UNIVERSITY OF CALIFORNIA, SAN DIEGO

Bcl6 and Blimp-1 regulate the differentiation of Follicular Helper T cells

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Biomedical Sciences

by

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2011

The Dissertation of Robert James Johnston is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2011

DEDICATION

To my family. They got me here.

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COMMON ABBREVIATIONS

T_{FH}: Follicular Helper T cell

Bcl6: B-cell CLL/lymphoma 6

Blimp-1: B lymphocyte-induced maturation protein 1

RV: retrovirus

IL: interleukin

CD: cluster of differentiation

IFN: interferon

SMtg or SM: SMARTA TCR transgenic $CD4^+$ T cell

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ACKNOWLEDGEMENTS

None of this work would have been possible without the support of many people. Foremost is my thesis advisor, Shane Crotty. Shane is the finest scientist I have ever met, and his insights and guidance underlie all of the work presented here. I have been extraordinarily lucky to be a part of Shane's lab, and I am deeply indebted to him for his mentorship.

I am also immensely grateful to all of my colleagues in the Crotty lab, especially:

Ish Yusuf, who showed me how to navigate life in the lab. Ish was a wonderful teacher, and it was her hard work that paved the way for our identification of Bcl6 and Blimp-1 as key regulators of T_{FH} differentiation.

Jeff Diamond, who worked side by side with me on many of the experiments in Chapters 3, 4, and 5. Jeff's energy was infectious, and he was always ready to take on tough projects and ask questions.

Daniel DiToro, who provided assistance with the experiments in Chapter 2, particularly by pulling off the last and most technically challenging experiment requested by the reviewers.

Megan McCausland, who taught me almost every technique I've ever used. I can't imagine where I would be without her training. I'll never have her speed or her hands, though.

Danelle Eto, whose patience and good spirits were inspiring. She always had time to answer a question, even the third time I asked it!

Mark Kroenke, with whom I've had many excellent and insightful conversations. I'll miss his thoughts on papers, and his comments on my own manuscripts.

Youn Soo Choi, whose superb work on ICOS and early T_{FH} differentiation helped me make sense of the STAT3 and STAT5 data in Chapters 4 and 5.

Mohammed Rafii-El-Idrissi Benhnia, who kept me on my toes and always made time for a friendly chat.

Robin Kageyama, who was a delight to work with. Robin also kept me from complete computer illiteracy.

Burt Barnett, who has to be the most committed and enthusiastic guy that came through the lab. I also thank him for being remarkably gracious when we discovered that we had spent several months studying an artifact.

Lindsay Crickard, who managed almost our entire mouse colony perfectly. She saved countless experiments by reminding me to order mice.

Chris Lao, whose lunchtime exploits were inspiring, and who burned Guy Fawkes in effigy with me.

Tania Escobar, whose cheerfulness brightened the entire lab.

And Priscilla Colby, who kept the lab from falling apart.

I thank the staff of the LIAI Imaging Facility and the Department of Laboratory Animal Care for their invaluable technical assistance.

I also thank the additional members of my thesis committee: Co-chair Steve Hedrick, Victor Nizet, Ananda Goldrath, and Kees Murre. Their advice and patience were instrumental at many points, and every thesis committee meeting was extraordinarily helpful. I also have to acknowledge Steve Schoenberger, who always made time for me when I came to him with questions. Steve was practically a sixth member of my thesis committee.

This work was done in collaboration with many outstanding scientists, who are acknowledged in each individual chapter.

Chapters 1, in part, is adapted from the material as it appears in *Nature Immunology*, volume 11: 114-120, 2010, with co-authors Shane Crotty and Steven P. Schoenberger. I am the second author of this publication.

Chapter 2, in full, is a reprint of the material as it appears in *Science*, volume 325: 1006-1010, 2009, with co-authors Amanda C. Poholek, Daniel DiToro, Isharat Yusuf, Danelle Eto, Burton Barnett, Alexander L. Dent, Joe Craft, and Shane Crotty. I am the first author of this publication.

Chapter 3, in full, is currently being prepared for submission for publication, with co-authors Jeffrey A. Diamond, Daniel DiToro, and Shane Crotty. I am the first author of this manuscript.

Chapter 4, in full, is currently being prepared for submission for publication, with co-authors Jeffrey A. Diamond, and Shane Crotty. I am the first author of this manuscript.

Chapter 5, in full, is currently being prepared for submission for publication, with co-authors Jeffrey A. Diamond and Shane Crotty. I am the first author of this manuscript.

Chapters 6, in part, is adapted from the material as it appears in *Nature Immunology*, volume 11: 114-120, 2010, with co-authors Shane Crotty and Steven P. Schoenberger. I am the second author of this publication. Figure 6.2 is adapted from the material as it appears in *Follicular Helper T cells (Tfh)*. Shane Crotty. Annual Review of Immunology, volume 29: 621-663, 2011.

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Shane Crotty, Robert J. Johnston, and Steven P. Schoenberger. *Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation.* 2010. **Nature Immunology** 11: 114-120.

Robert J. Johnston*, Amanda C. Poholek*, Daniel DiToro, Isharat Yusuf, Danelle Eto, Burton Barnett, Alexander L. Dent, Joe Craft, and Shane Crotty. *Bcl6 and Blimp-1 are Reciprocal and Antagonistic Regulators T Follicular Helper Cell Differentiation.* 2009. **Science** 325: 1006-1010. *, co-authors

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Manuscripts submitted or in preparation

Robert J. Johnston, Jeffrey A. Diamond, and Shane Crotty. *STAT5 is a negative regulator of Bcl6-mediated T_{FH} differentiation*. In preparation.

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ABSTRACT OF THE DISSERTATION

Bcl6 and Blimp-1 regulate the differentiation of Follicular Helper T cells

by

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Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2011

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$CD4^+$ T cells are potent regulators of adaptive immune responses. It is well known that there are at least four independent $CD4^+$ T cell effector subsets: T_H1, T_H2, T_H17, and T_{reg}. One critical function of $CD4^+$ T cells is to provide B cell help; however, it has been unclear if this help is provided by all $CD4^+$ T cells, by T_H2 cells, or by a fifth subset of effector cells. Because each effector subset is controlled by a master regulator (T-bet, GATA3, RORγt, and Foxp3 respectively), we hypothesized that if the $CD4^+$ T cells that provide B cell help constitute a distinct effector subset, they too would possess a master regulator.

Here, we show that the transcription factor Bcl6 is the master regulator of a subset of effector $CD4^+$ T cells that is specialized to provide B cell help: the Follicular Helper T cells (T_{FH}). $CD4^+$ T cells constitutively expressing Bcl6 differentiated fully

to T_{FH} *in vivo* and provided B cell help, resulting in increased germinal centers and enhanced antibody responses. Furthermore, $CD4^+$ T cells deficient in Bcl6 failed to differentiate into T_{FH} cells, and were unable to sustain the germinal center reaction. Multiple Bcl6 repression domains were required for optimal T_{FH} differentiation. Expression of Bcl6 was dependent on interaction with antigen-presenting cells, such as cognate B cells. Surprisingly, STAT3 signaling, which has been proposed to induce Bcl6 expression, was neither necessary nor sufficient to drive T_{FH} differentiation.

Negative regulation is also a critical component of most biological processes. Bcl6 and the transcription factor Blimp-1 are mutual antagonists, and we found that Blimp-1 was highly downregulated in T_{FH} cells. Forced expression of Blimp-1 blocked T_{FH} differentiation and help to B cells, but did not impair non-T_{FH} effector cell differentiation. STAT5 signaling collaborated with Blimp-1 *in vivo* to inhibit T_{FH} differentiation. Consequently, effector $CD4^+$ T cells lacking either Blimp-1 or STAT5 preferentially differentiated into T_{FH} cells *in vivo*.

These findings demonstrate that T_{FH} cells constitute a critically important and distinct $CD4^+$ T cell effector subset, and that Bcl6 and Blimp-1 play central but opposing roles in T_{FH} differentiation.

Chapter 1:

Introduction

1.1: Lymphocytes and immunity

In higher organisms, the immune response consists of both innate and adaptive components. The innate immune response is a front-line defense initiated by inflammation and by pattern recognition receptors that recognize highly conserved molecules expressed by pathogens but not by self. These receptors are germline-encoded and widely expressed, enabling the rapid detection and response to pathogens (1). However, because the innate immune response is not adaptable, many pathogens have developed ways to evade it. Lymphocytes, which drive the adaptive immune response, have evolved to overcome this shortcoming using antigen receptors. Unlike germline-encoded pattern recognition receptors, antigen receptors are the products of genetic recombination (2). Lymphocytes expressing randomly generated antigen receptors then undergo selection so that those lymphocytes that can recognize foreign but not self antigens survive. This results in a repertoire of lymphocytes that together are capable of recognizing millions of different foreign antigens with a high degree of specificity.

Lymphocytes consist primarily of $CD8^+$ T cells, $CD4^+$ T cells, and B cells. $CD8^+$ T cells, or cytotoxic T cells, are specialized to identify and kill host cells that have become infected with intracellular pathogens such as viruses (3). $CD4^+$ T cells, or helper T cells, are specialized to drive and shape the immune response by producing co-stimulation or "help" to other cells (4). B cells can differentiate into antibody-secreting cells (5). Antibodies are secreted forms of the B cell antigen receptor that

contribute to the immune response by binding antigens, which can result in the neutralization, opsonization, or death of a pathogen or infected cell.

The positioning of lymphocytes within lymphoid tissues is regulated by concentration gradients of chemoattractant cytokines (chemokines) (6). Expression of chemokine receptors enables lymphocytes to recognize chemokine gradients and to migrate into and within the regions containing the highest concentrations of the relevant chemokine. T cells and most dendritic cells express the chemokine receptor CCR7 (BLR2) and localize to T cell zones, areas with high concentrations of the CCR7 ligands CCL19 (MIP-3β) and CCL21 (SLC) (6). B cells express the chemokine receptor CXCR5 (BLR1), which attracts them to B cell follicles, which are areas where the CXCR5 ligand CXCL13 (BLC) is highly expressed (6). Antigen is transported into lymphoid tissues passively and by professional antigen presenting cells. Antigen presenting cells, which are activated by signals from the innate immune response, facilitate the circulation of antigen through lymphoid tissues to facilitate the activation and expansion of antigen-specific lymphocytes.

The need for lymphocyte expansion delays the adaptive immune response when an antigen is first encountered. However, exposure to antigen induces some lymphocytes to differentiate into memory cells, which are primed to rapidly proliferate and function if they encounter that antigen again (7). In addition, some antigen-specific B cells will continue to produce protective antibodies long after exposure to antigen (8). These memory lymphocytes and antibodies establish a state of immunity,

in which the immune system is capable of preventing or rapidly clearing a second infection.

One of the greatest achievements of modern medicine has been to marginalize infectious diseases by vaccination (9). Vaccines consist of harmless or weakened pathogens and antigens which enable the immune system to develop immunity to a pathogen without the attendant risks of the actual disease. The best example of the efficacy of vaccination is the elimination of *Variola major* and *Variola minor*, the causative viruses of smallpox, a disease estimated to be responsible for 200-300 million deaths in the 20^{th} century alone (10).

1.2: The germinal center reaction

Nearly every human vaccine induces a B cell response and the sustained production of protective antibodies (9, 11). B cell activation is initiated by recognition of cognate antigen through the B cell antigen receptor. Activated B cells subsequently migrate from the interior of the B cell follicle to the border of the T cell zone (12). There, they may encounter cognate $CD4^+$ T cells which can "help" the B cell by providing co-stimulation through surface co-receptors and secreted cytokines (12). Most helped B cells undergo a short period of proliferation and then terminally differentiate into short-lived plasma cells (antibody secreting cells), which elaborate a large secretory apparatus for producing relatively low affinity antibodies to quickly combat infection (7, 13). Alternatively, a small number of helped B cells enter into the germinal center reaction (14, 15).

Germinal centers are structures that transiently form within B cell follicles that consist of B cells, $CD4^+$ T cells, follicular dendritic cells and macrophages. Germinal center B cells have a minimal secretory apparatus, and are instead specialized for proliferation and somatic hypermutation of the B cell receptor (14, 15). B cell receptor mutation results in a population of B cells with varying affinities for a given antigen. These cells compete for help from helper $CD4^+$ T cells (discussed in greater detail below), with only the highest affinity B cells being selected for survival (16). After repeated rounds of this complex process, B cells capable of producing high affinity antibodies exit the germinal center reaction and differentiate into long-lived plasma cells and memory B cells (14, 15) (Fig. 1). These cells comprise the cellular components B cell-mediated immunity (7, 17).

A key regulator of germinal center B cell differentiation is the transcription factor B-cell CLL/lymphoma 6 (Bcl6), which was first identified as a proto-oncogene frequently expressed in non-Hodgkin's lymphomas as a result of chromosomal translocations (14, 18-21). Bcl6 protein was subsequently found to be highly expressed in germinal center B cells, where it functions as an inducer of proliferation and an inhibitor of the DNA damage response (14). Bcl6 suppresses a collection of DNA damage response genes and inducers of apoptosis and cell cycle arrest (p53, cyclin D2, ATR), while facilitating activation-induced cytidine deaminase (AID) expression in germinal center B cells (14). AID induces class switch recombination and somatic hypermutation (22), which would result in cell cycle arrest and apoptosis if these normal DNA damage responses were not suppressed by Bcl6 (14). Bcl6-

deficient mice lack germinal center B cells and affinity maturation (23-25). Bcl6-deficient B cells are nevertheless able to differentiate into plasma cells and secrete antibodies (24, 26), although long-term antibody responses in *Bcl6*$^{-/-}$ mice are severely decreased since the germinal center reaction is required to produce the majority of long-lived plasma cells (25). Constitutive expression of Bcl6 in B cells *in vivo* results in large germinal centers, again confirming the central role of Bcl6 in germinal center B cell differentiation (27).

Plasma cell differentiation, in contrast, critically depends on B lymphocyte-induced maturation protein 1 (Blimp-1, Blimp1, the product of the *Prdm1* gene) (28, 29) and the absence of Bcl6 (14, 29). Blimp-1's role in B cell development was first demonstrated when ectopic Blimp-1 expression was able to induce human B lymphoma cells to differentiate into cells having plasma cell features (30). Blimp-1 is highly expressed in plasma cells and controls many genes important for plasma cell differentiation (29, 31-33), including XBP-1, which induces formation of the secretory apparatus necessary for a high level of antibody production (34, 35). Blimp-1 also inhibits genes for cellular proliferation such as Myc and Bcl6, which thereby allows the terminal differentiation of plasma cells to a post-mitotic state (29, 36). These data indicate that Blimp-1 is a master regulator of plasma cell differentiation, with at most minor roles in germinal center B cell and memory B cell differentiation.

Bcl6 and Blimp-1 are antagonistic master regulators of B cell differentiation, with both playing essential roles in the development of humoral immunity (Fig. 1.1).

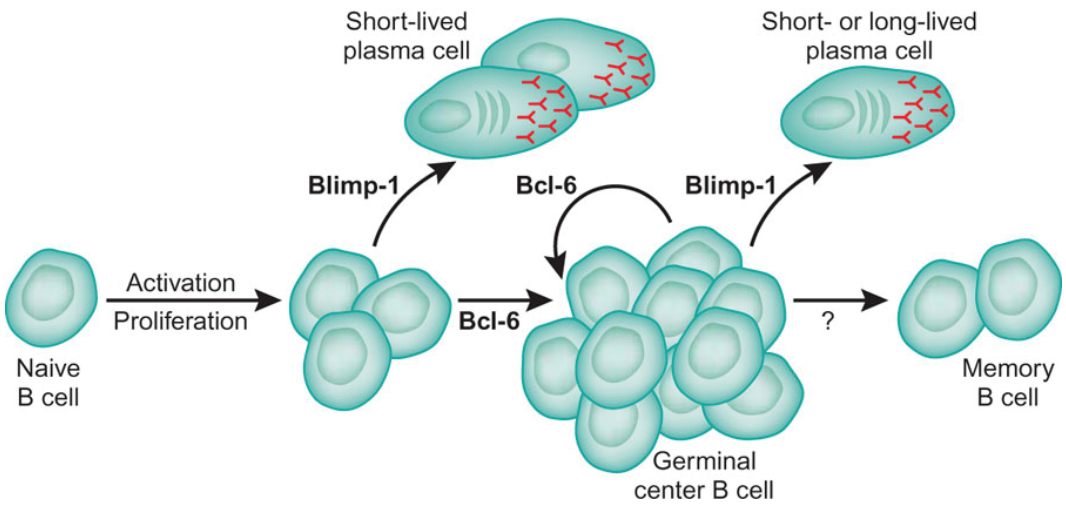

Figure 1.1: B cell differentiation and the roles of Bcl6 and Blimp-1.
Activated B cells can differentiate into plasma cells or germinal center B cells. Selected germinal center B cells further differentiate into plasma cells or memory B cells. Bcl6 antagonizes Blimp-1 and is a critial regulator of germinal center B cell differentiation and survival. Blimp-1 antagonizes Bcl6 and is a critical regulator of plasma cell differentiation and function.

Adapted from:

Shane Crotty, Robert J. Johnston, and Stephen P. Schoenberger. *Effectors and memories: Bcl6 and Blimp-1 in T and B lymphocyte differentiation.* Nature Immunology, vol. 11: 114-120, 2010.

1.3: Follicular Helper T cells

Effector $CD4^+$ T cells select from multiple phenotypes which are each specialized to drive a particular type of immune response. Viral infections usually induce the differentiation of type 1 $CD4^+$ T cells (T_H1 cells). T_H1 cells sustain an immune response that is optimal for combating intracellular pathogens such as viruses (4). In contrast, extracellular pathogens induce the differentiation of T_H2 effector cells (4). T_H17 cells are effector $CD4^+$ T cells that are distinguished by their production of the cytokine IL-17 (37). Regulatory T cells (T_{regs}) are also recognized as a distinct subset that suppresses immune responses (38).

The process by which a $CD4^+$ T cell differentiates into a particular type of effector cell has been extensively studied. Two transcription factors, T-bet and GATA3, were found to control T_H1 and T_H2 differentiation, respectively, and so were named "master regulators". It was later demonstrated that T_H17 and T_{reg} differentiation were controlled by the transcription factors RORγT and FoxP3, respectively (37, 38). Each of these master regulators was found to be under the control of a distinct set of cytokines and signal transduction molecules, and each was shown to drive the expression of a distinct gene program that determined the cell's effector functions (4). As a result, a consensus developed that in order to be considered an independent subset, a population of effector $CD4^+$ T cells must possess a unique master regulator that is induced by a distinct differentiation pathway.

Studies of human tonsils, which typically contain large germinal centers, revealed that some $CD4^+$ T cells expressed CXCR5 and so were able to migrate into B

cell follicles (39-41). Interestingly, these $CD4^+$ T cells expressed the germinal center B cell transcription factor Bcl6, and were superior to other $CD4^+$ T cells at providing B cell help *in vitro* (40-45). Because these $CD4^+$ T cells were specialized to co-localize with and provide help to B cells, they were named follicular helper T cells (T_{FH}) (40).

B cells elicit help from $CD4^+$ T cells by presenting cognate antigen epitopes bound to major histocompatibility complex (MHC) class II (16). Binding by the T cell's antigen receptor provides stimulatory signals to both the B cell and the T cell. Direct cell-cell contact is also required for the interaction between several coreceptors, including CD40L-CD40(16), ICOS-ICOSL (46), and Signaling Lymphocyte Activation Molecule (SLAM) family receptors (47), which also provide crucial survival and differentiation signals to both two cells. Stimulated $CD4^+$ T cells also produce cytokines that play crucial roles in the germinal center reaction. Interleukin 21 (IL-21) can induce expression of both Bcl6 and Blimp-1 in B cells, and so is thought to be essential for both plasma cell and germinal center B cell differentiation and survival (48-50). Interferon gamma (IFNγ) (51), IL-4 (51), and IL-17 drive B cell antibody class switching (52). B cells require sustained help from $CD4^+$ T cells through both cell-cell contact and cytokines to undergo the germinal center reaction.

1.4: Are T_{FH} cells a distinct subset?

It has long been unclear which $CD4^+$ T cells provide B cell help *in vivo*. T_H2 cells were long considered to be the B cell helpers because of *in vitro* studies and the

ability of the T_H2 cytokine IL-4 to induce B cell activation (53). Later studies suggested that any effector $CD4^+$ T cell could further differentiate into T_{FH} cells and drive the germinal center reaction. $CD4^+$ T cells differentiated *in vitro* into T_H1, T_H2, and T_H17 cells are all capable of providing B cell help *in vivo* (54-59). Distinguishing T_{FH} cells phenotypically was also difficult. All $CD4^+$ T cells transiently express CXCR5 after activation (60). Similarly, many other T_{FH} markers, such as ICOS, PD-1, and IL-21, are expressed by other effector $CD4^+$ T cells (60).

However, other evidence suggested that T_{FH} cells were independent of the known effector subsets (Fig. 1.2). T_H1, T_H2, and T_H17 effector differentiation can each be blocked without impairing the B cell response (61-64). The absence of SAP, a small adaptor protein encoded by the gene *sh2d1a*, causes X-linked lymphoproliferative syndrome, an immunodeficiency disease characterized in part by an inability to form germinal centers (65, 66). Using SAP-deficient mice, it has been shown that $CD4^+$ T cells are dependent on SAP to provide B cell help and drive germinal center formation (67). Yet $CD4^+$ T cell effector differentiation appeared normal in $SAP^{-/-}$ mice, suggesting that the differentiation of T_{FH} cells but not other effector $CD4^+$ T cells was uniquely dependent on SAP (67).

To determine if T_{FH} cells are a distinct effector subset, we examined the transcription factors that regulate T_{FH} differentiation. Surprisingly, we found that Bcl6 and Blimp-1, which were well known to be key regulators of B cell differentiation during the germinal center reaction, were critical regulators of T_{FH} differentiation (68).

These studies are described in the following chapters, which consist of both published (Chapter 2) and unpublished (Chapter 3-5) work.

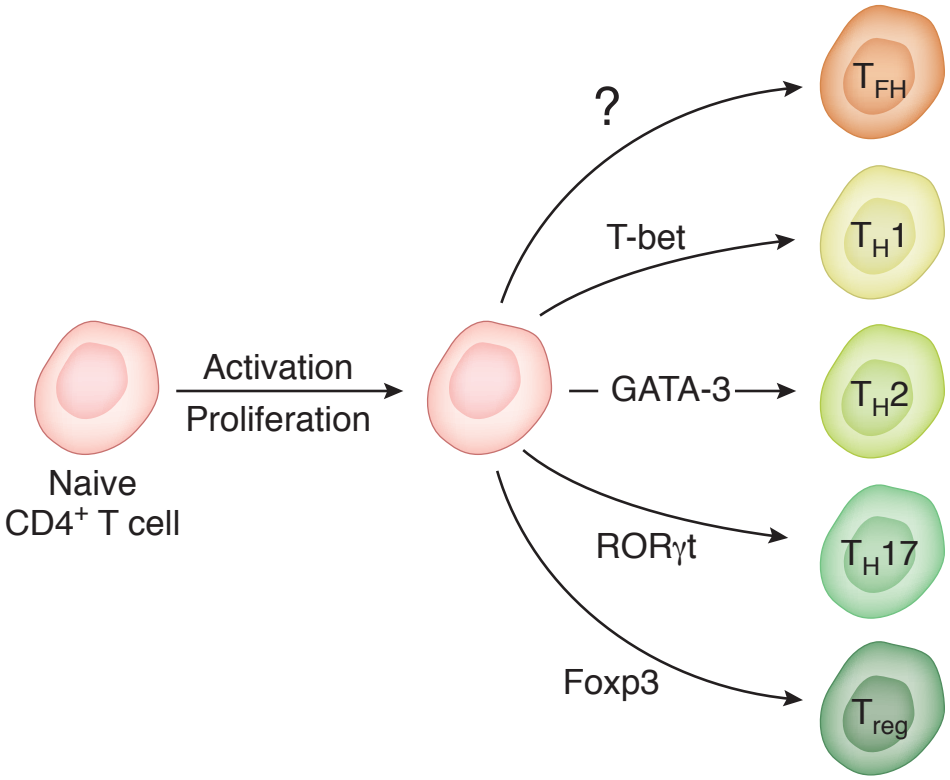

Figure 1.2: Effector CD4⁺ T cell differentiation.
Activated CD4⁺ T cells can differentiate into multiple distinct effector subsets. Each effector subset posseses a unique differentiation pathway and master regulator transcription factor (T-bet, GATA3, RORγT, and FoxP3). It was unclear if T_{FH} cells constituted a fifth effector subset, and no master regulator for T_{FH} differentiation had been identified.

Adapted from:

Shane Crotty, Robert J. Johnston, and Stephen P. Schoenberger. *Effectors and memories: Bcl6 and Blimp-1 in T and B lymphocyte differentiation.* Nature Immunology, vol. 11: 114-120, 2010.

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1.6: Acknowledgements

Chapter 1, in part, is adapted from the material as it appears in *Effectors and memories: Bcl6 and Blimp-1 in T and B lymphocyte differentiation.* Shane Crotty, Robert J. Johnston, and Stephen P. Schoenberger. Nature Immunology, vol. 11: 114-120, 2010. The dissertation author was the second author of this publication.

Chapter 2:

Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation

2.1: Abstract

Effective B cell-mediated immunity and antibody responses often requires help from $CD4^+$ T cells. It is thought that a distinct $CD4^+$ effector T cell subset, called T follicular helper cells (T_{FH}), provides this help; however the molecular requirements for T_{FH} differentiation are unknown. Here we show that expression of the transcription factor Bcl6 in $CD4^+$ T cells is both necessary and sufficient for *in vivo* T_{FH} differentiation and T cell help to B cells in mice. In contrast, the transcription factor Blimp-1, an antagonist of Bcl6, inhibits T_{FH} differentiation and help, thereby preventing B cell germinal center and antibody responses. These findings demonstrate T_{FH} are required for proper B cell responses *in vivo* and Bcl6 and Blimp-1 play central yet opposing roles in T_{FH} differentiation.

2.2: Results

Each lineage of effector $CD4^+$ T cells (T_H1, T_H2, T_H17, T_{reg}) is defined and controlled by a unique master regulator transcription factor (T-bet, GATA3, RORγt, and Foxp3, respectively) (*1*). A proposed fifth effector subset, T follicular helper cells (T_{FH}), is thought to provide help for the generation of B cell-mediated immune responses, including class switch recombination, germinal center differentiation, and affinity maturation (*2*). Here we have identified Bcl6 as a T_{FH} master regulator, and have found that germinal center formation does not occur in the absence of T_{FH}.

T_{FH} are well described phenotypically in humans, and more recently in mice, as expressing high levels of the chemokine receptor CXCR5, and molecules such as

ICOS, PD1, interleukin (IL)-21, and BTLA (*2-9*). Given that $CD4^+$ T cells can upregulate CXCR5 and/or ICOS after activation (*2, 10*), it is important to phenotypically distinguish T_{FH} from highly activated $CD4^+$ T cells. We identified T_{FH} in mice in the context of acute infection with lymphocytic choriomeningitis virus (LCMV) by adoptively transferring TCR transgenic T cells specific for the LCMV epitope gp66-77 in the context of MHC class II molecule $I\text{-}A^b$ (SMtg). T_{FH} were $CXCR5^{high}$ $ICOS^{high}$ $PD1^{high}$ $BTLA^{high}$ $CD200^{high}$ $SLAM^{low}$ (Fig. 2.1, A and B, and fig. 2.S1), capable of producing IL-21 (Fig. 2.1C). We confirmed these results for polyclonal LCMV-specific $CD4^+$ T cell responses (fig. 2.S2). CXCR5 is the receptor for the B cell follicle chemokine CXCL13 (*11*), and T_{FH} were selectively able to migrate in response to CXCL13 *in vitro* (Fig. 2.1D), consistent with the importance of CXCR5 for T_{FH} (*6, 12*).

To understand how T_{FH} differentiation is transcriptionally regulated, we performed gene expression microarray analysis of virus-specific T_{FH} and non-T_{FH} effector $CD4^+$ T cells (Fig. 2.1E, and figs. 2.S3 and 2.S4). Notably, the transcription factor B-cell CLL/lymphoma 6 (Bcl6) was strongly upregulated in T_{FH} (Fig. 2.1E). This is in agreement with previous reports of elevated Bcl6 expression in murine and human T_{FH} (*3-5, 8*). Furthermore, Blimp-1 (*Prdm1)* was the most downregulated transcription factor in T_{FH} (fig. 2.S4), consistent with a recent report (*13*). Bcl6 is essential for germinal center B cell differentiation (*14-16*), and Blimp-1 is well characterized as an antagonist of Bcl6 that can also be directly repressed by Bcl6 (*16-20*). Upregulation of Bcl6 mRNA (Fig. 2.1F) and the downregulation of Blimp-1

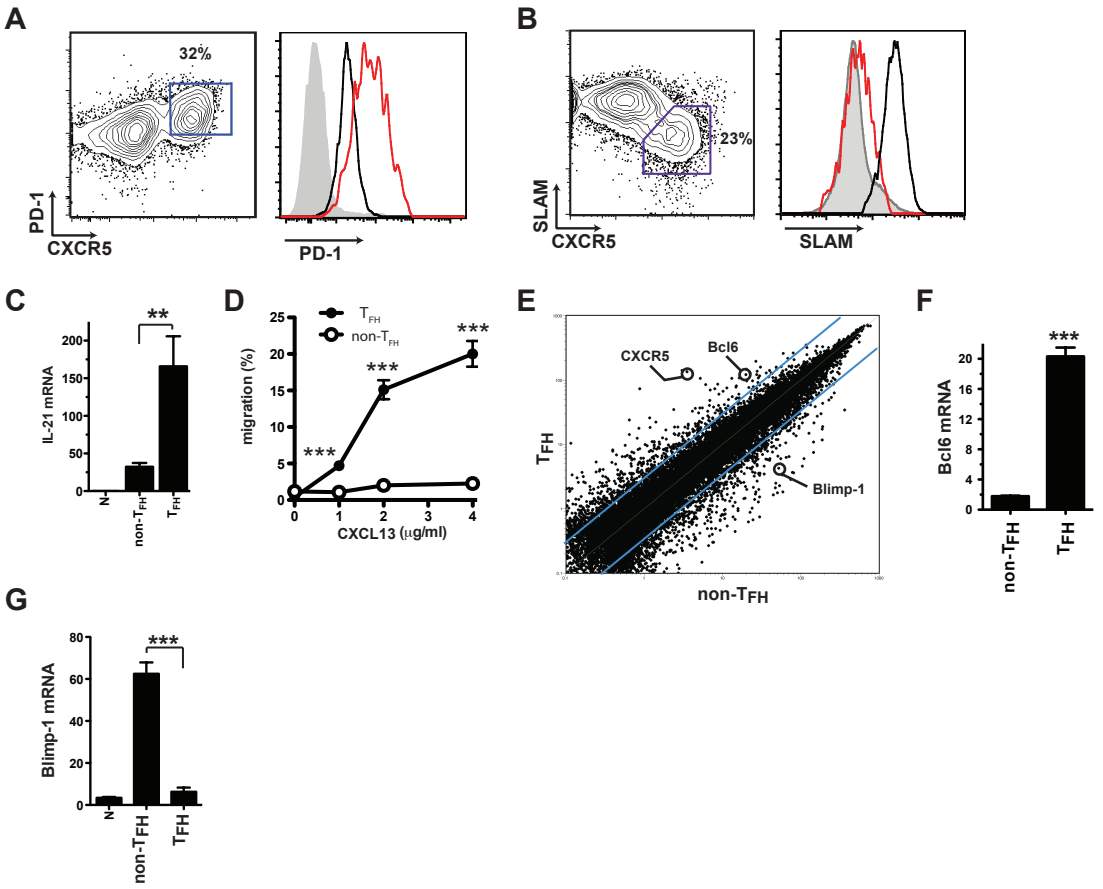

Figure 2.1: Bcl6 is a T_{FH}-specific transcription factor.
Naïve SMtg $CD4^+$ T cells were transferred into B6 mice. Splenocytes were analyzed 8 days after infection with LCMV, in all panels. SMtg expression of CXCR5 and (A) PD-1 and (B) SLAM (CD150). $SMtg^+$ ($CD45.1^+$) $CD4^+$ gated cells are shown. $CXCR5^{high}$ T_{FH} are boxed in FACS plots. Histogram overlays depict T_{FH} (red) as well as naïve $CD4^+$ T cells (gray) and $CXCR5^{low}$ non-T_{FH} SMtg (black). Data are representative of more than 10 independent experiments. (C) IL-21 mRNA in SMtg $CD4^+$ T cells, normalized to the β-actin mRNA level (x 10^{-4}). **, P = 0.008. (D) *In vitro* chemotaxis towards CXCL13 (BLC) by *ex vivo* SMtg $CD4^+$ T cells. Results are expressed as the percentage of $SLAM^{low}$ T_{FH} SMtg (filled circles) and $SLAM^{high}$ non-T_{FH} SMtg (open circles) that migrated in a transwell assay. ***, $P \leq 0.001$. 1μg P = 0.001, 2μg P = 0.0006. 4μg P = 0.0006. Data are representative of three independent experiments. n = 2/group. (E) Scatter plot of the average signal of biological replicates of T_{FH} vs. non-T_{FH} SMtg gene expression microarray data. Blue lines indicate three-fold changes in gene expression. 386 gene probes exhibited a > 3.0-fold increase in T_{FH}. Data from one of two independent experiments is shown. n = 2/group. Quantitative RT-PCR of (F) Bcl6 and (G) Blimp-1 mRNA expression, normalized to β- actin (x10^{-4}). ***, P < 0.0001. Data are representative of 4 independent experiments. n = 2/group. Error bars in all graphs depict SEM.

mRNA (Fig. 2.1F) was confirmed by quantitative PCR. Bcl6 protein expression was detected in germinal center $CD4^+$ T cells (fig. 2.S2), consistent with previous observations in human lymphoid tissue (*4, 21*).

While Bcl6 mRNA expression has been correlated with T_{FH}, no experimental data supporting a specific role for Bcl6 in T_{FH} differentiation have been reported. We expressed Bcl6 in SMtg $CD4^+$ T cells via a retroviral vector (RV) with a bicistronic mRNA co-expressing GFP (fig. 2.S5). Transduced Bcl6-RV^+ SMtg and control untransduced SMtg $CD4^+$ T cells were transferred into naive C57BL/6 hosts, which were subsequently infected with LCMV, and T_{FH} differentiation was examined (Fig. 2.2, A to E). Bcl6 expression drove nearly absolute T_{FH} differentiation *in vivo* (80-90%, Fig. 2.2, B to C), in contrast to T_{FH} differentiation in control untransduced (GFP^-) SMtg cells in the same mice (Fig. 2.2B), and in contrast to mice that received SMtg transduced with a control retrovirus expressing only GFP (GFP-RV^+) and untransduced SMtg in equal proportions (Fig. 2.2C). Comparably dramatic results were seen in studies where only Bcl6-RV^+ or GFP-RV^+ SMtg $CD4^+$ T cells were transferred into host mice (fig. 2.S6). Bcl-6 overexpression did not affect T cell expansion in vivo (fig. 2.S5). Constitutive expression of Bcl6 drove upregulation of CXCR5, PD-1, ICOS, CD200, and BTLA expression (Fig. 2.2, D to E, and fig. 2.S6), as well as the inhibition of SLAM and Blimp-1 (fig. 2.S6, and see below). These results indicated Bcl6 expression drives full T_{FH} differentiation *in vivo*.

T_{FH} differentiation is known to require the presence of B cells and is thought to require the presence of antigen-specific B cells (*6*). We thus hypothesized that Bcl6

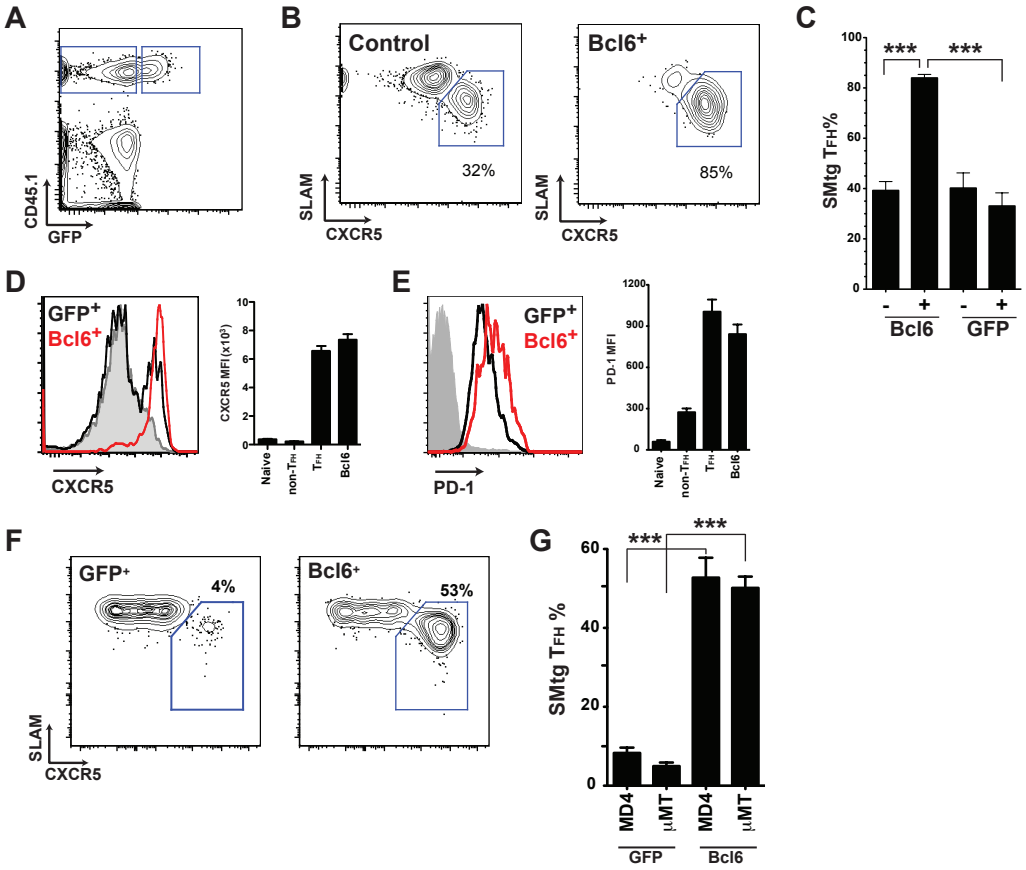

Figure 2.2: Bcl6 expression is sufficient for T_{FH} differentiation *in vivo*.
(A-C) Naive SMtg CD4$^+$ T cells were transduced with Bcl6-RV (Bcl6) or left untransduced (Control) and transferred into B6 mice subsequently infected with LCMV. (A) Gating of untransduced SMtg (GFP$^-$) and Bcl6-RV$^+$ SMtg (GFP$^+$) in the same host. CD4$^+$ B220$^-$ gate is shown. (B) T_{FH} (SLAM^{low} CXCR5^{high}, boxed) and non-T_{FH} (SLAM^{high} CXCR5^{low}) differentiation of untransduced SMtg (left) and Bcl6-RV$^+$ SMtg (right). (C) Quantitation of SMtg T_{FH} differentiation. Mice received Bcl6-RV$^+$ SMtg and untransduced SMtg, or GFP-RV$^+$ and untransduced SMtg. "-", untransduced. "+", transduced with indicated RV. ***, $P < 0.0001$. Data are representative of 3 independent experiments. n = 4/group. (D) CXCR5 and (E) PD-1 expression on naïve CD4$^+$ T cells (gray), GFP-RV$^+$ SMtg (black), and Bcl6-RV$^+$ SMtg (red). Bar graphs show MFI of naïve CD4$^+$ T cells, GFP-RV$^+$ SMtg non-T_{FH}, GFP-RV$^+$ SMtg T_{FH}, and Bcl6-RV$^+$ SMtg. For PD-1 MFI, non-T_{FH} vs T_{FH} or Bcl6-RV$^+$, $P < 0.05$; T_{FH} vs Bcl6-RV$^+$, $P > 0.05$. Data are representative of 3 independent experiments. n = 4-6/group. (F-G) GFP-RV$^+$ or Bcl6-RV$^+$ SMtg cells were adoptively transferred separately into B cell deficient mice (µMT), or HEL-specific BCR transgenic mice (MD4) on a µMT background. Host mice were subsequently infected with LCMV. n = 2 (GFP-RV$^+$ µMT), 6 (Bcl6-RV$^+$ MD4, and Bcl6-RV$^+$ µMT), or 8 (GFP-RV$^+$ MD4) per group, which are composites of three experiments. (F) Differentiation of SMtg CD4$^+$ T cells in MD4 BCR transgenic mice. T_{FH} (SLAM^{low} CXCR5^{high}) are boxed. CD4$^+$ B220$^-$ CD45.1$^+$ GFP$^+$ gate is shown. (G) Quantitation of SMtg T_{FH} differentiation. GFP-RV$^+$ vs Bcl6-RV$^+$ in MD4, ***, $P < 0.0001$. GFP-RV$^+$ vs Bcl6-RV$^+$ in µMT, ***, $P < 0.0001$.

expression induced by interaction with antigen-specific B cells could be the event that commits a T cell to T_{FH} differentiation. To test this, we examined whether Bcl6 expression in $CD4^+$ T cells was sufficient to drive T_{FH} differentiation in μMT B cell-deficient mice and in BCR transgenic mice of an irrelevant specificity (MD4, specific for hen egg lysozyme). GFP-RV^+ SMtg $CD4^+$ T cells failed to differentiate into T_{FH} in μMT or MD4 mice infected with LCMV (Fig. 2.2, F to G), demonstrating that T_{FH} differentiation in the context of a viral infection is dependent on the presence of antigen-specific B cells. In contrast, Bcl6-RV^+ SMtg cells differentiated into T_{FH} in the absence of antigen-specific B cells or even in the total absence of B cells (Fig. 2.2, F to G). These results indicate that cognate T-B interactions induce Bcl6 expression in $CD4^+$ T cells and that Bcl6 is sufficient to drive T_{FH} differentiation, even in the absence of such interactions.

T_{FH} are thought to provide B cell help *in vivo* (*2, 22*). We assessed the capacity of Bcl6-RV^+ SMtg $CD4^+$ T cells to help B cells *in vivo* by examining germinal center development in LCMV infected mice. Overexpression of Bcl6 increased the already robust frequency of germinal center B cells after LCMV infection (Fig. 2.3, A to B). As an additional measure of B cell help, we also examined the role of Bcl6 in T cell-dependent antibody production. Constitutive expression of Bcl6 in OT-II $CD4^+$ T cells enhanced NP-Ova serum IgG responses (Fig. 2.3C, and fig. 2.S7), which were sustained (Fig. 2.3C). Our results suggest Bcl6 was specifically enhancing T_{FH} differentiation and not skewing the T_H1/T_H2 profile of the $CD4^+$ T cells, as all IgG isotypes were enhanced in the mice receiving Bcl6-expressing OT-II $CD4^+$ T cells,

with the strong IgG1 dominance maintained (fig. 2.S7).

The preceding experiments demonstrated that Bcl6 expression was sufficient to drive the differentiation of functional T_{FH}. To test whether Bcl6 was also necessary for T_{FH} differentiation, we examined *Bcl6*$^{-/-}$ CD4^{+} T cells. *Bcl6*$^{-/-}$ mice have an abundance of highly activated CD4^{+} T cells (fig. 2.S8) and succumb to early mortality (*14, 15*). To circumvent these issues, we transferred *Bcl6*$^{+/+}$ or *Bcl6*$^{-/-}$ OT-II bone marrow into irradiated C57BL/6 recipients (fig. 2.S8). *Bcl6*$^{-/-}$ OT-II CD4^{+} T cells obtained from chimeric mice did not exhibit lymphoproliferation or spontaneous activation upon transfer into C57BL/6 mice (fig. 2.S8, D to F). *Bcl6*$^{-/-}$ or *Bcl6*$^{+/+}$ OT-II recipient mice were subsequently immunized with Ova in alum. Strikingly, *Bcl6*$^{-/-}$ OT-II CD4^{+} T cells did not differentiate into T_{FH} (Fig. 2.3D). We hypothesized that if T_{FH} are necessary for B cell help *in vivo*, a cell intrinsic CD4^{+} T cell block in T_{FH} differentiation should result in a failure to generate antigen-specific B cell responses, such as germinal center formation. To test this, we transferred *Bcl6*$^{-/-}$ or *Bcl6*$^{+/+}$ OT-II CD4^{+} T cells into *Icos*$^{-/-}$ mice, which have ineffective B cell help (*2, 23*). After NP-Ova immunization, *Icos*$^{-/-}$ mice that received *Bcl6*$^{-/-}$ OT-II CD4^{+} T cells were unable to form germinal centers, in contrast to mice that received wildtype OT-II CD4^{+} T cells (Fig. 2.3, E to G, and fig. 2.S9). These data demonstrated that Bcl6 is necessary for T_{FH} differentiation and that T_{FH} are necessary for germinal center formation. Altogether, these results indicated Bcl6 is a *bona fide* master regulator of T_{FH} differentiation *in vivo*.

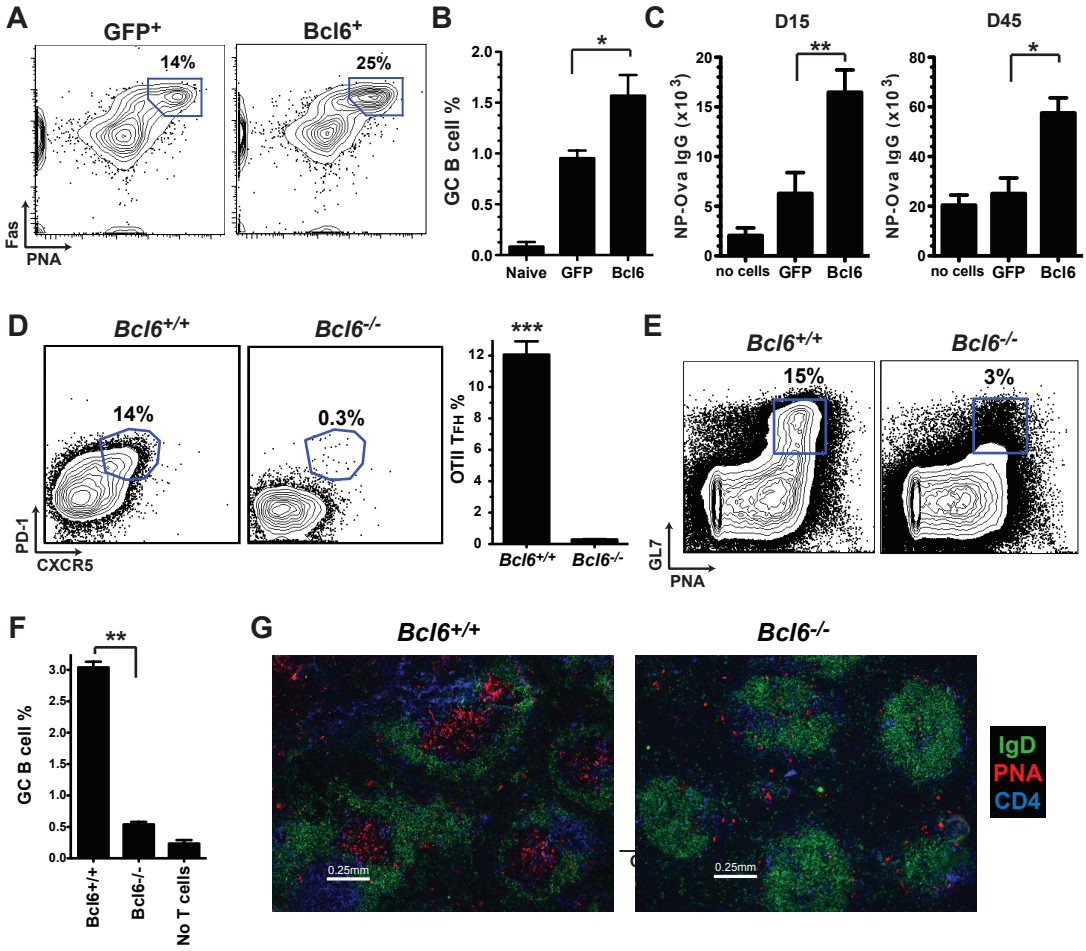

Figure 2.3: Bcl6 expression is necessary for inducing T_{FH} B cell help *in vivo*.
(A) Germinal center B cells (PNA^+ Fas^+, gated) in mice that received GFP-RV^+ or Bcl6-RV^+ SMtg $CD4^+$ T cells and were subsequently infected with LCMV. Analyzed at day 8. Gated on activated B cells ($B220^+$ IgD^{low}). (B) Frequency of germinal center B cells of total splenocytes . n = 4/group. Data are representative of 3 independent experiments. *, P = 0.029. (C) GFP-RV^+ or Bcl6-RV^+ OT-II $CD4^+$ T cells were transferred into B6 mice subsequently immunized with NP-Ova in alum. Control mice were immunized but received no OT-II cells. NP-Ova ELISA at day 15 and day 45. n = 6/group. Data are representative of 2 independent experiments. Day 15 endpoint ELISA titers, **, P = 0.008. Day 45 endpoint ELISA titers, *, P =0.017. (D) $Bcl6^{+/+}$ or $Bcl6^{-/-}$ OT-II $CD4^+$ T cells were transferred into congenically mismatched B6 mice subsequently immunized with Ova in alum. Splenocytes were analyzed 6 days after immunization. n = 4/group. Data are representative of 4 independent experiments. OT-II^+ $CD44^{high}$ gate is shown. Quantitation of OT-II T_{FH} differentiation is also shown. ***, P < 0.0001. (E-G) $Bcl6^{+/+}$ or $Bcl6^{-/-}$ OT-II $CD4^+$ T cells were co-transferred with B1-8 B cells into $Icos^{-/-}$ mice subsequently immunized with NP-Ova in alum. n = 2/group. Data are representative of 2 independent experiments. (E) Germinal center B cells (PNA^+ $GL7^+$, boxed) 7 days after immunization. $TCR\beta^-$ IgD^{low} gate is shown. (F) Quantitation of GC B cells as % of spleen. **, P = 0.0015. (G) Germinal center histology. Spleen sections are shown, stained with IgD (green), PNA (red), and CD4 (blue).

Blimp-1 is a known antagonist of Bcl6, capable of directly inhibiting Bcl6 expression in B and T cells (*17, 18*). Conversely, Blimp-1 expression can be inhibited by Bcl6 (*16-18, 20*). On the basis of our observations that Bcl6 drives T_{FH} cell differentiation and function, and because Blimp-1 was the single most downregulated transcription factor in T_{FH} by gene expression array analysis (Fig. 2.1E, fig. 2.S4) and qPCR (Fig. 2.1G), we hypothesized a role for Blimp-1 in blocking T_{FH} differentiation *in vivo*. We constructed a Blimp-1 retroviral expression vector, Blimp1-RV (fig. 2.S5), designed to express physiological levels of Blimp-1. Only $CD4^+$ T cells expressing low levels of the GFP reporter were used for *in vivo* experiments (fig. 2.S10A). Blimp-1 blocked Bcl6 protein expression in activated antigen-specific $CD4^+$ T cells *in vivo* (Fig. 2.4A). To determine the effects of Blimp-1 on T_{FH} differentiation, Blimp1-RV^+ SMtg $CD4^+$ T cells and untransduced control SMtg cells were mixed in equal proportion and transferred into host mice subsequently infected with LCMV. While we observed normal proliferation of Blimp-1-expressing SMtg $CD4^+$ T cells (fig. 2.S10), T_{FH} differentiation was severely abrogated, with an 80% reduction in T_{FH} frequency (Fig. 2.4, B to C). Blockade of T_{FH} differentiation by Blimp-1 was also observed when mice separately received Blimp1-RV^+ vs. GFP-RV^+ SMtg cells (fig. 2.S10, F to G). Constitutive expression of Blimp-1 inhibited acquisition of the T_{FH} phenotype: SLAM expression was increased (Fig. 2.4D) whereas CXCR5, ICOS, and PD-1 expression were all decreased (Fig. 2.4D, and fig. 2.S10). Inhibition of T_{FH} differentiation by Blimp-1 was physiological and specific, because the expression of SLAM, ICOS, and PD-1 by Blimp1-RV^+ SMtg $CD4^+$ T cells were equivalent to the

expression levels seen in wildtype activated non-T_{FH} SMtg $CD4^+$ T cells, and not naive cells (fig. 2.S10I). Blimp1-RV^+ and wildtype non-T_{FH} SMtg cells also expressed comparable amounts of the cytokines interferon (IFN)-γ and interleukin (IL)-2 (fig. 2.S11). High amounts of Blimp-1 expression can inhibit proliferation in B and T cells (*17, 24, 25*). The moderate level of Blimp-1 expression used in our experiments (fig. 2.S10E) did not affect proliferation *in vivo* (fig. 2.S10, C to D, and H), in agreement with previous *in vitro* studies (*26*) and our observation that non-T_{FH} $CD4^+$ T cells express 20-fold more Blimp-1 than T_{FH} and are still proliferative. Blimp-1 expression did not affect expression of T helper lineage-specific transcription factors Foxp3, GATA3, and RORγt (fig. 2.S11), indicating that Blimp-1 did not induce differentiation into other helper lineages. Collectively, these data suggest Blimp-1 acts specifically to repress Bcl6 and thus blocks T_{FH} differentiation.

Given that Blimp-1 is a physiological inhibitor of Bcl6 expression and T_{FH} differentiation *in vivo*, we performed an additional test of the necessity of T_{FH} for B cell help by transferring Blimp1-RV^+ OT-II and GFP-RV^+ OT-II $CD4^+$ T cells into SAP-deficient (*sh2d1a*$^{-/-}$) mice (SAP-deficient mice exhibit a $CD4^+$ T cell-intrinsic defect in germinal center formation (*27-29*)) subsequently immunized with NP-Ova. We observed germinal centers and anti-NP-Ova serum IgG in GFP-RV^+ OT-II $CD4^+$ T cell recipient mice after immunization (Fig. 2.4, E to F). Strikingly, although OT-II cell numbers were normal in Blimp1-RV^+ OT-II recipient mice (fig. 2.S12), germinal centers were reduced by 90% (Fig. 2.4, E). Constitutive Blimp-1 expression also inhibited the NP-Ova specific IgG response, reducing the serum antibody

concentration to only 16% of normal levels (Fig. 2.4F). All IgG isotypes were reduced (Fig. 2.S12), confirming that Blimp-1 was specifically inhibiting T_{FH} differentiation. These results demonstrated both that Blimp-1 inhibited $CD4^+$ T cell help to B cells and that T_{FH} are required for B cell help *in vivo*.

To confirm the biological role of Blimp-1 in inhibiting T_{FH} differentiation *in vivo*, we tested the ability of Blimp-1-deficient $CD4^+$ T cells to differentiate into T_{FH}. To avoid autoimmunity complications (*30, 31*), we deleted Blimp-1 (*prdm1*) *in vitro* in mature $prdm1^{fl/fl}$ $CD4^+$ T cells (*32*) using a Cre expressing RV. We transferred Cre^+ $SMtg^+$ $prdm1^{fl/fl}$ and control Cre^- $SMtg^+$ $prdm1^{fl/fl}$ $CD4^+$ T cells into mice and infected with LCMV. Deletion of *prdm1* substantially enhanced T_{FH} differentiation *in vivo* (Fig. 2.4G), without altering proliferation (fig. 2.S13). These data indicate that Blimp-1 expression *in vivo* normally restricts Bcl6 expression and T_{FH} differentiation. In sum, our results reveal that Bcl6 and Blimp-1 are reciprocal master regulators of T_{FH} differentiation, with T_{FH} differentiation *in vivo* requiring the presence of Bcl6 and the absence of Blimp-1.

There has been extensive speculation about a role for Bcl6 in T_{FH} differentiation, based on gene expression data from human (*2, 4*) and murine T_{FH} studies (*3, 8, 9, 13*). The data presented herein now directly demonstrate that Bcl6 specifically drives T_{FH} differentiation and is a *bona fide* master regulator. The relationship between T_{FH} and other $CD4^+$ T cell lineages has been a longstanding problem. The predominant $CD4^+$ T cell response to LCMV is T_H1 (fig. 2.S14), and it is notable that T-bet and IFNγ were still expressed in the T_{FH} *in vivo*, though at lower

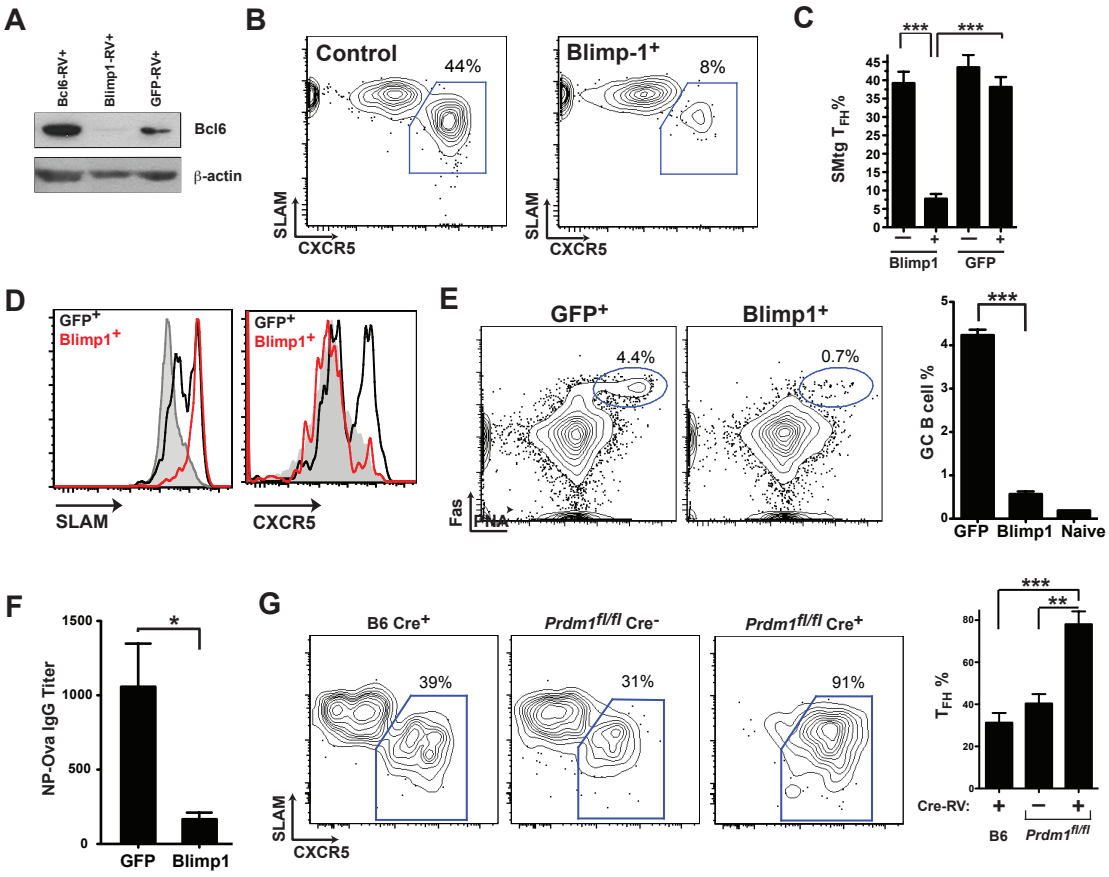

Figure 2.4: Blimp-1 and Bcl6 are antagonistic and reciprocal regulators of T_{FH} differentiation. (A) Immunoblot of Bcl6 protein expression (and β-actin control) in transduced SMtg CD4$^+$ T cells *in vivo*. (B) T_{FH} (SLAM^{low} CXCR5^{high}, boxed) differentiation of untransduced SMtg (left, "Control") and Blimp1-RV$^+$ SMtg (right, "Blimp-1$^+$") cells within a common host 8 days after LCMV infection. Gating is shown in fig. 2.S10B. (C) Quantitation of SMtg T_{FH} differentiation. "-", untransduced. "+", transduced with the indicated RV. ***, P < 0.0001. n = 4/group. Data are representative of 2 independent experiments. (D) SLAM and CXCR5 expression by naïve CD4$^+$ T cells (gray), GFP-RV$^+$ SMtg (black), and Blimp1-RV$^+$ SMtg (red). (E-F) GFP-RV$^+$ or Blimp1-RV$^+$ OT-II CD4$^+$ T cells were transferred into SAP-deficient mice subsequently immunized with NP-Ova in alum. n = 4/group. Data are representative of 3 independent experiments. (E) Germinal center B cells (PNA$^+$ Fas$^+$, gated) in mice that received GFP-RV$^+$ or Blimp1-RV$^+$ OT-II CD4$^+$ T cells. B220$^+$ IgD^{low} gate is shown. Quantitation of germinal center B cells in the spleen is also shown. ***, P < 0.0001. (F) NP-Ova IgG ELISA endpoint titers at day 10. *, P = 0.016. (G) Purified naive B6 and prdm1$^{fl/fl}$ CD45.2$^+$ CD4$^+$ T cells were transduced with SMtg-RV, with or without Cre-RV (Cre$^+$ or Cre$^-$), sorted, and transferred into CD45.1$^+$ mice subsequently infected with LCMV. FACS plots depict T_{FH} (CXCR5^{high} SLAM^{low} boxed) differentiation of control Cre$^+$ SMtg$^+$ B6 cells (left), Blimp-1 sufficient Cre$^-$ SMtg prdm1$^{fl/fl}$ cells (center), and Blimp-1 deficient Cre$^+$ SMtg prdm1$^{fl/fl}$ cells (right). CD4$^+$ CD45.1$^-$ CD44^{high} 7AAD$^-$ gate is shown. Quantitation of T_{FH} differentiation is also shown. Data are representative of two independent experiments. **, P = 0.002. ***, P = 0.0006. n = 4-5/group.

levels than in T_H1/non-T_{FH} LCMV-specific $CD4^+$ T cells (fig. 2.S14). These observations are consistent with a model where T_{FH} follow their own differentiation pathway, but are not an isolated lineage and can exhibit partial characteristics of T_H1/T_H2 polarization based on environmental conditions. This overlapping differentiation model would resolve the conundrum in the literature that neither T_H1 nor T_H2 nor T_H17 are required for B cell help *in vivo* (*8, 33, 34*), but that cells with T_H1 or T_H2 or T_H17 phenotypes can provide B cell help in vivo (*9, 35-39*).

The capacity for B cell help is a central attribute of $CD4^+$ T cells, and is a cornerstone of protective immunity. It is well known that in B cells Bcl6 and Blimp-1 are powerful antagonistic master regulators of germinal center B cell differentiation and plasma cell differentiation. The findings reported herein that Bcl6 and Blimp-1 also control T_{FH} differentiation illustrate the elegant utilization of the same antagonistic transcription factors to drive different functions in two lymphocyte populations differentiating in parallel: antigen-specific B cells and the T_{FH} that provide their help. Manipulation of these signaling pathways *in vivo* may have substantial therapeutic benefit for enhancing vaccines or, conversely, blocking autoantibody responses.

2.3: Supplementary Materials

Mice

C57BL/6J (B6), ICOS-deficient (*Icos*$^{-/-}$, B6.129S1-*Icos*^{tm1Flv}/J) (*5*), Blimp-1 conditional knockout (*Prdm1*$^{flox/flox}$), and B6 μMT mice were purchased from the Jackson Laboratory. SAP^{-} (*Sh2d1a*$^{-}$) (*1*), OT-II CD45.1^{+}, and SMtg CD45.1^{+} (SMtg = SMARTA. LCMV gp66-77 I-A^{b} specific) (*2*) mice were all fully backcrossed to B6 (determined > 99% pure by pan-genome microsatellite analysis at LIAI) and bred at LIAI. HEL BCR-transgenic mice (MD4) were bred on a μMT background (*3*). OT-II Thy1.1 and Thy1.2 were bred at the Yale School of Medicine. B1-8 mice (NP-specific B cells) were provided by M. Shlomchik, originally from K. Rajewsky (*4*). Bcl6-deficient animals (*6*) were bred to OT-II at the Yale School of Medicine. All animal experiments were conducted in accordance with approved animal protocols.

Retroviral vectors, transductions, and cell transfers

Bcl6 and Blimp-1 cDNAs were obtained from Open Biosystems (Bcl6 Clone ID: 6309948. Prdm1 Clone ID: 40048956), sequenced in full, and complete open reading frames were cloned into the retroviral expression vector pMIG-GFP. The Blimp-1 expression construct included the natural Kozak sequence upstream of the Blimp-1 open reading frame. For *prdm1*$^{fl/fl}$ experiments, CD4^{+} T cells were co-transduced with SMARTA TCR-RV (constructed using the 2A peptide linked design of Vignali (*9*)) and with Cre-RV (constructed using the NLS-Cre sequence of Rajewsky and colleagues (*10*)). Deletion of *Prdm1* by Cre-RV was confirmed to be

greater than 98% efficient by genomic DNA qPCR. Signals from primers targeting the final exon of *Prdm1* (GCTATGACTTTGGTGCTTGGGC and GACTGGATGGTGTGGTGTTTCTATC) were normalized to β-actin (*Actb*) (AGGCCAACCGTGAAAAG and GCGTGAGGGAGAGCATAG).

Virions were produced using the Plat-E cell line (*7*) as described (*8*). Naive $CD4^+$ T cells were purified from whole splenocytes by negative selection using magnetic beads (Miltenyi) and suspended in D-10 (DMEM + 10% fetal calf serum (FCS), supplemented with 2mM GlutaMAX (Gibco), and 100 U/mL Penicillin/Streptomycin (Gibco)) + 10 ng/mL hIL-2. 2 x 10^6 cells/well were stimulated in 24-well plates pre-coated with 0.5 mL anti-hamster IgG (Vector Laboratories) followed by anti-CD3 (clone 17A2, eBiosciences) and anti-CD28 (clone 37.51, eBiosciences), or directly coated with 0.5 mL of 8 μg/ml anti-CD3 and anti-CD28 (BioXcell). At 24 and 36 hours, cells were transduced as described (*8*), except centrifugations were at 30-34 °C. After a total of 72 hours of stimulation, the $CD4^+$ T cells were transferred into new wells in fresh D-10 + 10 ng/mL IL-2 for an additional 72 hours, and were split as needed. When used, control untransduced cells were subjected to the same *in vitro* stimulation and culture conditions as were used for transduced cells. Transduced cells were then purified by sorting $7AAD^-$ GFP^+ (and GFP^- as needed) cells on a FACSDiva or FACSAria (BD Biosciences).

Cell transfers into host mice were performed by intravenous injection via the retro-orbital sinus. Transferred cells were rested in host mice for 3-5 days before infection or immunization (*11*). For naïve SMtg $CD4^+$ T cell transfers, 6,000 cells

were transferred per mouse. For transduced SMtg $CD4^+$ T cell transfers, 25 x 10^3 cells were transferred. In the case of mixed experiments, 25 x 10^3 non-transduced SMtg (GFP^-) and 25 x 10^3 RV^+ SMtg cells were transferred into a common host (50 x 10^3 SMtg cells/mouse). For transduced OT-II $CD4^+$ T cell transfers, 250 x 10^3 cells were transferred per mouse. For experiments assessing *Bcl6*$^{-/-}$ $CD4^+$ T cells, whole splenocytes containing 0.5 - 1 x 10^6 $CD4^+$ T cells and, when specified, whole splenocytes containing 1 x 10^6 NP-specific B cells were transferred per mouse.

Infections and Immunizations

LCMV stocks were prepared and quantified as described (*8*). All infections were done by intraperitoneal injection of 1-2 x 10^5 plaque-forming units of LCMV Armstrong per mouse. NP-Ova in alum was prepared by mixing NP(19)-Ova (Biosearch Technologies) in PBS with Alum (Pierce) at a 3:1 ratio for 60 minutes at 4 °C. NP-Ova/Alum immunizations were done by intraperitoneal injection of 100 μg. Ova in alum was prepared and injected in a comparable manner. Splenocytes and serum were analyzed 8 days after infection or immunization, unless stated otherwise.

Bone marrow chimeras

Thy1.2 or Thy1.1 B6 recipient mice were irradiated with the equivalent of 1000 cGy (XRAD 320 Biological Irradiator, Precision X-ray, Inc.). Bone marrow from OT-II *Bcl6*$^{+/+}$ or OT-II *Bcl6*$^{-/-}$ mice was isolated with PBS and a syringe. Bone marrow cells were subjected to red cell lysis and magnetic separation to remove T

cells. Approximately 1 x 10^7 cells were transferred into congenically mismatched recipients immediately after irradiation. Eight weeks later, mice were bled to confirm hematopoietic reconstitution. Splenocytes from fully reconstituted bone marrow chimeras were subsequently transferred into new sets of congenically mismatched hosts as above, followed by immunization with Ova or NP-Ova in alum.

Flow Cytometry

Single-cell suspensions of spleen were prepared by standard gentle mechanical disruption. Surface staining for flow cytometry used monoclonal antibodies to SLAM (CD150, Biolegend); CD4, PD-1, CD200, ICOS, BTLA, CD62L, B220, CD45.1/2, and Fas (BD Biosciences); CD44, TCRβ and B220 (eBiosciences), as well as Biotin/FITC-labelled peanut agglutinin (PNA) and GL7 (Vector Laboratories and BD Pharmingen, respectively). CD43 staining was done with the 1B11 clone (*12*).

For most figures, CXCR5 staining was done using purified anti-CXCR5 (BD Pharmingen) for 1 hour, followed by biotinylated anti-rat IgG (Jackson Immunoresearch), and then APC-labelled streptavidin (Caltag Laboratories) in PBS + 0.5% BSA + 2% FCS + 2% Normal Mouse Serum on ice; and samples were then acquired without fixation. For Figure 3D *Bcl6*$^{-/-}$ experiments, T_{FH} staining was done using biotinylated anti-CXCR5 for 30 minutes at room temperature, followed by PE-Cy7-conjugated streptavidin (BD Biosciences) at 4 °C.

Intracellular cytokine staining (ICS) was done in most cases with a 5 hour peptide (2.0 μg/mL) stimulation (*8*). For ICS with PMA and ionomycin stimulation,

splenocytes were cultured with 20 ng/mL PMA and 1μM Ionomycin for 4 hours. Directly conjugated antibodies against IFN-γ and IL-2 (BD Pharmingen) were used. Intracellular IL-21 detection was done using an IL-21R-Fc chimeric protein (R&D Systems) followed by PE or APC-labelled anti-human IgG (Jackson Immunoresearch)(*13*).

MHC Class II tetramer was produced by oligomerizing biotinylated I-A^b-gp66 (DIYKGVYQFKSV (*14*)) monomer (NIH tetramer core) with APC-labelled streptavidin (Molecular Probes) following NIH tetramer core facility recommendations. To stain, gp66 I-A^b tetramer was incubated with splenocytes for 3 hours at 37 °C. Tetramer stained cells were washed twice and then stained on ice for additional markers prior to acquisition. Splenocytes intended for tetramer staining were obtained 10 days after LCMV infection.

Microscopy

For Figure S2, standard immunofluorescence histology was performed, as described previously (*8*), using a deconvolution confocal microscope (Marianas). For Figure 3G and S8, spleens were harvested and immediately frozen in OCT tissue-freezing medium. Sections were cut to 6μm thickness on a cryostat and stained for immunofluoresence using CD4-Pacific Blue or CD4-Alexa647 (eBioscience and BD Biosciences, respectively), biotinylated PNA (Vector Labs), IgD-FITC (BD Biosciences), and Streptavidin-Alexa555 (Invitrogen). 25x images were acquired on a Zeiss LSM 510 Meta Confocal. 10x images were acquired with an Olympus BX40

equipped with 100W mercury lamp (Olympus) and a SPOT RT CCD camera (Diagnostic Instruments) using fluorescence filters optimized for DAPI, FITC, Cy3, and Cy5 (Chroma). Images were acquired, processed to reduce background, pseudo-colored, and merged with Adobe Photoshop.

RNA, gene expression microarrays, and qPCR

Splenocytes were isolated and antigen-specific $CD4^+$ T cells were enriched using anti-CD45.1-FITC and anti-FITC magnetic bead purification (Miltenyi). T_{FH} and non-T_{FH} $CD4^+$ $CD45.1^+$ $TCR\beta^+$ $CD19^-$ $7AAD^-$ cells were then sorted on the basis of CXCR5 expression using a FACSAria (BD Biosciences). Approximately 1 x 10^6 cells from each condition (in duplicate) were sorted directly into RNALater (Ambion). Naive SMtg $CD4^+$ T cells were obtained from intact SMtg mice, sorting for $CD4^+$ $CD45.1^+$ $CD44^{low}$ $CD62L^{high}$ $7AAD^-$. RNA was isolated using Qiagen RNAeasy Mini spin columns (Qiagen), including QiaShredder and on-column digestion of genomic DNA. Some RNA samples were then concentrated using MinElute spin columns (Qiagen). RNA quality of all samples was confirmed by BioAnalyzer Nano gel (Agilent), then probes were generated by single round linear amplification of 30 ng RNA using the Ovation Pico system (Nugen) and used on Affymetrix 430 2.0 chips. Data was analyzed using Genespring 7 (Agilent) and signals were GC-RMA preprocessed. Gene normalized signals were used for optimal analysis (median signal = 1), and gene probes without a signal of > 0.5 normalized value in at least one of three conditions (Naive, non-T_{FH}, T_{FH}) were excluded from scatter plots. Raw

microarray signal data has been deposited at NCBI GEO. cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen) with oligo dT and random hexamer primed reactions that were then pooled. qPCR reactions were performed in triplicate using iTaq Sybr Green with Rox (BioRad) on a Roche Lightcycler 480, using primers described in Table 1.

Immunoblot

Bcl6 protein was detected by Western blot. Whole cell lysates were prepared using RIPA lysis buffer. Blots were probed with polyclonal anti-Bcl6 (#19, Santa Cruz Biosciences).

ELISAs

Anti-NP-Ova IgG was quantified by ELISA using NP(19)-Ova (Biosearch Technologies) as the capture antigen on 96-well Maxisorp microtiter plates (Nunc). Following incubation of sample serum or media, HRPO-conjugated goat anti-mouse IgG-γ (Caltag Laboratories) was used, or isotype specific secondary antibodies (anti-IgM, IgG1, IgG2a/c, IgG2b, IgG3. Caltag Laboratories).

Transwell Chemotaxis assay

All migration assays were performed in 24-well Transwell plates with a polycarbonate filter pore size of 5 μm (Corning Costar). $CD4^+$ T cells were purified from B6 host mice 8 days after SMtg transfer and LCMV infection. Cells were rested

for 60 min in migration medium (DMEM + 2% FCS) at 37 °C in 5% CO_2 prior the start of the assay. Recombinant mouse BLC/CXCL13 (R&D Systems) was serially diluted in migration medium and added to each of the lower wells while 1 x 10^6 $CD4^+$ T cells were added to the top chambers. Cells were allowed to migrate for 90 min at 37 °C in 5% CO_2. Migrated cells from each well were collected and cell counts performed using the Accuri C6 (Accuri Cytometers). Surface staining with anti-CD4, CD45.1, CD44, and SLAM antibodies was done to phenotype an aliquot of migrants by flow cytometry. Migration was calculated for SMtg T_{FH} ($CD4^+$ $CD45.1^+$ $CD44^{high}$ $SLAM^{low}$) and non-T_{FH} ($CD4^+$ $CD45.1^+$ $CD44^{high}$ $SLAM^{high}$) as the percentage of input cells that migrated at each chemokine concentration.

Statistical Analysis

Statistical tests were performed using Prism 5.0 (GraphPad). P-values were calculated using two-tailed unpaired Student's t tests with a 95% confidence interval. Error bars depict the standard error of the mean (SEM). For experiments in which GFP^+ and GFP^- SMtg cells were co-transferred into host mice and compared, paired Student's t tests were used.

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Table 2.1: Quantitative PCR (qPCR) Primers.

Gene	Forward Primer Sequence	Reverse Primer Sequence
Bcl6	CCTGTGAAATCTGTGGCACTCG	CGCAGTTGGCTTTTGTGACG
Blimp1	ACATAGTGAACGACCACCCCTG	CTTACCACGCCAATAACCTCTTTG
IL-21	GCTCCACAAGATGTAAAGGGGC	CCACGAGGTCAATGATGAATGTC
CXCR5	GACCTTCAACCGTGCCTTTCTC	GAACTTGCCCTCAGTCTGTAATCC
SLAM	AAAAGTGTCCGCATCCTCGTC	ATTGAAAGTGGTAGCCATCCTCC
PD-1	GCTCACTTCAGGTTTACCACAAGC	GCCCAACAGTAGGATTCAGGAGAC
ICOS	CAGGAGAAATCAATGGCTCGG	TTGGTCTTGGTGAGTTCGCAG
Foxp3	TCCAGGTTGCTCAAAGTCTTCTTG	AGGCTGCTGTTACGGGAATAGG
Gata3	ACAGAAGGCAGGGAGTGTGTGAAC	TTTTATGGTAGAGTCCGCAGGC
Rorc	TCTACGCTATGAGGAAGGAAGGC	GACTATGGAGGAGAAACAGGTCCC
T-bet	ACCAACAACAAGGGGGCTTC	CTCTGGCTCTCCATCATTCACC

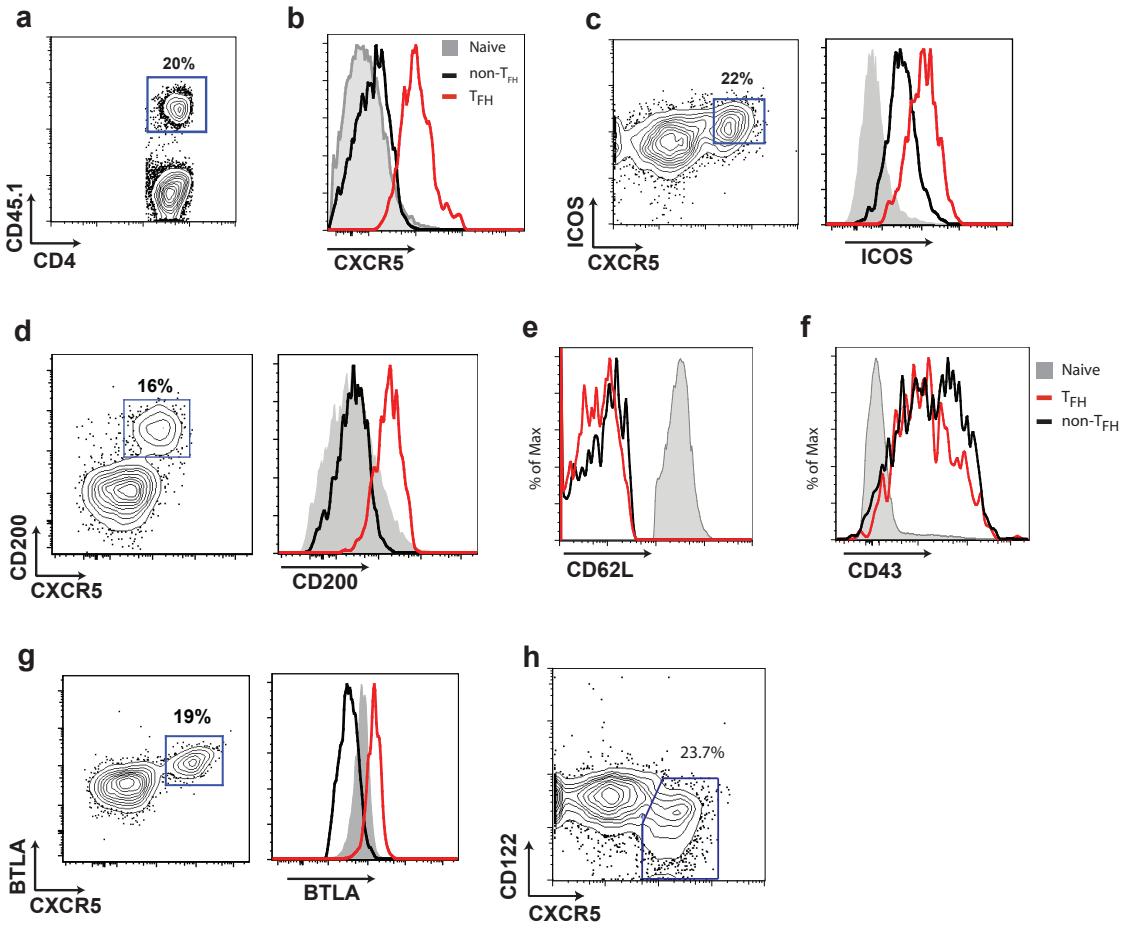

Figure 2.S1: Phenotypic characterization of T_{FH}.
Naïve SMtg $CD4^+$ T cells were transferred into B6 mice subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. (A) SMtg ($CD45.1^+$, boxed) and endogenous $CD4^+$ T cells (not boxed). $CD4^+B220^-$ gate is shown. Flow cytometry of (B) CXCR5, (C) ICOS, (D) CD200, (E) CD62L, (F) CD43, (G) BTLA, and (H) CD122 expression on SMtg $CD4^+$ T cells. FACS plots are gated on total SMtg $CD4^+$ T cells ($CD45.1^+CD4^+B220^-$). Histogram overlays are gated on naive $CD4^+$ T cells or SMtg $CD4^+$ T cells at day 8 after infection and depict $CXCR5^{high}$ T_{FH} (red), $CXCR5^{low}$ non-T_{FH} (black), and naïve $CD4^+$ T cells (gray). In conjunction with the data shown in Figure 2.1, these results indicate that T_{FH} are not simply highly activated cells, as they have specifically downregulated SLAM and CD122, which are activation markers (15, 16), and have upregulated the inhibitory receptor BTLA (17), which is downregulated on most non-T_{FH} effector $CD4^+$ T cells. The T_{FH} also have equivalent expression to non-T_{FH} of activation markers CD62L and CD43. Data are representative of more than 10 independent experiments.

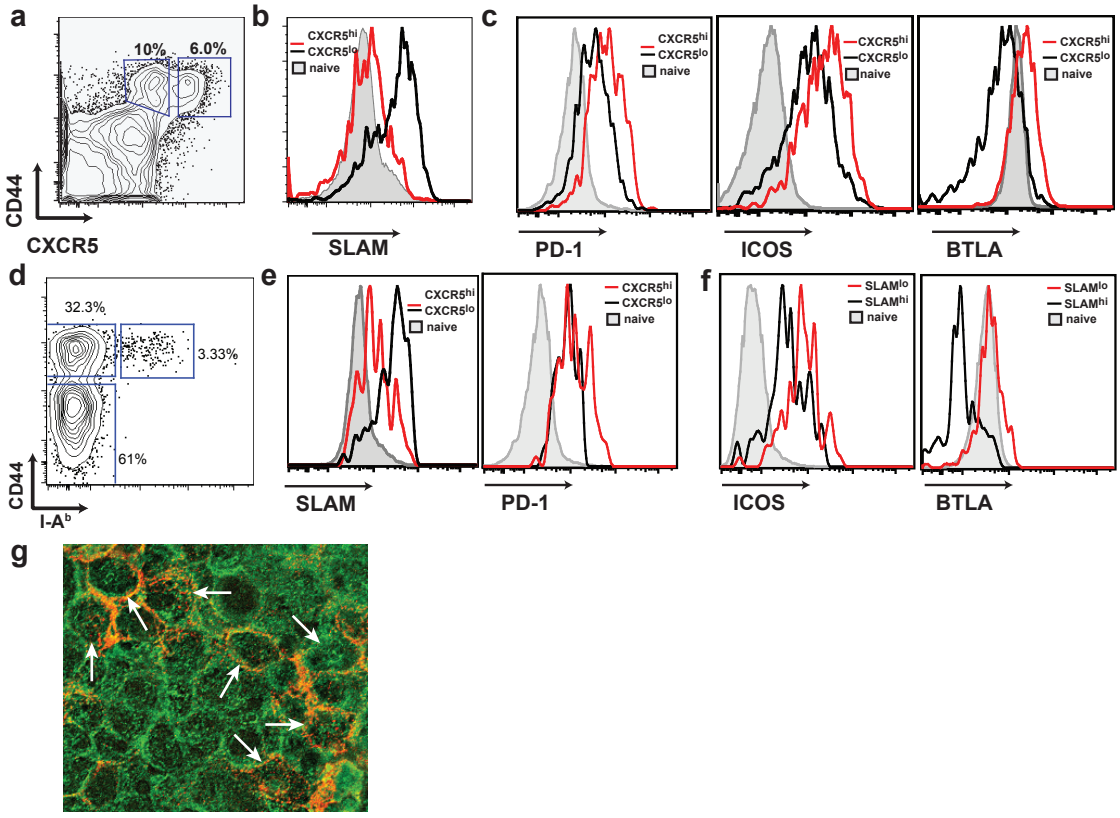

Figure 2.S2: T_{FH} phenotypic characterization of polyclonal LCMV-specific $CD4^+$ T cells. T_{FH} phenotyping of polyclonal LCMV-specific $CD4^+$ T cell responses provided results comparable to that obtained with SMtg $CD4^+$ T cells. (A) Identification of endogenous $CXCR5^{high}$ (right gate) and $CXCR5^{low}$ populations (left gate) of $CD44^{high}$ (activated) $CD4^+$ T cells in LCMV-infected C57BL/6 mice. $CD4^+$ $B220^-$ gate is shown. While all $CD44^{high}$ $CD4^+$ T cells expressed more CXCR5 than $CD44^{low}$ (naive) $CD4^+$ T cells, consistent with the published literature that CXCR5 is an activation marker in mice (18), a $CXCR5^{high}$ $CD44^{high}$ population was clearly distinguishable among the $CD44^{high}$ cells. (B) SLAM expression in $CD44^{high}$ $CXCR5^{high}$ T_{FH} (red), $CD44^{high}$ $CXCR5^{low}$ non-T_{FH} (black), and naive $CD4^+$ T cells (gray). Only $CD44^{high}$ $CXCR5^{high}$ were $SLAM^{low}$ and defined as T_{FH}. (C) Further phenotypic analysis of endogenous T_{FH}. Expression of PD-1, ICOS, and BTLA are shown on T_{FH} ($SLAM^{low}$, red), non-T_{FH} ($SLAM^{high}$, black) and naive $CD4^+$ T cells (gray). All expression patterns were comparable to that observed with SMtg transgenic $CD4^+$ T cells. Data are representative of more than 10 independent experiments. (D-F) The T_{FH} phenotype was also confirmed in polyclonal LCMV-specific CD4+ T cells using gp66-77 $I\text{-}A^b$ MHC-II tetramer staining to analyze C57BL/6 splenocytes 10 days after infection with LCMV. (D) Identification of MHC-II gp66-$tetramer^+$ $CD4^+$ T cells (top right gate). $CD4^+$ $B220^-$ gate is shown. (E) Phenotypic analysis of $tetramer^+$ T_{FH}, on the basis of CXCR5 staining. SLAM and PD-1 staining are shown. (F) Phenotypic analysis of tetramer+ T_{FH}, on the basis of SLAM staining. ICOS and BTLA staining are shown. Data are representative of 2 or more independent experiments. All expression patterns were comparable to that observed with SMtg transgenic $CD4^+$ T cells or polyclonal $CD44^{high}$ $CD4^+$ T cells at day 8 post-infection. (G) Immunofluorescence histology of spleen sections, 15 days after LCMV infection. Co-staining of a germinal center (PNA stain not shown) with anti-Bcl6 (green) and anti-CD4 (orange). All non-$CD4^+$ T cells are germinal center B cells, and all germinal center B cells are positive for intracellular BCL6. The majority of $CD4^+$ T cells (indicated by white arrows) are also positive for intracellular BCL6.

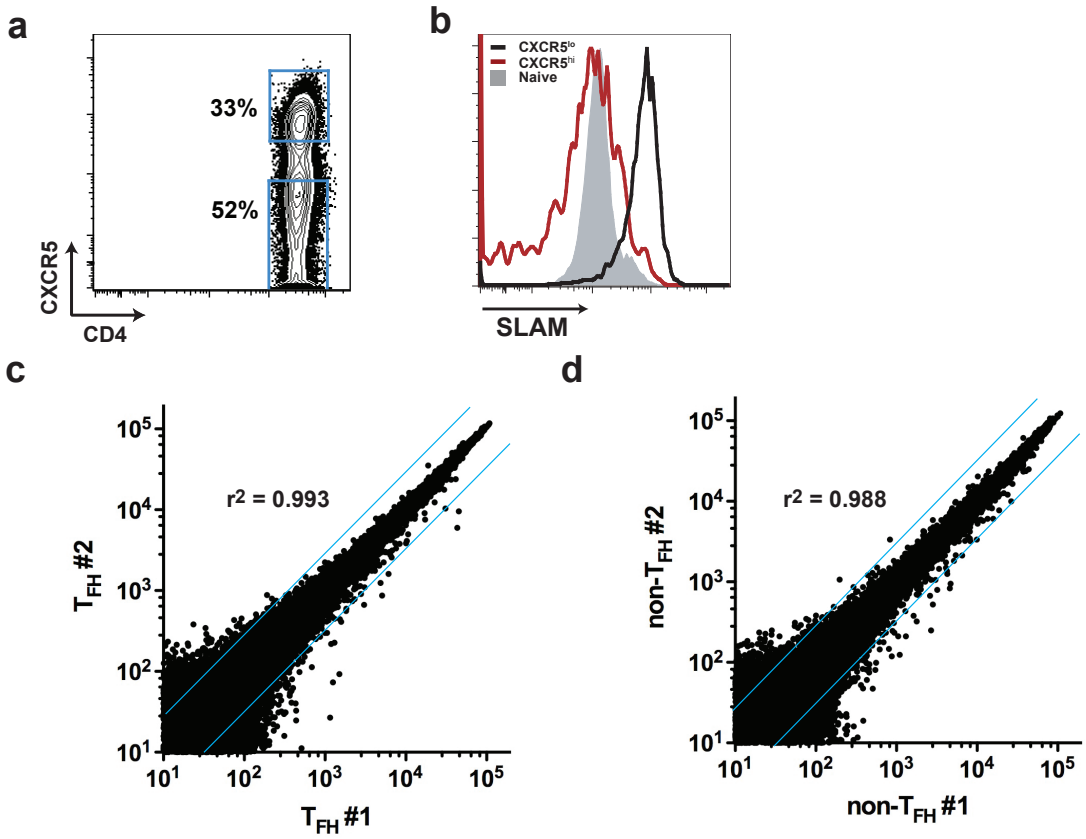

Figure 2.S3. Gene expression microarray analysis of *in vivo* antigen-specific T_{FH}.
SMtg $CD4^+$ T cells were transferred into C57BL/6 mice subsequently infected with LCMV. (A) SMtg T_{FH} (top gate) and non-T_{FH} (bottom gate) were then sorted on the basis of CXCR5 expression. $CD4^+$ $CD45.1^+$ TCR^+ $CD19^-$ $7AAD^-$ cell gate is shown. Biological replicate samples were collected for each condition (sample #1 and sample #2). (B) SLAM expression was confirmed on $CXCR5^+$ and $CXCR5^-$ SMtg $CD4^+$ T cells using an aliquot of cells in an independent stain prior to sorting the cells used in panel A. (C) Gene expression microarray analysis (as per Fig. 2.1E). Scatter plot of T_{FH} sample 1 vs T_{FH} sample 2 microarray data. Each gene probe is shown as an individual point. Replicate samples were highly correlated, $r^2 = 0.993$. (D) Non-T_{FH} sample 1 vs. non-T_{FH} sample 2 microarray data. Replicate samples were highly correlated, $r^2 = 0.988$.

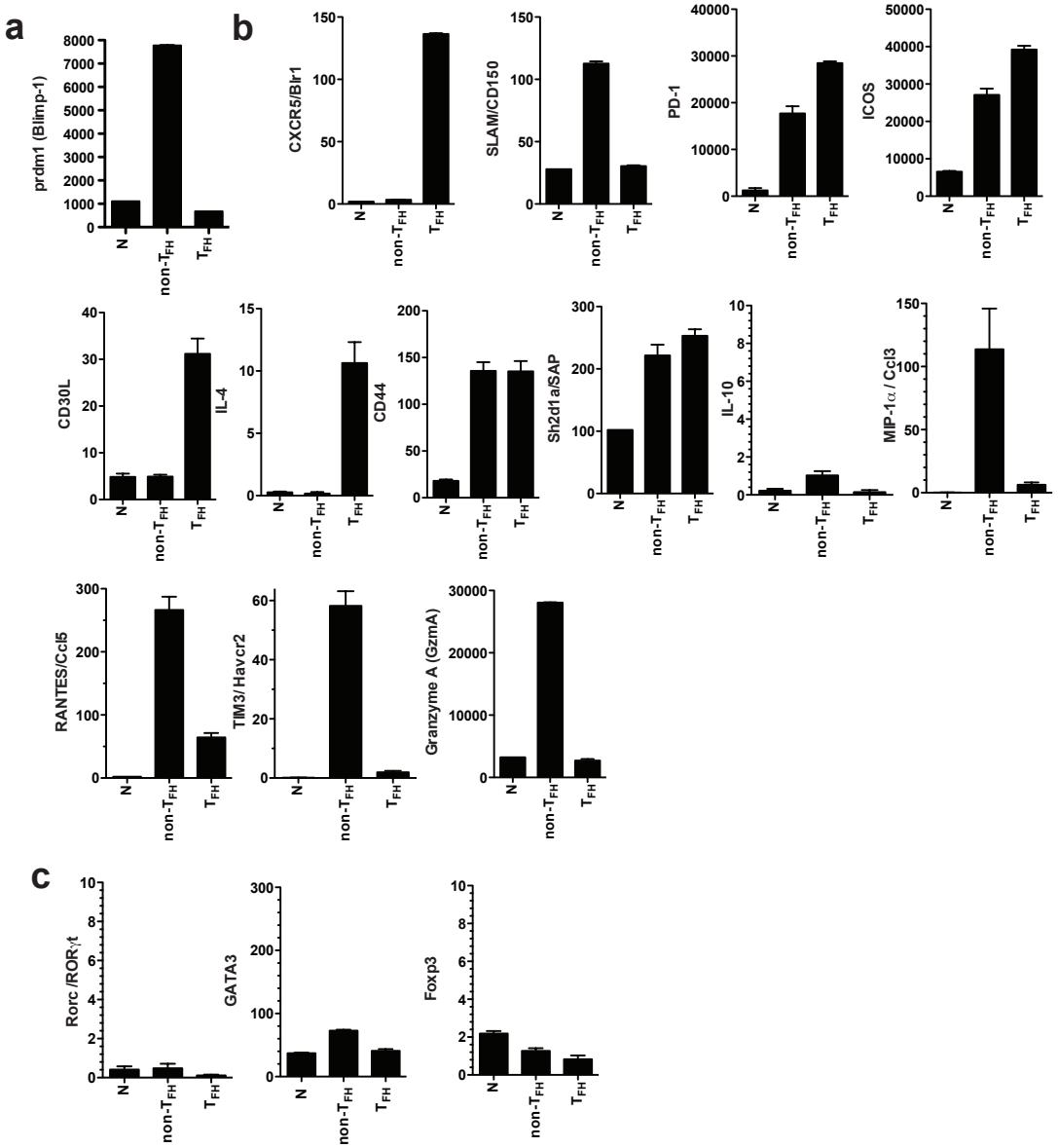

Figure 2.S4: Gene expression microarray analysis of *in vivo* antigen-specific T_{FH}.
(A-C) Gene expression microarray data of selected genes in naive $CD4^+$ T cells (N), SMtg non-T_{FH}, and SMtg T_{FH} from the cells described in Figure 2.S3.

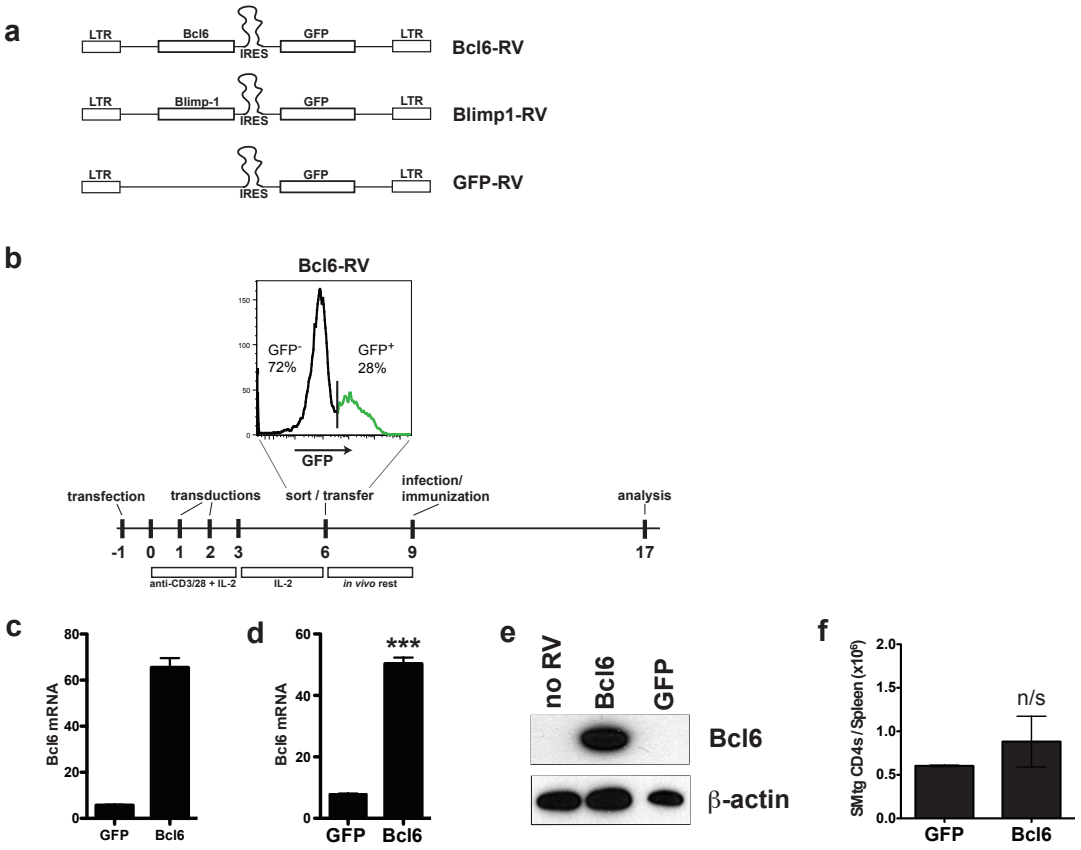

Figure 2.S5: Retroviral expression vectors and transduction schema.
(A) Bcl6 (Bcl6-RV), Blimp1 (Blimp1-RV) and control (GFP-RV, expressing only GFP) retroviral constructs. (B) A brief retroviral transduction and adoptive transfer schematic, with Bcl6-RV$^+$ FACS at the time of sort. Details provided in Methods. (C-D) Bcl6 qPCR using Bcl6-RV$^+$ (transduced) SMtg CD4$^+$ T cells (C) *in vitro* and (D) after transfer into C57BL/6 mice subsequently infected with LCMV. Day 8 post-infection, as per Figure 2.1. GFP-RV$^+$ CD4$^+$ T cells shown for comparison. (E) Bcl6 immunoblot with lysates from transfected and non-transfected Plat-E cells. (F) Expansion of Bcl6-RV$^+$ and GFP-RV$^+$ SMtg cells *in vivo*, day 8 after LCMV infection, as per Figure 2.2, A-E. Graph depicts total Bcl6-RV$^+$ or GFP-RV$^+$ SMtg CD4$^+$ T cells per spleen. No significant difference in expansion was observed between the groups (NS, not significant. $P > 0.05$).

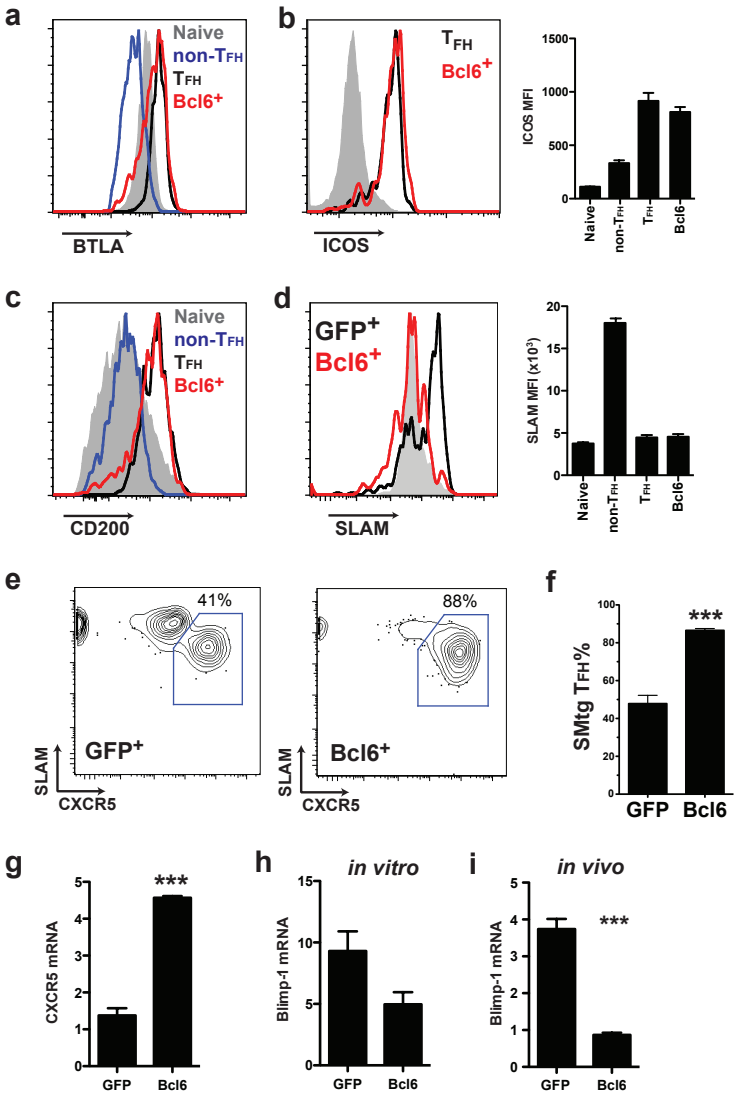

Figure 2.S6: Phenotypic analysis of Bcl6-RV$^+$ CD4$^+$ T cells *in vivo*.
In conjunction with the data shown in Figure 2.2, these data indicated Bcl6 expression drives full T_{FH} differentiation *in vivo*. Histogram overlays and MFI bar graphs depicting expression of (A) BTLA, (B) ICOS, (C) CD200, and (D) SLAM in naïve CD4$^+$ T cells (gray), GFP-RV$^+$ SMtg non-T_{FH} (blue), GFP-RV$^+$ SMtg T_{FH} (black), and Bcl6-RV$^+$ SMtg (red). Data shown are from the same experiments as shown in Figure 2.2. (E-F) Differentiation of GFP-RV$^+$ SMtg ("GFP$^+$") or Bcl6-RV$^+$ SMtg ("Bcl6$^+$") within independent hosts. All mice were infected with LCMV after the cell transfers. Experiments were performed comparably to those described in Figure 2.2, except mice received either GFP-RV$^+$ or Bcl6-RV$^+$ cells. n = 4/group. Data are representative of 6 independent experiments. (E) T_{FH} (SLAMlow CXCR5high, boxed) and non-TFH (SLAMhigh CXCR5low) differentiation of GFP-RV+ SMtg ("GFP$^+$") and Bcl6-RV$^+$ SMtg ("Bcl6$^+$"). (F) Quantitation of SMtg T_{FH} differentiation. ***, P = 0.0002. (G) CXCR5 qPCR in Bcl6-RV$^+$ and GFP-RV$^+$ B6 CD4$^+$ T cells cultured *in vitro*. ***, P < 0.0001. (H-I) Blimp-1 qPCR in Bcl6-RV$^+$ and GFP-RV$^+$ B6 CD4$^+$ T cells (H) cultured *in vitro* and (I) after transfer into C57BL/6 mice subsequently infected with LCMV.

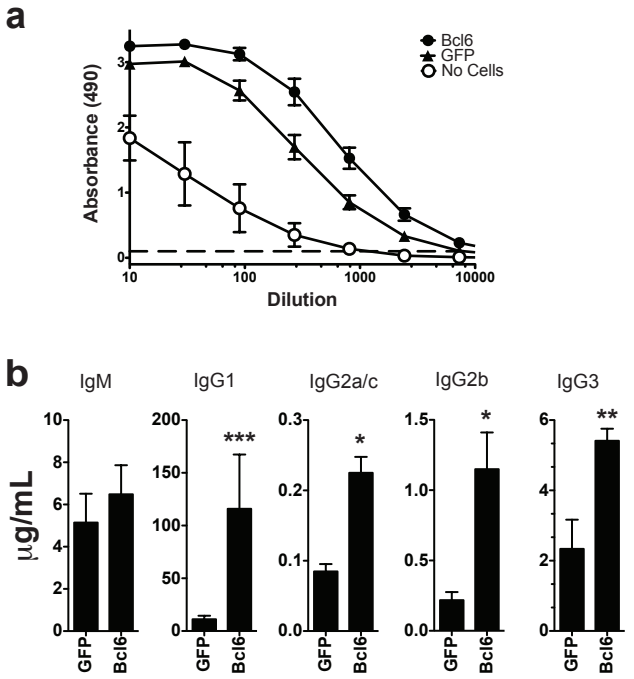

Figure 2.S7: Constitutive Bcl6 expression in CD4$^+$ T cells drives enhanced antibody responses *in vivo*.

Day 8 (A) IgG and (B) isotype-specific anti-NP-Ova ELISA data for samples from the Bcl6-RV$^+$ SMtg vs. GFPonly-RV$^+$ SMtg study described in Figure 2.3B to C. IgG1, ***, P = 0.0009. IgG2a/c, *, P = 0.019. IgG2b, *, P = 0.039. IgG3, **, P = 0.0065. NP-Ova specific IgM levels were not affected by constitutive expression of Bcl6, suggesting that IgM production is not T_{FH} dependent.

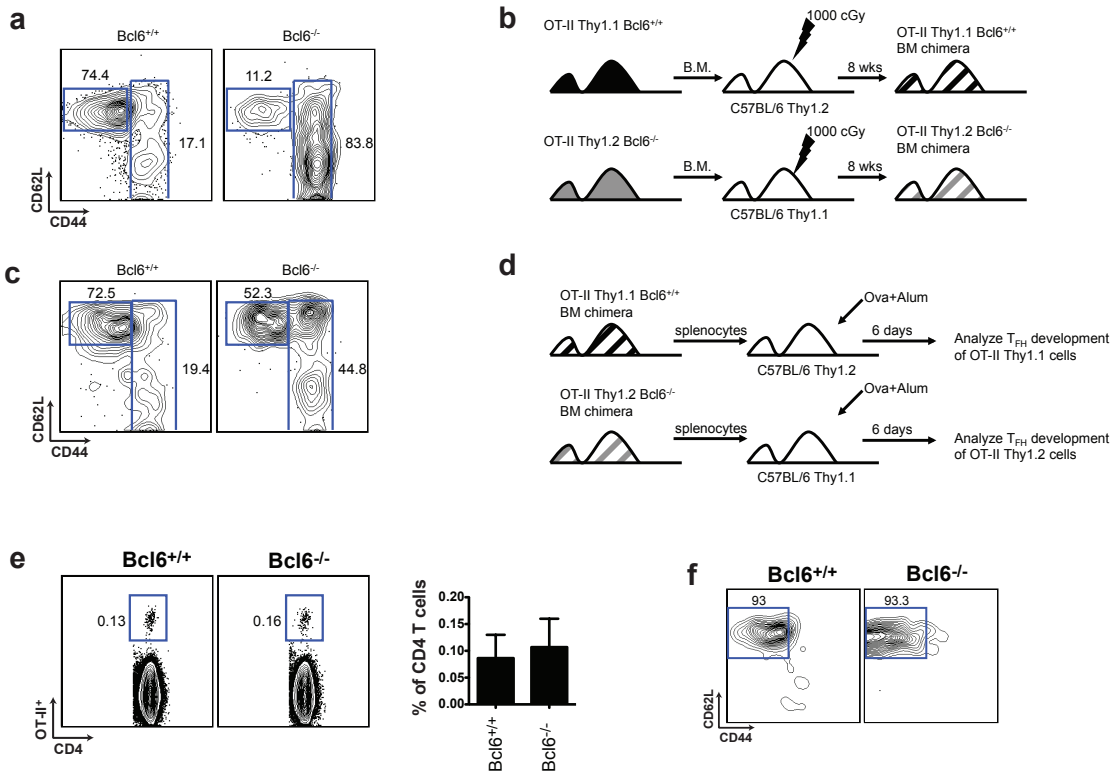

Figure 2.S8: Generation and characterization of Bcl6$^{-/-}$ OT-II CD4^{+} T cells.
(A) Activation state of Bcl6$^{+/+}$ and Bcl6-/- OT-II cells. Splenic CD4^{+} T cells from 3-4 week old mice are shown. (B) Diagram of bone marrow chimera generation. T-depleted bone marrow from OT-II Bcl6$^{+/+}$ and Bcl6$^{-/-}$ mice was transferred into irradiated, congenically mismatched C57BL/6 mice. (C) Activation state of Bcl6$^{+/+}$ and Bcl6$^{-/-}$ OT-II cells isolated from bone marrow chimeras after 8 weeks. (D) Splenocytes were transferred from bone marrow chimeras into congenically mismatched recipient mice for experiments. (E-F) Bcl6$^{-/-}$ OT-II CD4^{+} T cells obtained from chimeric mice did not exhibit lymphoproliferation or spontaneous activation after adoptive transfer. Cell frequencies and numbers (E) and activation state (F) of OT-II Bcl6$^{+/+}$ and Bcl6$^{-/-}$ splenic CD4^{+} T cells as described in Figure 2.3 but in animals that were not immunized.

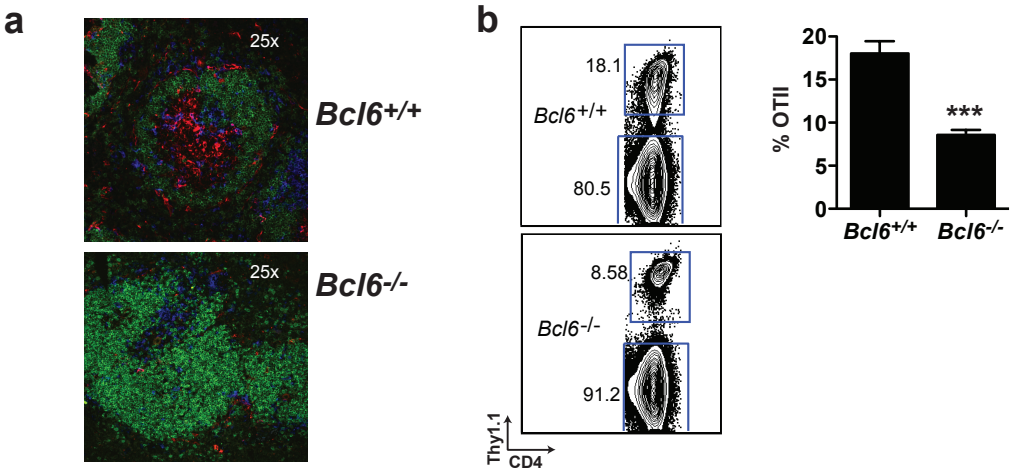

Figure 2.S9: Germinal center formation is dependent on Bcl6 expression by CD4$^+$ T cells. (A) High power images of germinal center histology, from the experiment described in Figure 2.3 E-G: Bcl6$^{+/+}$ or Bcl6$^{-/-}$ OT-II CD4$^+$ T cells were co-transferred with B1-8 B cells into Icos$^{-/-}$ mice subsequently immunized with NP-Ova in alum. Spleen sections were stained with IgD (green), PNA (red), and CD4 (blue). (B) Expansion of Bcl6$^{+/+}$ and Bcl6$^{-/-}$ OT-II CD4$^+$ T cells as described in Figure 2.3D. CD4$^+$ splenocytes are shown.

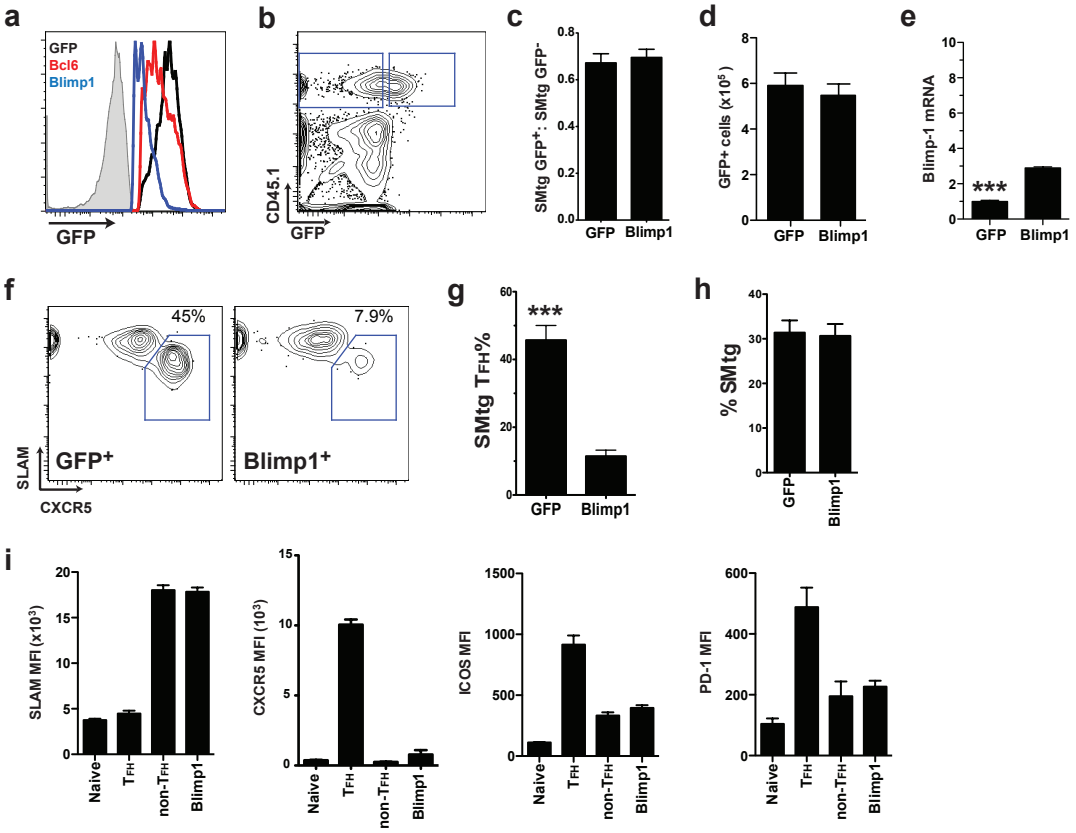

Figure 2.S10: Constitutive Blimp-1 expression selectively blocks T_{FH} differentiation of CD4$^+$ T cells *in vivo*.

(A) Histogram overlay of GFP expression in untransduced (gray), GFP-RV$^+$ (black), Bcl6-RV$^+$ (red), and Blimp1-RV$^+$ (blue) SMtg CD4$^+$ T cells *in vitro*, during cell sorting, prior to adoptive transfer. (B-I) All data shown is from experiments at day 8 after LCMV infection. (B) Cell gating for Figure 2.4B. CD4$^+$ B220$^-$ gate is shown. Transduced (right gate, GFP$^+$ CD45.1$^+$ SMtg cells. "Blimp1") and untransduced (left gate, GFP$^-$ CD45.1$^+$ SMtg cells. "Control") cells. (C-D) Quantitation of GFP-RV$^+$ and Blimp1-RV$^+$ SMtg cells *in vivo*, for the experiment shown in Figure 2.4B-C. (C) Ratio of transduced (Blimp1 or GFP) SMtg to GFP$^-$ (untransduced) SMtg. (D) Number of GFP-RV$^+$ or Blimp1-RV$^+$ SMtg CD4$^+$ T cells per spleen. n = 4/group. Data are representative of 2 independent experiments. (E) qPCR of Blimp-1 mRNA in total GFP-RV$^+$ SMtg (non-T_{FH} and T_{FH}) and total Blimp1-RV$^+$ SMtg from LCMV infected mice, normalized to the β–actin mRNA level (x 10^{-4}). ***, $P < 0.0001$. (F-I) Differentiation of GFP-RV$^+$ SMtg ("GFP$^+$") or Blimp1-RV$^+$ SMtg ("Blimp1$^+$") within independent hosts subsequently infected with LCMV. Experiments were performed comparably to those described in Figure 2.4, except mice only received either GFP-RV$^+$ or Blimp1-RV$^+$ cells. n = 4/group. Data are representative of more than 4 independent experiments. (F) Flow cytometry of T_{FH} (SLAM^{low} CXCR5^{high}, boxed) and non-T_{FH} (SLAM^{high} CXCR5^{low}) differentiation of GFP-RV$^+$ SMtg or Blimp1-RV$^+$ SMtg. (G) Quantitation of SMtg T_{FH} differentiation. ***, $P = 0.0002$. (H) SMtg expansion, as a percentage of CD4$^+$ T cells in the spleen. No difference was observed. (I) Expression levels (MFIs) of SLAM, ICOS, and PD-1 on naïve CD4$^+$ T cells, GFP-RV$^+$ SMtg non-T_{FH} ("non-T_{FH}"), GFP-RV$^+$ SMtg T_{FH} ("T_{FH}"), and Blimp1-RV$^+$ SMtg ("Blimp1"). No significant differences in expression were observed between GFP-RV$^+$ SMtg non-T_{FH} and total Blimp1-RV$^+$ SMtg for any of these proteins.

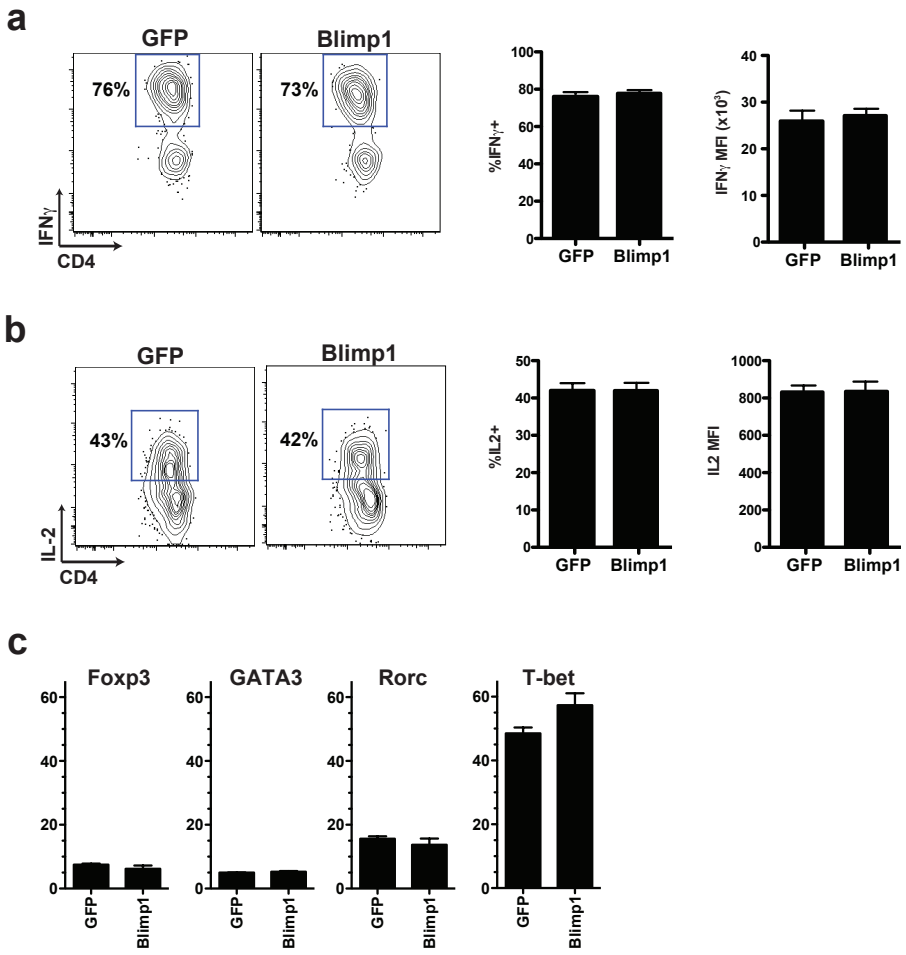

Figure 2.S11: Cytokine production by Blimp1-RV$^+$ SMtg CD4$^+$ T cells.
Blimp1-RV$^+$ and GFP-RV$^+$ SMtg cells were transferred into C57BL/6 mice subsequently infected with LCMV. Cells were analyzed at day 8 after infection. (A) Intracellular staining for IFNγ in GFP-RV$^+$ CXCR5^{low} non-T_{FH} and Blimp1-RV$^+$ CXCR5^{low} non-T_{FH}. Bar graphs show quantification of % IFNγ$^+$ non-T_{FH} SMtg, and IFNγ MFI. No differences were observed. (B) Intracellular staining for IL-2 in GFP-RV$^+$ CXCR5^{low} non-T_{FH} and Blimp1-RV$^+$ CXCR5^{low} non-T_{FH}. Bar graphs show quantification of % IL-2$^+$, and IL-2 MFI. No differences were observed. (C) Foxp3, GATA3, Rorc and T-bet qPCR in GFP-RV$^+$ and Blimp1-RV$^+$ non-T_{FH}, normalized to the β-actin mRNA level (x 10^{-4}). No differences were observed.

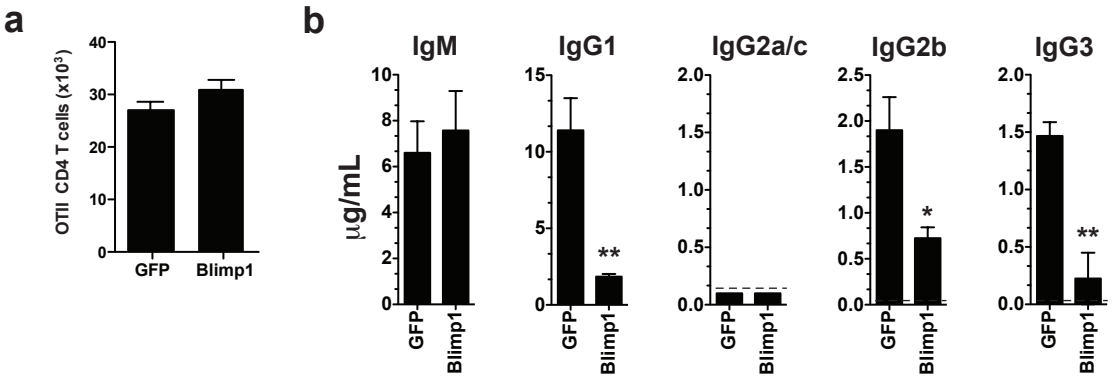

Figure 2.S12: Constitutive Blimp-1 expression in CD4$^+$ T cells prevents antigen-specific IgG responses of all isotypes.

Blimp1-RV$^+$ and GFP-RV$^+$ OT-II cells were transferred into SAP$^{-/-}$ mice subsequently immunized with NP-Ova in alum. Experiment is the same as Figure 2.4E to F. (A) Number of transduced OT-II cells per spleen. (B) Day 10 isotype-specific anti-NP-Ova antibody titers. IgM, not significant. IgG1, **, P = 0.0029. IgG2a/c, not detected. IgG2b, *, P = 0.017. IgG3, **, P = 0.0073. All IgG isotypes were reduced in Blimp1-RV$^+$ recipient mice. IgM levels were again unaffected, suggesting that IgM production is not T_{FH} dependent.

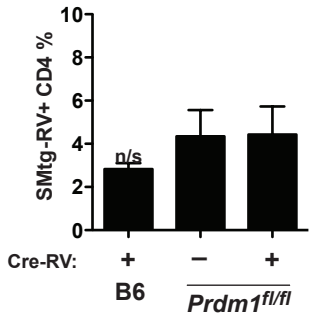

Figure 2.S13: RV-transduced Prdm1$^{fl/fl}$ CD4^{+} T cells proliferate normally.
C57BL/6 and Prdm1$^{fl/fl}$ CD4^{+} T cells were transduced and transferred into SAP-deficient CD45.1^{+} mice subsequently infected with LCMV, as described in Figure 2.4G. Expansion of the transduced CD4^{+} T cells after LCMV infection, as a percentage of total CD4^{+} T cells in the spleen. No significant differences were observed between the groups (NS, not significant. $P > 0.05$).

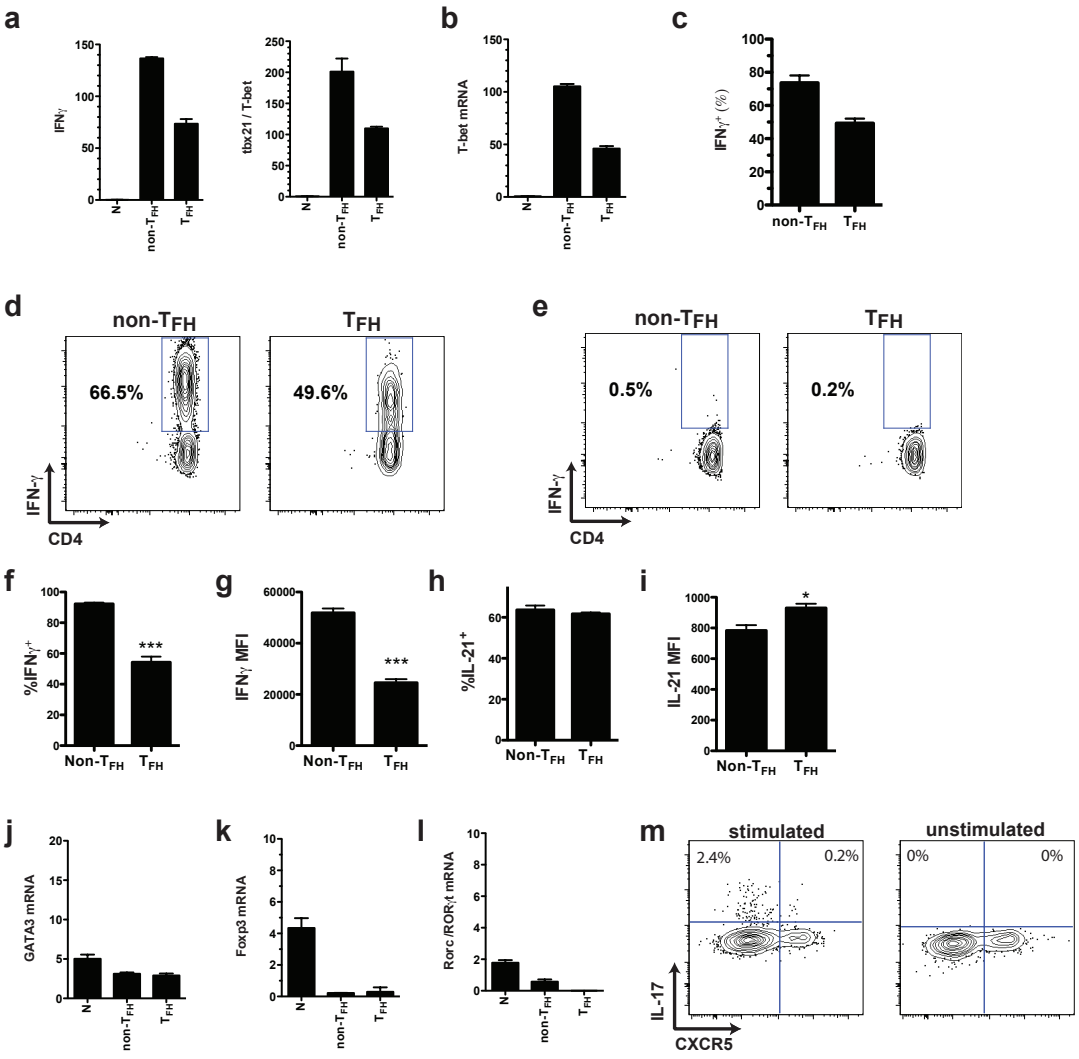

Figure 2.S14: Analysis of cytokines and T_H1, T_H2, T_{reg}, and T_H17 related genes in T_{FH}.
(A) Microarray signals of T_H1 genes IFNγ and T-bet (tbx21) are reduced in T_{FH} vs. non-T_{FH} *in vivo*. N = Naive SMtg $CD4^+$ T cells. (B) qPCR of T-bet from the same RNA, normalized to the β-actin mRNA level (x 10^{-4}). (C-E) The predominant $CD4^+$ T cell response to LCMV is T_H1 (19, 20). IFNγ expression in T_{FH} and non-T_{FH} after *ex vivo* stimulation with antigen presenting cells plus peptide (LCMV gp66-77). n = 4/group. Data are representative of 3 independent experiments. (C) Quantitation and (D) representative FACS plots of IFNγ intracellular cytokine staining in (D) peptide stimulated and (E) unstimulated SMtg $CD4^+$ T cells. (F-I) Cytokine production by SMtg $CD4^+$ T cells after *ex vivo* stimulation with PMA and ionomycin. n = 4/group. Data are representitive of 3 independent experiments. (F) % $IFN\gamma^+$ T_{FH} and non-T_{FH}. The percentage of $IFN\gamma^+$ cells was somewhat reduced in T_{FH}. (G) IFNγ MFI of $IFN\gamma^+$ cells in (F). The expression level (MFI) of IFNγ was somewhat reduced in T_{FH}, among $IFN\gamma^+$ cells. (H) % $IL\text{-}21^+$ in T_{FH} and non-T_{FH}. (I) IL-21 MFI of $IL\text{-}21^+$ cells in (H). (J-L) qPCR for the $CD4^+$ T cell transcription factors (J) GATA3 (normalized to the β-actin mRNA level (x 10^{-4})), (K) Foxp3 (normalized to the β-actin mRNA level (x 10^{-5})), (L) RORγt (normalized to the β-actin mRNA level (x 10^{-5})). (M) IL-17 intracellular cytokine staining in SMtg T_{FH} and non-T_{FH} 8 days after LCMV infection. No significant production was observed.

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40. We thank R. Kageyama, L. Crickard, K. Hansen, C. Kim, and K. Van Gunst for technical assistance. We thank S. Kaech for helpful discussions, and A. Haberman and S. Kerfoot for technical help. We acknowledge the NIH Tetramer Core for providing MHCII tetramer reagents. This work was supported by LIAI Institutional Funds, a Pew Scholar Award, a Cancer Research Institute Award, and NIH NIAID R01 072543 and NIAID R01 063107 to S.C. This work was funded by NIH Grants AR40072, AR44076, P30 AR053495, Rheuminations, Inc., the Arthritis Foundation, and the Connecticut Chapter of the Lupus Foundation of America to J.C. I.Y. and R.J. were supported by fellowships from the UCSD/LIAI Immunology NIH Training Grant. R.J. is a member of the UCSD Biomedical Sciences (BMS) graduate program. Microarray data deposited at NCBI GEO, GSE16697. Author contributions are as follows. LCMV T_{FH} identification and microarrays: I.Y. and S.C.; Bcl6-RV and Blimp1-RV experiments: R.J., D.D., B.B.; T_{FH} migration: D.E.; Blimp-1 conditional knockout experiments: D.D.; $Bcl6^{-/-}$ mice: A.D.; $Bcl6^{-/-}$ experiments: A.P.; S.C. wrote the manuscript with intellectual and editorial contributions from the other authors; project conception and experimental design: S.C. and J.C.

2.5: Acknowledgements

Chapter 2, in full, is a reprint of the material as it appears in *Science*, volume 325: 1006-1010, 2009, with co-authors Amanda C. Poholek, Daniel DiToro, Isharat Yusuf, Danelle Eto, Burton Barnett, Alexander L. Dent, Joe Craft, and Shane Crotty. The dissertation author was the first author of this publication.

Chapter 3:

Multiple Bcl6 repression domains are required for control of T_{FH} differentiation

3.1: Abstract

Follicular Helper T cells (T_{FH}) are effector $CD4^+$ T cells that are uniquely capable of driving the germinal center reaction. Recently, it was shown that the transcription factor Bcl6 is a master regulator of T_{FH} differentiation. Bcl6 is a transcriptional repressor that is known in B cells to recruit co-repressors to target genes through its bric-a-brac, tramtrack, broad-complex (BTB) functional domain. Another Bcl6 functional domain, repression domain two (RDII), functions independently to recruit the co-repressor MTA3 and has been shown to mediate repression of Blimp-1 in B cells. Blimp-1 is an inhibitor of Bcl6 and T_{FH} differentiation, and so is likely to be a key Bcl6 gene target in T_{FH} cells. However, it is unknown if RDII domain-mediated inhibition of Blimp-1 expression is sufficient to induce T_{FH} differentiation, or if additional Bcl6 gene regulation is needed. Using Bcl6 mutants with impaired co-repressor recruitment, we found that both the BTB domain and the RDII domain were required for optimal T_{FH} differentiation. Interestingly, impaired RDII domain function also resulted in a severe decline in $CD4^+$ T cell expansion. Ablation of Blimp-1 was not sufficient to restore expansion in cells with impaired Bcl6 co-repressor recruitment. These data indicate that Bcl6 regulates multiple downstream pathways to control T_{FH} differentiation, utilizing multiple Bcl6 functional domains.

3.2: Introduction

The transcriptional repressor B cell CLL/lymphoma 6 (Bcl6) is a key regulator of germinal center (GC) B cell differentiation and survival. In these cells, which undergo extensive somatic hypermutation and repeated rounds of division and selection to achieve affinty maturation, Bcl6 is crucial to potentiating proliferation and inhibiting DNA-damage induced cell death (1). Bcl6 represses the transcription factor Blimp-1, a master regulator of plasma cell differentiation and itself an antagonist of Bcl6 and the GC B cell gene program (1). Together, Bcl6 and Blimp-1 determine B cell fate by controlling the decision between GC B cell and plasma cell differentiation.

Bcl6 is also expressed by effector $CD4^+$ T cells within germinal centers, known as Follicular Helper T cells (T_{FH}) (2-6), and has recently been shown to control T_{FH} cell differention (7-9). As in B cells, Bcl6 and Blimp-1 are reciprocal regulators of $CD4^+$ T cell fate, with Blimp-1 driving the differentiation of non-T_{FH} effector cells such as T_H1- and T_H2-polarized cells (1). Aside from inihibiting Blimp-1, it is possible that Bcl6 has different functions in T cells and B cells. T_{FH} cells do not undergo affinity maturation, and gene expression analysis has found few similarities between GC B cells and T_{FH} cells beyond Bcl6 and the lack of Blimp-1 (1).

Other effector $CD4^+$ T cell master regulators (T-bet, GATA3, RORγT, and FoxP3) have been shown to control expression of effector cytokines, and to inhibit the expression of competing transcription factors (10). In some studies, Bcl6 has been found to inhibit expression of T-bet, GATA3, and/or RORγT (8, 9, 11, 12), though in other studies Bcl6-expressing T_{FH} cells were shown to express T-bet, GATA3, or

RORγT in T_H1-, T_H2-, and T_H17-polarizing conditions, respectively (7, 13) (14). Expression of IL-21, an important T_{FH} cytokine, is not dependent on Bcl6 (8). One report found that Bcl6 repressed a microRNA cluster capable of inhibiting B cell expression of CXCR5, the chemokine receptor that also enables T_{FH} to access B cell follicles and germinal centers (9). Yet constitutive expression of Bcl6 does not induce CXCR5 protein expression by $CD4^+$ T cells *in vitro* (Eto and Crotty, submitted). Thus, the mechanisms by which Bcl6 controls the T_{FH} lineage remain unclear.

Here, we have examined the contributions of individual Bcl6 repressor domains to T_{FH} differentiation and function. Bcl6 consists of a bric-a-brac, tramtrack, broad-complex (BTB) domain, a second repressor domain (RDII), and a DNA-binding zinc finger domain (1). A number of BTB domain-containing and other Bcl6 co-repressors are recruited to the BTB domain (15). The co-repressor MTA3, which mediates Blimp-1 repression (16), is recruited to the RDII domain (16, 17). This distinction enabled us to examine Bcl6 BTB domain-mediated gene regulation independently of the gene regulation exerted by Bcl6 via lifting Blimp-1-mediated gene repression. We found that disrupting the function of either the BTB domain or the RDII domain impaired T_{FH} cell differentiation. Surprisingly, in cells expressing a form Bcl6 with a mutated RDII domain, T_{FH} cell differentiation was not restored by the absence of Blimp-1. These results indicate that multiple Bcl6 repressor domains control T_{FH} cell differentiation, and that the role of the RDII domain extends beyond inhibition of Blimp-1.

3.3: Materials and Methods

Mice. C57BL/6J (B6) mice were purchased from the Jackson Laboratory. $SAP^{-/-}$, μMT, $Prdm1^{fl/fl}$, and SMARTA TCR transgenic (SM, specific for LCMV gp66-77 on $I\text{-}A^b$) mice were on a fully B6 background and were bred at LIAI. All animal experiments were conducted in accordance with approved animal protocols.

Retroviral vectors, transductions, and cell transfers. The green fluorescent protein (GFP)-expressing retroviral expression vector pMIG was used in gene expression experiments. Bcl6 cDNA was obtained from Open Biosystems (clone ID 6309948), and the complete open reading frame was cloned into pMIG, as previously described (7). Mutant Bcl6 retroviral vectors (RV's) were generated using site-directed mutagenesis of the the wildtype Bcl6 construct. BTB-mutant Bcl6 RV (BTB-mut) was generated by introducing N21K and H116A mutations (18). RDII-mutant Bcl6 RV (RDII-mut) was generated by three lysines within the KKYK acetylation motif (amino acids 376-379) with glutamines, resulting with a QQYQ motif that mimics acetylation (19). A Cre recombinase-expressing RV was generated using the NLS-Cre sequence of Rajewsky and colleagues (20), as previously described (7). Deletion of *Prdm1* in $Prdm1^{fl/fl}$ $CD4^+$ T cells transduced with Cre-RV has previously been shown to be greater than 98% (7).

The Open Biosystems GFP-expressing retroviral expression vector LMP was used in shRNA expression experiments (catalog number EAV4679). $CD4^+$ T cells transduced with unmodified LMP were rapidly rejected when transferred into B6 host mice subsequently infected with LCMV (Fig. 3.S1). Therefore, the puromycin

resistance gene (pac) and accompanying internal ribosomal entry sequence was removed (Fig. 3.S1). $CD4^+$ T cells transduced with this modified form of LMP were not appreciably rejected (Fig. 3.S1). To facilitate co-transduction experiments, a second modified form of LMP was generated in which GFP was replaced by mAmetrine1.1 (Fig. 3.S1). Bcl6 shRNA target sequences were selected from Open Biosystems. Three Bcl6 shRNA sequences were synthesized: shRNA-53, shRNA-54, and shRNA-55 (Table 3.1). shRNA-53 and Bcl6 shRNA-54 were cloned into GFP-expressing modified LMP, and Bcl6 shRNA-55 was cloned into mAmetrine1.1-expressing LMP.

Virions were produced using the Plat-E cell line (21), as previously described (7, 22). $CD4^+$ T cells were purified from the splenocytes of naïve mice by magnetic bead negative selection (Miltenyi, catalog number 130-090-861) and suspended in D-10 (DMEM + 10% fetal calf serum, supplemented with 2mM GlutaMAX (Gibco) and 100 U/mL Penicillin/Streptomycin (Gibco)) with 10 ng/mL recombinant human IL-2 and 50 μM β-Mercaptoethanol (BME). 2 x 10^6 cells per well were stimulated in 24-well plates pre-coated with 8 μg/mL anti-CD3 (clone 17A2, BioXcell) and anti-CD28 (clone 37.51, BioXcell). After 24 hours, cells were transduced as described (7, 22). Where necessary, cells were co-transduced by simultaneously transducing with two individual RV's. After a total of 72 hours of stimulation, the $CD4^+$ T cells were split 1:3 and transferred into new wells with fresh D-10, IL-2 and BME. After a further 72 hours, transduced cells were purified by sorting $7AAD^-$ GFP^+, $7AAD^-$ mAmetrine1.1^+, or $7AAD^-$ GFP^+ mAmetrine1.1^+ cells on a FACSDiva or FACSAria (BD Biosciences).

Cell transfers into host mice were performed as described (7), by intravenous injection via the retro-orbital sinus. Transferred cells were allowed to rest in host mice for 3-5 days before infection or immunization. 25 x 10^3 transduced SM cells were transferred into each mouse; these cells expanded as efficiently as 5 x 10^3 naïve SM cells (data not shown), suggesting that the activated and transduced SM cells contracted roughly 5 fold after adoptive transfer.

Infections. LCMV stocks were prepared and quantified as previously described (22). Infections were performed by intraperitoneal injection of 0.5-2 x 10^5 plaque-forming units of LCMV Armstrong per mouse.

Flow cytometry. Single-cell suspensions of spleen were prepared by standard gentle mechanical disruption. Surface staining for flow cytometry was done with monoclonal antibodies against SLAM (CD150, Biolegend) and CD4, CD8, CD44, CD62L, CD25, B220, Fas, and GL7 (eBiosciences). Stains were done for 30 minutes at 4 °C in PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide, unless specified otherwise.

CXCR5 staining was done as described (7), using purified anti-CXCR5 (BD Pharmingen or BioXcell) for 1 hour, followed by biotinylated anti-rat IgG (Jackson Immunoresearch), and then by APC- or PE-labelled streptavidin (Caltag Laboratories) at 4° C in PBS supplemented with 0.5% bovine serum albumin, 2% fetal calf serum, and 2% normal mouse serum. Samples were not fixed and were acquired immediately.

Intracellular staining for BCl6 was performed with a PE-conjugated monoclonal antibody to Bcl6 (BD Pharmingen) and the FoxP3 ICS kit buffers and protocol (eBioscience).

Immunoblot. Bcl6 protein was detected by Western blot, as previously described (7). Whole cell lysates were prepared using RIPA lysis buffer. Blots were probed with polyclonal anti-Bcl6 (C19, Santa Cruz Biosciences).

Statistical Analysis. Statistical tests were performed using Prism 5.0 (GraphPad). P values were calculated by two-tailed unpaired Student's t tests with a 95% confidence interval. Error bars depict the standard error of the mean. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

Table 3.1: Bcl6 shRNA sequences

	Sequence	Location
shRNA 53	GCTGTCAAAGAGAAGGCTTTA	bp 2629 – 2649 (3' UTR)
shRNA 54	CCGGCTCAATAATCTCGTGAA	bp 1428 – 1448 (ORF)
shRNA 55	CGGCCTGTTCTACAGTATCTT	bp 250 – 270 (ORF)

3.4: Bcl6 is expressed in naïve $CD4^+$ T cells

In previous studies we found Bcl6 mRNA expression in naïve $CD4^+$ T cells, challenging the notion that Bcl6's role in $CD4^+$ T cells was limited to effector differentiation (7, 23). At least one other effector $CD4^+$ T cell master regulator, GATA3, is also expressed in naïve $CD4^+$ T cells, and has multiple crucial roles in $CD4^+$ T cell development (24). It was therefore important to determine if Bcl6 protein was indeed expressed in naïve $CD4^+$ T cells. Our previous studies had measured Bcl6 expression by mRNA, which is a poor indicator of protein expression due to extensive post-transcriptional regulation of Bcl6 (1). To test for Bcl6 protein expression, we performed an immunoblot for Bcl6 in naïve ($CD44^{low}$ $CD25^{low}$ $CD62L^{high}$) $CD4^+$ T cells. Bcl6 protein was detected in naïve $CD4^+$ T cells, although at levels much lower than in germinal center B cells or T_{FH} cells purified from mice infected with Lymphocytic Choriomeningitis Virus (LCMV) (Fig. 3.1). The presence of Bcl6 in naïve cells raised the possibility that Bcl6 played a role in CD4 T cell development or homeostasis, and consequently gainsaid the use of germline Bcl6 knockout and transgenic systems to examine the role of Bcl6 in T_{FH} cell differentiation.

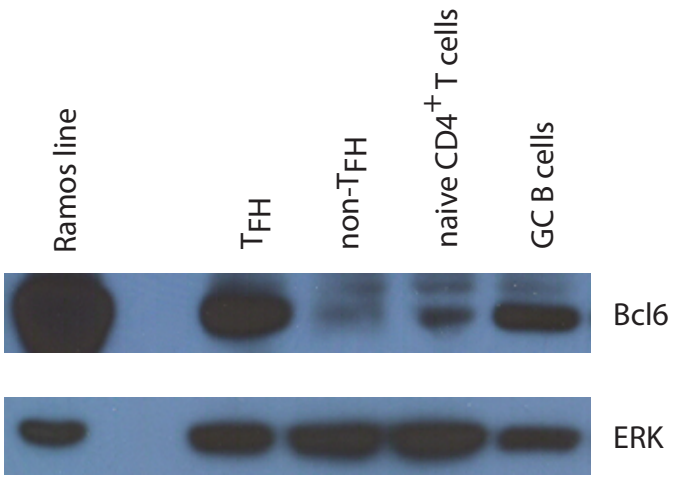

Figure 3.1: Bcl6 is expressed in naïve CD4$^+$ T cells.
Bcl6 protein expression was measured by Western blot in primary lymphocytes, with the Bcl6-expressing Ramos cell line as a positive control. GC B cells (B220$^+$ GL7$^+$ Fas$^+$), non-T_{FH} effector CD4$^+$ T cells (CD4$^+$ CD44^{high} CXCR5$^-$), and T_{FH} cells (CD4$^+$ CD44^{high} CXCR5$^+$) were purified from the spleens of C57BL/6 mice 8 days after infection with LCMV. Naïve CD4$^+$ T cells (CD4$^+$ CD44^{low} CD25$^-$) were purified from uninfected C57BL/6 mice.

3.5: Silencing Bcl6 in peripheral $CD4^+$ T cells inhibits T_{FH} differentiation, but non-T_{FH} differentiation

Previous studies have shown that $Bcl6^{-/-}$ $CD4^+$ T cells are unable to differentiate into T_{FH} cells (7-9). However, these studies all utilized germline Bcl6 knockout mice, making it possible that the absence of Bcl6 during thymic development, rather than during effector differentiation, was contributing to the inability of the cells to undergo T_{FH} differentiation. To test the importance of Bcl6 only after thymic development, we constructed retroviral vectors (RV's) expressing Bcl6-specific short hairpin RNAs in a natural shRNAmir context (Fig. 3.S1). We transduced peripheral SMARTA TCR-transgenic (SM) $CD4^+$ T cells, which recognize the LCMV gp66-77 epitope when presented by MHC class II $I\text{-}A^b$, and then adoptively transferred the transduced cells into C57BL/6 mice subsequently infected with LCMV. SM cells that were transduced with a control RV expressing only GFP (GFP-only) differentiated normally into a $Bcl6^-$ non-T_{FH} population and a $Bcl6^+$ T_{FH} population (Fig. 3.2A). In contrast, SM cells that were transduced with Bcl6 shRNA RV's ($shRNA^+$) did not express Bcl6 protein ($P < 0.001$) (Fig. 3.2B) and largely lost the ability to differentiate into T_{FH} cells (from 47% to 16%, $P = 0.002$) (Figure 3.2A). Non-T_{FH} differentiation was unaffected (Fig. 3.2A). shRNA-mediated knockdown of Bcl6 expression also blocked the of germinal center T_{FH} cells, which are distinguished by GL7 expression in an acute viral infection (25) ($P = 0.003$) (Fig. 3.2C). Germinal center formation in $shRNA^+$ SM hosts was significantly lower than in $GFP\text{-}only^+$ SM hosts ($P = 0.039$) (Fig. 3.2D) These data indicated that Bcl6 expression in activated

$CD4^+$ T cells is required for T_{FH} differentiation and T cell help to germinal centers, apart from any role Bcl6 may be playing earlier in T cell development. Thus, it was possible to design experiments that manipulated Bcl6 functionality during activation and effector differentiation but not elsewhere.

3.6: BTB domain-mediated Bcl6 gene regulation is required for optimal T_{FH} differentiation.

We then tested the contributions of individual Bcl6 functional domains to T_{FH} differentiation. Bcl6's BTB domain recruits most of the co-repressors known to interact with Bcl6 (15). Many of these co-repressors bind to a groove formed at the interface of the BTB domain dimer (18, 26). We made use of N21K and H116A mutations in the BTB domain, which have previously been shown to prevent co-repressor binding to the BTB domain dimer groove without impairing dimerization, DNA binding, or the recruitment of co-repressors to other regions of Bcl6 (18, 27). We transduced SM $CD4^+$ T cells with a RV expressing this mutant form of Bcl6 (BTB-mut), a RV expressing wildtype Bcl6 (Bcl6-WT), or the GFP-only control RV. Cells transduced with BTB-mut RV expressed the same amount of Bcl6 protein as cells transduced with Bcl6-WT RV (Fig. 3.S2). Transduced SM $CD4^+$ T cells were transferred into naïve C57BL/6 host mice subsequently infected with LCMV. Ectopic expression of wildtype Bcl6 drove T_{FH} cell differentiation, as previously reported (Fig. 3.3A) (7). Ectopic expression of BTB-mutant Bcl6 also enhanced T_{FH} differentiation (Fig. 3.3A). Interestingly, however, BTB-mut^+ SM cells did not differentiate into

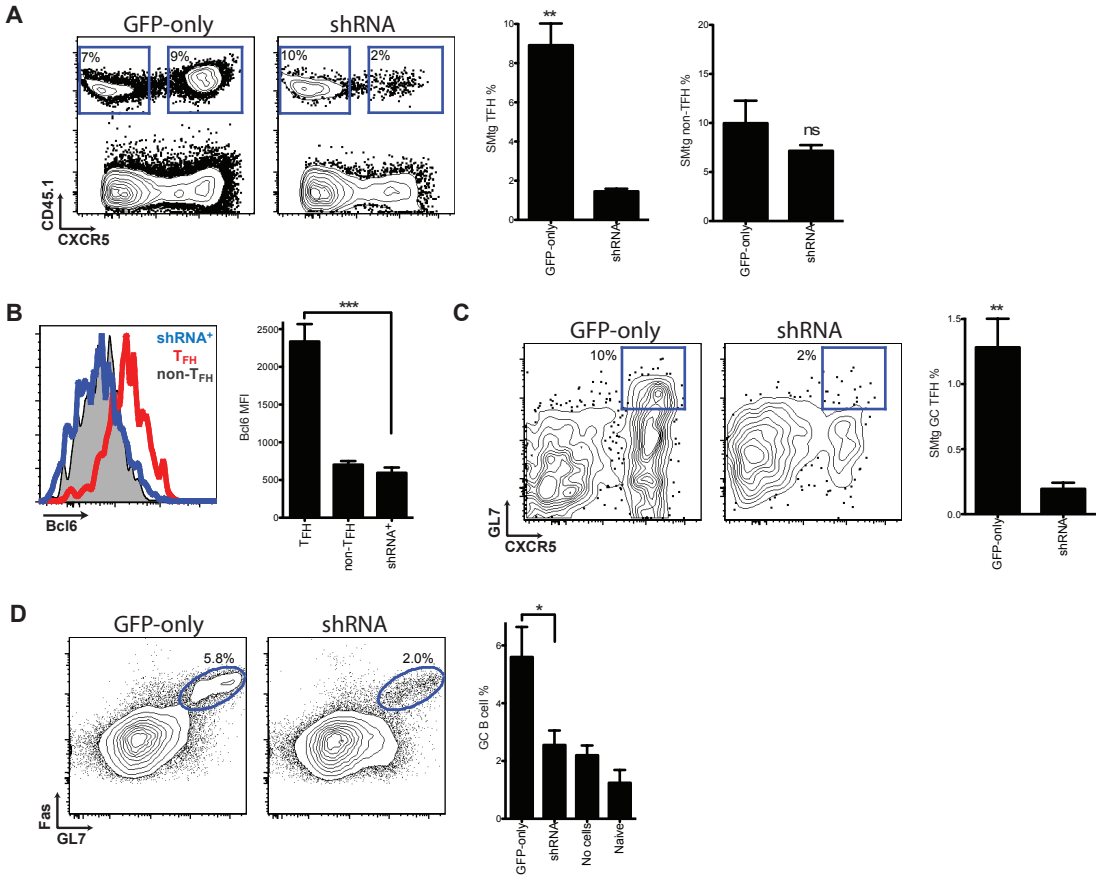

Figure 3.2: Silencing Bcl6 in peripheral CD4$^+$ T cells inhibits T_{FH} differentiation, but not non-T_{FH} differentiation.

SMtg CD4$^+$ T cells were transduced with an empty control RV (GFP-only), or were co-transduced with two Bcl6 shRNA RV's (shRNA). Transduced SMtg cells were adoptively transferred into C57BL/6 host mice that were subsequently infected with LCMV. Other mice received no cells but were still infected with LCMV (No cells) or were not infected (Naïve). Splenocytes were analyzed 6 days after infection. Data are representative of 3 independent experiments; n = 4 per group. (A) SMtg T_{FH} differentiation. FACS plots are gated on CD4$^+$ T cells. SMtg T_{FH} cells (CD45.1$^+$ CXCR5$^+$) and SMtg non-T_{FH} cells (CD45.1$^+$ CXCR5$^-$) are boxed. Quantification of SMtg T_{FH} and SMtg non-T_{FH} as a percentage of total CD4$^+$ T cells is also shown. (B) Histogram overlay of Bcl6 expression in shRNA-transduced SMtg cells (CD4$^+$ CD45.1$^+$ GFP$^+$ Ametrine$^+$), GFP-only-transduced SMtg T_{FH} cells (CD4$^+$ CD45.1$^+$ GFP$^+$ CXCR5$^+$), and GFP-only-transduced SMtg non-T_{FH} cells (CD4$^+$ CD45.1$^+$ GFP$^+$ CXCR5$^-$). Quantification of Bcl6 MFI is also shown. (C) SMtg GC T_{FH} differentiation. FACS plots are gated on transduced SMtg cells (CD4$^+$ CD45.1$^+$ GFP$^+$, or CD4$^+$ CD45.1$^+$ GFP$^+$ Ametrine$^+$). GC T_{FH} cells (CXCR5$^+$ GL7$^+$) are boxed. Quantification of SMtg GC T_{FH} as a percentage of total CD4$^+$ T cells is also shown. (D) Germinal center B cell differentiation in mice receiving SMtg cells transduced with the indicated RV's. FACS plots are gated on B220$^+$ cells. Germinal center B cells (GL7$^+$ Fas$^+$) are circled. Quantification of germinal center B cells as a percentage of total B cells is also shown.

T_{FH} cells as efficiently as Bcl6-WT^+ SM cells ($P < 0.001$) (Fig. 3.3A). Because changes in antigen-specific $CD4^+$ T cell abundance can affect T_{FH} differentiation (data not shown), we examined the expansion of the transduced SM cells in their host mice. We found no difference between BTB-mut^+ SM cells and GFP-$only^+$ or Bcl6-WT^+ SM cells ($P > 0.05$, Fig. 3.3B). Therefore, BTB domain co-repressor recruitment contributed to T_{FH} differentiation.

Under normal conditions, T_{FH} differentiation is dependent on the presence of cognate B cells (7, 28). Ectopic Bcl6 expression overcomes this requirement, suggesting that interaction with cognate B cells is a key step in the induction or maintenance of Bcl6 expression (7). To further test the importance of Bcl6 BTB domain-mediated regulation, we transferred GFP-$only^+$, Bcl6-WT^+, or BTB-mut^+ SM cells into B cell deficient μMT mice that were subsequently infected with LCMV. As expected, GFP-$only^+$ SM cells were largely unable to differentiate into T_{FH} cells, while Bcl6-WT^+ SM cells did differentiate into T_{FH} cells (Fig. 3.3C). BTB-mut^+ SM cells differentiated into T_{FH} as efficiently as Bcl6-WT^+ SM cells (Fig. 3.3C).

We also transferred BTB-mut^+ SM cells into $SAP^{-/-}$ mice, whose endogenous $CD4^+$ T cells are unable drive the germinal center reaction due to an inability to differentiate into germinal center T_{FH} cells (23, 29) (30). In $SAP^{-/-}$ hosts, BTB-mut^+ SM cells initiated germinal centers as effectively as Bcl6-WT^+ SM cells (Fig. 3.3D). Taken together, these data suggested that while Bcl6 BTB domain activity is required for optimal T_{FH} cell differentiation, other Bcl6 domains were the major contributers to T_{FH} cell differentiation and function.

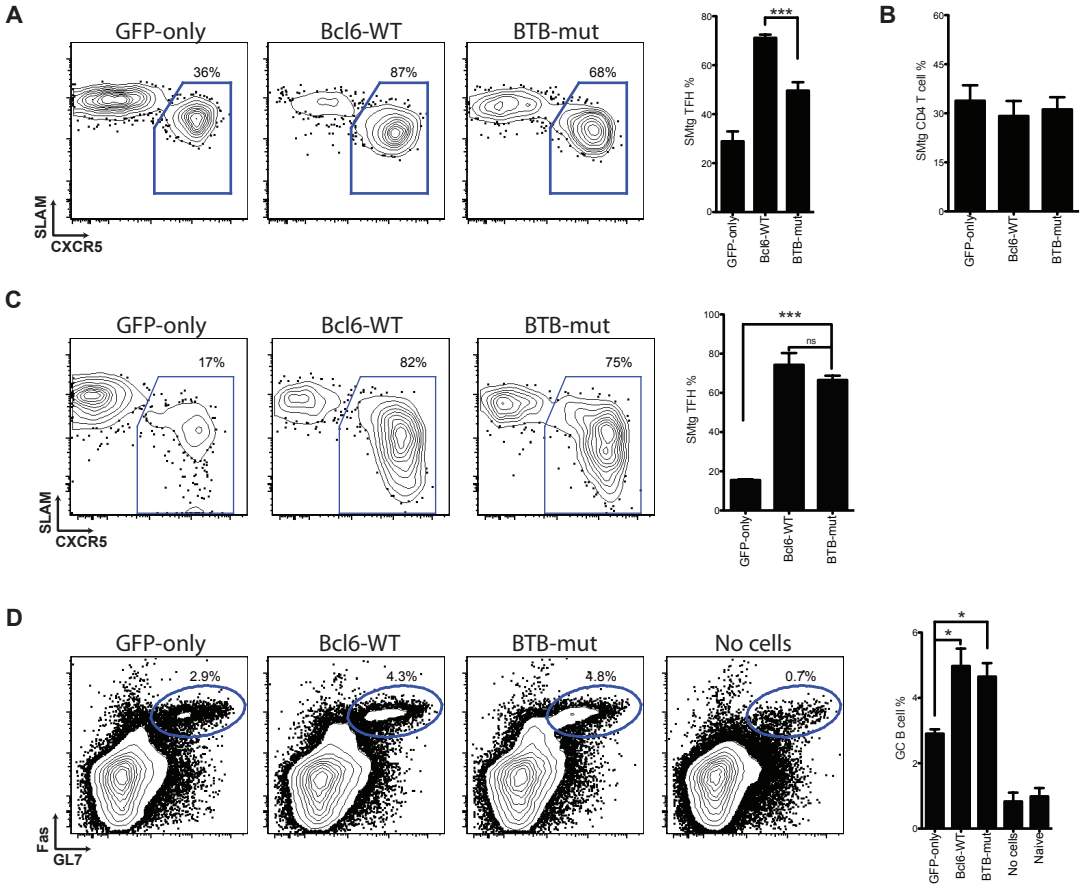

Figure 3.3: BTB domain-mediated Bcl6 gene regulation is required for optimal T_{FH} differentiation.

SMtg $CD4^+$ T cells were transduced with an empty control RV (GFP-only), a wildtype Bcl6 RV (Bcl6-WT), or a RV expressing a mutant form of Bcl6 in which BTB-mediated co-repressor recruitment is impaired (BTB-mut). Transduced SMtg cells were adoptively transferred into host mice that were subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. (A-B) SMtg T_{FH} differentiation (A) and SMtg cell expansion (B) in C57BL/6 host mice. FACS plots are gated on GFP^+ $SMtg^+$ $CD4^+$ T cells. T_{FH} cells ($CXCR5^+$ $SLAM^{low}$) are boxed. Quantification shows the percentage of SMtg cells that were T_{FH} (A) and the percentage of $CD4^+$ T cells that were $SMtg^+$ (B). Each group is a composite of 4 independent experiments; n = 12-20 per group. (C) SMtg T_{FH} differentiation in μMT (B cell-deficient) host mice, gated and calculated as in (A). Each group is a composite of two independent experiments; n = 5-6 per group. (D) Transduced SMtg cells were adoptively transferred into $SAP^{-/-}$ mice subsequently infected with LCMV. Other mice received no cells but were infected (No cells) or were not infected (Naive). FACS plots are gated on B cells, with germinal center B cells ($GL7^+$ Fas^+) circled. Quantitation shows germinal center B cells as a percentage of total B cells.

3.7: RDII domain-mediated Bcl6 gene regulation is required for T_{FH} cell persistence

The previous results suggested that the BTB domain was not the only Bcl6 domain that has a role in T_{FH} differentiation. The second Bcl6 repressor domain, the RDII domain, recruits the co-repressor MTA3 (16) and mediates repression of *Prdm1*, the gene which encodes Blimp-1 (16, 17). Blimp-1 is an antagonist of Bcl6 in both B cells and T cells, and is a potent inhibitor of T_{FH} differentiation (1, 7). Because of this, we hypothesized that impairing Bcl6 RDII domain-mediated gene regulation would strongly inhibit T_{FH} cell differentiation. The RDII domain contains an acetylation site, and a mutation that mimics constitutive acetylation of Bcl6 has the effect of inhibiting RDII-mediated co-repressor recruitment and gene regulation (16, 19).

We constructed a mutant form of Bcl6 with this RDII domain mutation (RDII-mut) and used this in the SM retroviral expression system described above. SM cells transduced with the RV containing RDII-mutant Bcl6 (RDII-mut$^+$) expressed Bcl6 at the same level as Bcl6-WT$^+$ SM cells (Fig. 3.S2). SM cells transduced with the GFP-only control RV differentiated normally into T_{FH} cells, and approximately 6% of the splenic CD4$^+$ T cell population in C57BL/6 host mice were SM T_{FH} cells (Fig. 3.4A). In contrast, RDII-mut$^+$ SM cells failed to differentiate into T_{FH}, with RDII-mut$^+$ SM T_{FH} cells making up only about 0.1% of the splenic CD4$^+$ T cell compartment ($P < 0.001$) (Fig. 3.4A). Surprisingly, RDII-mut$^+$ SM cells also failed to develop into non-T_{FH} effector cells. Expansion of the RDII-mut$^+$ SM cells was reduced more than 99% relative to the GFP-only$^+$ SM control group ($P = 0.001$) (Fig. 3.4A,B). The

transduction efficiency of the RDII-mutant Bcl6 RV was normal, and RDII-mut$^+$ SM cells persisted normally *in vivo* prior to infection (Fig 3.4C and data not shown). Taken together, these data indicated that Bcl6 RDII domain-mediated gene regulation was indeed essential to T_{FH} differentiation. The loss of non-T_{FH} differentiation in RDII-mutant Bcl6-expressing CD4$^+$ T cells was in contrast with the normal non-T_{FH} differentiation seen in Bcl6-silenced cells (Fig. 3.2). This suggests that the mutant form of Bcl6 was sufficient to induce cells to commit to T_{FH} differentiation, but that in the absence of RDII domain-mediated gene regulation these would-be T_{FH} cells were unable to persist.

3.8: Blimp-1 ablation does not restore T_{FH} differentiation in the absence of RDII domain-mediated Bcl6 gene regulation

Only a handful of Bcl6 target genes have been shown to be regulated by the RDII domain. One RDII domain target is Blimp-1 which, in addition to its role as an inhibitor of Bcl6 expression and T_{FH} differentiation, is potently anti-proliferative when expressed at high levels (7, 31). Consequently, we hypothesized that the failure of RDII-mut$^+$ SM cells to expand and differentiate into T_{FH} cells was due to a failure to repress Blimp-1 expression. To test this, we used Prdm1$^{fl/fl}$ SM cells, which lose Blimp-1 expression and differentiate preferentially into T_{FH} cells when transduced with a RV expressing Cre recombinase (Cre-RV) (Fig. 3.5A and (7)). We transduced Prdm1$^{fl/fl}$ SM cells with the Bcl6-WT, RDII-mut or GFP-only RV, and co-transduced other Prdm1$^{fl/fl}$ SM cells with Cre-RV in addition to the GFP-only, Bcl6-WT, or RDII-

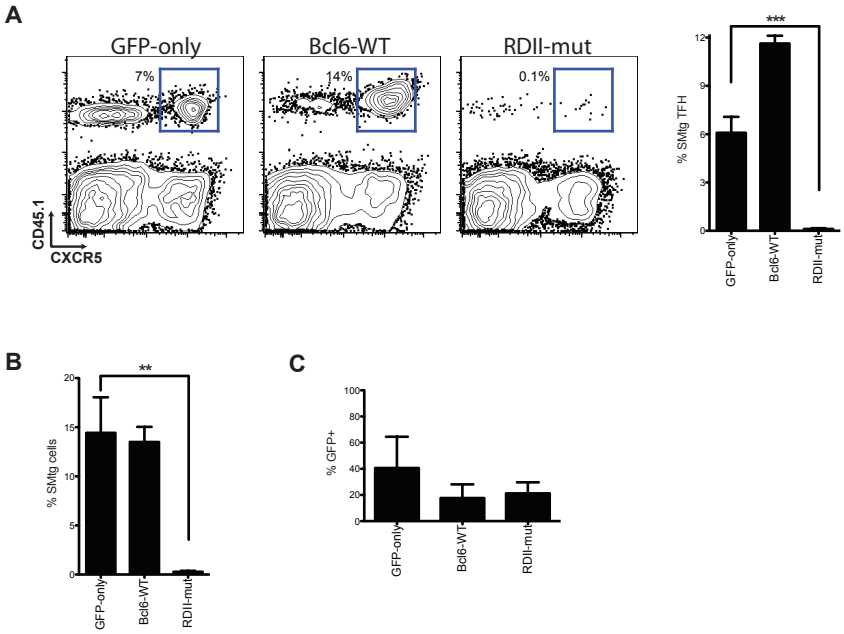

Figure 3.4: RDII domain-mediated Bcl6 gene regulation is required for T_{FH} cell persistence. SMtg $CD4^+$ T cells were transduced with an empty control RV (GFP-only), a wildtype Bcl6 RV (Bcl6-WT), or a RV expressing a mutant form of Bcl6 in which RDII-mediated co-repressor recruitment is impaired (RDII-mut). Transduced SMtg cells were adoptively transferred into C57BL/6 host mice that were subsequently infected with LCMV. SMtg T_{FH} and non-T_{FH} differentiation were analyzed 8 days after infection. FACS plots are gated on $CD4^+$ T cells. SMtg T_{FH} cells ($CD45.1^+$ $CXCR5^+$) are boxed. Quantification shows the percentage of SMtg cells that were T_{FH} (A) and the percentage of $CD4^+$ T cells that were SMtg cells ($CD45.1^+$) (B). Data are representative of 4 independent experiments; n = 4 per group. (C) Percentage of SMtg cells that were successfully transduced (GFP^+) with the indicated RV, prior to purification and adoptive transfer.

mut RV. Surprisingly, Blimp-1 ablation had no effect on RDII-mut^{+} SM cells, which remained unable to expand and differentiate into T_{FH} (Fig. 3.5B). This date suggests that in addition to Blimp-1, the Bcl6 RDII domain regulates at least one additional gene is essential for cell survival.

3.9: Discussion

Bcl6 is a master regulator of T_{FH} cell differentiation. Bcl6-deficient CD4^{+} T cells fail to differentiate into T_{FH} cells (7-9), and constitutive expression of Bcl6 enhances T_{FH} differentiation (6). We and others have considered models of T_{FH} differentiation in which Bcl6 expression is induced in activated CD4^{+} T cells to induce and maintain the T_{FH} gene program (1, 32, 33). However, CD4^{+} T cells from *Bcl6*$^{-/-}$ mice are not only unable to differentiate into T_{FH}; these cells are highly susceptible to spontaneous activation (12, 34), even when specific for an irrelevant antigen (7). This, along with indications of Bcl6 expression in naïve CD4^{+} T cells in two previous studies (7, 23), led us to examine Bcl6 expression in CD4^{+} T cells more closely. We have now confirmed that Bcl6 protein is expressed in naïve CD4^{+} T cells, suggesting that Bcl6 may have a role earlier in CD4^{+} T cell development. Additional studies are needed to more fully characterize the functions of Bcl6 in T cells.

Because of the possibility that Bcl6 is active in CD4^{+} T cells prior to T_{FH} differentiation, careful controls were required to in our examination of the role of Bcl6 in T_{FH} differentiation. Rather than utilize Bcl6$^{-/-}$ CD4^{+} T cells, which lack Bcl6 both

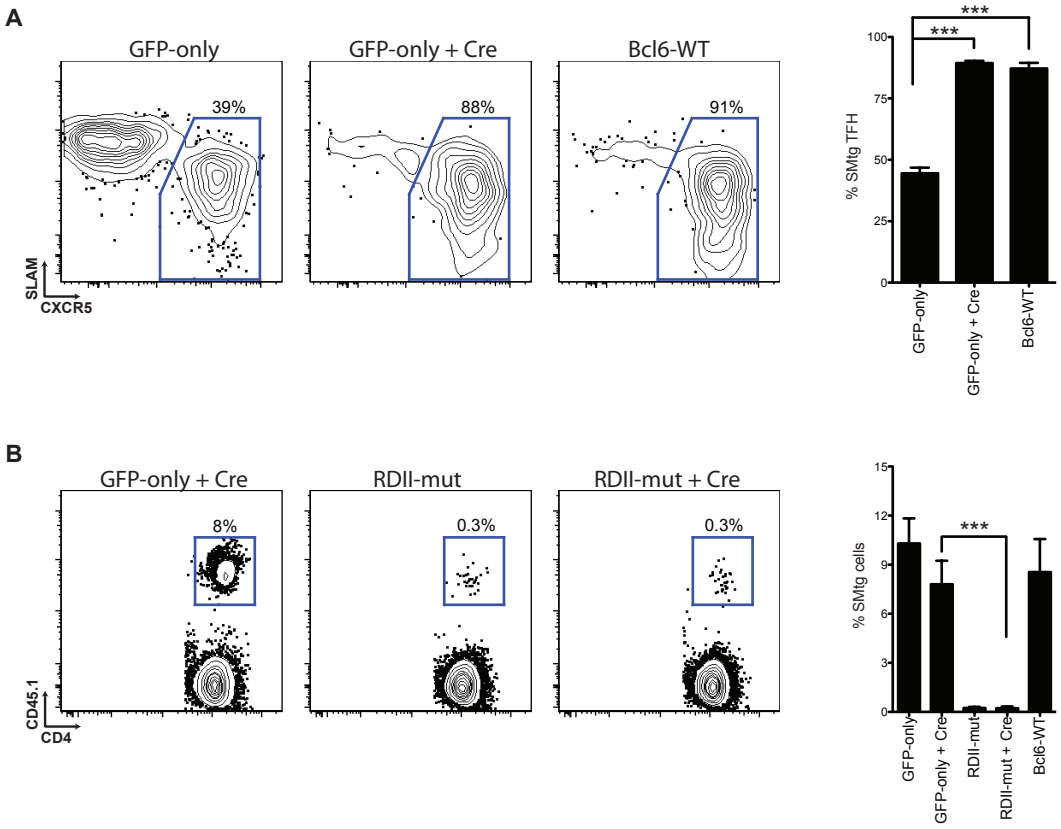

Figure 3.5: The absence of Blimp-1 does not enable T_{FH} differentiation in the absence of RDII domain-mediated Bcl6 gene regulation.
$Prdm1^{fl/fl}$ $SMtg^{+}$ $CD4^{+}$ T cells were (co-)transduced with the an empty control RV (GFP-only), a Cre recombinase RV (Cre), a wildtype Bcl6 RV (Bcl6-WT), and/or a RV expressing a mutant form of Bcl6 in which RDII-mediated co-repressor recruitment is impaired (RDII-mut). Transduced SMtg cells were adoptively transferred into C57BL/6 host mice subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. Data are representative of two independent experiments; n = 5 per group. (A) SMtg T_{FH} differentiation. FACS plots are gated on $CD4^{+}$ $CD45.1^{+}$ GFP^{+} (for GFP-only and Bcl6-WT groups) or on $CD4^{+}$ $CD45.1^{+}$ GFP^{+} $Ametrine^{+}$ cells (for GFP-only + Cre group). T_{FH} cells ($CXCR5^{high}$ $SLAM^{low}$) are boxed. Quantification shows the percentage of SMtg cells that were T_{FH} cells. (B) SMtg cell expansion. FACS plots are gated on $CD4^{+}$ T cells. SMtg ($CD45.1^{+}$) cells are boxed. Quantification shows the percentage of $CD4^{+}$ T cells that were SMtg cells.

during T cell development and during effector differentiation, we transduced fully developed, peripheral $CD4^+$ T cells with retroviruses expressing Bcl6-specific shRNA's. These retroviruses reduced Bcl6 expression in effector $CD4^+$ T cells to background levels, and potently inhibited T_{FH} differentiation. Therefore, by modulating Bcl6 expression at the point of effector differentiation, it was possible to separate the role of Bcl6 in T_{FH} cells from any role for Bcl6 in $CD4^+$ T cell development.

Our understanding of the mechanisms by which Bcl6 regulates T cell differentiation has been based largely on studies of Bcl6 in B cells. This approach has met with some success: Blimp-1 is a key inhibitor of Bcl6 in B cells, and has been found to play a similar role in in $CD4^+$ T cells (7) and possibly in $CD8^+$ T cells (35, 36). We have proposed that Bcl6 and Blimp-1 constitute a regulatory axis for effector and memory cell differentiation that is shared by all lymphocytes (1). However, Bcl6 is known to target different genes in different cell types (37), and the T_{FH} gene program has little in common with the germinal center B cell gene program (7). A more thorough understanding of Bcl6 gene targets and functions in T cells was needed.

Here, we have examined the roles of individual Bcl6 functional domains in T_{FH} differentiation. Both BTB domain- and RDII domain-mediated gene regulation was required for optimal T_{FH} differentiation. Surprisingly, however, the BTB domain had a relatively small role in T_{FH} differentiation. It was not required to overcome the cognate

B cell requirement for T_{FH} differentiation, and BTB-mut$^+$ CD4$^+$ T cells drove the germinal center reaction as well as Bcl6-WT$^+$ CD4$^+$ T cells. This sharply contrasts with studies of Bcl6 in germinal center B cells, where BTB domain-mediated gene regulation is essential (38). Given this result, and the importance of the RDII domain to repression of Blimp-1, we hypothesized that RDII domain-mediated gene regulation would be crucial to T_{FH} differentiation. Indeed, we found that RDII-mut$^+$ CD4$^+$ T cells were unable to differentiate into, or survive as, T_{FH} cells. However, ablation of Blimp-1 did not restore T_{FH} differentiation in RDII-mut$^+$ cells, indicating that the RDII domain has multiple gene targets that are antagonistic to the T_{FH} gene program.

Surprisingly, impairing the Bcl6 RDII domain also inhibited CD4$^+$ T cell expansion. There are two possible explanations for this result. One is that RDII-mutant Bcl6 was sufficient to induce commitment to T_{FH} differentiation, but not sufficient to regulate expression of survival or proliferation factors necessary for T_{FH} persistence. The other possible interpretation is that Bcl6 RDII domain-mediated gene regulation is required in all CD4$^+$ T cells shortly after activation, which would be consistent with the expression of Bcl6 in naïve CD4$^+$ T cells. In either case, it is clear that the Bcl6 RDII domain is an essential mediator of CD4$^+$ T cell effector responses, and additional studies of Bcl6 gene targets and functions in T cells are needed to more fully understand T_{FH} and non-T_{FH} effector cell differentiation.

3.10: Supplementary Material

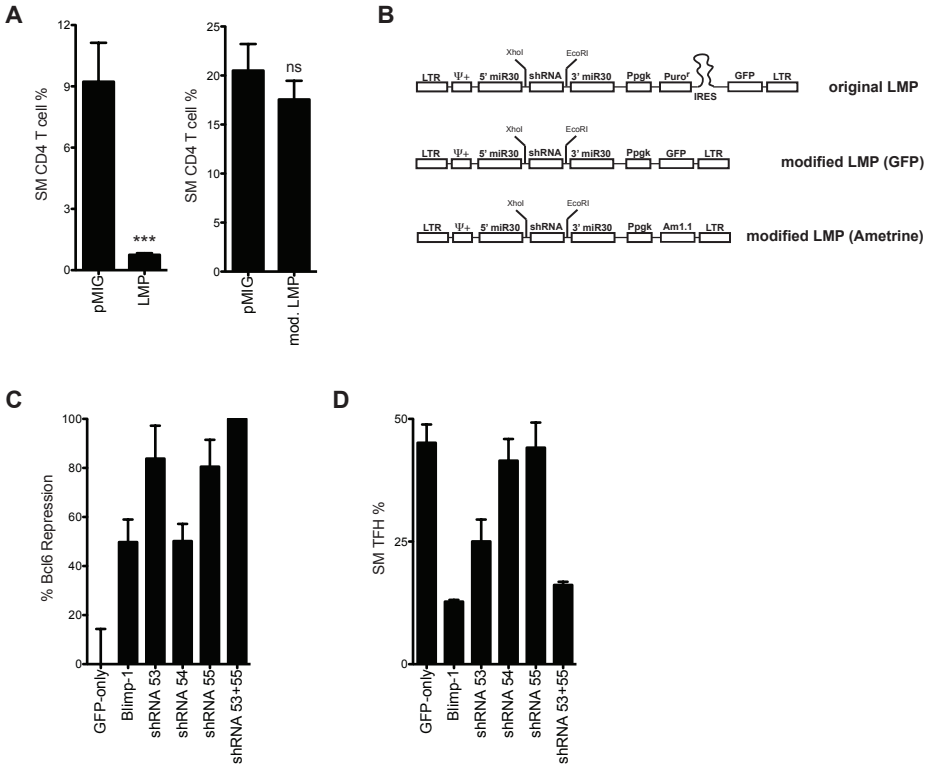

Figure 3.S1: Modification of the LMP shRNA expression vector and Bcl6 shRNA validation. (A-B) SMtg CD4$^+$ T cells were transduced with pMIG RV or with LMP RV and adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. In a second experiment, SMtg CD4$^+$ T cells were transduced with pMIG RV or with modified LMP RV, in which pac (puromycin resistance) and an associated IRES sequence was removed. Quantitation shows SMtg cells as a percentage of total CD4$^+$ T cells. (C-D) SMtg CD4$^+$ T cells were transduced with GFP-only RV, Blimp1 RV, or with modified LMP RVs expressing Bcl6 shRNA sequences as indicted. Transduced SMtg cells were adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV. Quantitation shows (C) the percentage loss of Bcl6 MFI, relative to GFP-only$^+$ T_{FH} and non-T_{FH} cells, and (D) the percentage of SMtg cells that were T_{FH} (CXCR5$^+$). Data are representative of 3 or more independent experiments. n = 4/group. Error bars in all graphs depict SEM.

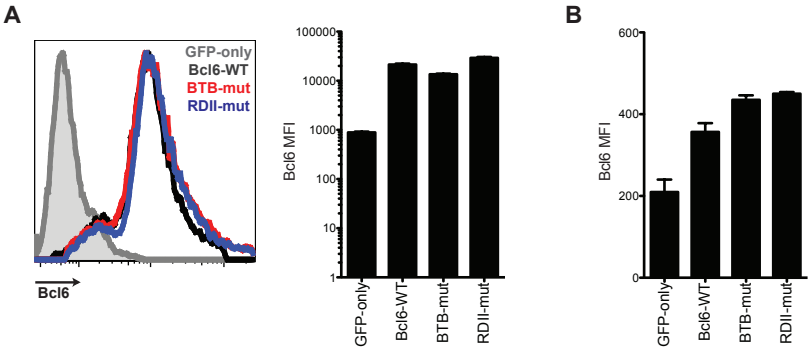

Figure 3.S2: Mutant Bcl6 RV expression.
(A) Plat-E cells were transfected with GFP-only, Bcl6-WT, BTB-mut, or RDII-mut plasmid DNA. Bcl6 expression was measured by intracellular staining 48 hours after transfection. Histogram overlay shows relative Bcl6 expression. Quantitation shows Bcl6 MFI. (B) SMtg $CD4^+$ T cells were transduced with GFP-only, Bcl6-WT, BTB-mut, or RDII-mut RV. Transduced cells were adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. SMtg cell Bcl6 expression was measured by intracellular staining. Quantitation shows Bcl6 MFI. Error bars in all graphs depict SEM.

3.11: References

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Chapter 3.12: Acknowledgements

Chapter 3, in full, is being prepared for submission for publication with co-authors Jeffrey A. Diamond, Daniel DiToro, and Shane Crotty. The dissertation author is the first author of this manuscript.

We thank Lindsay Crickard, Cheryl Kim, Kurt Van Gunst, and Anthony Jose for technical assistance. mAmetrine1.2 plasmid DNA and technical advice were kindly provided by Dario Vignali.

Chapter 4:

T_{FH} differention is not dependent on STAT3

4.1 Abstract

Follicular helper T cells (T_{FH}) are the $CD4^+$ T cell effector subset that is specialized to provide B cell help. T_{FH} differentiation is controlled by the transcription factor Bcl6, which is necessary and sufficient for T_{FH} differentiation and function *in vivo*. It has been proposed that Bcl6 expression is induced by STAT3-mediated IL-6 and IL-21 signals, akin to the cytokine and STAT-driven differentiation pathways of other effector subsets. Here, we have examined role of STAT3 signaling in T_{FH} differentiation. STAT3 was important for optimal T cell activation and proliferation. Surprisingly, however, STAT3-deficient $CD4^+$ T cells differentiated normally into T_{FH} cells, expressed IL-21, and drove germinal center formation in response to acute viral infection or protein immunization. Furthermore, constitutive STAT3 signaling was unable to drive T_{FH} differentiation. These results indicate that STAT3 does not have a central role in T_{FH} differentiation or function.

4.2: Introduction

Follicular Helper T cells (T_{FH}) are effector $CD4^+$ T cells that express the chemokine receptor CXCR5, enabling them to migrate into B cell follicles and provide help to germinal center B cells (1). It was recently shown that T_{FH} cells possess a distinctive gene program (2, 3), and that the transcription factor B cell CLL/lymphoma 6 (Bcl6) is both necessary and sufficient for T_{FH} differentiation *in vivo* (3-5). Bcl6 expression is antagonized by the transcription factor Blimp-1, which is highly expressed in non-T_{FH} effector subsets such as T_H1 and T_H2 cells (4, 6). Like $Bcl6^{-/-}$

cells, Blimp-1-expressing non-T_{FH} effector cells are unable to drive the germinal center reaction, indicating that T_{FH} cells are uniquely capable of providing help to germinal center B cells (4). These results demonstrated that T_{FH} cells were a distinct effector subset, but did not fully elucidate the T_{FH} differentiation pathway.

Other $CD4^+$ T cell effector subsets are defined in large part by the cytokines, Signal Transducers and Activators of Transcription (STATs), and transcription factors that constitute their differentiation pathways (7). STAT4-mediated IL-12 and IFNγ signaling induces T-bet expression and T_H1 differentiation. IL-4 signals through STAT6 to drive GATA3 expression and T_H2 differentiation. T_H17 differentiation is controlled RORγT, which is induced by TGFβ and IL-6 signaling through STAT3.

Recently, it has been proposed that STAT3-mediated IL-6 and/or IL-21 signals are required for optimal T_{FH} differentiation or function (3, 8-12). Other studies have found that T_{FH} differentiation is dependent on sustained interaction with cognate antigen presenting cells, typically B cells (4, 13, 14). It has been unclear if and how these cell-cell interactions and STAT-mediated cytokine signals might collaborate to induce Bcl6 expression and commitment to the T_{FH} gene program, and several competing models of T_{FH} differentiation have been proposed (1, 15-19).

Here, we have examined the role that STAT3 in T_{FH} differentiation. Surprisingly, we found that STAT3 signaling is neither necessary nor sufficient for T_{FH} differentiation. Constitutive STAT3 signaling did not drive enhanced T_{FH} differentiation or germinal center B cell differentiation *in vivo*. Mice whose T cells lack STAT3 ($STAT3^{fl/fl}$ CD4-cre^+) generated fewer T_{FH} cells due to a general decline

in the quality of the T cell response, but antigen-specific STAT3-deficient $CD4^+$ T cells were fully competent to differentiate into functional T_{FH} cells. These data indicated that STAT3 is an important regulator of T cell activation and expansion but not of T_{FH} differentiation.

4.3: Materials and Methods

Mice.

C57BL/6J mice and $Prdm1^{fl/fl}$ mice (20) were purchased from the Jackson Laboratory. CD4-cre mice were purchased from Taconic. SMARTA TCR transgenic $CD45.1^+$ mice (SM, specific for LCMV gp66-77 on $I\text{-}A^b$) (21), OTII TCR transgenic $CD45.1^+$ mice (OTII, specific for Ovalbumin 323-339 on $I\text{-}A^b$) (22), and $SAP^{-/-}$ mice (23) were on a full C57BL/6J background and bred at LIAI. $STAT3^{fl/fl}$ mice were generated and backcrossed onto a C57BL/6J background by Akira and colleagues (24). $STAT3^{fl/fl}$ mice were crossed to CD4-cre mice and then to SM or OTII mice at LIAI. Whole-genome microsatellite analysis was performed through the UCLA Southern California Genotyping Consortium. For $STAT3^{fl/fl}$ CD4-cre^+ mice, 94 of 884 (10.6%) descriptive single nucleotide polymorphisms (SNPs) were not consistent with a C57BL/6J background. Of these SNPs, a cluster of 12 on chromosome 3 was attributable to the Taconic CD4-cre mouse background. All animal experiments were conducted in accordance with approval animal protocols.

Retroviral vectors, transductions, and cell transfers

Gene expression experiments were performed with the green fluorescent protein (GFP)-expressing retroviral expression vector pMIG as well as a modified form of pMIG that expressed the fluorescent protein mAmetrine1.2 (25). STAT3-expressing retroviruses (RVs) were designed using previously described sequences for wildtype, constitutively active (A661C + N663C), and dominant negative (Y705F) variants (26, 27).

Virions were produced using the Plat-E cell line (28), as previously described (29). $CD4^+$ T cells were purified from the splenocytes of naïve mice by magnetic bead negative selection (Miltenyi) and suspended in D-10 (DMEM + 10% fetal calf serum, supplemented with 2mM GlutaMAX (Gibco) and 100 U/mL Penicillin/Streptomycin (Gibco)) with 10 ng/mL recombinant human IL-2 and 50 μM β-Mercaptoethanol (BME). 2 x 10^6 cells per well were stimulated in 24-well plates pre-coated with 8 μg/mL anti-CD3 (clone 17A2, BioXcell) and anti-CD28 (clone 37.51, BioXcell). After 24 hours, cells were transduced as described (29). Where necessary, cells were co-transduced by simultaneously transducing with two individually prepared retrovirus cultures. After a total of 72 hours of stimulation, the $CD4^+$ T cells were split and transferred into new wells with fresh media, IL-2, and BME. After a further 72 hours, transduced cells were purified by sorting on a FACSDiva or FACSAria (BD Biosciences). In some experiments, cells that were not transduced but were treated similarly were used as controls.

Cell transfers into host mice were performed as described (4), by intravenous injection via the retro-orbital sinus. 2.5 x 10^4 transduced SM cells or 2.5 x 10^5 OTII

cells were transferred into each mouse. Transferred cells were allowed to rest in host mice for 3-5 days before infection or immunization.

Infections and Immunizations

LCMV stocks were prepared and quantified as previously described (29). Mice received 5 x 10^4 plaque-forming units of LCMV Armstrong per mouse. For NP-Ova immunizations, mice received 100 μg of NP(17)-Ova (Biosearch Technologies) in PBS mixed with 10 μg lipopolysaccharide (Sigma) and alum (Pierce). Infections and immunizations were performed by intraperitoneal injection.

Flow cytometry

Single-cell suspensions of spleen were prepared by standard gentle mechanical disruption. Surface staining for flow cytometry was done with monoclonal antibodies against SLAM (CD150, Biolegend) and CD4, CD8, CD45.1, CD44, CD62L, CD25, PD-1, B220, Fas, and GL7 (eBiosciences). Surface stains were done for 30 minutes at 4 °C in PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide, unless specified otherwise.

CXCR5 staining was done using purified anti-CXCR5 (BD Pharmingen or BioXcell) for 60 minutes, followed by biotinylated anti-rat IgG (Jackson Immunoresearch), and then by APC- or PE-labeled streptavidin (Caltag Laboratories) at 4° C in PBS supplemented with 0.5% bovine serum albumin, 2% fetal calf serum,

and 2% normal mouse serum, as previously described (4). Samples were not fixed and were acquired immediately.

Intracellular cytokine staining was done as described (29), after stimulation with 20 ng/mL PMA (Sigma) and 1 μM Ionomycin (Sigma) in the presence of 2 μg/mL brefeldin-A (BD Biosciences) for 4 hours. Directly conjugated antibodies against IFNγ and IL-2 (BD Pharmingen) were used. For IL-21, staining was done using an IL-21R-FC chimeric protein (R&D Systems) followed by PE- or APC-labeled anti-human IgG (Jackson Immunoresearch) (4). Intracellular phospho-STAT staining was done with pSTAT1 (pY701) and pSTAT3 (pY705) antibodies and the Phosflow protocol III for mouse splenocytes (BD Biosciences).

Samples were acquired using a C6 Flow Cytometer (Accuri) or an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star).

ELISAs

Anti-NP-Ova IgG was quantified by ELISA as described (4), using NP(17)-Ova (Biosearch Technologies) as the capture antigen on 96-well Maxisorp microtiter plates (Nunc). Following incubation of sample serum or media, HRPO-conjugated goat anti-mouse IgGγ (Caltag Laboratories) was used.

Statistical Analysis

Statistical tests were performed using Prism 5.0c (GraphPad). P values were calculated by two-tailed unpaired Student's t tests with a 95% confidence interval.

Error bars depict the standard error of the mean (SEM). *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

4.4: STAT3 signaling does not drive T_{FH} differentiation *in vivo*.

First, we tested the ability of STAT3 signaling to drive T_{FH} differentiation *in vivo*. We transduced SMARTA TCR-transgenic (SM) $CD4^+$ T cells, which recognize an LCMV glycoprotein epitope bound by MHC class II I-A^b, with RVs that expressed only GFP (GFP-only), wildtype STAT3 (STAT3-WT), or a constitutively active mutant of STAT3 (STAT3-CA). STAT3-WT^+ and GFP-$only^+$ SM cells had high levels of phosphorylated STAT3 after stimulation with IL-6, but not without stimulation (Fig. 4.1A). STAT3-CA^+ SM cells had high levels of phosphorylated STAT3 with or without cytokine stimulation (Fig. 4.1A).

Transduced SM cells were adoptively transferred into C57BL/6J mice that were subsequently infected with lymphocytic choriomeningitis virus (LCMV). Constitutive STAT3 signaling had no effect on T_{FH} differentiation (Fig. 4.1B). GFP-$only^+$ and STAT3-WT^+ SM cells differentiated into T_{FH} normally as well (Fig. 4.1B). STAT3-CA^+ SM cell expansion and germinal center B cell differentiation were also unaffected (Fig. 4.1D-E). These results suggested that STAT3 signaling did not drive T_{FH} differentiation or function *in vivo*, challenging the notion that STAT3 was a key regulator of the T_{FH} gene program.

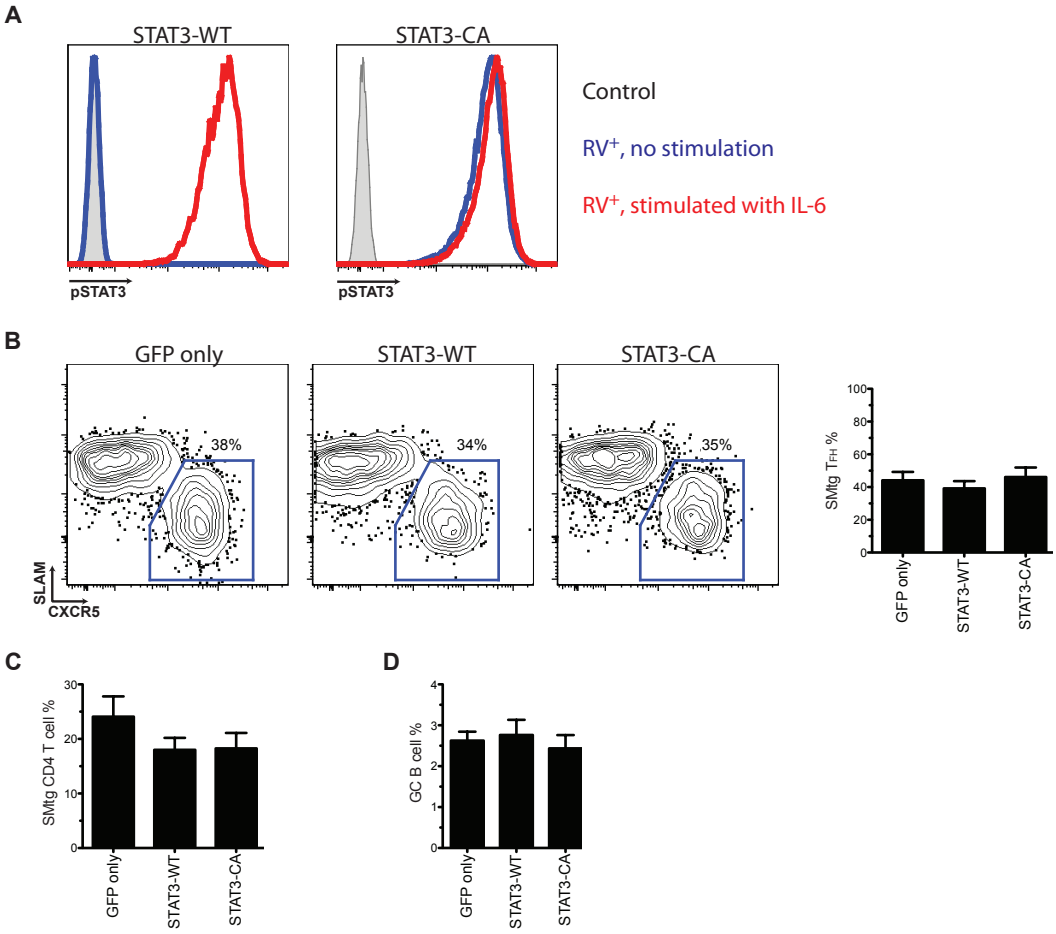


Figure 4.1: STAT3 signaling does not drive T_{FH} differentiation *in vivo*.
SMARTA TCR-transgenic (SM) cells were transduced with retroviral vectors (RVs) expressing only GFP (GFP-only), wildtype STAT3 (STAT3-WT), or a constitutively active mutant form of STAT3 (STAT3-CA). (A) STAT3 phosphorylation. SM cells transduced with the indicated RV were immediately stained for phosphorylated STAT3 (Control), or were first incubated with IL-6 (red) or with no cytokine (blue) and then stained. Data are representative of 2 independent experiments. n = 2-3/group. (B-E) Transduced SM cells were adoptively transferred into C57BL/6 mice that were subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. Data are a composite of 3 independent experiments. n = 13/group. (B) SM cell T_{FH} differentiation. FACS plots are gated on SM cells ($CD4^+$ $CD45.1^+$), with T_{FH} cells ($CXCR5^{high}$ $SLAM^{low}$) boxed. (C) Quantitation of SM cell expansion as a percentage of total $CD4^+$ T cells. (D) Quantitation of germinal center B cells ($GL7^+$ Fas^+ $B220^+$) as a percentage of total splenocytes. An uninfected C57BL/6J mouse (Naïve) is also shown. Error bars in all graphs depict SEM.

4.5: STAT3 is required for optimal T cell activation.

Because T_{FH} differentiation and persistence is dependent on cell-cell interactions, it was possible that STAT3 signaling might be necessary, but not sufficient, to drive T_{FH} differentiation. To test this hypothesis, we transduced SM cells with a RV expressing a dominant negative form of STAT3 (STAT3-DN). STAT3-DN^+ $CD4^+$ T cells had no detectable phosphorylation of STAT3 in response to IL-6 stimulation (Fig. 4.S1). Surprisingly, T_{FH} differentiation and function were not impaired in STAT3-DN^+ SM cells (Fig. 4.2A). Again, these results argued against a model of T_{FH} differentiation in which STAT3 plays a central role. However, these results appeared to conflict with previous report that T_{FH} differentiation or function was defective in mice whose T cells lacked STAT3(3, 11), and we could not exclude the possibility that the STAT3-DN^+ cells retained a low level of STAT3 activity, or that STAT3 activity prior to RV transduction was sufficient to enable T_{FH} differentiation.

Consequently, we next examined $STAT3^{fl/fl}$ CD4-cre^+ (STAT3 CKO) mice, with $STAT3^{fl/+}$ CD4-cre^+ mice as controls (Fig. 4.S2). We found that STAT3 CKO mice had fewer T_{FH} cells and fewer germinal center B cells after LCMV infection (Fig. 4.S2), consistent with a previous study (3). However, it also appeared that STAT3 CKO mice had fewer non-T_{FH} cells (Fig. 4.S2). Indeed, STAT3 CKO mice had substantially fewer effector $CD4^+$ and $CD8^+$ T cells than did control mice (Fig. 4.2B-C). This indicated that STAT3 was an important mediator of the total T cell response, as recently demonstrated by O'Shea and colleagues (30). It was therefore

possible that the reduction in T_{FH} and germinal center B cell numbers in STAT3 CKO mice was a consequence of this general T cell defect, rather than the result of a specific requirement for STAT3 in Bcl6 expression or T_{FH} differentiation.

In an attempt to distinguish between the effects of STAT3 on overall T cell activation and on T_{FH} differentiation, we analyzed T_{FH} cells as a percentage of activated ($CD44^{high}$) $CD4^+$ T cells, rather than as a percentage of total $CD4^+$ T cells. We found that T_{FH} differentiation was normal among activated $CD4^+$ T cells in STAT3 CKO mice (Fig. 4.2D). Differentiation of germinal center T_{FH} cells, which are marked by GL7 expression after acute viral infection (6), was also normal (Fig. 4.2E). Taken together, these data suggested that STAT3 was not specifically required for T_{FH} differentiation, but we could not exclude the possibility that a direct role for STAT3 in T_{FH} differentiation was being masked by the general T cell defect.

4.6: STAT3 is not required for T_{FH} differentiation and function.

Next, we transferred small numbers of STAT3 CKO SM cells into wildtype C57BL/6J host mice that were subsequently infected with LCMV. STAT3-deficient SM cells differentiated normally into T_{FH} and germinal center T_{FH} cells (Fig. 4.3A-B), confirming that STAT3 was not required for T_{FH} differentiation.

IL-21 is a key effector cytokine produced by T_{FH} cells. Because IL-21 production is reported to be dependent on STAT3 signaling (30), it was unlikely that T_{FH} cells would be fully functional if a lack of STAT3 prevented IL-21 production. Therefore we examined IL-21 production in STAT3-deficient $CD4^+$ T cells. As

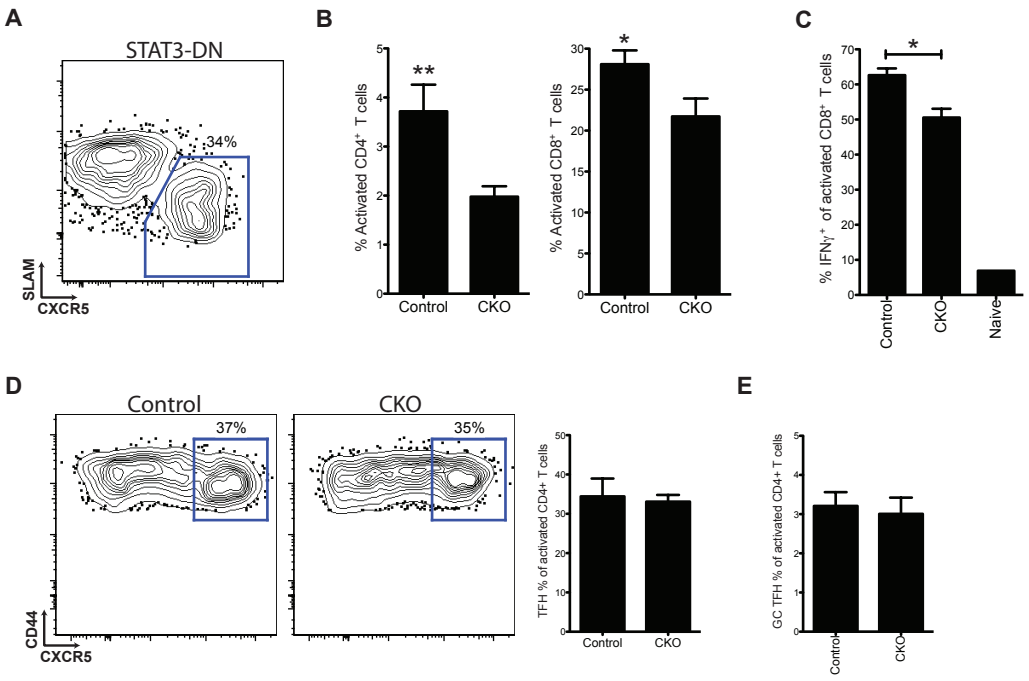


Figure 4.2: STAT3 CKO mice generate T_{FH} cells, but do not respond optimally to immune challenge.

(A) SM cells were transduced with STAT3-DN RV and adoptively transferred into C57BL/6 mice that were subsequently infected with LCMV in the same experiments depicted in Fig. 4.1. Splenocytes were analyzed 8 days after infection. FACS plots are gated on $CD4^+$ SM^+ ($CD45.1^+$) cells, with T_{FH} cells ($CXCR5^{high}$ $SLAM^{low}$) boxed. Quantitation is of SM T_{FH} cells as a percentage of total SM cells. Data are a composite of 3 independent experiments. n = 13/group. (B-E) $STAT3^{fl/fl}$ CD4-cre^+ (CKO) mice and $STAT3^{fl/+}$ CD4-cre^+ (Control) mice were infected with LCMV. Splenocytes were analyzed 8 days after infection. Data are a composite of 4 independent experiments (2 independent experiments for (E)). n = 16-21/group. (B) T cell activation. Quantitation is of activated ($CD44^{high}$) $CD4^+$ T cells (left) and $CD8^+$ T cells (right) as a percentage of total splenocytes. **, P = 0.002. *, P = 0.044. (C) $CD8^+$ T cell cytokine production. Splenocytes were stimulated with PMA and Ionomycin in the presence of BFA. Quantitation is of $IFN\gamma^+$ cells as a percentage of activated ($CD44^{high}$) $CD8^+$ T cells. *, P = 0.016. (D) T_{FH} differentiation. FACS plots are gated on activated $CD4^+$ T cells ($CD44^{high}$), with T_{FH} cells ($CXCR5^{high}$) boxed. Quantitation is of T_{FH} cells as a percentage of activated $CD4^+$ T cells. (E) Germinal center T_{FH} differentiation. Quantitation is of germinal center T_{FH} cells ($CD4^+$ $CD44^{high}$ $CXCR5^{high}$ $GL7^{high}$) as a percentage of activated $CD4^+$ T cells. Error bars in all graphs depict SEM.

reported, STAT3-deficient $CD4^+$ T cells cultured in T_H17-polarizing conditions *in vitro* failed to produce IL-17 or IL-21 (Fig. 4.S3) (30). Surprisingly, however, STAT3-deficient SM cells analyzed *ex vivo* after LCMV infection produced IL-21 as well as control SM cells (Fig. 4.3C). Endogenous $CD4^+$ and $CD8^+$ T cells from STAT3 CKO mice also produced IL-21 *ex vivo* (Fig. 4.S3). These results suggested that STAT3 was required for IL-21 production in T_H17 cells, but not in effector $CD8^+$ T cells or T_{FH} cells in T_H1-polarizing conditions.

Because the ultimate function of T_{FH} cells is to help germinal center B cells, we tested the ability of STAT3-deficient SM cells to drive the germinal center reaction. We transferred STAT3-deficient or control SM cells into $SAP^{-/-}$ mice, whose endogenous $CD4^+$ T cells are unable to provide help to GC B cells (31). After LCMV infection, STAT3-deficient SM cells expanded and drove germinal center B cell differentiation as well as control SM cells (Fig. 4.3D). These results indicated that STAT3-deficient $CD4^+$ T cells were fully competent to function as T_{FH} cells during an acute viral infection.

We also tested the necessity of STAT3 in T_{FH} differentiation and function in a protein immunization system. We transferred STAT3 CKO or control OTII TCR-transgenic $CD4^+$ T cells, which recognize an Ovalbumin epitope bound by MHC class II $I\text{-}A^b$, into C57BL/6J mice subsequently immunized with NP-Ova. STAT3-deficient OTII cells differentiated into T_{FH} cells as well as control OTII cells, and drove normal germinal center reactions and antibody responses (Fig. 4.3 E-G).

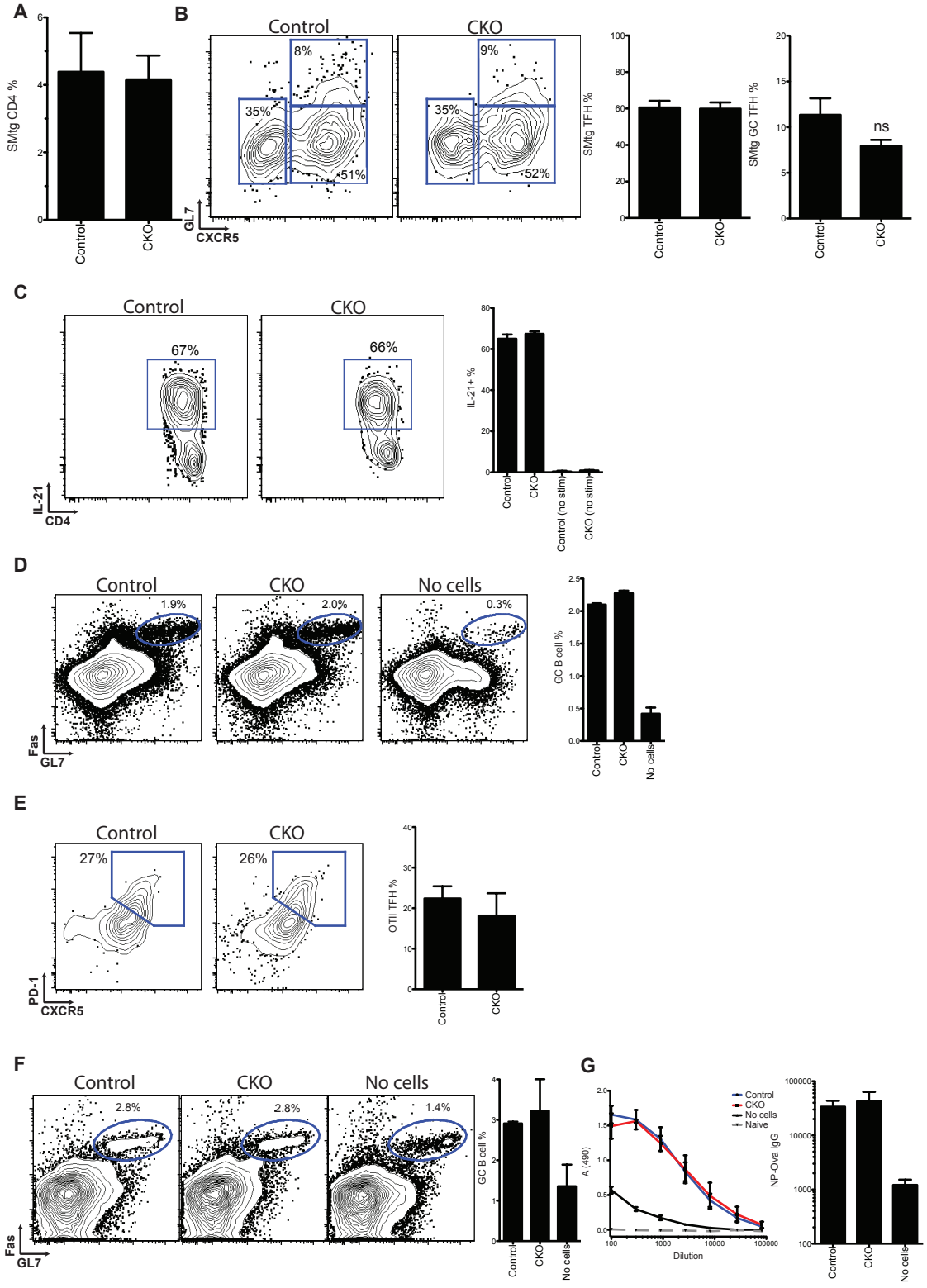
These results indicated that while STAT3 was clearly an important mediator of T cell proliferation and survival, STAT3 was not specifically required for full T_{FH} differentiation or function.

4.7: Discussion

T_{FH} cells have recently been recognized as a distinct subset of effector $CD4^+$ T cells controlled by the transcription factor Bcl6 (Crotty, in press and ref.(16, 19)). However, the signals regulating Bcl6 expression have been unclear. Several studies reported defects in T_{FH} differentiation in the absence of STAT3 or the cytokines IL-6 and IL-21, which signal through STAT3 (3, 8, 9, 11). Consequently, a leading model of T_{FH} differentiation has been that Bcl6 expression is induced by IL-6 and IL-21 signaling through STAT3. This model is consistent with the current $CD4^+$ T cell paradigm, in which effector differentiation is governed primarily by STAT-mediated cytokine signaling. However, we and others have reported that T_{FH} differentiation is largely intact in the absence of IL-6 and/or IL-21, and that stimulating $CD4^+$ T cells in the presence of IL-6 and/or IL-21 does not induce expression of Bcl6 or CXCR5 protein (Eto et al, submitted and ref. (12)). In humans, IL-6 and IL-21 have not been shown to drive T_{FH} differentiation (32), and patients with defective STAT3 signaling appear to have normal numbers of T_{FH} cells (33, 34).

To resolve these conflicting data, we tested the ability of STAT3 signaling to drive T_{FH} differentiation or function *in vivo*. We found no effect on T_{FH} differentiation and germinal center formation in mice receiving SM $CD4^+$ T cells expressing a

Figure 4.3: STAT3-deficient $CD4^+$ T cells in immunocompetent mice differentiate normally into fully functional T_{FH} cells.
(A-C) $STAT3^{fl/+}$ $CD4\text{-}cre^+$ (Control) or $STAT3^{fl/fl}$ $CD4\text{-}cre^+$ (CKO) SM cells were transferred into C57BL/6 mice subsequently infected with LCMV. Splenocytes were analyzed 6 days after infection. Data are representative of 3 independent experiments. n = 4-5/group. (A) SM cell expansion. Quantitation is of SM cells ($CD45.1^+$ $CD4^+$) as a percentage of total splenocytes. (B) SM T_{FH} differentiation. FACS plots are gated on SM cells, with non-T_{FH} cells ($CXCR5^{low}$), T_{FH} cells ($CXCR5^{high}$ $GL7^{low}$), and germinal center T_{FH} cells ($CXCR5^{high}$ $GL7^{high}$) boxed. Quantitation is of SM T_{FH} cells as a percentage of total SM cells (left) and of SM GC T_{FH} cells as a percentage of total SM cells (right). (C) SM cell IL-21 production. Splenocytes were stimulated with PMA and Ionomycin in the presence of BFA. FACS plots are gated on SM cells ($CD4^+$ $CD45.1^+$), with $IL\text{-}21^+$ cells boxed. Quantitation is of IL-21 producing SM cells as a percentage of total SM cells, with IL-21 production in the absence of stimulation shown as a control. (D) Control SM cells, STAT3 CKO SM cells, or no cells were transferred into $SAP^{-/-}$ mice that were subsequently infected with LCMV. Splenocytes were analyzed 8-10 days after infection. FACS plots are gated on $B220^+$ cells, with germinal center B cells ($GL7^+$ Fas^+) circled. Quantitation is of germinal center B cells as a percentage of total B cells. An uninfected C57BL/6 mouse (Naïve) is also shown. Data are representative of 2 independent experiments. n = 4/group (2/group for mice receiving no SM cells). (E-G) Control or STAT3 CKO OTII TCR-transgenic $CD4^+$ T cells were adoptively transferred into C57BL/6 mice that were subsequently immunized with NP-Ova. One group of mice received no OTII cells but was still immunized with NP-Ova (No cells). Splenocytes and serum were analyzed 8-10 days after immunization. Data are representative of 2 independent experiments. n = 10/group. (E) OTII T_{FH} differentiation. FACS plots are gated on OTII cells ($CD4^+$ $CD45.1^+$), with T_{FH} cells ($CXCR5^{high}$ $PD\text{-}1^{high}$) boxed. Quantitation is of OTII T_{FH} cells as a percentage of total OTII cells. (F) Germinal center B cells. FACS plots are gated on B cells ($B220^+$), with germinal center B cells ($GL7^+$ Fas^+) circled. Quantitation is of germinal center B cells as a percentage of total B cells. (G) anti-NP-Ova antibody production. NP-Ova specific IgG ELISA and endpoint titers are shown. Error bars in all graphs depict SEM.


A
SMtg CD4 %
Control
CKO
B
Control
CKO
8%
35%
51%
9%
35%
52%
GL7
CXCR5
SMtg TFH %
SMtg GC TFH %
ns
C
Control
CKO
67%
66%
IL-21
CD4
IL-21+ %
Control (no stim)
CKO (no stim)
D
Control
CKO
No cells
1.9%
2.0%
0.3%
Fas
GL7
GC B cell %
E
Control
CKO
27%
26%
PD-1
CXCR5
OTII TFH %
F
Control
CKO
No cells
2.8%
2.8%
1.4%
Fas
GL7
GC B cell %
G
Control
CKO
No cells
Naive
A (490)
Dilution
NP-Ova IgG

constitutively active variant of STAT3. This led us to examine the requirement for STAT3 in T_{FH} differentiation. SM cells expressing a dominant negative variant of STAT3 differentiated normally into T_{FH} cells. STAT3 T cell conditional knockout mice had impaired T_{FH} generation and fewer germinal centers, as previously reported (3), but also had reduced numbers of effector $CD4^+$ and $CD8^+$ T cells. We noted that T_{FH} differentiation was normal if only activated $CD4^+$ T cells were considered, leading us to hypothesize that STAT3 deficiency was reducing effector T cell numbers overall, but not specifically impairing T_{FH} differentiation. To test this hypothesis, we used an adoptive transfer system to study the differentiation and function of STAT3-deficient TCR transgenic $CD4^+$ T cells in wildtype host mice. We found that STAT3-deficient T cells were able to differentiate normally into T_{FH} cells that were fully capable of producing IL-21 and of driving the germinal center reaction. These data demonstrated that STAT3 was not required for T_{FH} differentiation or function.

T_{FH} differentiation requirements appear to be fundamentally different from those of other effector subsets. T_{FH} cells arise in varying conditions and are adaptable: T_{FH} cells exhibit partial T_H1 polarization during viral infections (4), partial T_H2 polarization during helminth infections (35, 36), and partial T_H17 polarization in mice with experimental autoimmune encephalitis (37). This flexibility probably enables T_{FH} cells to control isotype class switching (38). It is unlikely that STAT3 signaling drives T_{FH} differentiation in each of these conditions and specifies T_H1, T_H2 or T_H17 polarization as appropriate. It is possible that multiple STATs can collaborate with cell-cell signals to induce T_{FH} differentiation and specify T_{FH} polarization. This model

would explain how T_{FH} cells rapidly differentiate during a variety of different immune responses and acquire the capacity to optimally shape the antibody response.

4.8: Supplementary Material

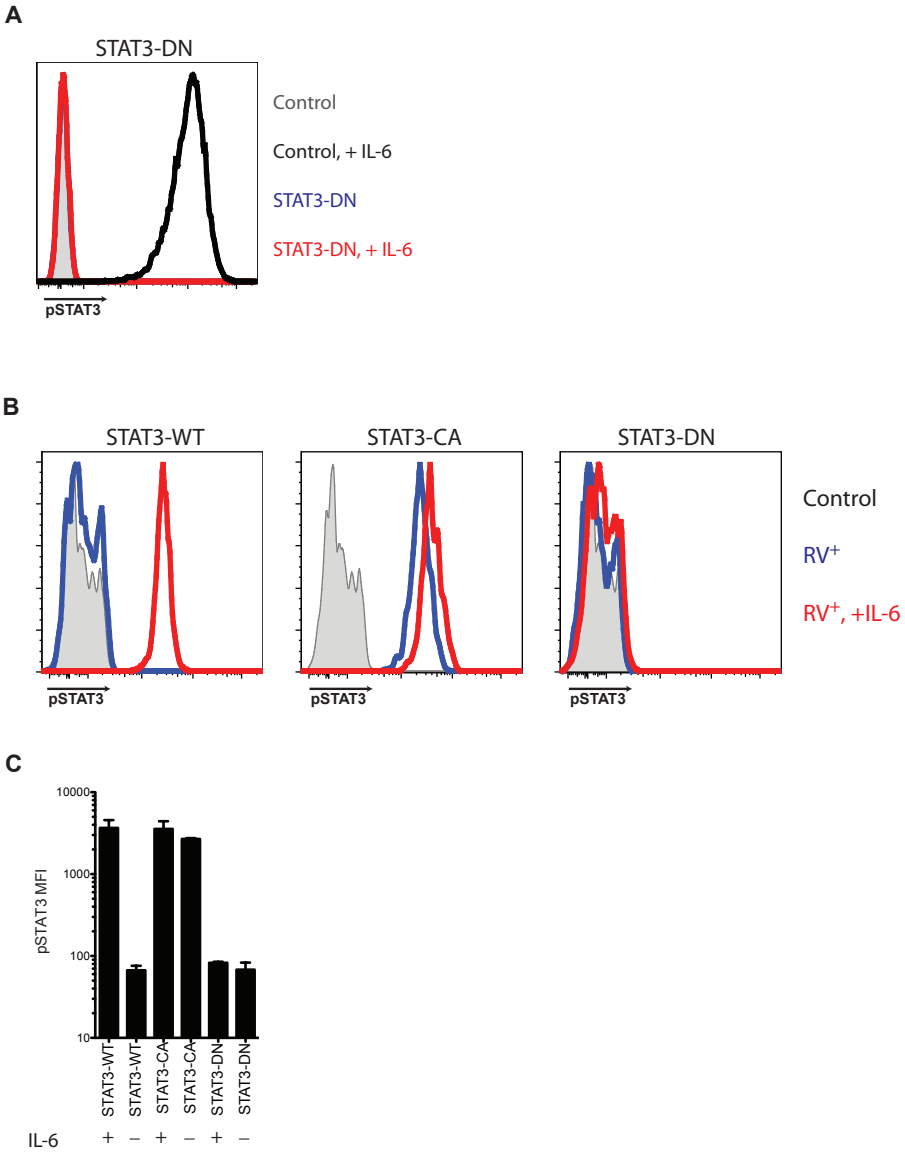


Figure 4.S1: STAT3 mutant retroviruses.
(A) SM cells were transduced with STAT3-DN or untransduced (Control). Transduced cells and untransduced cells were stimulated with IL-6 or not stimulated, and then stained for phosphorylated STAT3. Histogram is gated on SM cells ($CD4^+$ $CD45.1^+$). Data are representative of 2 independent experiments. n = 2 / group. (B-C) SM cells were transduced with STAT3-WT, STAT3-CA, and STAT3-DN RVs and adoptively transferred into C57BL/6 mice that were subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. Data are representative of 1 experiment. n = 2 / group. (B) STAT3 activity. Splenocytes transduced with the indicated RV were stimulated with IL-6 (red) or not stimulated (blue). Unstimulated endogenous $CD4^+$ T cells were also analyzed (Control, gray). Histograms are gated on SM cells ($CD4^+$ $CD45.1^+$). (C) Quantitation of phosphorylated STAT3 MFI. Error bars depict SEM.

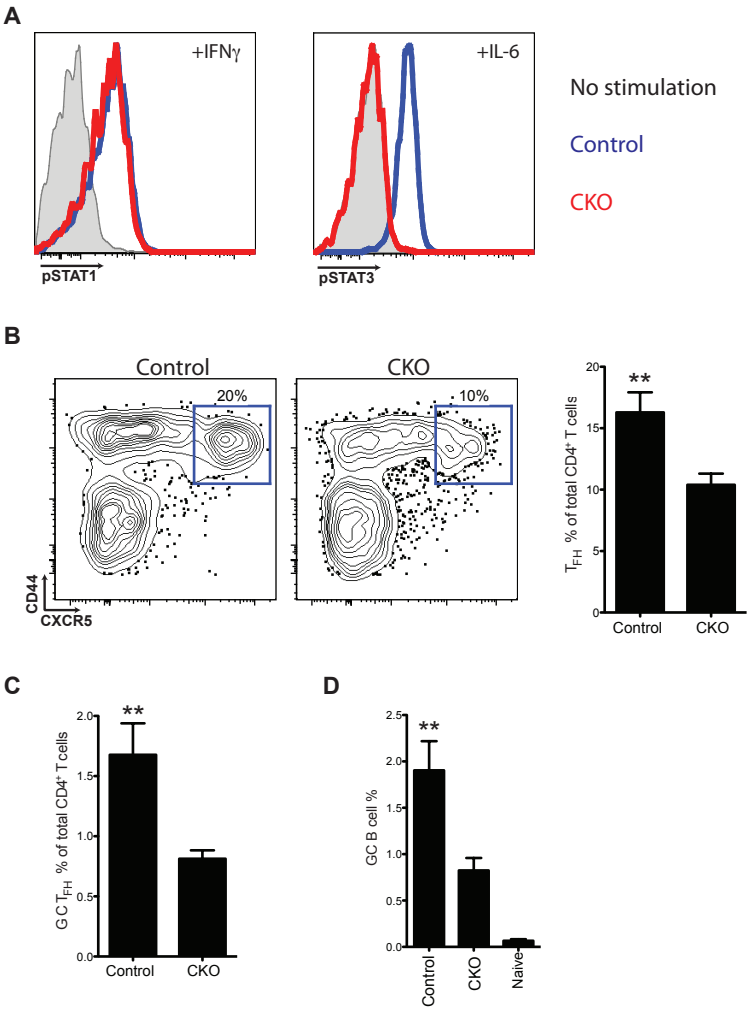


Figure 4.S2: STAT3 CKO mice.
(A) Splenocytes from STAT3$^{fl/+}$ CD4-cre^{+} (Control, blue) and STAT3$^{fl/fl}$ CD4-cre^{+} (CKO, red) mice were stimulated with IFNγ (left) or IL-6 (right) and stained of phosphorylated STAT1 or STAT3, respectively. Unstimulated control splenocytes are also shown (No stimulation, gray). Data are representative of 2 independent experiments. (B-D) Control and STAT3 CKO mice were infected with LCMV. Splenocytes were analyzed 8 days after infection. Data are a composite of 3 independent experiments; n = 12 (Control) or 20 (CKO). (B) Effector CD4^{+} T cell differentiation. FACS plots are gated on CD4^{+} T cells, with T_{FH} cells (CD44^{high} CXCR5^{high}) boxed. Quantiation is of T_{FH} cells as a percentage of CD4^{+} T cells. **, P = 0.0018. (C) Germinal center T_{FH} cell differentiation. Quantiation of germinal center T_{FH} cells (CD44^{high} CXCR5^{high} GL7^{high}) as a percentage of CD4^{+} T cells. **, P = 0.0025. (D) Germinal centers. Quantitation is of germinal center B cells (Fas^{+} GL7^{high}) as a percentage of B cells. An uninfected C57BL/6 mouse is also shown (Naïve). Control vs. CKO, **, P = 0.0022. Error bars in all graphs depict SEM.

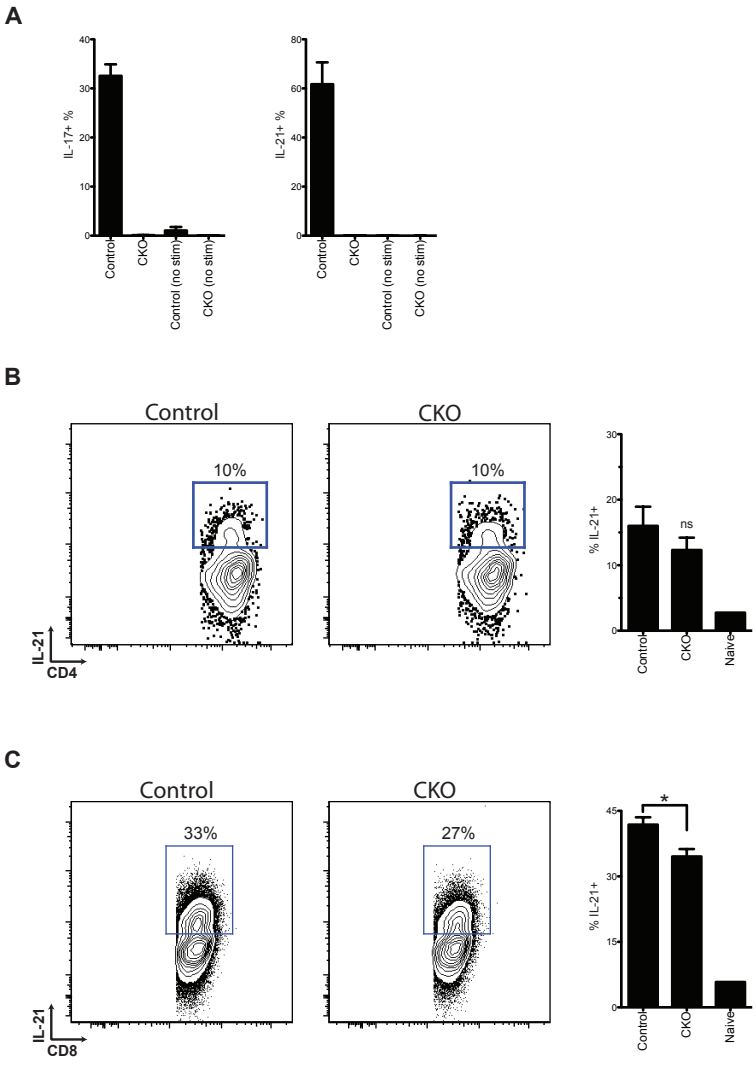


Figure 4.S3: IL-21 production in the absence of STAT3.
(A) Naïve $CD4^+$ T cells ($CD4^+$ $CD62L^+$ $CD44^{low}$ $NK1.1^-$ $CD25^-$) were purifed from $STAT3^{fl/+}$ CD4-cre^+ (Control) and $STAT3^{fl/fl}$ CD4-cre^+ (CKO) mice and cultured in T_H17-polarizing conditions *in vitro*. Quantitation is of IL-17^+ (left) and IL-21^+ (right) cells as a percentage of $CD4^+$ T cells. n = 2/group. Data are representative of 2 independent experiments. (B-C) Control and STAT3 CKO mice were infected with LCMV, and splenocytes were analyzed 8 days after infection. n = 3 (Control) or 7 (CKO). Data are representative of 2 independent experiments. FACS plots are gated on $CD4^+$ T cells (B) or $CD8^+$ T cells (C), with IL-21^+ cells boxed. Quantitation is of IL-21^+ cells as a percentage of activated ($CD44^{high}$) $CD4^+$ and $CD8^+$ T cells, respectively. Stimulated cells from an uninfected C57BL/6 mouse (Naïve) are also shown. For $CD8^+$ T cells, *, P = 0.031. Error bars in all graphs depict SEM.

4.9: References

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4.10: Acknowledgements

Chapter 4, in full, is being prepared for submission for publication with co-authors Jeffrey A. Diamond and Shane Crotty. The dissertation author is the first author of this manuscript.

We thank Youn Soo Choi, Robin Kageyama, Lindsay Crickard, Cheryl Kim, Kurt Van Gunst, Anthony Jose, and Wenbin Xiao for technical assistance. STAT3 plasmids were provided by Toshi Kawakami. $STAT3^{fl/fl}$ CD4-cre^{+} mice were provided

by Gisen Kim with the permission of Kiyoshi Takeda. mAmetrine1.2 plasmid DNA and technical advice were kindly provided by Dario Vigali.

Chapter 5:

STAT5 is a negative regulator of Bcl6-mediated T_{FH} differentiation

5.1: Abstract

Follicular helper T cells (T_{FH}) constitute the $CD4^+$ T cell effector subset that is specialized to provide germinal center B cell help. T_{FH} differentiation is driven by the transcription factor Bcl6, and recent studies have identified cytokine and cell-cell signals that are required for optimal expression of Bcl6. However, the mechanisms underlying the negative regulation of T_{FH} differentiation have been less clear. Here, we show that STAT5 was a key inhibitor of T_{FH} differentiation and function. Constitutive STAT5 signaling in activated $CD4^+$ T cells selectively blocked T_{FH} differentiation, resulting in a collapse of the germinal center reaction. Conversely, STAT5-deficient $CD4^+$ T cells (mature $STAT5^{flox/flox}$ $CD4^+$ T cells transduced with a Cre-expressing retrovirus) rapidly upregulated Bcl6 expression and preferentially differentiated into T_{FH} cells. STAT5 signaling failed to inhibit T_{FH} differentiation in the absence of the transcription factor Blimp-1, a direct repressor of Bcl6 expression and T_{FH} differentiation. These results demonstrate that STAT5 and Blimp-1 collaborate to negatively regulate T_{FH} differentiation.

5.2: Introduction

Follicular Helper T cells (T_{FH}) are a distinct subset of effector $CD4^+$ T cells that is specialized to migrate into follicles and provide B cell help (1). The transcription factor Bcl6 is a master regulator of T_{FH} differentiation *in vivo* (2-4). As discussed in Chapter 4, multiple studies have elucidated the signals that induce Bcl6 expression and T_{FH} differentiation. However, it has been unclear what signals

negatively regulate T_{FH} differentiation. One repressor of Bcl6 expression is Blimp-1, a transcription factor that is highly expressed in non-T_{FH} effector $CD4^+$ T cells and is an inhibitor of T_{FH} differentiation (2, 5-7).

Antagonism between Bcl6 and Blimp-1 is a common feature of lymphocyte differentiation (8). In B cells, it was found that STAT3 drives Blimp-1 expression and STAT5 drives Bcl6 expression (9-11), but the opposite has also been reported (12). Given that STAT3 is proposed to induce Bcl6 expression in $CD4^+$ T cells, we hypothesized that STAT5 may also be a regulator of T_{FH} differentiation. Here, we have investigated the role of STAT5 in T_{FH} differentiation. We found that STAT5 signaling blocked Bcl6 expression and T_{FH} differentiation, and that the absence of STAT5 signaling potentiated T_{FH} differentiation. STAT5-mediated inhibiton of T_{FH} differentiation was largely dependent on Blimp-1. These results reveal an important negative regulator of T_{FH} differentiation.

5.3: Materials and Methods

Mice.

C57BL/6J mice and $Prdm1^{fl/fl}$ mice (13) were purchased from the Jackson Laboratory. SMARTA TCR transgenic $CD45.1^+$ mice (SM, specific for LCMV gp66-77 on $I\text{-}A^b$) (14) were on a full C57BL/6J background and bred at LIAI. $STAT5^{fl/fl}$ mice were generated by Hennighausen and colleagues (15), backcrossed to the C57BL/6J background by Farrar and colleagues (University of Minnesota) and re-derived at LIAI. $STAT5^{fl/fl}$ mice and $Prdm1^{fl/fl}$ mice were crossed to SM mice at LIAI.

Whole-genome microsatellite analysis was performed through the UCLA Southern California Genotyping Consortium. 862 of 884 (97.5%) descriptive single nucleotide polymorphisms were consistent with a C57BL/6J background for $STAT5^{fl/fl}$ mice. All animal experiments were conducted in accordance with approval animal protocols.

Retroviral vectors, transductions, and cell transfers

Gene expression experiments were performed with green fluorescent protein (GFP)-expressing retroviral expression vector pMIG as well as with a modified form of pMIG that expressed the flourescent protein mAmetrine1.2 (16). STAT5-expressing retroviruses were designed using previously described sequences for wildtype and constitutively active (H299R + S711F) STAT5b (17). Cre recombinase-expressing retrovirus was designed using the NLS-Cre sequence of Rajewsky and colleagues (18), as previously described (2).

Virions were produced using the Plat-E cell line (19), as previously described (20). $CD4^+$ T cells were purified from the splenocytes of naïve mice by magnetic bead negative selection (Miltenyi, catalog number 130-090-861) and suspended in D-10 (DMEM + 10% fetal calf serum, supplemented with 2mM GlutaMAX (Gibco) and 100 U/mL Penicillin/Streptomycin (Gibco)) with 10 ng/mL recombinant human IL-2 and 50 μM β-Mercaptoethanol (BME). 2 x 10^6 cells per well were stimulated in 24-well plates pre-coated with 8 μg/mL anti-CD3 (clone 17A2, BioXcell) and anti-CD28 (clone 37.51, BioXcell). After 24 hours, cells were transduced as described (20). Where necessary, cells were co-transduced with two RVs. After a total of 72 hours of

stimulation, the $CD4^+$ T cells were split and transferred into new wells with fresh D-10, IL-2 and BME. After a further 72 hours, transduced cells were purified by sorting on a FACSDiva or FACSAria (BD Biosciences). In some experiments, cells that were not transduced but were treated similarly were used as controls.

Cell transfers into host mice were performed as described (2), by intravenous injection via the retro-orbital sinus. 2.5×10^4 or 2.0×10^5 transduced SM cells were transferred into each mouse for day 8 and day 4 experiments, respectively. Transferred cells were allowed to rest in host mice for 3-5 days before infection or immunization.

Infections and Immunizations

LCMV stocks were prepared and quantified as previously described (20). Mice received 5×10^4 or 2×10^5 plaque-forming units of LCMV Armstrong per mouse for day 8 and day 4 experiments, respectively. Infections were performed by intraperitoneal injections.

Flow cytometry

Single-cell suspensions of spleen were prepared by standard gentle mechanical disruption. Surface staining for flow cytometry was done with monoclonal antibodies against SLAM (CD150, Biolegend) and CD4, CD8, CD45.1, CD44, CD62L, CD25, PD-1, B220, Fas, and GL7 (eBiosciences). Surface stains were done for 30 minutes at 4 °C in PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide, unless specified otherwise.

CXCR5 staining was done as described (2) for day 8 experiments, using purified anti-CXCR5 (BD Pharmingen or BioXcell) for 60 minutes, followed by biotinylated anti-rat IgG (Jackson Immunoresearch), and then by APC- or PE-labelled streptavidin (Caltag Laboratories) at 4° C in PBS supplemented with 0.5% bovine serum albumin, 2% fetal calf serum, and 2% normal mouse serum. For day 4 experiments, CXCR5 staining was done using biotinylated anti-CXCR5 (BD Biosciences) for 30 minutes, followed by APC- or PE-labelled streptavidin (Caltag Laboratories) at 4° C. Samples were not fixed and were acquired immediately.

Intracellular cytokine staining was done as described (20), after stimulation with 20 ng/mL PMA (Sigma) and 1 μM Ionomycin (Sigma) in the presence of 2 μg/mL brefeldin-A (BD Biosciences) for 4 hours. Directly conjugated antibodies against IL-2 (BD Pharmingen) were used. For IL-21, staining was done using an IL-21R-FC chimeric protein (R&D Systems) followed by PE- or APC-labelled anti-human IgG (Jackson Immunoresearch) (2). Intracellular staining for Bcl6 was performed with an Alexa647-conjugated monoclonal antibody to Bcl6 (BD Pharmingen) and the FoxP3 ICS kit buffers and protocol (eBioscience).

Samples were acquired using a C6 Flow Cytometer (Accuri) or an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star).

Statistical Analysis

Statistical tests were performed using Prism 5.0c (GraphPad). P values were calculated by two-tailed unpaired Student's t tests with a 95% confidence interval.

Error bars depict the standard error of the mean. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

5.4: STAT5 signaling blocks T_{FH} differentiation and function

We first tested the ability of STAT5 to regulate T_{FH} differentiation *in vivo* using retroviral expression vectors (RVs) that expressed wildtype STAT5b (STAT5-WT), a constitutively active mutant of STAT5b (STAT5-CA) (17), or only GFP (GFP-only). We adoptively transferred transduced SMARTA TCR transgenic $CD4^+$ T cells (SM cells) into C57BL/6J mice that were subsequently infected with LCMV. STAT5-WT^+ and STAT5-CA^+ SM cell expansion was normal (Fig. 5.1A). However, STAT5-CA^+ SM cells largely failed to differentiate into T_{FH} cells (78% decrease, $P < 0.0001$, Fig. 5.1B). Overexpression of wildtype STAT5 moderately inhibited T_{FH} differentiation as well (30% decrease, $P = 0.004$, Fig. 5.1B). Mice that received STAT5-CA^+ SM cells had substantially fewer germinal center B cells than mice receiving GFP-$only^+$ SM cells (71% reduction, $P < 0.0001$, Fig. 5.1C).

STAT5 signaling is known to induce expression of the T_{reg} master regulator FoxP3 in thymocytes (21). However, STAT5-WT^+ and STAT5-CA^+ SM cells were indistinguishable from wildtype non-T_{FH} effector cells. Constitutive STAT5 signaling did not impair production of the T_H1 cytokine IFNγ and did not induce expression of FoxP3 (Fig. 5.1D-E). Taken together, these results suggested that STAT5 signaling was a potent yet selective inhibitor of T_{FH} differentiation.

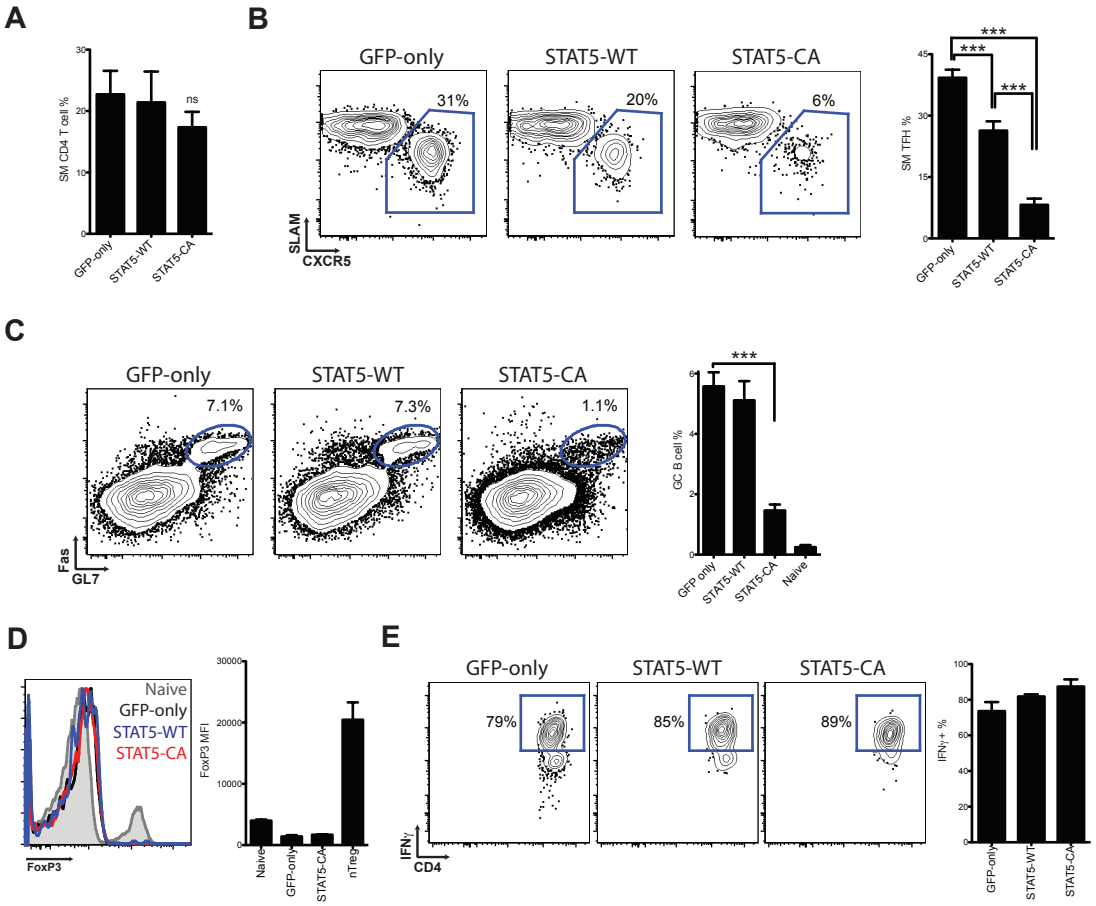

Figure 5.1: STAT5 signaling inhibits T_{FH} differentiation and function *in vivo*.
CD45.1$^+$ SMARTA TCR transgenic (SM) cells were transduced with retroviral vectors (RVs) expressing only GFP (GFP-only), wildtype STAT5b (STAT5-WT), or a constitutively active mutant form of STAT5b (STAT5-CA). Transduced SM cells were adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. Data are a composite of 4 independent experiments where n = 12-16/group (A-D) or are representative of 2 independent experiments n = 4/group (D-E). (A) SM cell expansion. Quantitation shows SM cells as a percentage of all CD4$^+$ T cells. (B) SM T_{FH} differentiation. Representative FACS plots are gated on SM cells (CD4$^+$ CD45.1$^+$), with T_{FH} cells (CXCR5^{high} SLAM^{low}) boxed. Quantitation shows SM T_{FH} cells as a percentage of all SM cells. ***, GFP-only vs. STAT5-WT, P = 0.0002; ***, GFP-only vs. STAT5-CA, P < 0.0001; ***, STAT5-WT vs. STAT5-CA, P < 0.0001. (C) Germinal center B cell differentiation. Representative FACS plots are gated on B cells (B220$^+$), with GC B cells (Fas$^+$ GL7$^+$) circled. Quantitation shows germinal center B cells as a percentage of all B cells. Uninfected C57BL/6J mice (Naïve) are also shown. ***, P < 0.0001. (D) SM cell FoxP3 expression. Representative histogram is gated on SM cells or on CD4$^+$ T cells from an uninfected C57BL/6J mouse (Naïve). Quantitation shows FoxP3 MFI, with CD25$^+$ nT_{regs} from an uninfected C57BL/6J mouse also shown as a control (nT_{reg}). (B) SM cell non-T_{FH} effector function. Splenocytes were stimulated with PMA and Ionomycin. Representative FACS plots are gated on SM cells, with IFNγ$^+$ cells boxed. Quantitation shows IFNγ$^+$ SM cells as a percentage of all SM cells. Error bars in all graphs depict SEM.

5.5: Absence of STAT5 signaling potentiates T_{FH} differentiation

If STAT5 is an inhibitor of T_{FH} differentiation, then reduced STAT5 signaling during $CD4^+$ T cell activation should enhance T_{FH} differentiation. However, the absence of STAT5 during thymic development results in disregulated T cell activation (22, 23). To circumvent this potential complication, we deleted STAT5 in mature peripheral $STAT5^{fl/fl}$ SM $CD4^+$ T cells with a RV expressing Cre recombinase (Cre). Deletion of STAT5 in this manner did not impair activation or expansion *in vitro* (data not shown).

Transduced (Cre^+, STAT5-deficient) or untransduced $STAT5^{fl/fl}$ SM cells were adoptively transferred into C57BL/6J host mice that were subsequently infected with LCMV. STAT5 mediates signaling by IL-2 and IL-7, cytokines that are important drivers of T cell survival and proliferation (24). Surprisingly, Cre^+ $STAT5^{fl/fl}$ SM cells expanded as well as untransduced $STAT5^{fl/fl}$ SM cells, and expressed equivalent high levels of CD44 and low levels of CD62L (Fig. 5.2A-B).

Deletion of STAT5 strongly enhanced T_{FH} differentiation (103% increase, $P < 0.0001$, Fig. 5.2C), a result reminiscent of that obtained by constitutive expression of Bcl6 (2). Expression of IL-21, a T_{FH} effector cytokine necessary for optimal germinal center formation (1), was not impaired by the lack of STAT5 signaling (Fig. 5.2D). IL-2 production was modestly reduced in STAT5-deficient SM cells due to the reduced frequency of non-T_{FH} cells, which produced IL-2 at a higher frequency than did T_{FH} cells (Fig. 5.2E-F). These data demonstrated that STAT5 was a physiological

inhibitor of T_{FH} differentiation, and that T_{FH} proliferation and survival were not dependent on STAT5-mediated cytokine signaling.

5.6: Absence of STAT5 signaling drives Bcl6 expression and early commitment to T_{FH} differentiation

T_{FH} differentiation is typically aborted in the absence of cognate B cells (2, 25), which led us to propose that interaction with cognate B cells induces Bcl6 expression and commitment to T_{FH} differentiation (8). However, recent studies have shown that other antigen-presenting cells are capable of driving T_{FH} differentiation (26), and that T_{FH} differentiation begin during priming by dendritic cells in the T cell zone (Choi et al, submitted). Consequently, we examined the effect of STAT5 deficiency on this early stage of effector cell differentiation.

Cre^{+} and untransduced $STAT5^{fl/fl}$ SM cells were adoptively transferred into C57BL/6J host mice that were subsequently infected with LCMV. Splenic SM cells were analyzed 4 days after infection. STAT5-deficient SM cells expanded as well as wildtype SM cells (Fig. 5.3A). Interestingly, the absence of STAT5 led to a dramatic increase in Bcl6 expression (123% increase, $P = 0.0012$, Fig. 5.3B). As at the peak of the response (Fig. 5.2), T_{FH} differentiation was also substantially increased (128% increase, $P = 0.0007$, Fig. 5.3C).

During T cell priming, non-T_{FH} cells but not T_{FH} cells express high levels of CD25, the high-affinity subunit of the IL-2 receptor (Choi et al, submitted). Consistent with previous reports (27, 28), we found that STAT5-deficiency impaired CD25

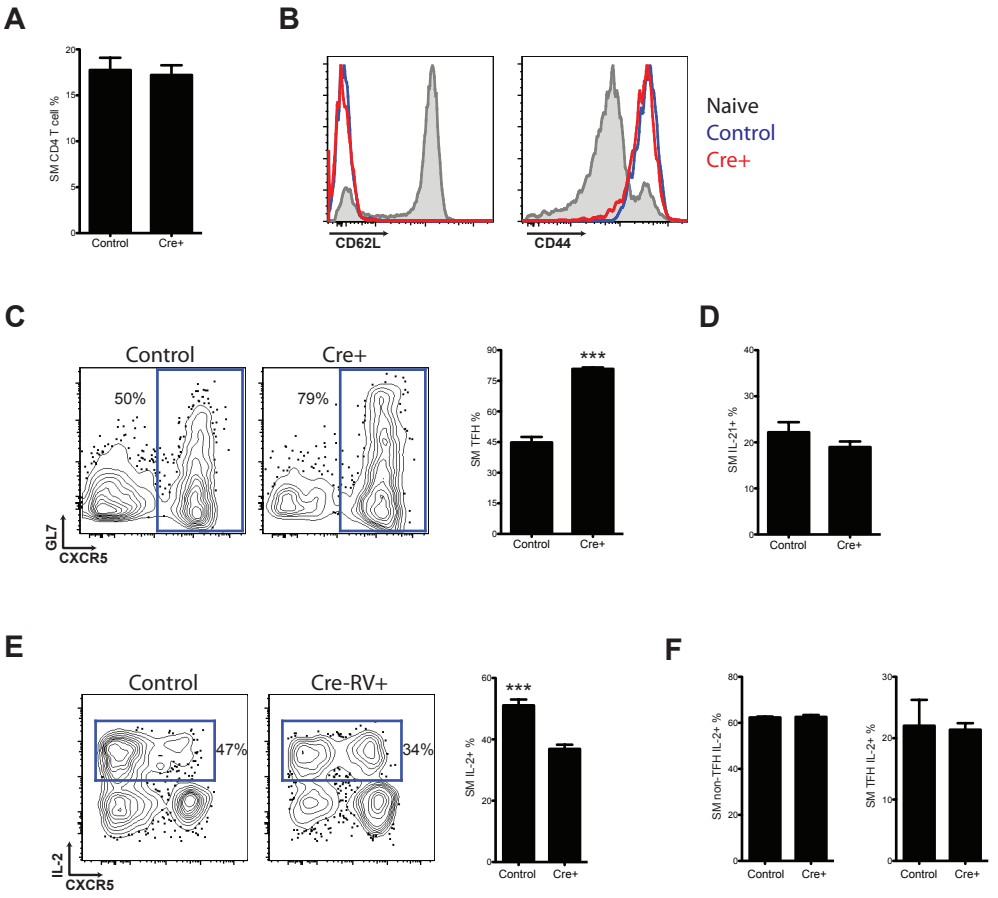

Figure 5.2: STAT5-deficient CD4$^+$ T cells preferentially differentiate into T_{FH} cells *in vivo*. STAT5$^{fl/fl}$ SM cells were transduced with a vector expressing Cre recombinase (Cre), or were not transduced but treated similarly (Control). Transduced and untransduced SM cells were adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. Data are a composite of 2 independent experiments. n = 8/group. (A) SM cell expansion. Quantitation shows SM cells (CD4$^+$ CD45.1$^+$) as a percentage of all CD4$^+$ T cells. (B) SM cell activation. Representative histograms are gated on SM cells or on CD4$^+$ T cells from an uninfected C57BL/6J mouse (Naïve). (C) SM T_{FH} differentiation. Representative FACS plots are gated on SM cells, with T_{FH} cells (CXCR5^{high}) boxed. Quantitation shows SM T_{FH} cells as a percentage of all SM cells. ***, $P < 0.0001$. (D) SM cell IL-21 production. Quantitation shows the percentage of SM cells that produced IL-21 after stimulation with PMA and Ionomycin in vitro. (E-F) SM cell IL-2 production. Representative FACS plots are gated on SM cells after stimulation with PMA and Ionomycin in vitro, with IL-2$^+$ cells boxed. Quantitation shows (E) IL-2$^+$ SM cells as a percentage of all SM cells as well as (F) the percentages of non-T_{FH} (CXCR5^{low}) and T_{FH} SM cells that produced IL-2. Error bars in all graphs depict SEM.

expression, resulting in a loss of $CD25^{high}$ $CXCR5^{low}$ non-T_{FH} cells (65% decrease, P = 0.0002, Fig. 5.3D-E). These results suggested that STAT5 signaling was an inhibitor of Bcl6 expression and T_{FH} differentiation, and a positive regulator of non-T_{FH} effector cell differentiation.

5.7: STAT5-mediated inhibition of T_{FH} differentiation is dependent on Blimp-1

Constitutive STAT5 signaling did not induce T_{reg} differentiation or impair non-T_{FH} differentiation, indicating that STAT5 selectively inhibited T_{FH} differentiation (Fig. 5.1). Because STAT5 can regulate myriad signaling pathways in lymphocytes, it was important to identify which STAT5-dependent pathway was responsible for blocking T_{FH} differentiation. One key function of STAT5 in $CD4^+$ T cells is to mediate signaling by the cytokine IL-2 (24), which may induce expression of Blimp-1 (29). Our observation that expression of the high affinity IL-2 receptor is lost in the absence of STAT5 led us to hypothesize that STAT5 signaling inhibited Bcl6 expression and thus T_{FH} differentiation through expression of Blimp-1.

We transduced $Prdm1^{fl/fl}$ SM cells with the STAT5-CA RV, which expressed GFP, and a variant of the Cre RV which expressed the fluourescent protein Ametrine. STAT5-CA^+ Cre^+, STAT5-CA^+, Cre^+, and untransduced SM cells were purified and adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV (Fig. 5.4A). Transduced and untransduced SM cells expanded equivalently in response to infection (Fig. 5.4B). Deletion of Blimp-1 (Cre^+) resulted in constitutive T_{FH} differentiaton (Fig. 5.4C), as previously reported (2). As before, STAT5-CA RV

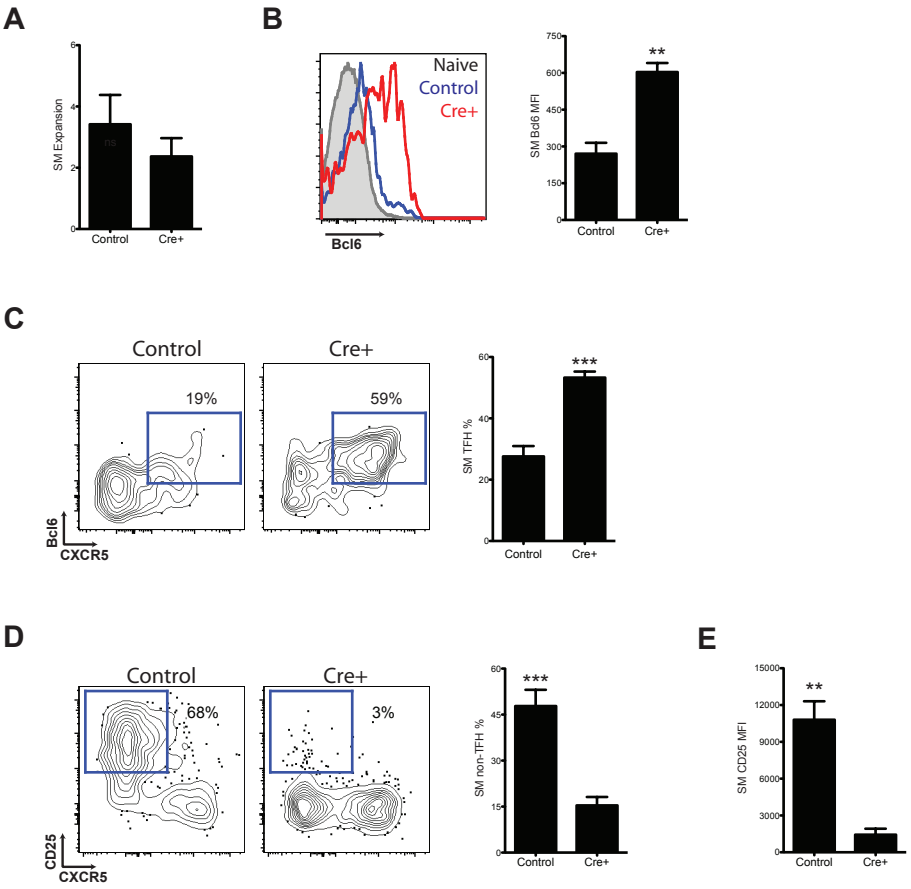

Figure 5.3: STAT5 deficiency rapidly drives Bcl6 expression and decreased CD25 expression. $STAT5^{fl/fl}$ SM cells were transduced as described in Figure 5.2. Transduced and untransduced SM cells were adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV. Splenocytes were analyzed 4 days after infection. Data are representative of (A) or a composite of (B-E) 2 independent experiments. n = 8/group. (A) SM cell expansion. Quantitation shows SM cells ($CD4^+$ $CD45.1^+$) as a percentage of all $CD4^+$ T cells. (B) SM cell Bcl6 expression. Representative histogram is gated on SM cells or on $CD4^+$ T cells from an uninfected C57BL/6J mouse (Naïve). Quantitation shows SM cell Bcl6 MFI in a representative experiment. **, P = 0.0012. (C) SM T_{FH} differentiation. Representative FACS plots are gated on SM cells, with T_{FH} ($CXCR5^{high}$ $Bcl6^{high}$) boxed. Quantitation shows SM T_{FH} cells as a percentage of all SM cells. ***, P < 0.0001. (D) SM cell CD25 expression. Representative FACS plots are gated on SM cells, with $CD25^{high}$ non-T_{FH} cells ($CXCR5^{low}$) boxed. Quantitation shows $CD25^{high}$ non-T_{FH} SM cells as a percentage of all SM cells. ***, P < 0.0001. (E) SM cell CD25 MFI in a representative experiment. **, P = 0.0010. Error bars in all graphs depict SEM.

inhibited T_{FH} differentiation (Fig. 5.4C). However, co-transduced SM cells, which had constitutive STAT5 signaling but were also Blimp-1 deficient, differentiated far more readily into T_{FH} cells (176% increase, $P < 0.0001$, Fig. 5.4D). STAT5-CA^+ Blimp-1 deficient SM cells differentiated into T_{FH} cells nearly as preferentially as Blimp-1 deficient SM cells (Fig. 5.4D). Differentiation of germinal center T_{FH} cells, which can identified by GL7 staining in an acute viral infection (7), was also restored in STAT5-CA^+ SM cells by the absence of Blimp-1 (104% increase, $P = 0.0006$, Fig. 5.4E). These data indicated that the primary mechanism of STAT5-mediated inhibition of T_{FH} differentiation was the induction of Blimp-1, a direct repressor of Bcl6.

5.8: Discussion

In this study, we found that STAT5 was a potent and selective inhibitor of T_{FH} differentiation, and that the absence of STAT5 resulted in substantially increased Bcl6 expression and commitment to T_{FH} differentiation. Previously, relatively little has been known about the negative regulation of T_{FH} differentiation. We reported that Blimp-1 repressed Bcl6 and thus inhibited T_{FH} differentiation, analagous to the antagonistic relationship between Bcl6 and Blimp-1 that is well documented in B cells and likely exists in $CD8^+$ T cells (8). In B cells, Bcl6 and Blimp-1 are regulated by two STATs, STAT3 and STAT5; these STATs appear to act reciprocally, such that STAT3 induces Bcl6 while STAT5 induces Blimp-1 or *vice versa*. In $CD4^+$ T cells, a similar relationship between STAT3 and STAT5 appears to exist during T_H17 effector cell differentiation, during which STAT3 drives IL-17 production and T_H17

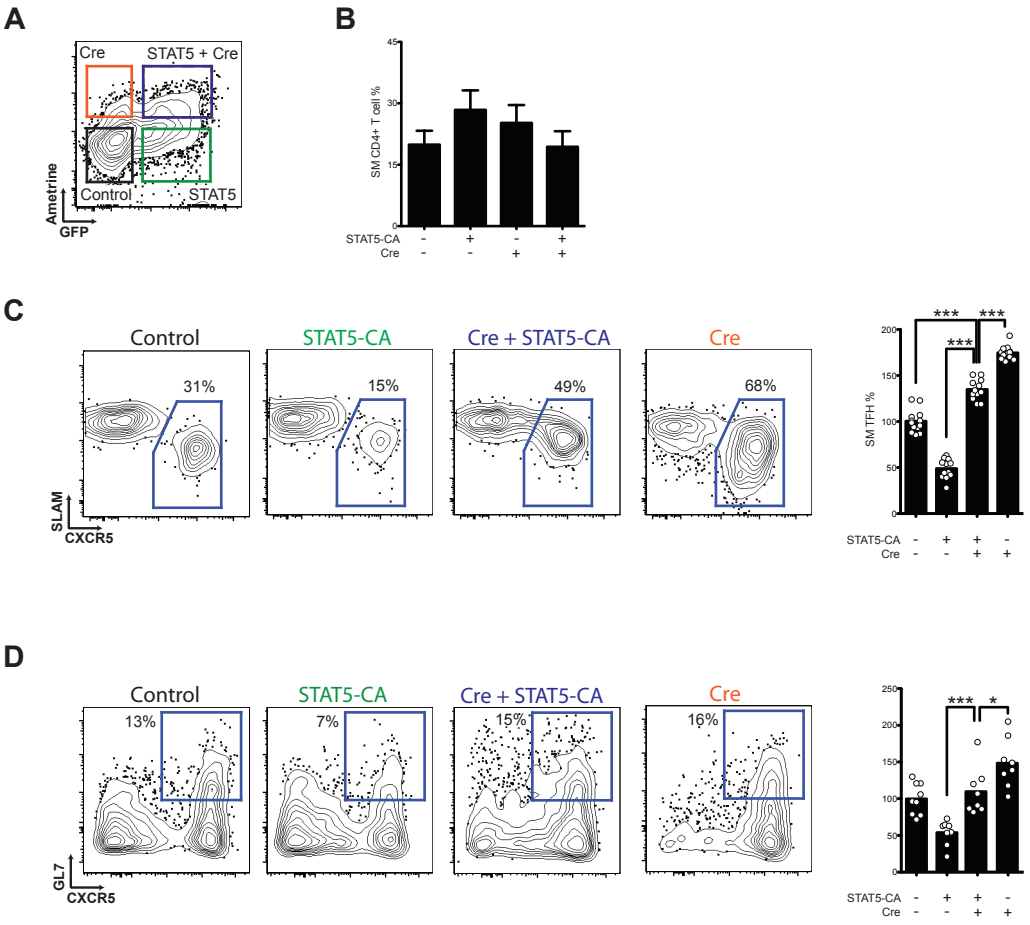

Figure 5.4: STAT5-mediated inhibition of T_{FH} differentiation is dependent on Blimp-1. $Prdm1^{fl/fl}$ SM cells were transduced with STAT5-CA and/or retrovirus expressing Cre recombinase and the fluorescent protein Ametrine (Cre), or were not transduced but treated similarly (Control). Data are a composite of 3 (A-C) or 2 (D) independent experiments. n = 12/group (A-C) or 8/group (D). (A) SM cell GFP and Ametrine expression in vitro. Representative FACS plot is gated on viable ($7AAD^{low}$) cells. (B-D) Transduced and untransduced SM cells were adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV. Splenocytes were analyzed 8 days after infection. (B) SM cell expansion. Quantitation shows SM cells as a percentage of all $CD4^+$ T cells. (C) SM T_{FH} differentiation. Representative FACS plots are gated on SM cells, with T_{FH} cells ($CXCR5^{high}$) boxed. Quantitation shows SM T_{FH} cells as a percentage of all SM cells. Data have been normalized so that the mean Control SM T_{FH} percentage for each experiment is set at 100%. ***, $P < 0.0001$. (D) Germinal center T_{FH} differentiation. Representative FACS plots are gated on SM cells, with germinal center T_{FH} cells ($CXCR5^{high}$ $GL7^{high}$) boxed. Quantitation shows SM germinal center T_{FH} cells as a percentage of all SM cells. Data have been normalized so that the mean Control SM T_{FH} percentage for each experiment is set at 100%. ***, $P < 0.0001$. *, $P = 0.0330$. Error bars in all graphs depict SEM.

differentiation (30, 31), while STAT5 blocks IL-17 production and T_H17 differentiation (32). Given that STAT3 signaling has been proposed to induce Bcl6 expression and T_{FH} differentiation, it is possible that STAT5 antagonizes STAT3-mediated induction of Bcl6 expression during T_{FH} differentiation.

STAT5 may repress T_{FH} differentiation by directly repressing Bcl6 expression; alternatively, STAT5 may induce expression of a Bcl6 repressor, such as Blimp-1, which is induced by IL-2 signaling (29, 33, 34). We found that STAT5 inhibition of T_{FH} differentiation was largely dependent on Blimp-1. Additionally, we recently found that early commitment T_{FH} differentiation was characterized in part by reduced expression of CD25 relative to Blimp-1^+ non-T_{FH} effector cells (Choi et al, submitted). Taken together, these findings support a model of T_{FH} differentiation in which activated $CD4^+$ T cells rapidly bifurcate into T_{FH}- and non-T_{FH}-committed subsets, with STAT5-mediated IL-2 signaling inducing Blimp-1 expression and non-T_{FH} differentiation.

Given the importance of IL-2 and other STAT5-signaling cytokines in T cell proliferation and survival, it was surprising that STAT5-deficient T_{FH} cells expanded normally. Indeed, it was recently demonstrated that effector $CD8^+$ T cell differentiation was disrupted in the absence of STAT5 (35). The same study, however, reported that the overall size of the effector $CD4^+$ T cell effector population was unaffected by STAT5 deficiency. Thus, it seems likely that STAT5 is not required for the $CD4^+$ T cell effector response *per se*, but is necessary to properly balance T_{FH} and non-T_{FH} differentiation. These results also suggest that T_{FH} cells are less dependent on

STAT5-mediated survival signals than other $CD4^+$ T cells. It is possible that expression of Bcl6, which is well known to enhance cell survival and proliferation in germinal center B cells (8), may be responsible for T_{FH} cell expansion in the absence of STAT5 signaling.

These data confirm that T_{FH} cells constitute a distinct subset of effector $CD4^+$ T cells. T_{FH} differentiation is driven by Bcl6, which appears to be induced primarily by ICOS-ICOSL and IL-6/IL-21 signaling during priming. T_{FH} differentation is blocked by Blimp-1, which appears to be induced in $CD4^+$ T cells by STAT5-mediated IL-2 signaling. Given the importance of T_{FH} responses to immune protection, and the role of dysregulated T_{FH} responses in a number of autoimmune diseases, further elucidation of the signals that regulate T_{FH} differentiation may be of substantial benefit in the development of immunotherapies.

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5.10: Acknowledgements

We thank Youn Soo Choi, Robin Kageyama, Lindsay Crickard, Cheryl Kim, Kurt Van Gunst, and Anthony Jose for technical assistance. Michael Farrar kindly provided STAT5 plasmids and technical advice. $STAT5^{fl/fl}$ mice were also provided by Michael Farrar, with the permission of Lothar Hennighausen. mAmetrine1.2 plasmid DNA was provided by Dario Vigali. We thank BD Biosciences, and especially Erika O'Donnell and David Ernst, for generously providing anti-Bcl6 monoclonal antibodies.

Chapter 5, in full, is being prepared for submission for publication with co-authors Jeffrey A. Diamond and Shane Crotty. The dissertation author is the first author of this manuscript.

Chapter 6:

Conclusions

6.1: T_{FH} cells constitute a distinct $CD4^+$ T cell effector subset regulated by Bcl6 and Blimp-1

$CD4^+$ T cells can differentiate into different effector subsets (such as T_H1, T_H2, T_H17, and T_{reg} cells) with different effector functions, enabling them to tailor the immune response (1). $CD4^+$ T cells also provide help to B cells, enabling the formation of germinal centers and the differentiation of memory B cells, and long lived plasma cells (2). Yet T_H2, T_H1, and T_H17 $CD4^+$ T cells are not required for germinal center formation and B cell help (3-5). Along with gene expression profiling and *in vitro* B cell help assays, these findings led to the hypothesis that specialized follicular B helper T cells (T_{FH}) are a fifth $CD4^+$ T cell effector subset (6-8). However, unlike the other effector lineages, no T_{FH} lineage-specifying master regulator transcription factor (i.e., T-bet, GATA3, RORγt, Foxp3) had been identified (9).

We and others have now shown that Bcl6 is a master regulator of T_{FH} cells (Chapter 2) (10-12). Constitutive expression of Bcl6 in $CD4^+$ T cells drove near absolute T_{FH} differentiation *in vivo* (10). These T_{FH} induced larger germinal centers and higher antigen-specific antibody titers (10). In contrast, $Bcl6^{-/-}$ $CD4^+$ T cells were unable to differentiate into T_{FH} *in vivo*, demonstrating that Bcl6 is necessary for T_{FH} differentiation (10-12). *Bcl6*$^{-/-}$ $CD4^+$ T cells became activated and proliferated in response to protein immunization, but were unable to drive germinal center formation (10-12). These results confirmed that T_{FH} are a distinct subset of helper $CD4^+$ T cells, revealed that Bcl6 is a T_{FH} master regulator both necessary and sufficient for T_{FH}

differentiation, and demonstrated that T_{FH} are uniquely capable of driving the germinal center reaction.

While T_{FH} express high amounts of Bcl6 (9), the remaining antigen-specific $CD4^+$ T cells (non-T_{FH}) express high amounts of Blimp-1 (10, 13, 14), indicating that Bcl6 versus Blimp-1 expression may be a central cell fate decision of differentiating $CD4^+$ T cells. Constitutive expression of Blimp-1 blocks Bcl6 expression and T_{FH} differentiation but does not block proliferation and differentiation of non-T_{FH} $CD4^+$ T cells (10). Like *Bcl6*$^{-/-}$ $CD4^+$ T cells, Blimp-1 expressing $CD4^+$ T cells cannot induce germinal center formation, resulting in dramatic reductions in antibody titers (10). Conversely, Blimp-1-deficient $CD4^+$ T cells preferentially differentiate into T_{FH} cells *in vivo* (10). Therefore, in $CD4^+$ T cells, Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T_{FH} differentiation (10).

6.2: Bcl6 and Blimp-1 as regulators of effector cell fate

Bcl6 and Blimp-1 were first characterized as antagonistic regulators of B cell differentiation (see Chapter 1), and are now recognized to be key regulators of $CD4^+$ T cell differentiation as well. In addition, recent studies have convincingly shown that Bcl6 and Blimp-1 are important regulators of effector $CD8^+$ T cell function, proliferation, and conversion to memory (15-20). Thus Bcl6 and Blimp-1 control the differentiation of $CD8^+$ T cells, $CD4^+$ T cells, and B cells, and several commonalities in the effects of these two transcription factors can be identified (Fig. 6.1).

Virus-specific Blimp-1-deficient $CD8^+$ T cells failed to differentiate into $KLRG1^{hi}$ $IL\text{-}7R^{lo}$ cells (18, 19), also known as short-lived effector cells (SLECs) due to their effector functions and limited ability to survive and convert into memory cells (21). Instead, Blimp-1-deficient $CD8^+$ T cells preferentially differentiate into $KLRG1^{lo}$ $IL\text{-}7R^{hi}$ memory precursor effector cells (MPECs)(18, 19), which exhibit better survival than SLECs and have the potential to convert into memory $CD8^+$ T cells (21). Blimp-1-deficient $CD8^+$ T cells expressed lower amounts of effector molecules important for cytotoxicity, such as granzyme B (18-20). Nonetheless, Blimp-1-deficient $CD8^+$ T cells possessed sufficient effector functions to control and clear acute lymphocytic choriomeningitis virus (LCMV) or influenza infections (18, 19). Taken together, these studies elegantly demonstrated that Blimp-1 expression is required for terminal differentiation of effector $CD8^+$ T cells (18, 19).

Blimp-1-deficient $CD8^+$ T cells preferentially differentiate into memory $CD8^+$ T cells (15, 18, 19), and Bcl6 is upregulated in memory $CD8^+$ T cells (22). Bcl6-deficient $CD8^+$ T cells proliferate poorly and are less able to acquire a memory-like phenotype (23). Conversely, Bcl6-overexpressing $CD8^+$ T cells generated a larger memory cell population (24). In contrast, Bcl6-deficient $CD8^+$ T cells proliferate poorly and are less able to acquire a memory-like phenotype (23). Bcl6-overexpressing $CD8^+$ T cells generated a larger memory cell population (24).

These data indicate that in both $CD8^+$ and $CD4^+$ T cells, Blimp-1 is critical for most terminal effector cell differentiation. Terminal differentiation is characterized by low proliferative potential and high effector molecule secretion. Blimp-1-deficient

$CD8^+$ T cells are diverted to a memory precursor differentiation pathway with enhanced proliferative potential, and have reduced effector molecule production. Blimp-1-deficient $CD4^+$ T cells are diverted away from terminal T_H1 or T_H2 cell differentiation and towards T_{FH} differentiation (10), and exhibit increased proliferative potential (15, 16). These proliferation and secretion commonalities of Blimp-1-expressing T cells are again shared with the B cell lineage where high Blimp-1-expressing B cells are terminally differentiated effectors (plasma cells) with low proliferative potential and high effector molecule production (antibodies).

6.3: Bcl6 and Blimp-1 transcriptional repression

While the gene targets regulating these effects in B cells are well understood, it has been unclear how Bcl6 and Blimp-1 function at the level of transcriptional repression in T cells.

Bcl6. As discussed in chapter 3, a key aspect of Bcl6 biology is that Bcl6 dimers repress transcription only in combination with co-repressors, and there are many co-repressors that Bcl6 can partner with. Most co-repressors bind to or near the Bcl6 BTB/POZ domain, including BCoR (25, 26), N-CoR (27, 28), SMRT (27, 28), CtBP (29), BAZF (30), PLZF (31), MIZ1 (32), and others. At least some of these partners can also form mixed complexes (29, 31). The ETO (*runx1t1*) co-repressor binds to the Zn finger domain of Bcl6 (33). The metastasis-associated protein 3 (MTA3) co-repressor binds to the RDII of the Bcl6 protein (RDII) (34, 35). Utilization of these many different co-repressors allows combinatorial targeting of Bcl6 to

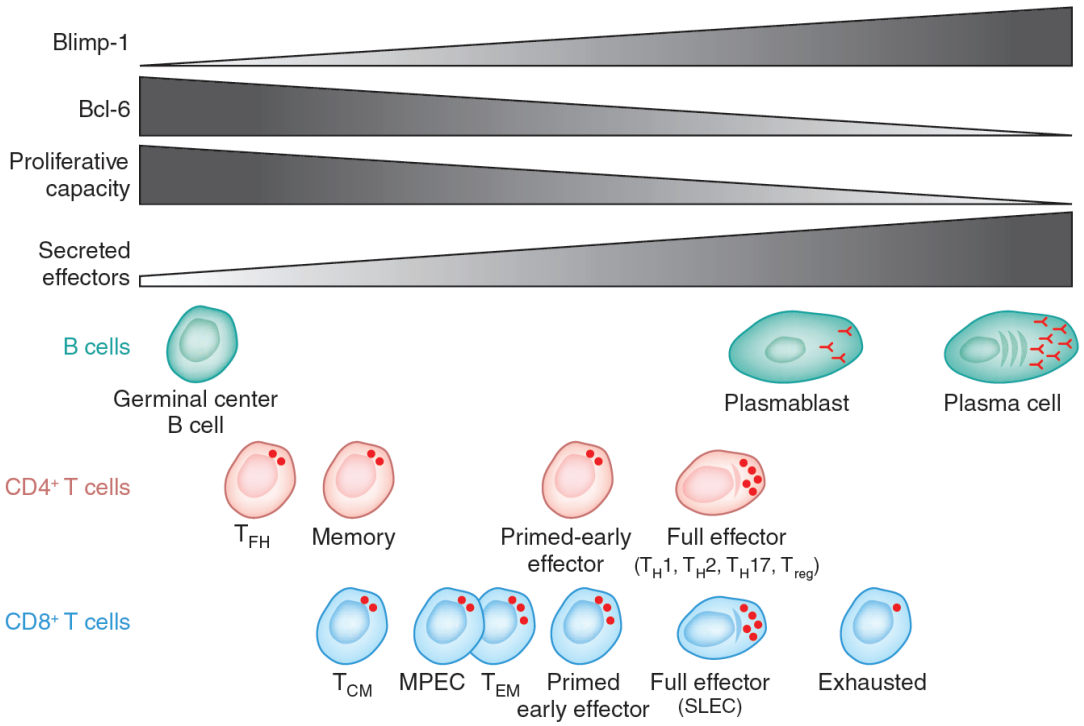

Figure 6.1: Functional impacts of differing levels of Bcl6 and Blimp-1 expression.
Bcl6 and Blimp-1 expression levels are balanced differentially in different lymphocytes at distinct differentiation stages, as indicated. Lymphocytes expressing higher amounts of Bcl6 exhibit increased proliferative capacity, but less secretory capacity. Lymphocytes expressing higher amounts of Blimp-1 exhibit reduced proliferative capacity and increased secretory capacity. Note: while exhausted CD8 T cells have high levels of Blimp-1 and reduced proliferative capacity, they do not have increased secreted effectors.

Adapted from:

Shane Crotty, Robert J. Johnston, and Stephen P. Schoenberger. *Effectors and memories: Bcl6 and Blimp-1 in T and B lymphocyte differentiation.* Nature Immunology, vol. 11: 114-120, 2010.

different collections of genes in different cell types at different times. For example, Bcl6 inhibits Blimp-1 via interactions with MTA3 (34, 36), and AP-1 (37). Bcl6 protein inhibition of the *Bcl6* gene instead depends on CtBP (29). It is also important to note that Bcl6 mRNA levels are a poor indicator of Bcl6 protein expression (38), and a poor indicator of Bcl6 function, as Bcl6 is controlled by a wide array of post-transcriptional regulatory mechanisms, including absence of translation at the mRNA level (38), as well as protein phosphorylation (39), acetylation (40), and co-factor-mediated degradation (41). This allows for intensive levels of signal integration by Bcl6, as the functional amount of Bcl6 protein in a cell can be heavily influenced by many transcriptional and post-transcriptional activities, in combination with changes in the availability of co-repressors.

In B cells, whole genome ChIP-on-chip analysis has revealed that Bcl6 regulates a very different set of genes in primary germinal center B cells and diffuse large B cell lymphoma (42). Of 3,345 genes bound by Bcl6, less than half of those targets were bound in both cell types (42), indicating that differential co-repressor availability likely has dramatic effects on Bcl6 gene targeting. There are only a smattering of obvious gene expression changes conserved between Bcl6 expressing T_{FH} $CD4^+$ T cells and Bcl6-expressing germinal center B cells, beyond inhibition of Blimp-1 (10, 43). This suggests that different Bcl6 co-repressors are present in T_{FH} and germinal center B cells, collaborating to suppress different constellations of genes in the two cell types. There are also only limited similarities in the gene expression changes between Bcl6-expressing T_{FH} $CD4^+$ T cells and Blimp-1-deficient $CD8^+$ T

cells (which express elevated levels of Bcl6)(10, 19), again suggesting that the specific gene expression changes controlled by the Bcl6 - Blimp1 regulatory axis are more different than similar in B cells, $CD4^+$ T cells, and $CD8^+$ T cells, and are therefore probably highly influenced by the available co-repressors.

To characterize the roles of Bcl6's repressor domains in T_{FH} differentiation, we used retroviral vectors expressing variants of Bcl6 with a mutant BTB domain, a mutant RDII domain, or no mutations (see Chapter 3). Because of Blimp-1's importance as a physiological inhibitor of Bcl6 expression and T_{FH} differentiation (44), it was possible that T_{FH} differentiation would remain intact if we disrupted the majority of Bcl6's co-repressor recruitment but not it's ability to recruit MTA3 and thus repress *Prmd1*. Indeed, we found that a form of Bcl6 that was unable to recruit most BTB-associated co-repressors could drive T_{FH} differentiation, though not as efficiently as wildtype Bcl6. In contrast, expression of a form of Bcl6 with a mutated RDII domain blocked T_{FH} differentiation. Interestingly, expression of RDII-mutant Bcl6 also prevented the expansion of non-T_{FH} effector $CD4^+$ T cells. This suggested that the activities of Bcl6's BTB and zinc finger domains were still sufficient to commit cells to T_{FH} differentiation, but that T_{FH} cells could not persist in the absence of RDII-mediated gene regulation. Taken together, these data indicate that Bcl6 functions through multiple co-repressors and all of its functional domains to control T_{FH} differentiation and persistence. Bcl6-mediated T_{FH} gene regulation is likely to be complex, and further studies are needed to identify Bcl6 gene targets in $CD4^+$ T cells.

Blimp-1. Blimp-1 target genes are best characterized in B cells. Blimp-1 gene targets in B cells can be placed into three functional categories: inhibition of proliferation (Myc (45, 46) and E2F1 (47)), induction of secretory machinery (XBP-1 (47)); and inhibition of the germinal center B cell program (Bcl6 (47), Pax5 (48), CIITA (49, 50)). Blimp-1 is also known to exert a dose dependent antiproliferative effect (10, 51, 52)). Id3, a pro-proliferation transcription factor (53) expressed in T_{FH}- (10), germinal center B cells (47), and Blimp-1-deficient $CD8^+$ T cells (19), appears to be one common Blimp-1 target in both B and T cells and may be a key component of the anti-proliferative effects of Blimp-1. Blimp-1 induction of the secretory apparatus (via XBP-1 and possibly other mechanisms) is most dramatic for plasma cells (54), but also occurs in T cells (55), as terminally differentiated effector T cells are specialized producers of cytokines and other secreted products. In both plasma cells and terminally differentiated effector T cells the cellular metabolism is optimized for protein production and secretion instead of DNA synthesis and proliferation.

Substantially less is known about Blimp-1 gene targets in T cells. One target that is known is IL-2 (51, 56, 57), a critical cytokine for T and B cell proliferation and differentiation. T_{FH} may produce more IL-2 than non-T_{FH} (10, 12), consistent with the lower Blimp-1 levels in T_{FH} (10). Blimp-1-deficient $CD8^+$ T cells also exhibited increased IL-2 production (18, 19). Memory CD8 T cells are also frequently characterized by their ability to produce high amounts of IL-2. IL-2 has been reported to induce Blimp-1 expression (57), and the likely importance of IL-2 for non-T_{FH}

effector $CD4^+$ T cell differentiation (Choi et al, submitted) suggests that Blimp-1 repression of IL-2 production may act as a negative feedback loop.

Because Bcl6 and Blimp-1 are reciprocal antagonists, the direct effects of Bcl6 and the indirect effects of lifting Blimp-1 repression on gene expression ought to be considered in tandem. Further characterization of Blimp-1 regulatory activity may be as important as identification of Bcl6 gene targets to determine the mechanisms by which Bcl6 drives T_{FH} differentiation.

6.4: T_{FH} differentiation

Several models for T_{FH} differentiation have been proposed. The first and simplest was that T_{FH} differentiation occurs via a fully independent differentiation pathway, analogous to T_H1, T_H2, T_H17, and inducible T_{reg} (iT_{reg}) differentiation pathways (4). In support of this model, there is evidence that Bcl6 can inhibit T_H1, T_H2, and/or T_H17 gene expression (11, 12, 58, 59). This model also fit well with conventional T_H schematics.

After we discovered the roles of Bcl6 and Blimp-1 in T_{FH} and non-T_{FH} differentiation, we proposed a second model, in which T_{FH} differentiation pathway was not fully independent of T_H1, T_H2, T_H17, or iTreg differentiation (10, 60). In this model, a $CD4^+$ T cell would be primed by a dendritic cell and acquire early T_H1, T_H2, T_H17, iTreg, or unbiased T_H0 cell characteristics. The primed $CD4^+$ T cell (early effector) could then further polarize to a Blimp-1^{high} full effector T_H1 (ref (10)), T_H2 (ref (10, 13)), T_H17, or iTreg cell (16). Alternatively, the primed $CD4^+$ T cell would

encounter a cognate B cell, which would induce Bcl6 expression and T_{FH} differentiation.

Several lines of evidence point suggested that this second model was a more accurate representation of the *in vivo* biology. First, T_{FH} are observed to produce T_H1 (10, 61-63), T_H2 (10, 13, 61, 63-65), or T_H17 (66) cytokines. T_H1, T_H2 or T_H17 cytokine production likely enables T_{FH} to specify the antibody isotypes produced during the B cell response (63). Nevertheless, T_{FH} produce T_H1, T_H2, or T_H17 cytokines at lower amounts than non-T_{FH} (10, 12, 13), which express Blimp-1 (10, 13). Therefore, Bcl6-expressing T_{FH} appear to have some T_H1, T_H2, or T_H17 characteristics that were probably acquired at the initial dendritic cell priming, but those characteristics are partially subdued in the presence of Bcl6. Second, T_{FH} were not observed in the absence of cognate B cells (10, 67), indicating that interaction of a primed $CD4^+$ T cell with a cognate B cell was a key signal to induce T_{FH} differentiation (10, 60, 67). T_{FH} differentiation could be rescued in B cell-deficient mice by expression of Bcl6 in antigen-specific $CD4^+$ T cells, suggesting that cognate B cells specifically induce Bcl6 expression in $CD4^+$ T cells (10).

Additional studies have now led us to refine this model further. We recently found that T_{FH} differentiation begins during T cell priming by dendritic cells in the T cell zone, rather than during later T-B interactions (Choi et al, submitted). This early stage of T_{FH} differentiation is independent of B cells but requires ICOS-ICOSL signaling, which is also an essential component of the T-B interaction that drives T_{FH} differentiation (Choi et al, submitted). Recently differentiated T_{FH} cells expressed a

lower level of IL-2Rα (CD25) than non-T_{FH} cells, suggesting that IL-2 signaling may play an important role in antagonizing ICOS-mediated induction of T_{FH} differentiation (Choi et al, submitted). This model of early T_{FH} differentiation is supported by our finding that STAT5 – which mediates IL-2 signaling – was a potent inhibitor of T_{FH} differentiation, but that STAT5 signaling was largely unable to block T_{FH} differentiation in the absence of Blimp-1 (see Chapter 5). Taken together, these data suggest that T_{FH} differentiation is positively regulated by ICOS signaling during priming by dendritic cells, and negatively regulated by extended IL-2 signaling, which may drive Blimp-1 expression through STAT5. Interaction with cognate B cells, rather than inducing Bcl6 expression and T_{FH} differentiation, may instead be required to maintain Bcl6 expression and thus enable T_{FH} cells to provide sustained help inside germinal centers (Fig. 6.2).

Notably absent in this third model of T_{FH} differentiation is a role for cytokines in the induction of Bcl6 expression. The differentiation pathways of other effector subsets are controlled by cytokines, and it has been proposed that T_{FH} differentiation is driven by STAT3-mediated IL-6 and IL-21 signaling. However, we have found that STAT3 signaling is neither necessary nor sufficient to drive T_{FH} differentiation and function during T_H1- and T_H2-polarized immune responses (see Chapter 4). Similarly, studies have now shown that the absence of IL-6 and/or IL-21 has only a limited effect on T_{FH} differentiation (Eto et al, submitted and ref. (68-70)). These data suggest that Bcl6 expression and T_{FH} differentiation may not be positively regulated by STAT-mediated cytokine signaling. In this case, cytokines such as IL-12, IL-4, and IL-17

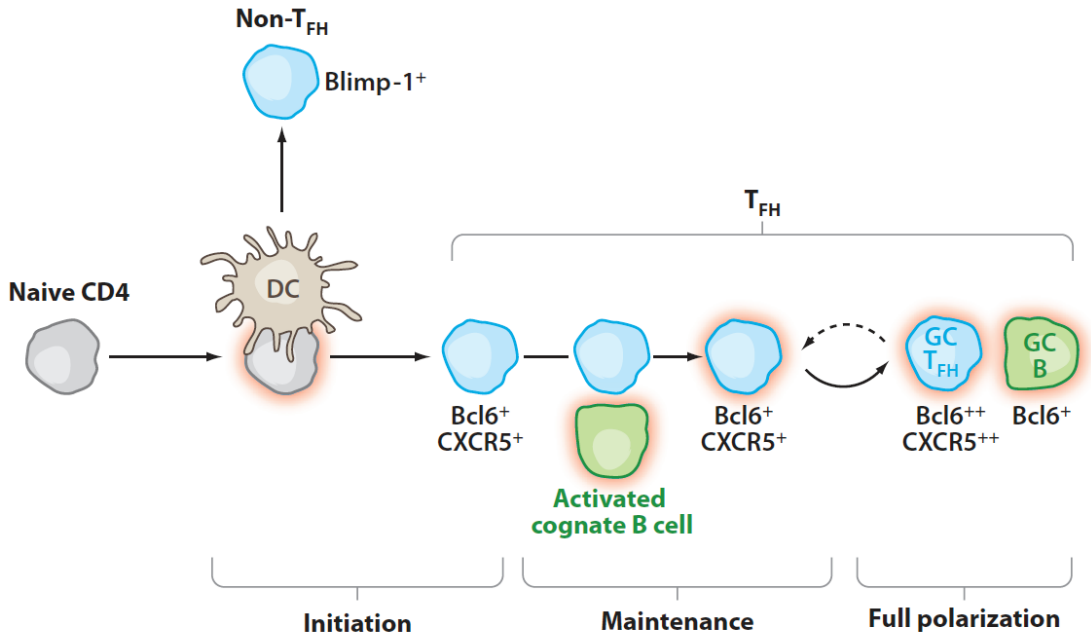

Figure 6.2: Integrated model of T_{FH} differentiation.
Bcl6 is the central regulator of T_{FH} differentiation. T_{FH} differentiation appears to occur in 3 stages: initiation, maintenance, and full polarization. Underlying each of these stages is a requirement for the continued expression of Bcl6. Alternatively, Blimp-1 is expressed, resulting in the differentiation of non-T_{FH} effector $CD4^+$ T cells, such as T_H1, T_H2 and T_H17 cells. See text for details.

Adapted from:

Shane Crotty. *Follicular Helper CD4 T cells (Tfh)*. Annual Review of Immunology, vol. 29: 621-63, 2011.

may collaborate with ICOS signaling to polarize differentiating T_{FH} cells. This is consistent with observations that T_{FH} cells possess some T_H1, T_H2 or T_H17 effector functions and gene expression similarities in T_H1, T_H2 and T_H17-type immune responses, respectively. Consequently, it is possible that STAT3, which is an essential regulator of T_H17 differentiation, may be required for full effector function and persistence of T_{FH} in T_H17-polarizing conditions. Further studies are needed to clarify the roles of STAT signaling in T_{FH} differentiation.

6.5: Summary

$CD4^+$ T cell help to B cells is a critical component of the adaptive immune response and the development of immunity. It was unclear if $CD4^+$ T cells specialized in B cell help constitute a fifth effector subset. We found that Bcl6 and Blimp-1 are antagonistic regulators of T_{FH} differentiation, and that T_{FH} are a distinct subset that uniquely capable of providing help to germinal center B cells. However, our current understanding of T_{FH} cells does not fit easily into the effector cell paradigm. Unlike other types of effector $CD4^+$ T cell, T_{FH} cells differentiate during many different types of immune response. T_{FH} cells also mirror the gene programs and phenotypes of non-T_{FH} effector responses. This adaptability is clearly important, as it enables the development of robust and tailored antibody responses to a wide range of pathogens.

Additional studies are still needed to enhance our understanding of T_{FH} cells. Most importantly, the signals that drive T_{FH} differentiation are not fully elucidated. Unlike other effector cells, T_{FH} have yet to be fully differentiated using defined

cofactors *in vitro*; this noteworthy failure strongly implies that our understanding of T_{FH} regulation is incomplete. It is these regulatory signals that must be manipulated if T_{FH}-targeted therapies are to be developed.

The ability to modulate T_{FH} differentiation – and consequently antibody responses – would be a potent therapeutic tool. Enhancing T_{FH} responses may contribute to the development of future vaccines. Conversely, repressing T_{FH} cells may block the production of autoantibodies, which contribute to a substantial number of autoimmune diseases. Because T_{FH} cells are a distinct effector subset, it may be possible to manipulate T_{FH} differentiation and persistence with enough precision to leave other components of the immune system largely unaffected.

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6.7: Acknowledgements

Chapter 6, in part, is adapted from the material as it appears in *Effectors and memories: Bcl6 and Blimp-1 in T and B lymphocyte differentiation.* Shane Crotty, Robert J. Johnston, and Stephen P. Schoenberger. Nature Immunology, vol. 11: 114-120, 2010. The dissertation author was the second author of this publication.

Figure 6.2 is adapted from the material as it appears in *Follicular Helper CD4 T cells (T_{FH}).* Shane Crotty. Annual Review of Immunology, vol. 29: 621-63, 2011.