textit{in vivo}. We addressed this issue by analyzing the T cell-dependent B cell response to the protein antigen PE in mice lacking specific CD4\(^+\) T cell subsets. As expected, PE-specific GC B cell production required Tfh cells. However, Tfh-, Th1-, or Th17-deficient mice produced as many PE-specific isotype-switched plasmablasts as wild-type mice. This response depended on CD4\(^+\) T cell expression of CD40L and antigen presentation by B cells. These results indicate that many CD4\(^+\) T cell subsets can promote plasmablast formation by providing CD40 signals to B cells.

Non-Tfh subsets that promote plasmablast formation lack CXCR5 expression. Therefore how might non-Tfh cells traffic to the T-B border to provide B cell help? One possibility is G-protein-coupled receptor EBI2 encoded by the \textit{Gpr183} gene, which is induced in antigen-specific CD4\(^+\) T cells upon their activation by DCs (160). EBI2 expression allows for migration to the outer T cell zone where CD4\(^+\) T cells interact with activated B cells in an antigen-specific manner within 24 hours after the initiation of the immune response (136, 137). We propose a model whereby DCs activate antigen-specific CD4\(^+\) T cells early after an immune response, leading to their migration to the T-B border. Once there, these undifferentiated CD4\(^+\) T cells deliver CD40 signals to activated B cells, driving them to proliferate and become extrafollicular PBs. This work is significant because better understanding the process of CD4\(^+\) T cell help for generating antibody-secreting B cells is critical for producing more efficacious vaccines.
References


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