Receptor revision in CD4 T cells is influenced by follicular helper T cell formation and germinal center interactions

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Abstract

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T cells produce diverse antigen receptors, including some with self-reactive specificities, through a process of somatic gene rearrangement. During the course of T cell development in the thymus, central tolerance eliminates self-reactive T cells. Self-reactive T cells that escape from the thymus can be tolerized in the periphery by multiple mechanisms including anergy and deletion. Peripheral CD4 T cells expressing Vβ5 undergo tolerance to an endogenous superantigen encoded by mouse mammary tumor virus 8 (Mtv-8) by either deletion or T cell receptor (TCR) revision. Revision is a process by which surface expression of the $V\beta5^{+}$ TCR is downregulated and recombination activating genes are expressed to drive rearrangement of the endogenous TCRβ locus, effecting cell rescue through the expression of a newly generated, non

self reactive TCR. Previous research has conclusively demonstrated the extrathymic nature of revision, but the regulatory context in which this process takes place remains unclear. Revision is known to occur in the germinal center (GC), a site in which B cells and T cells interact to promote memory B cell formation and high-affinity antibody responses. GCs have a highly specific structure and require distinct cell interactions, which could contribute to a regulatory environment for revision. In this thesis, we present evidence that revising T cells phenotypically and functionally resemble follicular helper T cells, the T cells found in GCs, and that GCs are required for efficient revision. The GC is known to provide a confined space in which B cell antigen receptors undergo selection. Our data extend the role of this selective microenvironment into the arena of T cells, suggesting this fluid structure also provides a regulatory environment in which TCR revision can safely take place.

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List of Abbreviations

Ab	antibody
Ag	antigen
AID	activation-induced deaminase
APC	antigen presenting cell
B6	C57BL/6J
B6 CD45.1^{+}	B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ CD45.1^{+}
Bcl-6	B cell leukemia/lymphoma 6
BCR	B cell receptor
Blimp-1	B lymphocyte-induced maturation protein 1
BSA	bovine serum albumin
BTLA	B- and T-lymphocyte attenuator
CD40L	CD40 ligand
CGG	chicken gamma globulin
d	days
DC	dendritic cell
DP	double positive
E0.5	embryonic day 0.5
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
GC	germinal center
GFP	green fluorescent protein
HBSS	Hanks balanced salt solution

HPRT	hypoxanthine guanine phosphoribosyltransferase
HRP	horseradish peroxidase
hrs	hours
i.p.	intraperitoneal
i.v.	intravenous
ICOS	inducible T cell co-stimulator
Ig	immunoglobulin
IL	interleukin
KLH	keyhole limpet hemocyanin
LN	lymph node
LPS	lipopolysaccharide
MFI	median fluorescence intensity
MHC	major histocompatibility complex
mLN	mesenteric lymph node
mTEC	medullary thymic epithelial cell
Mtv	mouse mammary tumor virus
nonTg	non transgenic
NP	4-hydroxy-3-nitrophenylacetyl
nt	nucleotide
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
PD-1	programmed death-1
PerL	peritoneal lavage

qPCR	quantitative Real Time PCR
Rag	recombination activating gene
RBC	red blood cells
RSS	recombination signal sequence
SAP	SLAM associated protein
SEM	standard error of the mean
SP	single positive
superAg	superantigen
TCR	T cell receptor
TCRβδ$^{-/-}$	TCR β and δ deficient
TdT	terminal deoxynucleotidyl transferase
Tfh	follicular helper T cell
Tg	transgenic
Th	T helper
Treg	regulatory T cell
Tx	thymectomized
V(D)J	Variable, Diversity and Joining gene segments
wks	weeks
WT	wildtype

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Chapter One: Introduction

Introduction to T cells and immunity

In order for the immune system to efficiently respond to a diverse array of pathogens, it must respond with specificity and rapidity. The innate immune system achieves a rapid response by utilizing effectors that activate quickly in response to pathogen challenge, generally by recognizing conserved features of pathogens. Recognition by the innate immune system leads to inflammatory responses and activation of the adaptive immune system (1).

The adaptive immune response consists of activity by B and T lymphocytes. Both cell types express receptors responsive to a variety of antigens (Ags). B cells, which develop in the bone marrow, produce membrane-bound B cell receptors (BCRs) and secreted antibodies (Ab) that recognize a wide range of Ags, from sugars and lipids to proteins and other macromolecules. In contrast, T cell receptors (TCRs), expressed by thymus-derived T cells, are exclusively membrane-bound and limited to recognition of Ag presented on the surface of a cell (1). In addition, BCRs and Abs undergo diversification following Ag recognition to achieve a higher affinity for Ag, whereas TCRs cannot. B cell-mediated immunity, or humoral immunity, predominantly consists of targeting microbes for destruction through production of Ab (1). T cell-mediated immunity, or cellular immunity, is more complex.

Conventional T cells, or those with an αβ TCR, can be divided into two groups, each defined by the coreceptor expressed by that cell type. CD8 T cells, or cytotoxic T lymphocytes, combat infection primarily by killing infected cells. CD4 T cells, or helper T (Th) cells, promote B cell or CD8 T cell responses and produce cytokines to induce a variety of responses.

Antigen presentation

For a T cell to recognize Ag, peptide must be presented by a major histocompatibility complex (MHC) on another cell. T cell Ag recognition is restricted to peptide presented by self-MHC. There are two classes of MHC molecules: class I, which presents to CD8 T cells, and class II, which presents to CD4 T cells (2, 3). MHC class specificity is mediated in part by the TCR and in part by the CD4 and CD8 coreceptors (reviewed in 4). MHC molecules on the surface of a cell include an extracellular peptide-binding groove that is the site of Ag binding and presentation (1). MHC class I molecules, which present peptide from nuclear or cytosolic proteins, are expressed on all nucleated cell types; whereas MHC class II molecules, which present exogenous Ag, are expressed only on Ag-presenting cells (APCs) such as dendritic cells (DCs) or B cells (5, 6). For presentation on MHC class II molecules, an APC endocytoses Ag that then undergoes proteolytic degradation inside endocytic vesicles. MHC class II molecules are transported to those vesicles carrying an invariant peptide in the peptide-binding groove, and processed peptides replace invariant peptide on MHC class II inside the vesicle. After assembly of MHC class II and peptide, the vesicle is transported to the cell membrane and peptide-MHC expressed on the cell surface (6, 7). Peptide-MHC class II can then be recognized by the TCR of a CD4 T cell. Productive TCR signaling requires both TCR-MHC interactions and a second signal through a costimulatory molecule such as CD28, which interacts with CD80 or CD86 on a B cell or DC (8).

V(D)J recombination

Diverse B cell and T cell Ag receptors are encoded by genes that are the product of somatic gene recombination. Ag receptor genes in the germline require recombination to produce

functional transcripts, as they contain groups of variable (V), diversity (D), and joining (J) segments (9, 10). D segments are only present in the immunoglobulin (Ig) heavy chain and TCRβ loci. These loci also contain multiple constant regions, which do not undergo recombination. V, D, and J segments are flanked by recombination signal sequences (RSSs), which are repeats featuring conserved heptamer and nonamer sequences and an intermediate spacer sequence of either 12 or 23 bp for RSS-12 and RSS-23 respectively (9). A complex of the proteins recombination activating gene 1 (RAG1) and RAG2 binds DNA at RSS nonamer sequences and cleaves at heptamer sequences (9).

In the process of RAG-mediated recombination, first RAG binds to one RSS sequence, and then to a second. If RAG first binds an RSS-12 site, it will preferentially bind RSS-23 for the second, and vice versa. After binding of both RSS sequences, cleavage occurs. Then the protein terminal deoxynucleotidyl transferase (TdT) adds additional nontemplated nucleotides (known as N nucleotides) to coding gene fragments, and the segments are joined by non-homologous end-joining. As a result, some of the V, D, and J segments are excised such that a rearranged Ag receptor gene in a B or T cell has one V segment, one D segment, and one J segment (9). Nucleotide addition by TdT and DNA repair provide further Ag receptor diversity (9).

This gene recombination process provides B and T cells with a wide variety of immune specificities that can contribute to an adaptive response. However, every RAG-mediated recombination event involves double stranded breaks in DNA, and errors in V(D)J recombination can lead to leukemia or lymphoma (9). Given the risks inherent in this process, it must be precisely regulated in order to be safe. Several mechanisms contribute to the regulation of RAG-mediated recombination. First, expression of both *Rag1* and *Rag2* is required for significant DNA cleavage. Expression of *Rag2*, required for a catalytically active RAG complex,

is very tightly regulated and limited to lymphoid cells at specific stages of development. In addition, chromatin inaccessibility prevents cleavage of RSS sequences by RAG (9), protecting from off-target cleavage.

V(D)J recombination is also regulated through precise ordering of events. Rearrangement occurs first on the Ig heavy chain or TCRβ chain loci, with D-J recombination preceding V-DJ. If rearrangement produces a functional Ig heavy chain or TCRβ, rearrangement on the other allele is blocked, in a process known as allelic exclusion (11, 12). Only then does V-J recombination of Ig light chain or TCRα chain loci occur. This minimizes expression of multiple Ag receptors on B cells and T cells (11). In addition, Ig V-DJ_H rearrangement is limited to B cells, and TCR V-DJ_β rearrangement to T cells.

T cell development and central tolerance

T cell progenitors migrate from the bone marrow to the thymus (13), where they become known as thymocytes. The most immature, or double negative, thymocytes express neither the CD4 or CD8 coreceptors, nor the TCR. Thymocytes begin development at the boundary between the cortex and medulla in the thymus, migrate to the outer cortex and then back through the cortex, finishing development in the medulla (14).

Rag expression is induced in double negative thymocytes, and the gene segments of the TCRβ chain are rearranged. Once an in-frame TCRβ rearrangement is completed, a pre-TCR is expressed, consisting of the rearranged TCRβ and an invariant pre-Tα, associated with CD3 (15). At this stage the T cell undergoes β selection to determine whether the TCRβ chain can signal, whereupon *Rag* expression is downregulated (11). Pre-TCR signaling appears to be ligand-independent (16). If β selection is successful, further TCRβ chain rearrangement is

blocked, *Rag* is reexpressed, and TCRα recombination begins (15). At this time, the thymocyte also begins to express both CD4 and CD8, and is known as a double positive (DP) thymocyte. While allelic exclusion is complete for the TCRβ chain locus, TCRα allelic exclusion is less strict, resulting in some cases of expression of two TCRα chains on one T cell (17). Upon successful TCRα rearrangement and surface expression of an αβ TCR, DP thymocytes migrate through the cortex and undergo positive selection and termination of *Rag* expression (14). In the positive selection process, the TCR interacts with self-MHC molecules on thymic epithelial cells. A T cell with a TCR that weakly interacts with self-MHC will survive positive selection, whereas those with TCRs that do not interact with self-MHC undergo apoptosis (4, 14). After positive selection, DP thymocytes downregulate CD8 expression, and then either maintain CD4 expression or switch to CD8 expression (4, 18). At this stage, single positive (SP) thymocytes must undergo negative selection, or central tolerance.

Negative selection is the process by which thymocytes undergo selection to eliminate thymocytes with self-reactive TCRs. In this process, TCRs interact with tissue specific Ags expressed on the surface of medullary thymic epithelial cells (mTECs). DCs also present Ag acquired from mTECs to thymocytes (19). Any SP thymocytes with TCRs that bind with high affinity or avidity to self-peptide-self-MHC are deleted. T cells that survive negative selection then leave the thymus, capable of recognizing foreign Ag presented on self-MHC, but tolerant to a variety of self-Ag (14). Some cells that recognize self-Ag during negative selection become regulatory T cells (Treg), which enter the periphery and suppress immune responses (20). As a result of sequential selection processes in the thymus, ~99% of all thymocytes die during development (21). The wastefulness inherent in T cell development indicates that appropriate selection is extremely important for a healthy immune response.

T cell peripheral tolerance

Although the majority of self-reactive T cells are eliminated through negative selection, those that escape must undergo tolerance in the periphery in order to avoid autoimmunity. There are several mechanisms in place promoting tolerance in the lymphoid periphery. T cells entering the periphery as recent thymic emigrants undergo a period of further maturation, shown by their phenotype and function that are distinct from those of more mature naive T cells (22). This maturation period is likely in place to tolerize T cells to commensal and self-Ag.

Another tolerance mechanism is induction of anergy. Typically, during an immune response to foreign Ag, a T cell will receive TCR stimulation as well as costimulatory signals through CD28 (8). Conversely, during an autoimmune response, TCR stimulation generally occurs without costimulation. As a protective mechanism, T cells with prolonged TCR stimulation but no costimulation become anergic, or nonresponsive (20).

Ag stimulation of TCRs in the absence of costimulation or repeated Ag stimulation can also induce tolerance through deletion, or activation-induced cell death. One of two apoptotic pathways is typically induced in this process. In the cell intrinsic pathway, activation of the pro-apoptotic protein Bim induces apoptosis through mitochondrial permeability and activation of caspase 9 (23). In the cell extrinsic pathway, stimulation increases expression of death receptors, such as Fas, and their ligands. Engagement of receptors and ligands induces caspases, leading to apoptosis (23).

Peripheral tolerance can also be induced by Treg inhibition of lymphocytes and innate immune cells. The suppressive activity of Treg is mediated predominantly by cytokines. Treg express high levels of interleukin 2 receptor (IL-2R), taking up IL-2 and limiting IL-2 available to promote survival of other T cells (24), and actively secrete immunosuppressive cytokines,

such as IL-10, IL-35 and TGF-β. In addition, Treg may suppress DCs. Treg can develop both as previously described, and also as induced Treg in the periphery, induced by Ag in the presence of IL-2 and TGF-β (24).

Secondary Ag receptor rearrangement and peripheral modification

B and T cells expressing self-reactive Ag receptors are typically eliminated by negative selection during the course of development, or through anergy or deletion in the periphery. However, there is evidence that tolerance can instead be induced by secondary rearrangements. In a process known as BCR editing, immature B cells with self-reactive BCRs upregulate *Rag1* and *Rag2* to undergo secondary rearrangement of Ig light chains and replace the BCR. This process occurs exclusively in the bone marrow (25). T cells can also undergo receptor editing in the thymus. In response to a cortically expressed Ag, they internalize the TCR, leading to *Rag* expression and secondary TCRα rearrangement (26).

Somatic hypermutation is a well-understood process of Ag receptor modification in which B cells interacting with T cells in the specific microenvironment of the germinal center (GC) mutate their BCRs and undergo selection for BCR affinity, leading to the formation of a population of B cells with high affinity BCRs. This process requires expression of activation induced cytidine deaminase (AID), another genome mutator that can potentially induce genomic instability (27).

These three processes share the features that more functional Ag receptors are formed through the expression of a DNA mutagen in a confined microenvironment. The specific localization may explain how these processes can occur safely with minimal risk of DNA damage and oncogenesis.

T cell receptor revision

TCR revision is a peripheral tolerance process in which surface TCR expression is lost in response to interaction with a self-Ag, and a new TCR is rearranged and expressed on the cell surface (28). There are a variety of models of TCR revision in both mice (28-35) and humans (36-39). Most models involve TCRβ rearrangement, but some affect TCRα. Mouse models of revision include those induced by myelin (35) or pancreatic islet cell Ag (34), and those induced by exogenous (31) and endogenous (40-42) superantigens (superAg). In humans, evidence of revision has been found both in healthy donors (36, 37) and in patients with defects in DNA damage repair (38, 39). CD8 T cells are excluded from revision in the majority of models. Revision can protect against autoimmunity (35), but also can produce autoreactive TCRs (34). In our lab, we study revision induced when a $V\beta5^+$ TCR on a CD4 T cell interacts with the endogenous superAg encoded by mouse mammary tumor virus (Mtv) 8.

Mouse mammary tumor virus superantigens

Mtvs are retroviruses that can infect mice through breast milk transmission, or are found stably integrated as proviruses in the mouse genome (43). C57BL/6J (B6) mice carry integrants of Mtv-8, -9, -17, and -30 (40). These proviruses encode superAg, which are primarily transcribed in B cells and CD8 T cells (44). SuperAg, unlike conventional Ag, bind to MHC class II outside of the peptide-binding groove and interact with TCRβ (45). B cell expression of Mtv transcripts increases upon lipopolysaccharide (LPS) stimulation, along with MHC class II expression (46). Each Mtv superAg stimulates certain Vβ rearrangements, and in most cases targets those T cells for deletion (47). Most Mtvs induce proliferation and deletion of both CD4

and CD8 T cells expressing the responsive Vβ (47-49). The Mtv superAg that induces TCR revision in our model, Mtv-8, is extremely weak, activating $V\beta5^+$ T cells, and inducing deletion only of CD4 T cells. It is not expressed in the thymus, and therefore does not affect thymic selection (47).

TCR revision in our model

Mtv-8-induced revision can be effectively studied using Vβ5 transgenic (Tg) B6 mice. These mice have a Vβ5 TCR rearrangement encoded in the germline, and all T cells exit the thymus with Vβ5 paired to endogenous TCRα chains (40, 50). Most Mtv-8-reactive $V\beta5^+$ CD4 T cells become anergic and are deleted, leading to an age-dependent decline in the CD4:CD8 T cell ratio in Vβ5 Tg B6 mice (40, 50). Fewer cells undergo TCR revision, in which interaction of the $V\beta5^+$ TCR with Mtv-8 leads to downregulation of TCR surface expression, induction of *Rag* and TdT expression, and rearrangement of endogenous TCRβ chain genes (51). The endogenous TCRβ is expressed on the cell surface, and the T cell is no longer responsive to Mtv-8. $TCR\beta^+V\beta5^-$ CD4 T cells accumulate in these mice with age (50).

Cells that have completed revision, referred to as post-revision T cells, are functional memory cells. They undergo lymphopenia-induced proliferation and also proliferate in response to microbial Ag (52). In addition, they recognize Ag in a self-MHC restricted manner and become effectors after pathogen challenge (52). However, post-revision T cells are unusual in that they are skewed to a Th17 phenotype, do not become Treg (53), and have elevated steady-state proliferation when compared to other memory T cells (52).

Revising T cells can be identified using mice in which green fluorescent protein (GFP) is expressed under the control of the *Rag2* promoter, which will be referred to as Rag2p-GFP mice

(54). Although they no longer express *Rag*, recent thymic emigrants maintain GFP expression for up to 3 weeks (wks) after thymic egress (55), so to differentiate thymic from extrathymic *Rag* re-expression, we thymectomize Rag2p-GFP Tg mice and analyze their peripheral T cells >3 wks later. When GFP^+ and GFP^- $V\beta5^+$ CD4 T cells were sorted from thymectomized Vβ5 Tg Rag2p-GFP Tg mice 5 wks after thymectomy, the GFP^+ cells expressed *Rag1* and *Rag2* (42). These cells also contained TCRβ V-DJ recombination intermediates, confirming that *Rag* expression in peripheral T cells does lead to properly regulated gene rearrangement (42). In addition, the requirement of *Rag* for TCR revision has been confirmed using a mouse model in which *Rag* is deleted in peripheral T cells. In the absence of peripheral *Rag* expression, revision does not occur (56).

Further evidence substantiates the idea that TCR revision does not occur in the thymus. SP thymocytes in Vβ5 Tg mice are exclusively $V\beta5^+$. Furthermore, $V\beta5^-$ CD4 T cells accumulate in thymectomized mice, indicating that $V\beta5^-$ T cells in Vβ5 Tg mice do not originate in the thymus (42, 50). The TCR repertoire of post-revision T cells recapitulates the $V\beta5^-$ TCR repertoire in TCR nonTg mice with little variation between mice, indicating that post-revision T cells do not develop from a rare founder population. However, post-revision TCRs are molecularly distinct in that they have shorter N regions than thymically derived TCRs, but longer than those found in embryos, which lack TdT expression (41). In addition, $V\beta5^+$ CD4 T cells that are transferred into an Mtv-8^+ adoptive host become $V\beta5^-$ over time (56). Overall, the body of research on TCR revision shows that this is a process of functional gene rearrangement, occurring in the lymphoid periphery.

Evidence that revising T cells undergo some form of selection can be found in analysis of the affects of death pathways on revision. Deficiency in the death receptor Fas leads to increased

accumulation of post-revision T cells (57). Bim deficiency leads to an increased frequency of revising T cells (58). These data indicate that revising and post-revision T cells are culled through death pathways, suggesting that selection is involved in the revision process. However, these studies do not illustrate the mechanism of selection.

Using the Rag2p-GFP Tg model, previous lab members investigated the localization and surface phenotype of revising T cells. In splenic sections, revising T cells localize predominantly in or near GCs. They also express CXCR5, a chemokine receptor associated with GCs (59). Analysis of possible requirements for revision has shown that revision does not occur in the absence of B cells, indicating that B cells are the APCs presenting Mtv-8 to revising T cells. In addition, the costimulatory molecules CD28 and inducible T cell co-stimulator (ICOS) are required for revision (57). However, in contrast to TCR revision, deletion does not require B cells, CD28, or ICOS. Altogether, the phenotype and requirements for revising T cells suggest that revision occurs in the GC, and that revising T cells may be follicular helper T cells (Tfh).

T helper differentiation

CD4 T cells differentiate into a variety of Th subsets. Effector CD4 T cells include Th1, Th2, and Th17 cells (60). Tfh are an additional CD4 T cell subset dependent on the transcription factor B cell leukemia/lymphoma 6 (Bcl-6). Effector T cells require B lymphocyte-induced maturation protein-1 (Blimp-1), a mutually antagonistic repressor of Bcl-6 (61). In addition, there is evidence that T-bet, the transcription factor that induces formation of Th1 cells, binds to Bcl-6 and inhibits its function (62). For some time, the understanding in the field was that differentiation of Th lineages was terminal. However, recent research on the interactions of different transcription factors, and on the ability of Tregs to differentiate from different effector

T cell types, indicates that Th differentiation is plastic and T cells can transition between different states (63).

The germinal center reaction

Lymph nodes (LNs) and the white pulp of spleen contain specialized zones that promote cell interactions. The major areas are the T cell zone and the B cell follicle, which border at the T cell – B cell boundary (64). T cells trafficking from the blood move to the T cell zone, facilitated by expression of the chemokine receptor CCR7. Both B cells and CD4 T cells migrate to the B cell follicle when they upregulate expression of CXCR5 and downregulate CCR7. CD4 T cells upregulate CXCR5 after priming by DCs (61). These T cells accumulate along the T cell – B cell boundary and interact with B cells, further upregulating CXCR5 and migrating deeper into the follicle. Within the follicle, sites of T cell – B cell interaction become GCs, in which cell interactions promote somatic hypermutation and affinity maturation.

When a B cell is activated by uptake of Ag, it migrates to the T cell – B cell boundary following chemokine gradients (64). Then the B cell begins interacting with Ag-specific CD4 T cells through TCR-MHC and costimulatory interactions. CD40 – CD40 ligand (CD40L) costimulation and cytokine production allow the T cell to stimulate the B cell, leading to B cell proliferation and the early stages of GC differentiation, including Ig class-switch (65). Abs belong to one of five classes, each of which corresponds to a heavy chain constant region. Naive B cells express IgM or IgD, and class-switch to IgG, IgA, or IgE, all of which promote distinct effector functions (66).

The full GC reaction develops between 4 and 7 days after initial B cell activation by Ag (64). In the GC reaction, B cells cycle between two different zones of the GC, the dark zone and

the light zone. B cells in the dark zone proliferate and undergo somatic hypermutation, in which point mutations are induced in Ig genes by AID. In the light zone, B cells with mutated Ig interact with follicular DCs and CD4 T cells to undergo selection for high affinity Ig (65, 67). As a result, B cells with high affinity receptors survive; these B cells differentiate either into Ab-secreting plasma cells or memory B cells.

Germinal centers and Tfh

The CD4 T cells interacting with B cells in the GC are known as Tfh. Generation of Tfh requires a highly specific gene expression pattern, interaction with DCs and B cells, and localization to the GC (61). Tfh differentiation is induced by the transcription factor Bcl-6 and inhibited by Blimp-1 (68). Tfh secrete the cytokines IL-4, IL-6, and IL-21. IL-21 in particular is associated with Tfh function, but production of IL-4 by Tfh promotes heavy chain class-switch (61, 69). On the cell surface, Tfh express the chemokine receptor CXCR5 that allows them to migrate into B cell follicles, as well as the death receptor PD-1. Costimulatory markers ICOS, CD40L, and OX40 contribute to Tfh function (69). Tfh also express B- and T-lymphocyte attenuator (BTLA) on the cell surface (61).

Tfh exist in two separate stages of differentiation. Pre-Tfh, induced by interaction with DCs, are found at the T cell – B cell boundary and express intermediate levels of Bcl-6, CXCR5, and PD-1 (70). GC Tfh are further differentiated, found in the GC, and express elevated levels of Bcl-6, CXCR5, PD-1, and other Tfh markers (61). The intracellular adaptor protein SLAM associated protein (SAP) is not necessary for B cell – T cell interactions to form, but it is required to stabilize long-lived interactions of B cells and T cells in the GC, which are necessary for GC Tfh formation (71, 72).

Goals of this thesis

Thus far, the evidence for the existence of TCR revision is substantial. However, much work remains to characterize its regulation. There are two clear dangers to Ag receptor rearrangement: the capacity of RAG to induce DNA damage and oncogenesis, and the rearrangement of self-reactive receptors. The precise regulation of *Rag* expression and thymic selection mitigate these dangers during T cell development, but thus far it is not clear which, if any, factors do so during TCR revision.

The goal of this thesis is to put TCR revision into the context of known aspects of CD4 T cell biology to address how this process is regulated to minimize the potential risks. Based on previous research in the lab, we hypothesized that revising T cells are Tfh and that GCs are required for revision. A GC requirement for revision would indicate that *Rag* expression is regulated by confinement to a specialized microenvironment, and also suggest a possible mechanism of selection through Ag-specific interactions with B cells. In this thesis, we show that revising T cells can act as Th cells for a B cell Ab response, and they adopt a Tfh-like surface, cytokine, and transcription factor phenotype. In addition, Bcl-6 is required for efficient revision whereas Blimp-1 inhibits it, and SAP-mediated interactions are required for completion of revision. Experiments attempting to increase the frequency of revision by enhancing Mtv-8 expression have no effect, indicating that the GC requirement for revision is a specific GC requirement, rather than an activated B cell requirement. Overall, these data show that revision does require GCs, and that T cells become Tfh during the course of revision.

Chapter Two: Materials and Methods

Mice

Wildtype (WT) B6 (B6 CD45.2$^+$), B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ CD45.1$^+$ (B6 CD45.1$^+$), B6.129S2(Cg)-*Cxcr5*^{tm1Lipp}/J (CXCR5$^{-/-}$, reference 73), and B6.129P2-*Tcrb*^{tm1Mom}*Tcrd*^{tm1Mom}/J (TCR βδ$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred at the University of Washington. TCR βδ$^{-/-}$ mice have no cells expressing αβ or γδ TCRs (74). Vβ5 Tg mice have a rearranged Vβ5.2 TCR encoded in the germline (50), and B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II Tg) mice are Tg for genes encoding an αβ TCR recognizing $OVA_{323-339}$ peptide in the context of I-A^b (75). Both Vβ5 Tg and OT-II Tg B6 mice were bred in-house and maintained as heterozygotes. Rag2p-GFP Tg mice (54), which are Tg for GFP under the control of the *Rag2* promoter, were originally a gift from M. Nussenzweig (The Rockefeller University) and have been backcrossed at least 12 generations on the B6 background in our lab. Mtv-8$^+$ and Mtv$^-$ mice were derived by intercross/backcross breeding of Vβ5 Tg mice to a male WLC-0 mouse provided by D. Morris (University of California, Irvine, CA). WLC-0 mice are wild-derived and Mtv$^-$, whereas B6 mice express Mtvs -8, -9, -17, and -30 (40). Bcl-6$^{+/-}$ B6 (76) mice were provided by A. Dent (Indiana University, Indianapolis, IN). Blimp-1$^{gfp/+}$ B6 mice, in which a knock-in allele expresses GFP and non-functional Blimp-1 protein (77), were provided by P. Greenberg (University of Washington, Seattle, WA). SAP$^{-/-}$ B6 (71) mice were provided by P. Stein (Northwestern University, Chicago, IL). Genotyping of mice was performed by flow cytometry on peripheral blood lymphocytes (PBL), or by polymerase chain reaction (PCR) on tail snips. Mice were maintained under specific pathogen free conditions, except for TCR βδ$^{-/-}$ mice, which were maintained as helicobacter free due to susceptibility to mild inflammatory

bowel disease (78). All experiments were conducted in accordance with the University of Washington Institutional Animal Care and Use Committee.

Breeding

CXCR5$^{-/-}$, SAP$^{-/-}$, and TCR βδ$^{-/-}$ mice were maintained as homozygotes. Bcl-6$^{+/-}$ and Blimp-1$^{gfp/+}$ mice were maintained by breeding heterozygotes to B6 mice, as the null phenotype for both lines is embryonic lethal (77, 79). To obtain embryos for fetal liver, heterozygote intercross matings were performed. One or two females were added to a cage with one male between 5 and 6 pm and separated at 8 am the following morning (for Blimp-1^{gfp}) or the day after (for Bcl-6). For overnight matings, females were checked for vaginal plugs at the time of separation. A vaginal plug consists of secretions from a male mouse, typically appears as a whitish mass or visible clear secretions, and remains at least 8 hours (hrs) after breeding (80). For two day matings, females were not checked for plugs. Instead, 12-14 days (d) after breeding they were checked for pregnancy by inspection of the abdomen. We denote the morning of plugging as embryonic d (E)0.5.

Thymectomy

Eight-wk-old mice were anesthetized with tribromoethanol (Sigma-Aldrich, St. Louis, MO) at approximately 9 mg / 20 g body weight. Carprofen was administered subcutaneously at 0.1 mg / 20 g body weight as an analgesic prior to and every 12 hrs for 48 hrs after surgery. An incision was made from sternum to neck and the underlying tissue blunt dissected. The thymus was then removed from the top of the chest cavity using suction through a glass pipet, and the wound closed using surgical staples. Mice were kept warm and under observation until regaining

consciousness. Staples were removed 1 wk later and mice sacrificed 4-6 wk later. Thymectomy was verified upon sacrifice by staining for CD4 and CD8 expression in cells from any tissue remaining in the thymic region.

Mixed radiation chimeras

Bone marrow was isolated from the hind leg bones of adult donors, dispersed into a single-cell suspension, and T cell depleted by incubation with Ab to Thy1.2 (13.4.6), CD8 (3.168.6), and CD4 (RL172K) for 30 min on ice followed by a 45 min incubation of Low-Tox-M rabbit complement (CedarLane, Burlington, ON, Canada) at 37°C. For fetal liver chimeras, Vβ5 Tg CD45.1^{+} Bcl-6$^{+/+}$ control embryos and those from Vβ5 Tg CD45.2^{+} Bcl-6$^{+/-}$ intercross matings were collected on ~ E16. Livers were teased into single-cell suspension and resuspended in Hanks balanced salt solution (HBSS). Tail DNA was isolated using the Gentra Puregene Tissue Kit (Qiagen, Valencia, CA) and Vβ5 and Bcl-6 genotypes were identified by PCR. Vβ5 primers were: forward 5'-ACG TGT ATT CCC ATC TCT GG; reverse 5'-CTG TTC ATA ATT GGC CCG A. Bcl-6 primers were forward: 5′-CCA GCC AAC CTG AAG ACC CAC AC, reverse WT: 5′-TGT GGA TGC GCA GAT GGC TCT TCA GAG, and reverse mutant: 5′-AAA TGT GTC AGT TTC ATA GCC TGA AGA ACG (81). The Vβ5 PCR consisted of 15 minutes (min) at 94°C, 35 cycles of 30 seconds (s) at 94°C, 30 s at 61°C, and 30 s at 72°C, followed by 10 min at 72°C. The Bcl-6 PCR consisted of 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 63°C, and 30 s at 72°C, followed by 10 min at 72°C.

Genotyping of Blimp-1$^{gfp/gfp}$ embryos (which we were unable to use for mixed chimeras) was completed using PCR for Blimp-1 expression. Blimp-1 primers were: forward 5'-GGC AAG ATC AAG TAT GAG TGC, reverse WT 5'-TGA GTA GTC ACA GAG TAC CCA, and

reverse mutant 5'-GCG GAA TTC ATT TAA TCA CCC A. The Blimp-1 PCR consisted of 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by 10 min at 72°C.

For both bone marrow and fetal liver chimeras, Vβ5 Tg WT and null samples were mixed at the indicated ratios and resuspended in HBSS. For one round of Bcl-$6^{-/-}$ fetal liver chimeras, the Vβ5 Tg WT sample was fetal liver; for the other it was bone marrow. In the latter round, the WT:null ratio was adjusted from 1:1 to 3:1 to account for the lower hematopoietic potential of bone marrow (82). Irradiation was completed using a gamma irradiator with ^{137}Cs. Cells ($5x10^6$ total) were injected into the lateral tail vein of sublethally irradiated (400 rad) $CD45.2^+$ TCR $\beta\delta^{-/-}$ B6 mice. For bone marrow, donors and recipients were sex matched. Irradiation was completed either 6-8 (bone marrow) or 1 (fetal liver) hr prior to injection, and irradiated mice were maintained on antibiotic water from 1 d before irradiation until 14 d after. The antibiotic cocktail consisted of 2 mg/mL neomycin (Sigma-Aldrich) and 1 mg/mL polymyxin B (Gibco, Carlsbad, CA), changed weekly.

Cell preparation

PBL and sera were collected by retroorbital bleed using heparinized (for PBL isolation) or non-heparinized (for serum acquisition) capillary tubes by certified personnel. Spleen, thymus, and peripheral (axial, brachial, inguinal) and mesenteric (m) LNs were collected by dissection. For collection of peritoneal lavage (PerL), the outer layer of skin was opened without disturbing the peritoneal cavity, then 5 mL HBSS were injected into the peritoneal cavity, the cavity was washed by gentle massage, and the liquid was collected using a Pasteur pipet. Single-cell suspensions were prepared from spleen, thymus and LNs. Red blood cells (RBC) were removed

from spleens and PBL by water lysis. Cells were counted on a hemocytometer using 0.1% Trypan Blue.

Surface staining

Fc receptors were blocked with anti-CD16/32 (2.4G2; BD Pharmingen). Cells were surface stained in HBSS containing 1% bovine serum albumin (BSA) using fluorochrome-conjugated or biotinylated Abs, all purchased from BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), or eBioscience (San Diego, CA). Abs were specific for mouse BTLA (8F4), CD4 (RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD11b (M1/70), CD19 (1D3, MB19.1), CD35 (8C12), CD44 (IM7), CD45R/B220 (RA3-6B2), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD73 (TY/11.8), CD80 (16-10A1), CD95 (Jo2), CD126/IL-6Rα (D7715A7), CD127/IL-7Rα (A7R34), CCR7 (4B12), CXCR5 (2G8), GL-7 (GL7), ICOS (7E.17G9), αIgD (11-26c), αIgM (eB121-15F9), MHC class II (M5/115.15.2), OX40 (OX-86), panTCRβ (H57-597.13), PD-1 (J43), and Vβ5 (MR9-4). Staining with biotinylated Abs was followed by fluorescein isothiocyanate- (eBioscience), allophycocyanin- (eBioscience), or Brilliant Violet 421- (BioLegend) conjugated streptavidin. Abs recognizing Vβ5 and TCRβ do not cross block. Unless indicated otherwise, cells were stained for 20 min at 4°C. For CXCR5 and CCR7, cells were stained for 30 min at 37°C. In the majority of experiments, cells were stained in 96-well plates. Cells were stained first with Abs specific for chemokine receptors, second with the remainder of the surface stain, and third with streptavidin. In all cases, cells were washed twice between stains. After staining, cells were washed once and resuspended in PBS with 1% fetal bovine serum (FBS) and 100 U/mL DNase to prevent cell clumping.

Transcription factor staining

Surface stained cells were fixed with 200 μL freshly prepared Fixation/Permeabilization solution (eBioscience) for 30 min on ice, washed twice in 1X Permeabilization buffer (eBioscience), then stained with Alexa Fluor 647-conjugated anti-Bcl-6 (K112-91) from BD Biosciences for 1 hr at room temperature. Alexa Fluor 647-conjugated IgG1κ (BD Biosciences) was used as an isotype control. Stained cells were washed once in 1X permeabilization buffer and resuspended in PBS with 1% FBS and 100 U/mL DNase.

Flow cytometry and cell sorting

Flow cytometry data were collected on a FACSCanto or LSRII (Becton Dickinson, Franklin Lakes, NJ) and analyzed using FlowJo software (Tree Star, Ashland, OR). For cell sorting, enriched CD4 T cells were obtained using an EasySep Negative Selection Mouse $CD4^+$ T cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada). The enrichment cocktail consisted of biotinylated Abs specific for CD8, CD11b, CD11c, CD19, CD45R, CD49b, and TER119, and separation was accomplished with the use of magnetic beads. After enrichment, cells were surface stained and sorted using a FACSAria II (Becton Dickinson).

In vitro Th2 CD4 T cell differentiation

Sorted naive CD4 T cells were cultured in the presence of irradiated APCs for 3 d in complete RPMI 1640 medium containing 10% FBS, 10 mM Hepes, 4 mM L-glutamine, and 50 mM 2-mercaptoethanol at 37°C in the presence of 30-500 ng/mL anti-CD3 (145-2C11; BD Biosciences), 1 μg/mL anti-CD28 (37.51; eBioscience), 50 ng/mL rIL-4, 50 μg/mL anti-IFN-γ,

and 50 μg/mL anti-IL-12/IL-23 (all from eBioscience). Cultured cells were sorted based on CD4 expression, and RNA was isolated as described below (83).

Immunizations and serum collection

Vβ5 Tg Mtv^+ B6 and Mtv^- mice were immunized intraperitoneally (i.p.) with 100 μg 4-hydroxy-4-nitrophenyl acetyl-keyhole limpet hemocyanin (NP-KLH) or NP-chicken gamma globulin (CGG, Biosearch Technologies, Novato, CA) in a 1:1 solution with Imject Alum (Thermo Scientific, Waltham, MA). Protein was added dropwise to alum while gently vortexing, then the emulsion was mixed on a rotator for ~30 min. A drop was added to HBSS, and if the drop held together that was taken as confirmation that an emulsion had successfully formed. Secondary Ab responses were induced by immunization with 20 μg NP-CGG 21 d later. B6 recipients of 1-3x10^6 OT-II T cells were immunized with 100 μg NP-ovalbumin in alum at the base of the tail, and responding OT-II T cells sorted from the draining inguinal nodes 6.5-7.5 d later. Sera were collected 7, 14, and 21 d post immunization. After collection, blood sat at room temperature for 30-60 min to clot, and samples were spun down for 5 min at 3000 RPM in a microcentrifuge at room temperature. Sera were collected and stored at ⁻20°C.

Ab quantification

For enzyme-linked immunosorbent assays (ELISAs), 96-well ELISA plates (Thermo Scientific, Waltham, MA) were coated overnight with 4 μg/mL NP(4)- and NP(23)-BSA from Biosearch Technologies, washed 3 times in PBS-Tween, blocked overnight with PBS/1% BSA, then washed 3 times. Sera from d 7, 14, or 21 post primary immunization or d 7 post secondary immunization were added to plates in triplicate at dilutions of 1:100, 1:1000, and 1:10,000 and

incubated for 1 hr in PBS/1%BSA, followed by 5 washes in PBS-Tween. Anti-NP Abs were detected with goat anti-mouse IgG and IgM (Southern Biotech, Birmingham, AL) conjugated to horseradish peroxidase (HRP) used at a dilution of 1:6000 in PBS/1%BSA. Plates were incubated with Ab for 1 hr followed by 7 washes in PBS-Tween. The chromogenic substrate 3,3',5,5'-tetramethylbenzidine (Becton Dickinson) was used for detection. After 15 min, the reaction was stopped using 1M H_3PO_4 and plates were read at OD450 and OD570 on an iMark microplate reader (BioRad, Hercules, CA). Absorbance vs detection was plotted, and ratios for NP(4)-BSA:NP(23)-BSA were calculated from the 1:100 dilution.

Natural Ab quantification

Sera were collected from Mtv-8^+ and Mtv^- mice at 1 month intervals for 3-4 months. Mice ranged from 8 to 17.5 wks of age at the first time point. ELISA was performed using the Mouse Ig Isotyping ELISA Ready-SET-Go! Kit (eBioscience). ELISA plates (96-well) were coated overnight with anti-mouse IgM, IgG1, IgG2a, or IgG2b, washed 3 times PBS/Tween, blocked for 2 hrs with PBS/0.1%Tween/1% BSA, then washed 3 times PBS/Tween. Sera from the first and last time point for each mouse (3-4 months apart) were added to plates in triplicate at dilutions of 1:2000, 1:20,000, and 1:200,000 in PBS/0.1%Tween/1%BSA and incubated for 1 hr, followed by 5 washes with PBS/Tween. Abs were detected with anti-mouse Ig(H+L) conjugated to HRP used at a dilution of 1:250. Abs were incubated for 1 hr followed by 7 washes PBS/Tween. For detection, 3,3',5,5'-tetramethylbenzidine was added to plates. After 15 min, the reaction was stopped using 1M H_3PO_4 and plates were read at OD450 and OD570 on an iMark microplate reader. Absorbance vs detection was plotted, and ratios of old:young calculated for the 1:2000 dilution.

Injection of sheep red blood cells

Sheep red blood cells (SRBC) purchased in 40% blood/60% Alsevers Solution (Lonza, Basel, Switzerland) were washed and resuspended in PBS at a concentration of ~25x10^8 cells/mL. Vβ5 Tg mice were injected intravenously (i.v.) with 200 μL SRBC or PBS every 2 wks for a total of 6 injections.

Injection of agonistic anti-CD40

Vβ5 Tg mice were injected i.p. with one of two clones of anti-CD40, and injections were repeated 3 times at 3 wk intervals. The clones FGK4.5 (UCSF Monoclonal Antibody Core), which is broadly activating, and 1C10, which specifically stimulates mature B cells, were used (84, 85). 1C10 was obtained from the Clark lab, which isolated the Ab from a hybridoma cell line (86).

Injection of B-1 B cells

PerL was isolated from TCR nonTg mice and injected i.v. in a 200 μL volume of HBSS into Vβ5 Tg recipients, with one recipient per donor. Each recipient received 3 injections, with 3-6 wks elapsed between injections. At the time of injection, aliquots of PerL cells were stained to measure the % B-1 B cells of injected cells using the markers CD11b, CD5, αIgM, CD19, αIgD, and B220. Controls were injected i.v. with 200 μL PBS.

Lipopolysaccharide blast formation

Vβ5 Tg mice were injected i.p. with 50 μg LPS purchased from Calbiochem (La Jolla, CA) or 200 μL PBS. Injection was repeated 3 and 6 wks later. For in vitro blast formation, B

cells were enriched from Mtv-8$^+$ and Mtv$^-$ splenocytes using an EasySep Negative Selection Mouse B cell Isolation Kit (Stem Cell Technologies; Abs for depletion were specific for CD4, CD8α, CD11b, CD43, CD49b, CD90.2, Ly-6C/G, and Ter119) and cultured for 3 d in complete RPMI 1640 medium containing 10% FBS, 10 mM Hepes, 4 mM L-glutamine, and 50 μM 2-mercaptoethanol at 37°C in the presence of 50 μg/mL LPS.

Quantitative (q)PCR and semi quantitative PCR

Total RNA was extracted from purified cells using the RNeasy Micro kit (Qiagen) and first-strand cDNA synthesis was performed with oligo(dT) primers using the SuperScript III Reverse Transcriptase kit and protocol (Invitrogen, Grand Island, NY). For qPCR, an ABI 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA) and Power SYBR Green PCR Master Mix (Applied Biosystems) were used. PCRs were performed in 96-Well Optical Reaction Plates (Applied Biosystems) and consisted of 2 min at 50°C, and a 10 min 95°C denaturation step, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, and then a dissociation stage (15 s at 95°C, 30 s at 60°C, and 15 s at 95°C). The following primers were used: Bcl-6 forward 5'-GGA AGT TCA TCA AGG CCA GT, reverse 5'-GAC CTC GGT AGG CCA TGA, for a 114 nucleotide (nt) product; Blimp-1 forward 5'-AAA CTC CAT GAC CTC GCT ATG AC, reverse 5'-CAC CCT CAC CTC TGC ACT GA, for a 90 nt product; IL-4 forward 5'-CAT CGG CAT TTT GAA CGA GGT CA, reverse 5'-CTT ATC GAT GAA TCA GGC ATC G, for a 240 nt product (87); hypoxanthine guanine phosphoribosyltransferase (HPRT) forward 5'-GTT GTT GGA TAT GCC CTT GAC, reverse 5'-CAA CTT GCG CTC ATC TTA GGC, for a 111 nt product. Bcl-6, IL-4, and HPRT primers are intron-spanning; the

Blimp-1 primers are not. Reactions were run in triplicate and results for Bcl-6, Blimp-1, and IL-4 were normalized to the internal HPRT control using the $2^{-\Delta CT}$ method.

Semi-quantitative PCR was performed on serial 3-fold dilutions of cDNA starting with 150 ng cDNA. Samples of cDNA were quantified on a NanoDrop 1000 (Thermo Scientific, Waltham, MA). Mtv-8 primers amplify the Mtv-8 superAg and were: forward 5'-TCT AAA CAA TTC GGA GAA CTC; reverse 5'-AAG ACT TGG ATA AGT TCC A, for an 890 nt product. The Mtv-8 PCR consisted of 15 min at 95°C, 31 cycles of 1 min at 94°C, 1 min at 40°C, and 1 min 30 s at 72°C, followed by 10 min at 72°C. PCR using the HPRT primers described for qPCR was performed as a control for quantitation, starting with 10 ng cDNA. The HPRT PCR consisted of 5 min at 94°C, 31 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by 10 min at 72°C. Samples were run on a 2% agarose gel that was previously stained with SYBR Safe (Life Technologies, Carlsbad, CA). Bands were quantified relative to the lowest dilution of Mtv-8^{+} blast cDNA on a ChemiDoc XRS+ (BioRad, Hercules, CA) using Image Lab 4.1 software.

Statistics

Graphs were made using GraphPad Prism. An unpaired or paired (mixed chimeras in Figs. 11-15, 17, and 20 only) Student's *t* test was used to calculate p values. Asterisks denote significant p values. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. If no p value is listed, there is no statistically significant difference between samples.

Chapter Three: Revising T cells Provide T Cell Help and Adopt a Tfh Phenotype

Introduction

Data indicating that TCR revision requires B cells and costimulatory signals and occurs in the GC suggest that revising T cells may be Tfh. While B cell – T cell interactions in the GC are known to be crucial for promoting affinity maturation and somatic hypermutation in B cells, thus far no research has been done to investigate the effect that revising T cells have on GC B cells. We hypothesized that revising T cells can provide B cell help to enhance formation of memory B cells and production of high-affinity Ab. To address this hypothesis, we analyzed memory B cell populations in aged Vβ5 Tg Mtv^+ and Mtv^- mice and Ag-specific Ab production in immunized Vβ5 Tg Mtv^+ and Mtv^- mice. We also analyzed secondary Ab responses to determine whether the completion of revision has an effect on T cell help. Our results show that Vβ5 Tg Mtv^+ mice have enhanced B cell memory formation and primary Ab responses, but that this enhancement does not extend to secondary Ab responses. Given that revision occurs in the GC and revising T cells function as Tfh, we hypothesized that revising T cells adopt a Tfh phenotype. Our results show that revising T cells have a surface, cytokine, and transcription factor phenotype resembling that of Tfh.

Results

The size of mLNs and the proportion of pre-Tfh and GC Tfh increase in Vβ5 Tg mice in an Mtv-8-dependent manner

One striking phenotype in Vβ5 Tg mice is an Mtv-8-dependent increase in mLN size compared to TCR nonTg mice (Fig. 1A). GC B cell, pre-Tfh and GC Tfh populations (defined as shown in Fig. 1B – D) are elevated as a percent of total B or T cells in Vβ5 Tg mice compared to their TCR nonTg littermates (Fig. 1B – D). Analysis of the same phenotype in Vβ5 Tg Mtv^- mice indicates that the increased proportions of pre-Tfh and GC Tfh are Mtv-8-dependent (Fig. 1 C, D), but the GC B cell phenotype is not (Fig. 1B).

Mtv-8^+ Vβ5 Tg mice have elevated age-dependent increases in Ab production

Natural Ab formation and class-switch can be measured over time in mice by sampling serum at various ages and measuring production of Abs of different classes. When IgM and IgG2a levels were measured in the same Mtv-8^+ and Mtv^- mice at young and old ages, Mtv^- mice had elevated production of both classes of Ab at both time points (Fig. 2A, B). However, the change in Ab levels from young to old time points was slightly greater in Mtv-8^+ mice (Fig. 2A, B).

Mtv^+ Vβ5 Tg mice have elevated formation of memory B cells with mutated Ig

$CD73^+CD80^+$ memory B cells constitute an elevated percentage of B cells in Vβ5 Tg Mtv^+ B6 mice relative to TCR nonTg and Vβ5 Tg Mtv^- mice (Fig. 3). CD35 expression can be used to distinguish memory B cells with mutated ($CD35^-$) and unmutated ($CD35^+$) Ig genes (88). Memory B cells from Vβ5 Tg Mtv^+ mice have a higher frequency of mutated than unmutated Ig,

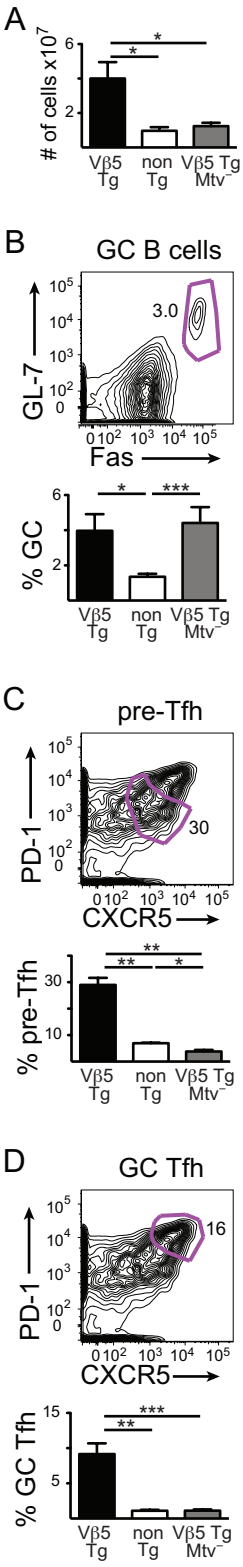


Figure 1. The size of mLNs and the proportion of pre-Tfh and GC Tfh increase in Vβ5 Tg mice in an Mtv-8 dependent manner.
A) Total number of cells in the mLN from Vβ5 Tg Mtv^+ (black), TCR nonTg Mtv^+ (white), and Vβ5 Tg Mtv^- (gray) mice ranging from 20-29 wks of age. (B – D) Representative flow cytometric plots show GC gating on mLN cells from a 22 wk old Vβ5 Tg mouse. B) B cells are gated as $CD4^-$ and $CD19^+$ or $B220^+$ and (C and D) T cells are gated as $CD4^+$ and $CD19^-$ or $B220^-$. Numbers next to gates represent the percentage of cells in that gate. mLN cells from Vβ5 Tg Mtv^+, TCR nonTg Mtv^+, and Vβ5 Tg Mtv^- mice ranging from 20-29 wks of age were analyzed for B) % GC of B cells and C) % pre-Tfh and D) GC Tfh of CD4 T cells. Bars indicate mean +/- standard error of the mean (SEM) from 3-12 mice per group in 2-6 independent experiments.

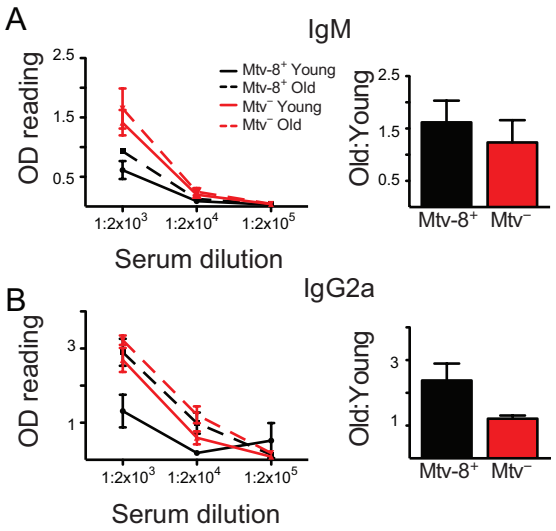


Figure 2. Mtv$^-$ mice have elevated natural Ab production but a reduced increase in Ab production with age when compared to Mtv-8$^+$ mice.
Left: titrations of A) IgM and B) IgG2a from Vβ5 Tg Mtv$^+$ (black) and Mtv$^-$ (red) mice at young and old time points. Right: ratio of A) IgM and B) IgG2a levels in old:young mice at the 1:2000 dilution. Bars indicate mean +/- SEM from 2 mice per group in 1 experiment. Experiment done in collaboration with Katie Deets.

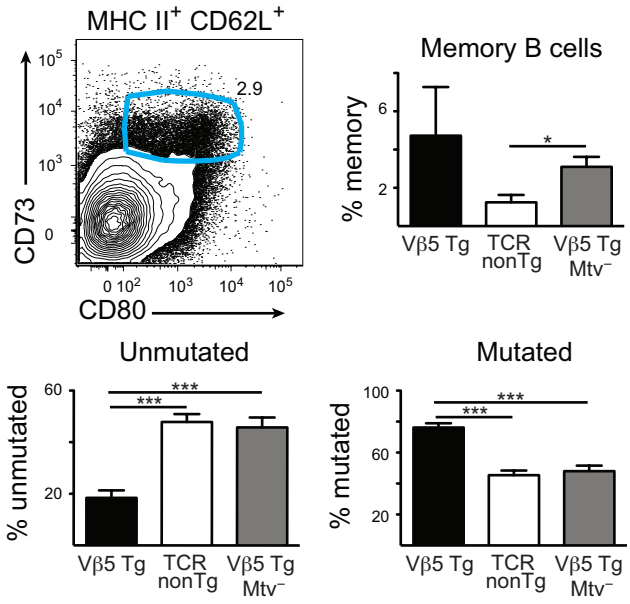


Figure 3. Mtv$^+$ Vβ5 Tg mice have elevated formation of memory B cells with mutated Ig. Upper left: representative flow cytometric plot of gating on CD73$^+$CD80$^+$ memory B cells from mLN of a 32 wk old Vβ5 Tg Mtv$^+$ mouse. Upper right: % memory (CD62L$^+$MHC class II$^+$CD73$^+$CD80$^+$), lower left: % memory with unmutated Ig (CD35$^+$), and lower right: % memory with mutated Ig (CD35$^-$) of B220$^+$ B cells in mLN from Vβ5 Tg Mtv$^+$ (black), TCR nonTg Mtv$^+$ (white), and Vβ5 Tg Mtv$^-$ (gray) mice ranging from 28-45 wks of age. Bars indicate mean +/- SEM from 4-8 mice per group in 5 independent experiments.

whereas TCR nonTg and Vβ5 Tg Mtv$^-$ mice have the reverse phenotype (Fig. 3).

Mtv$^+$ Vβ5 Tg mice have elevated high affinity Ab production during a primary Ab response, but not during a secondary response

Ab production was analyzed 7 d after primary immunization with NP-KLH (Fig. 4A). At this time point, Vβ5 Tg Mtv$^+$ mice produced lower levels of NP-specific IgM and IgG when compared to Vβ5 Tg Mtv$^-$ mice (Fig. 4B). A similar pattern was found 14 and 21 d after immunization, when Vβ5 Tg Mtv$^+$ mice produced higher levels of NP-specific IgM and lower levels of total NP-specific IgG when compared to Vβ5 Tg Mtv$^-$ mice. Interestingly, of this total NP-specific IgG, high-affinity IgG constituted a higher proportion in Vβ5 Tg Mtv$^+$ mice at both time points (Fig. 4B). However, 7 d after secondary immunization with NP-CGG (Fig. 5A), in addition to lower total NP-specific IgG, Vβ5 Tg Mtv$^+$ mice produced lower levels of high-affinity IgG (Fig 5B). Thus, the Mtv-8-dependent increase in GC Tfh in Vβ5 Tg mice is correlated with increases in Ab affinity maturation in primary but not in secondary Ab responses.

Revising T cells have a Tfh-like localization, surface phenotype, and cytokine and transcription factor expression pattern

The correlation between Mtv-8 expression and GC function indicates that revising T cells function as Tfh, in which case revising T cells and Tfh should have a similar phenotype. Revising T cells were identified as GFP$^+$Vβ5$^+$ peripheral CD4 T cells in thymectomized (Tx) Rag2p-GFP Tg Vβ5 Tg mice, and post-revision T cells were identified as GFP$^-$Vβ5$^-$TCRβ$^+$ peripheral CD4 T cells (Fig. 6A). CCR7 is a chemokine receptor expressed by cells in the T cell

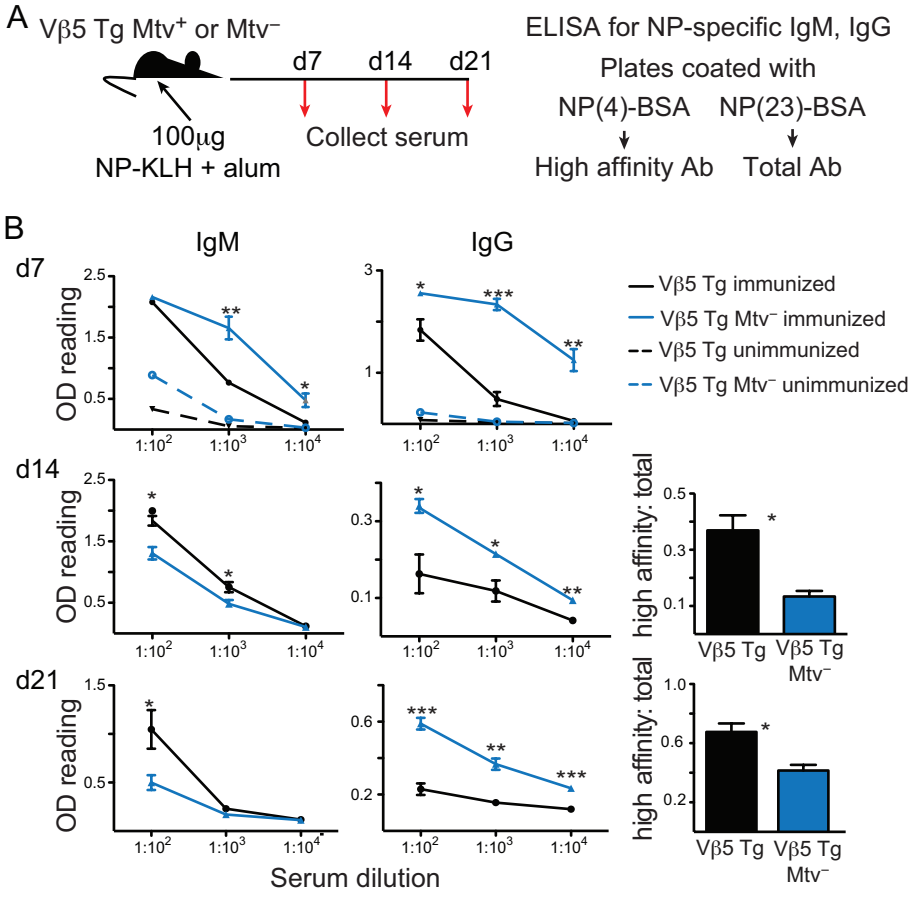


Figure 4. Mtv$^+$ Vβ5 Tg mice have elevated levels of high affinity Ab production during a primary Ab response.
A) Experimental scheme for immunization and characterization of high and low affinity NP-specific Ab. B) Left and middle: titrations of NP-specific IgM and IgG Ab and right: ratio of high affinity IgG to total IgG (1:100 dilution) in sera from Vβ5 Tg Mtv$^+$ (black) and Mtv$^-$ (blue) mice ranging from 15-17 wks of age 7-21 d after primary immunization with NP-KLH. Bars indicate mean +/- SEM from 3-4 mice per group in 1 experiment. Experiments were done in collaboration with Katie Deets.

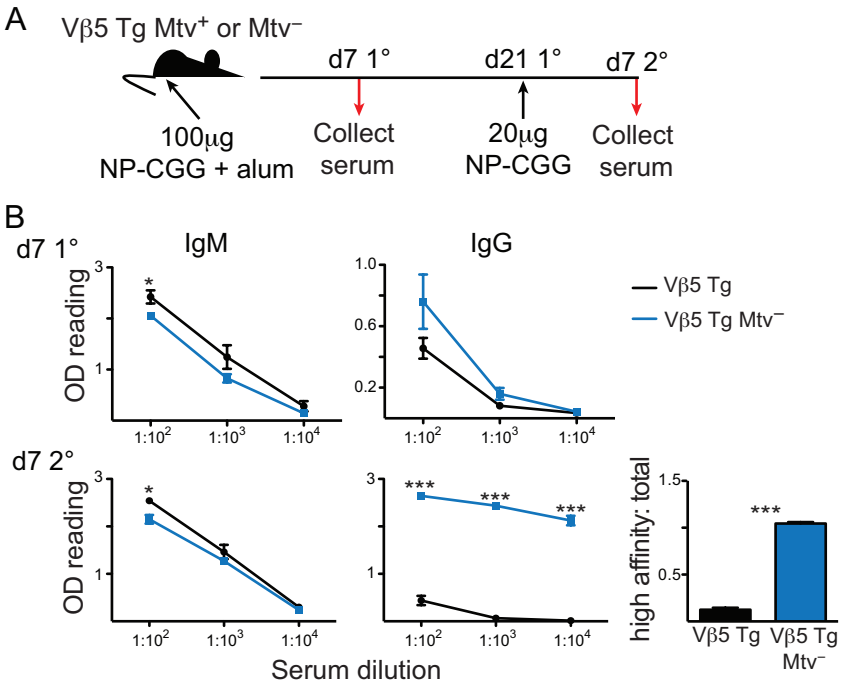


Figure 5. Mtv^{+} Vβ5 Tg mice have reduced levels of high affinity Ab production during a secondary Ab response.
A) Experimental scheme for immunization. B) Left and middle: titrations of NP-specific IgM and IgG Ab and right: ratio of high affinity IgG to total IgG (1:100 dilution) in sera from Vβ5 Tg Mtv^{+} (black) and Mtv^{-} (blue) mice ranging from 12-16 wks of age 7 d after primary (top) or secondary (bottom) immunization with NP-CGG. Bars indicate mean +/- SEM from 3-4 mice per group in 1 experiment. Experiments were done in collaboration with Katie Deets.

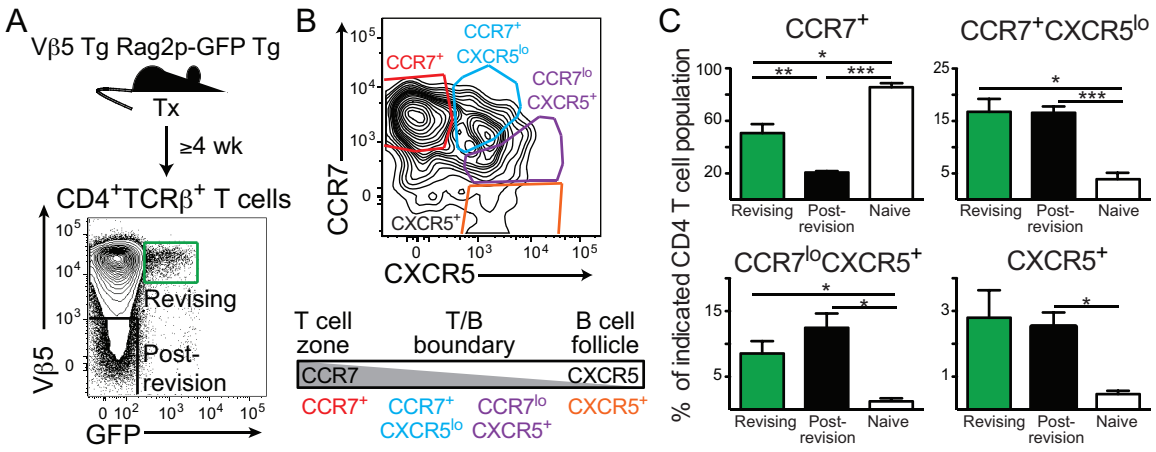


Figure 6. Revising T cells tend to localize to the T cell – B cell boundary.
A) Peripheral CD4^{+}TCRβ^{+} cells from Rag2p-GFP Tg Vβ5 Tg mice Tx 4-6 wks previously were identified as revising or post-revision T cells. B) Top: representative flow cytometric plot shows CCR7 and CXCR5 expression by revising T cells. Bottom: scheme for localizing lymphocytes within secondary lymphoid organs based on their chemokine receptor expression pattern. C) Percentage of revising (green), post-revision (black), or naive (white) CD4 T cells expressing the indicated levels of chemokine receptors. Naive CD4 T cells were defined as CD44^{lo} Vβ5^{+} CD4 T cells from 5-8 wk old Vβ5 Tg mice. Bars indicate mean +/- SEM from 4 pools of 2-3 mice per group in 3 independent experiments.

zone of secondary lymphoid organs, whereas CXCR5 is expressed by cells that migrate into the B cell follicle, the site of GCs (reviewed in 64). Intermediate levels of these receptors suggest localization at the T cell – B cell boundary (Fig. 6B). While naive CD4 T cells are primarily $CCR7^{+}$, revising and post-revision T cells express a range of CCR7 and CXCR5 levels (Fig. 6C). Hence, revising T cells likely localize to the T cell – B cell boundary of follicles.

Revising T cells display a Tfh phenotype (Fig. 7) for the markers PD-1, BTLA, IL-6 receptor (IL-6R) α, ICOS, OX40, and CD40L (61, 69). Post-revision T cells also express high levels of PD-1 and ICOS (Fig. 7). Expression of IL-6Rα on naive T cells (Fig. 7) is consistent with previous data (89). Revising T cells express IL-4 RNA at a higher level than naive T cells, but at a lower level than Th2 skewed T cells (Fig. 8A, B). Tfh differentiation requires the transcriptional repressor Bcl-6 and is inhibited by Blimp-1, its mutually antagonistic repressor (68). Revising T cells and Tfh have similar high Bcl-6 and low Blimp-1 RNA expression patterns (68), whereas post-revision T cells have lower Bcl-6 and higher Blimp-1 expression (Fig. 8B). In line with this RNA expression, revising T cells express Bcl-6 protein, but low (naive T cell) levels of T-bet (Fig. 8C), known to bind to Bcl-6 and inhibit its activity (62). Thus, revising T cells have a phenotype resembling that of Tfh.

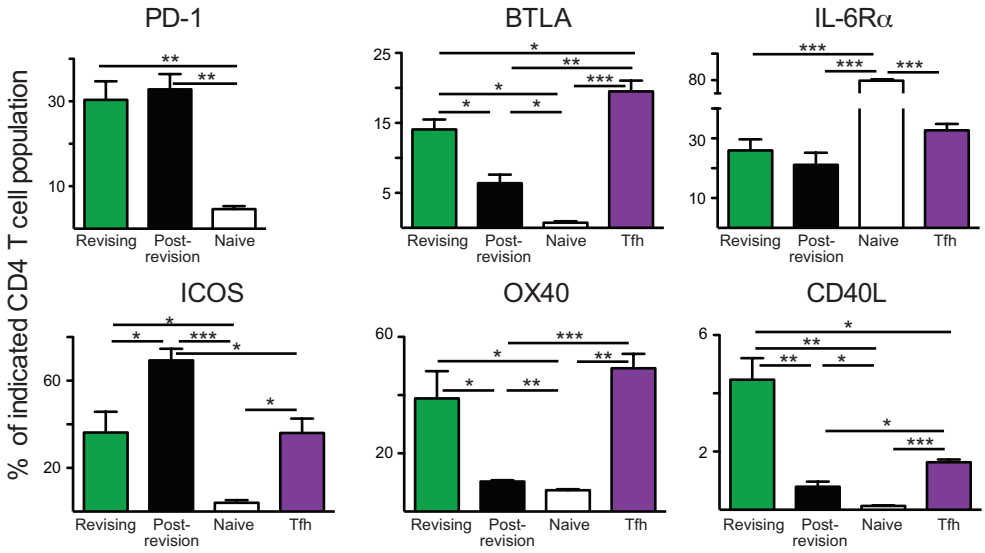


Figure 7. Revising T cells have a Tfh-like surface phenotype.
Percentage of revising (green), post-revision (black), naive (white, defined as in Fig. 6) or Tfh (purple) CD4 T cells expressing the indicated Tfh-associated markers. Tfh control was GC Tfh from mLN of Vβ5 Tg mice 7 d after NP-CGG immunization i.p. Bars indicate mean +/- SEM from 3-8 pools of 1-4 mice per group in 2-4 independent experiments.

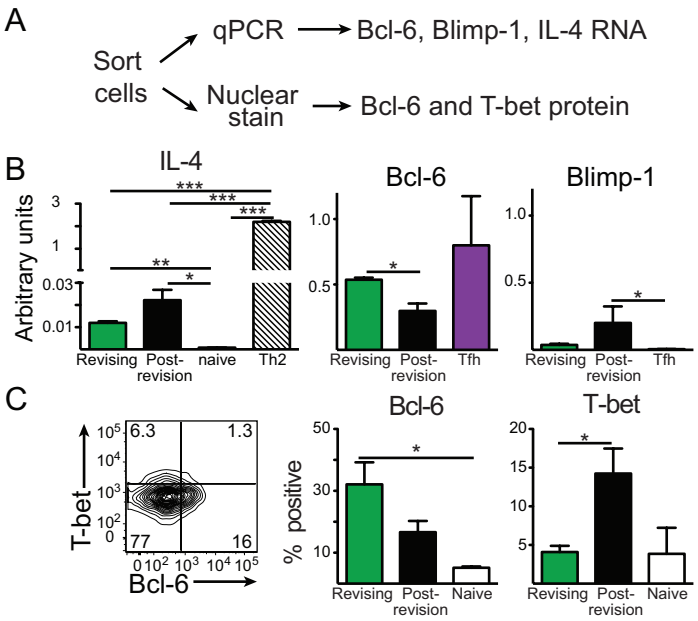


Figure 8. Revising T cells have a Tfh-like cytokine and transcription factor phenotype. A) Experimental scheme for qPCR and intranuclear staining. B) Left to right: IL-4, Bcl-6, and Blimp-1 RNA levels were measured in revising (green), post-revision (black), naive (white, defined as in Fig. 6), Th2 (striped) or Tfh (purple) CD4 T cells using qPCR. $2^{-\Delta CT}$ values were calculated for triplicate samples and normalized to HPRT. Tfh control is OT-II T cells ~7 d after NP-ovalbumin immunization in vivo. C) Left: representative flow cytometric plot of Bcl-6 and T-bet staining on revising T cells. Middle and right: graphs depict protein expression of Bcl-6 and T-bet. Naive control (white) was splenic $CD44^{lo}$ CD4 T cells from TCR nonTg mice. Bars indicate mean +/- SEM from 1-2 pools of 4-9 mice per group in 2 independent experiments.

Discussion

Revising T cells function as Tfh and adopt a Tfh phenotype. Consistent with the Tfh-like phenotype and function of revising T cells, the enhanced proportion of Tfh in Vβ5 Tg mice is Mtv-8-dependent. The Mtv-8-independence of the enriched GC B cell population was surprising. However, the memory B cell phenotype and high affinity Ab levels indicate that Mtv-8 does influence GC function. As CD4 T cells from Vβ5 Tg Mtv$^-$ mice do not undergo Mtv-8-mediated deletion, these mice have an elevated number of CD4 T cells when compared to Vβ5 Tg Mtv$^+$ mice (50). Consequently, they have more cells with the potential to become Tfh, which likely explains the elevated frequency of GC B cells in Vβ5 Tg Mtv$^-$ mice.

Consistent with this phenotype, Vβ5 Tg Mtv$^-$ mice have elevated natural Ab and Ag-specific Ab production. However, when the change in class-switched natural Ab over time or the ratio of high affinity:total Ab is measured, Vβ5 Tg Mtv$^+$ mice have the advantage, despite their lower total levels of Ab. Overall, these data indicate that GC responses occur normally in Vβ5 Tg Mtv$^-$ mice, but that the enhanced high-affinity Ab production in Vβ5 Tg mice is Mtv-8-dependent.

The combination of enhanced primary Ab response and diminished secondary response in Vβ5 Tg Mtv$^+$ mice suggests that the T cells activated in the primary response are either deleted or undergo revision and consequently no longer provide Ag-specific help for a secondary Ab response. In contrast, Vβ5 Tg Mtv$^-$ mice undergo normal secondary responses, presumably because their CD4 T cells neither delete nor revise. Our data on IL-4 and IL-6R expression by revising T cells also suggest that these cells function as Tfh. GC Tfh express IL-6R, and IL-6 produced by APCs contributes to Bcl-6 expression and the differentiation and maintenance of Tfh populations (61, 64). IL-4 production by GC Tfh promotes Ab class-switch, GC B cell

survival, and GC maintenance (61, 69). Overall, these data indicate that revising T cells are functional Tfh that promote affinity maturation and GC maintenance.

While the chemokine receptor expression pattern of revising T cells does demonstrate skewing towards CXCR5 expression, expression levels of this chemokine receptor are highly heterogeneous, indicating that there may be some variation in localization of revising T cells. In addition, the high frequency of revising T cells with intermediate expression of CXCR5 and CCR7 indicates that many revising T cells localize to the T cell – B cell boundary, the site where pre-Tfh are found.

While revising T cells have a distinctive Tfh phenotype, post-revision T cells become functional memory T cells able to undergo productive immune responses (52). Our data are consistent with the memory phenotype of post-revision T cells and show that they have a phenotype distinct from that of revising T cells. In particular, chemokine receptor expression suggests they exit the GC and traffic to other tissues, consistent with previous findings (40, 52).

The elevated PD-1 and ICOS expression on post-revision T cells was unexpected. PD-1 is expressed on activated T cells, dampens TCR signaling, and can tolerize autoreactive T cells (90). TCR revision has the potential to produce autoreactive specificities like those eliminated by negative selection in the thymus. There is a two-fold increase in the population of revising T cells in the absence of Bim (58), a protein that contributes to the apoptosis of T cells that fail negative selection; the increase in revision in the absence of Bim likely results from a lack of negative selection (91). These data, in combination with the Th17 skewing of post-revision T cells (53), indicate that revision involves TCR selection that is less stringent than thymic selection, in which case PD-1 expression could help tolerize post-revision T cells. ICOS expression protects activated T cells from apoptosis and promotes homeostatic proliferation and

T cell survival (92). Due to deletion of CD4 T cells, Vβ5 Tg mice are CD4 lymphopenic, and post-revision T cells undergo extensive homeostatic proliferation in a lymphopenic setting (52). PD-1 expression on post-revision T cells may promote tolerance, and ICOS may promote their survival and homeostatic proliferation.

Overall, the data in this chapter show that revising T cells provide T cell help to B cells, and by completing revision, they lose their ability to act as Ag-specific helpers. Revising T cells also adopt a Tfh phenotype and likely localize to the T cell – B cell boundary. These data also provide further evidence that revising and post-revision T cells have distinct phenotypes and functions.

Portions of this chapter and Figs. 1 and 3-8 were previously published (93).

Chapter 4: Germinal Centers are Required for Efficient TCR Revision

Introduction

The data in Chapter 3 demonstrate that revising T cells provide Ag-specific T cell help and adopt a Tfh phenotype. However, it is unclear from these data whether revising T cells adopt a Tfh phenotype solely to provide T cell help, or whether the GC microenvironment is required for revision to take place. We hypothesized that GCs are required for revision, and addressed this hypothesis both through experiments intended to increase the frequency of GCs and through genetic models. Our results show that enhancing GC formation with SRBC, agonistic Ab, or LPS injection does not affect revision. However, the frequency of post-revision T cells is reduced in the absence of Bcl-6, a transcription factor required for Tfh formation, and conversely is increased in the absence of its repressor, Blimp-1. Long-lived B cell – T cell interactions mediated by the adaptor protein SAP are required for the efficient completion of revision. These data show that GCs are required for efficient revision, though experimental induction of enhanced GCs does not affect revision.

Results

Increasing the frequency of activated B cells, GC cells, or B-1 B cells does not enhance TCR revision

Mtv surface expression increases along with MHC class II expression on activated B cells (46). It is therefore possible that TCR revision is selectively induced by Mtv^{high} activated B cells, and that any apparent GC requirement for TCR revision simply reflects an activated B cell requirement. To address this, expression of RNA specific for the Mtv-8 superAg was analyzed in non-activated splenic B cells and LPS-activated blasts from Mtv-8^+ and Mtv^- mice. Mtv-8^+ B cells and blasts express similar levels of Mtv-8 RNA (Fig. 9A). As a proxy for Mtv-8 surface expression, mice were injected with LPS to investigate the effect of elevated B cell MHC class II expression on the accumulation of post-revision T cells (defined as $V\beta5^-TCR\beta^+$ in Fig. 6A). Two wks after a second injection, MHC class II expression was higher on a per cell basis on B cells from LPS- than from PBS-injected mice (Fig. 9B). However, there was no difference in the accumulation of post-revision T cells between LPS- and PBS-injected mice 8 wks after a third injection (Fig. 9B), indicating that B cell activation is not sufficient to enhance TCR revision.

Based on the Tfh-like phenotype and function of revising T cells, we wanted to investigate whether increasing the frequency of GCs could also enhance revision. In addition, the frequency of post-revision T cells is elevated in PerL (Fig. 10A), a population in which the primary APCs are B-1 B cells that have high expression of MHC class II (94). Based on these observations, experiments were undertaken to increase the frequency of GCs by injection of SRBC or agonistic anti-CD40 Ab and to increase the frequency of B-1 B cells by adoptive transfer of PerL cells. Injection of SRBC (95) or the clone FGK4.5 of anti-CD40 did increase GC formation in the short term, but did not increase the accumulation of post-revision T cells in the

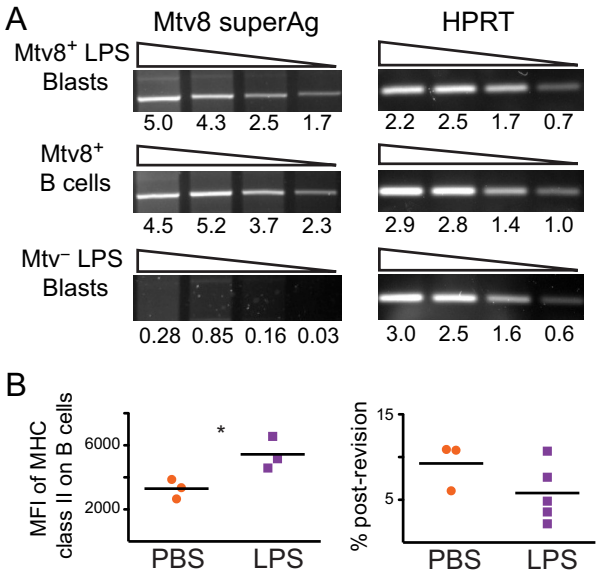
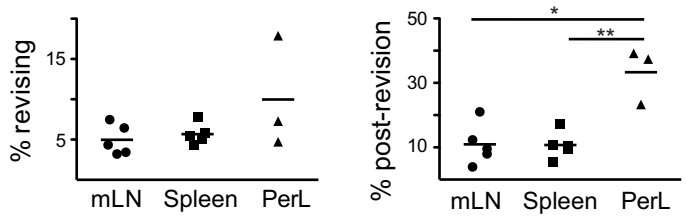


Figure 9. Increasing the frequency of activated B cells does not enhance TCR revision. A) RNA was isolated from Mtv-8$^+$ and Mtv$^-$ B cells and LPS blasts. Semi-quantitative PCR was performed on serial 3-fold dilutions of cDNA for expression of Mtv-8 (left), with HPRT used as a quantification control (right). Dilutions for Mtv-8 expression started from 150 ng, and dilutions for HPRT expression started from 10 ng of cDNA. Numbers below gel images represent quantification of band intensity. Data are representative of 3 experiments. B) Mice were injected i.p. with LPS (purple squares) or PBS (orange circles) at 3 wk intervals. Left: median fluorescence intensity (MFI) of MHC class II was measured on CD19$^+$ B cells from mLN 2 wk after the second injection. Right: the percentage post-revision of CD4 T cells in mLN was measured 8 wks after the third injection. Data are from 3-5 mice and representative of 2 independent experiments. Experiments were done in collaboration with Kelvin Sze.

A



B

	SRBC	anti-CD40 (FGK4.5)	anti-CD40 (1C10)	B-1 B cells
Short term (1-2 wks after 1 injection)				
GC B	↑	↑		
GC T	↑	—		
Post-revision	—	↓		▲
Long term (5-7 wks after 3-6 injections)				
GC B	—	—	▲	—
GC T	—	—	↓	↓
% CD44⁺ of Vβ5⁺ CD4 T cells	—	↓	↓	↓
Revising	—	—	↓	↓
Post-revision	—	↓	↓	↓

↑ Increase ▲ Slight Increase — No change ↓ Decrease

Figure 10. Enhancing GC formation or the frequency of B-1 B cells fails to increase the accumulation of post-revision T cells.
A) The percentage revising (left) or post-revision (right) of CD4 T cells in mLN (circles), spleen (squares), or PerL (triangles) from 20-29 wk old Vβ5 Tg mice. Data are from 3-5 mice and representative of 5 independent experiments. B) Table depicting the effects of inoculation with SRBC, agonistic anti-CD40 Ab (clones FGK4.5 and 1C10), or B-1 B cells on GC formation and revision, both short- and long-term. Key to table is at bottom. Table summarizes data from 3-8 mice per group, representative of 1-2 independent experiments for each condition. Experiments were done in collaboration with Kelvin Sze.

long-term (Fig. 10B). FGK4.5 is an agonistic Ab that activates NK cells as well as B cells (84). Because this could result in off-target effects, an alternate clone of anti-CD40 that specifically stimulates mature B cells, 1C10, was also used (85). Like FGK4.5, 1C10 did not increase the accumulation of post-revision T cells (Fig. 10B). Likewise, injection of B-1 B cells failed to promote long-term accumulation of post-revision T cells, though it may have induced a short-term spike in the frequency of post-revision T cells (Fig. 10B). Overall, these data indicate that the frequency of post-revision T cells cannot be enhanced by inducing elevated GC formation or MHC class II expression.

Bcl-6 is required for efficient revision, whereas Blimp-1 inhibits it

Given the antagonistic relationship between Bcl-6 and Blimp-1 and the impact of these transcription factors on Tfh formation, revision was studied in the absence of both proteins. Because Bcl-6$^{-/-}$ B6 embryos die late in gestation (79), recipients were reconstituted with a mixture of congenically marked Vβ5 Tg WT and Bcl-6$^{-/-}$ fetal liver (Fig. 11A). The kinetics of revision are impacted by high levels of irradiation (57), so chimeras were made in sublethally irradiated TCR βδ$^{-/-}$ hosts. To evaluate the effect of Bcl-6 deficiency on Tfh formation, two stages of Tfh differentiation were analyzed; PD-1^{int}CXCR5^{int} pre-Tfh and more differentiated PD-1^{+}CXCR5^{+} GC Tfh (61). In mixed fetal liver chimeras, both populations were dramatically reduced in the absence of Bcl-6 (Fig. 11B).

We analyzed activation and deletion of Bcl-6 deficient CD4 T cells by determining the CD4:CD8 T cell ratio and % Vβ5^{+}CD44^{hi} of CD4 T cells in mixed fetal liver chimeras. Vβ5^{+} CD4 T cells become activated (CD44^{hi}) with exposure to Mtv-8 (Fig. 12A), and can then be deleted or undergo revision. The percent Vβ5^{+}CD44^{hi} of CD4 T cells is reduced in Mtv^{-}

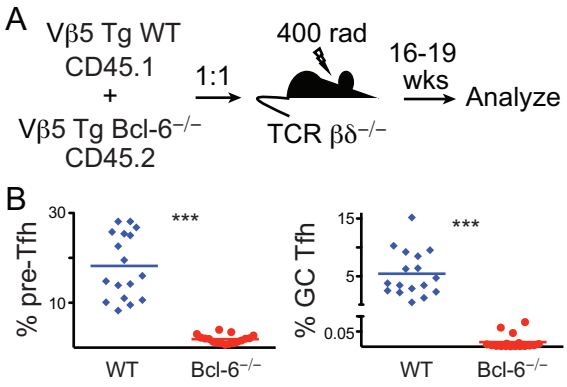


Figure 11. Bcl-6 is required for Tfh formation.
A) Vβ5 Tg WT (blue diamonds) and Bcl-$6^{-/-}$ (red circles) CD4 T cells from mLN of mixed fetal liver chimeras were analyzed 16-19 wks post reconstitution for the B) % pre-Tfh and GC Tfh. Data are from 17 mice comprising 2 independent groups of chimeras.

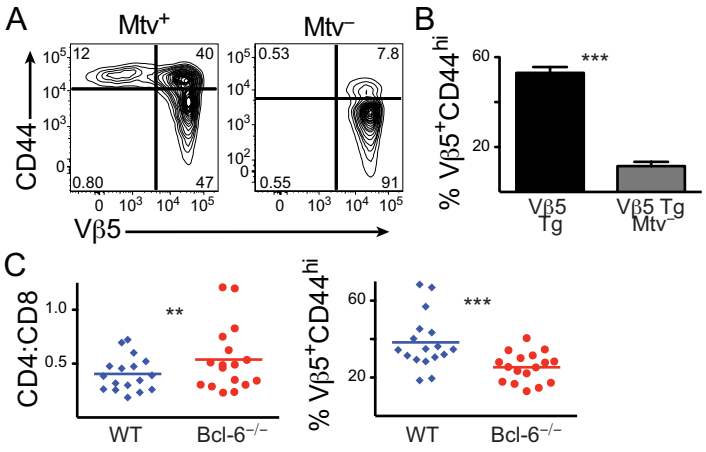


Figure 12. Mtv-8-dependent activation and deletion of CD4 T cells are decreased in the absence of Bcl-6.

A) Representative flow cytometric plots of Vβ5 and CD44 expression on mLN CD4 T cells from 25 wk old Mtv^{+} and Mtv^{-} mice. B) % Vβ5^{+}CD44^{hi} of CD4 T cells in mLN from 20-29 wk old Mtv^{+} (black) and Mtv^{-} (gray) mice. Data are from 5-6 mice per group in 8 independent experiments. C) Vβ5 Tg WT (blue diamonds) and Bcl-6$^{-/-}$ (red circles) T cells from mLN of mixed fetal liver chimeras were analyzed 16-19 wks post reconstitution for the CD4:CD8 T cell ratio and % Vβ5^{+}CD44^{hi} of CD4 T cells. Data are from 17 mice comprising 2 independent groups of chimeras.

mice (Fig. 12B). Deletion and the percent $V\beta5^{+}CD44^{hi}$ of CD4 T cells were both decreased in the absence of Bcl-6 (Fig. 12C).

To analyze revision in the absence of Bcl-6, we took advantage of the fact that $V\beta5^{lo}TCR\beta^{+}$ cells are revision intermediates that become post-revision T cells in adoptive hosts (58). Thus, we analyzed $V\beta5^{lo}TCR\beta^{+}$ revising T cells and $V\beta5^{-}TCR\beta^{+}$ post-revision T cells, which are subpopulations confined to CD4 T cells from Mtv^{+} mice (Fig. 13A). Both the revising and post-revision T cell populations were decreased in the absence of Bcl-6 (Fig. 13B), indicating that efficient initiation and completion of revision require this transcription factor.

The defect in activation of Bcl-6 deficient $V\beta5^{+}$ CD4 T cells led to the question of whether formation of $CD44^{hi}$ memory T cells is affected in the absence of Bcl-6. Memory T cells found in a LN can be divided into two broad categories, effector and central memory. Effector memory T cells can home to sites of infection and respond rapidly after TCR stimulation, whereas central memory T cells circulate through lymphoid organs and become effectors later in an immune response (96, 97). While both populations are $CD44^{hi}$, they can be distinguished through CCR7 and CD62L expression in both the CD4 (96) and CD8 (98) T cell populations. Central memory T cells express both markers, whereas effector memory do not (99). In the case of CD4 T cells, effector memory T cells derive from effector T cells, and central memory T cells derive from Tfh and memory precursors (96).

We analyzed memory CD4 and CD8 T cell populations in the Vβ5 Tg WT/Bcl-$6^{-/-}$ mixed fetal liver chimeras, and found that formation of both effector (Fig. 14A) and central (Fig. 14B) memory CD4 T cells was impaired in the absence of Bcl-6. Bcl-6 deficient CD8 T cells have a similar phenotype (Fig. 14A, B). These data were consistent with previous research on formation of memory CD4 and CD8 T cells in the absence of Bcl-6 (100, 101).

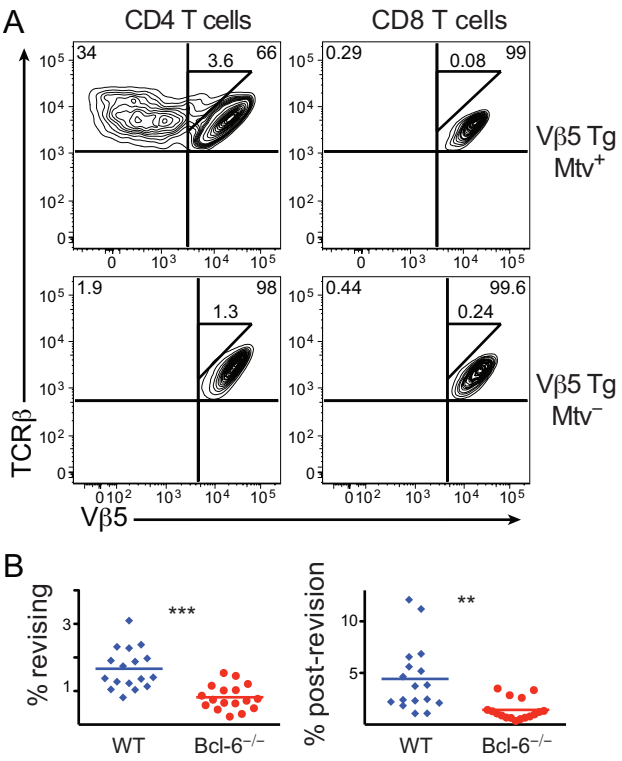


Figure 13. Bcl-6 is required for efficient revision.
A) mLN cells from 28 wk old Vβ5 Tg Mtv^{+} and Mtv^{-} mice were stained in separate experiments for CD4, CD8, Vβ5, and TCRβ. Revising T cells were identified as CD4^{+}TCRβ^{+}Vβ5^{lo} cells (triangular gate) and post-revision T cells were identified as CD4^{+}TCRβ^{+}Vβ5^{-} cells (upper left quadrant gate). Numbers in upper corners represent the % of cells in that quadrant; numbers above triangular gate represent the % of cells in that gate. B) Vβ5 Tg WT (blue diamonds) and Bcl-6$^{-/-}$ (red circles) CD4 T cells from mLN of mixed fetal liver chimeras were analyzed 16-19 wks post reconstitution for the % revising and post-revision. Data are from 17 mice comprising 2 independent groups of chimeras.

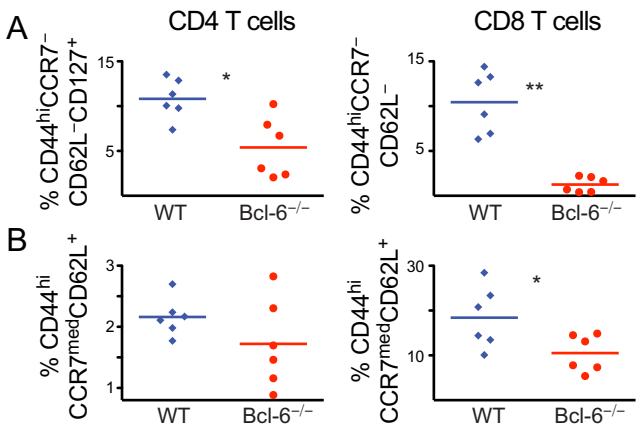


Figure 14. Memory T cell formation is impaired in Bcl-6$^{-/-}$ T cells.
T cells of Vβ5 Tg WT (blue diamonds) and Bcl-6$^{-/-}$ (red circles) origin from mixed fetal liver chimeras were analyzed for the % A) effector memory and B) central memory cells within CD4 (left) and CD8 (right) T cell compartments. Data are from 6 mice from a single group of chimeras.

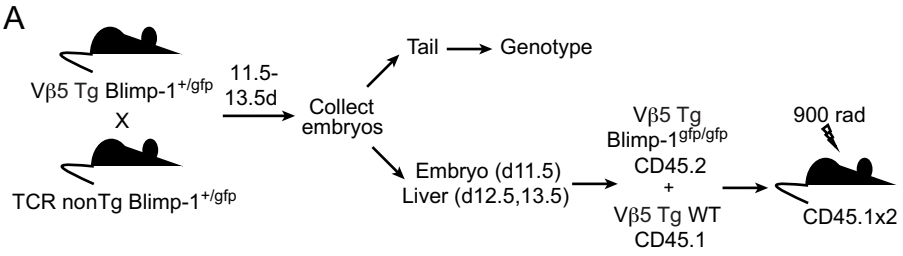
Because Blimp-1 is a mutually antagonistic repressor of Bcl-6, we also wanted to study revision in the absence of Blimp-1. We attempted to make mixed chimeras of Vβ5 Tg Blimp-$1^{gfp/gfp}$ (77) and WT fetal liver (Fig. 15A), but had no Vβ5 Tg Blimp-$1^{gfp/gfp}$ embryos survive to E12.5, having genotyped 16 embryos at that age. At E11.5, there were Vβ5 Tg Blimp-$1^{gfp/gfp}$ embryos remaining (Fig. 15B). The liver is not yet hematopoietic at E11.5 (82, 102, 103), so we injected dispersed whole embryos into irradiated recipients along with Vβ5 Tg WT bone marrow. As of 12 wks later, no Blimp-$1^{gfp/gfp}$ cells had survived (Fig. 15C). At E11.5, hematopoiesis occurs in the aorta-gonad-mesonephros and yolk sac (102), so it is probable that there were too few hematopoietic stem cells per embryo to survive reconstitution. Of note, we did successfully obtain TCR nonTg Blimp-$1^{gfp/gfp}$ embryos, though we have no explanation for the difference in survival between Vβ5 Tg and TCR nonTg embryos.

In light of these difficulties, the effect of Blimp-1 on revision was analyzed in Vβ5 Tg Blimp-$1^{gfp/+}$ mice. GC B and Tfh populations may be slightly increased (Fig. 16A), while revising T cells are not affected, and there is an increase in the frequency of post-revision T cells in Blimp-$1^{gfp/+}$ mice (Fig. 16B). Consistent with the congruence between Tfh and revising T cells, Bcl-6 and Blimp-1 regulate TCR revision antagonistically.

CXCR5 is not required for TCR revision

Because CXCR5 is required for migration of T cells into the B cell follicle, the site of GCs, we wanted to analyze the effect of eliminating CXCR5 expression on revision. Irradiated recipients were reconstituted with congenically marked Vβ5 Tg WT and $CXCR5^{-/-}$ bone marrow (Fig. 17A). The frequencies of GC B cells (analyzed in Vβ5 Tg $CXCR5^{-/-}$ mice) and GC T cells (defined as GL-$7^{+}Fas^{+}$, analyzed in chimeras) were both decreased in the absence of CXCR5

A



B

	Vβ5 Tg			TCR nonTg		
Blimp-1	+/+	+/gfp	gfp/gfp	+/+	+/gfp	gfp/gfp
Expected	12.5%	25%	12.5%	12.5%	25%	12.5%
E11.5 (37)	13.5%	10.8%	16.2%	18.9%	32.4%	8.1%
E12.5 (16)	25.0%	43.8%	0%	12.5%	12.5%	6.3%
E13.5 (39)	23.1%	33.3%	0%	15.4%	20.5%	7.7%
TOTAL (92)	55.2%			44.8%		

C

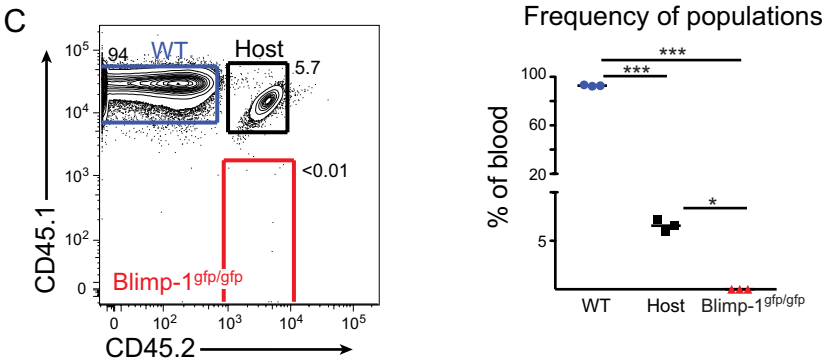


Figure 15. Vβ5 Tg B6 Blimp-1$^{gfp/gfp}$ embryos die too early for use in fetal liver chimeras. A) Experimental scheme for making mixed fetal liver chimeras. B) Expected (shaded purple) and actual (shaded green) frequencies of E11.5-E13.5 embryos for each possible genotype in a Vβ5 Tg Blimp1$^{+/gfp}$ x TCR nonTg Blimp1$^{+/gfp}$ cross. Numbers in parentheses represent the total number of embryos in that group. C) Left: representative flow cytometric plot of CD45.1 and CD45.2 expression on singlet gated blood from a CD45.1xCD45.2 recipient of E11.5 fetal liver 12 wk after irradiation and reconstitution. Numbers next to gates represent the % of cells in that gate. Right: graph depicts % CD45.1$^+$ (WT), CD45.1$^+$CD45.2$^+$ (host), and CD45.2$^+$ (Blimp-1$^{gfp/gfp}$) in blood of mixed chimeras 12 wk after injection of E11.5 embryos. Data are from 3 mice reconstituted with cells from 3 individual embryos.

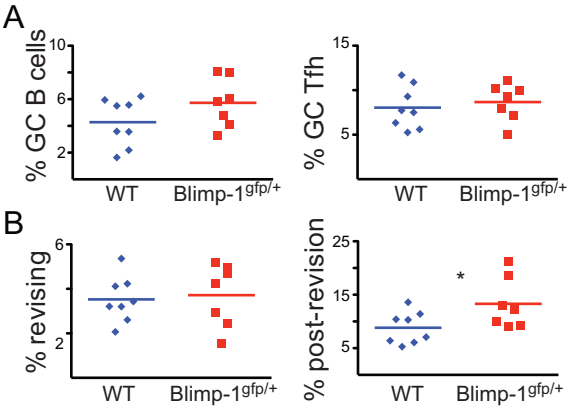


Figure 16. Blimp-1 inhibits the completion of revision.
Cells from mLN of 15-24 wk old Vβ5 Tg WT (blue diamonds) and Blimp-1$^{gfp/+}$ (red squares) mice were analyzed for A) % GC of B cells and % GC Tfh of CD4 T cells and B) % revising and post-revision of CD4 T cells. Data are from 7-8 mice per group in 5 independent experiments.

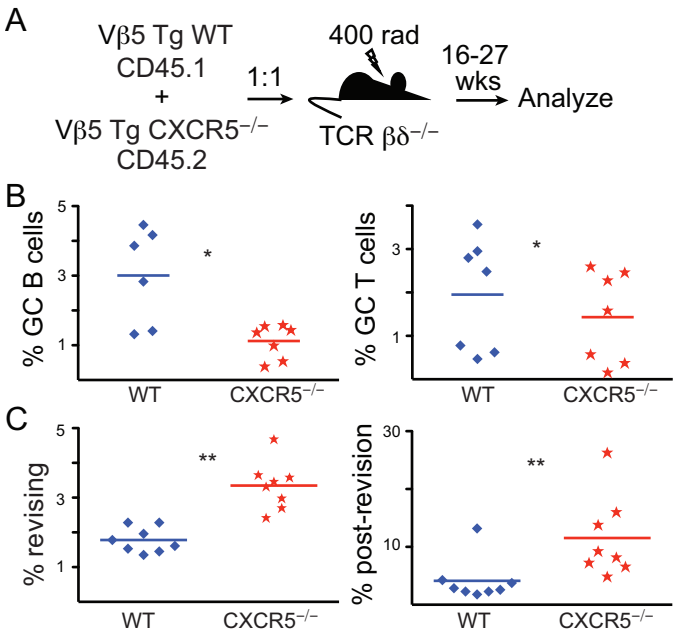


Figure 17. CXCR5 is not required for TCR revision.
A) Chimeras were made of Vβ5 Tg WT (blue diamonds) and CXCR5$^{-/-}$ (red stars) bone marrow in sublethally irradiated TCR βδ$^{-/-}$ hosts. mLN T cells from chimeras were analyzed 16-27 wks post reconstitution. B) Percentage GC (GL-7^{+}Fas^{+}) of CD19^{+} B cells from 11-31 wk old WT and CXCR5$^{-/-}$ mice and of CD4 T cells from chimeras. C) CD4 T cells in mLN of chimeras were analyzed for % revising and post-revision. Data are from 6-8 mice, representative of 2 independent groups of chimeras and 3-7 independent experiments.

(Fig. 17B). However, the frequencies of revising and post-revision T cells were increased in the absence of CXCR5 (Fig. 17C). While migration to the B cell follicle is disrupted in CXCR5$^{-/-}$ mice, extrafollicular GCs form in the T cell zone (104), allowing GC dependent processes to take place in the absence of CXCR5. Consequently, these results are unlikely to be useful for advancing our understanding of TCR revision.

SAP is required for efficient completion, but not initiation, of revision

SAP is an intracellular adaptor protein that interacts with SLAM receptors to stabilize interactions between B and T cells in the GC (71). Mice deficient in SAP have reduced Tfh formation and a severely diminished GC Tfh compartment (72). Analysis of Vβ5 Tg WT and SAP$^{-/-}$ mice confirms the reduction in both Tfh populations (Fig. 18A). Deletion may be slightly decreased in the absence of SAP, and there is a marked decrease in the frequency of Vβ5^{+}CD44^{hi} CD4 T cells (Fig. 18B). The post-revision T cell population is diminished in the absence of SAP, whereas there is no effect on revising T cells (Fig. 18C), indicating that the completion of revision requires long-lived interactions between B cells and T cells, but initiation does not.

The decreased Vβ5^{+}CD44^{hi} CD4 T cell population led to the question of whether memory T cells are affected in the absence of SAP. The frequency of effector memory CD4 T cells may be decreased in the absence of SAP; however, this phenotype does not extend to the central memory population (Fig. 19).

To further understand the activation phenotype, irradiated recipients were reconstituted with congenically marked Vβ5 Tg WT and SAP$^{-/-}$ bone marrow (Fig. 20A). Deletion was decreased and the proportion of Vβ5^{+}CD44^{hi} CD4 T cells was increased in SAP$^{-/-}$ CD4 T cells in the presence of WT CD4 T cells (Fig. 20B). In combination with the deletion and CD44

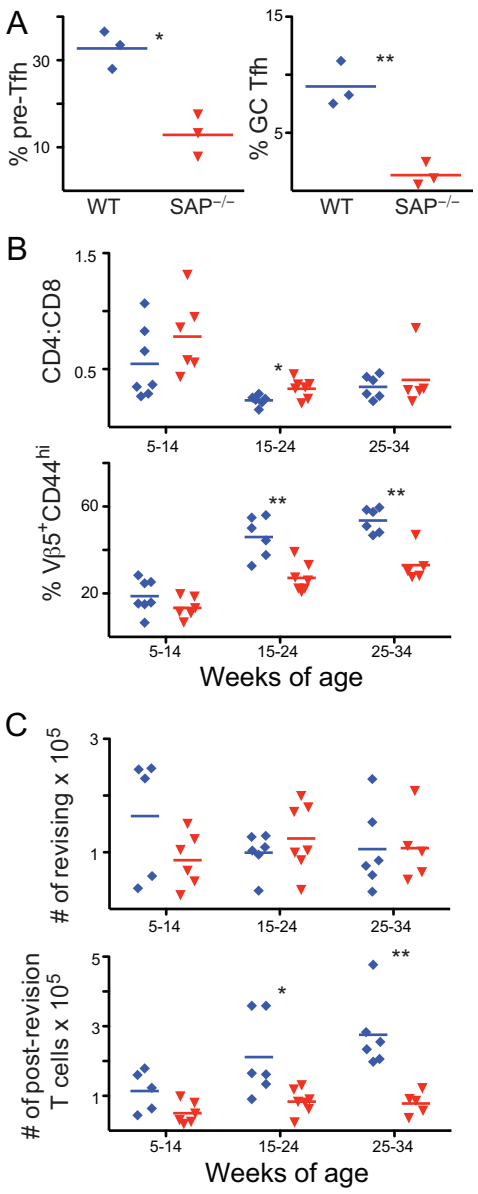


Figure 18. SAP is required for efficient completion of revision.
A) CD4 T cells from mLN of 20-29 wk old Vβ5 Tg WT (blue diamonds) and SAP$^{-/-}$ (red triangles) mice were analyzed for % pre-Tfh and GC Tfh. mLN T cells from 5-34 week old Vβ5 Tg WT and SAP$^{-/-}$ mice were analyzed for the B) CD4:CD8 T cell ratio and % Vβ5^{+}CD44^{hi} of CD4 T cells and C) total number of revising and post-revision CD4 T cells. Data are from 3-7 mice per group in 5-9 independent experiments.

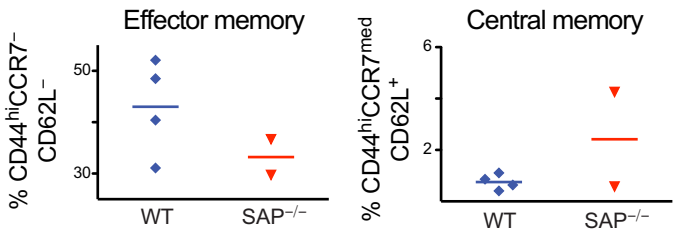


Figure 19. Effector but not central memory CD4 T cell formation is impaired in SAP$^{-/-}$ mice.
CD4 T cells from mLN of 17-26 wk old Vβ5 Tg WT (blue diamonds) and SAP$^{-/-}$ (red triangles) mice were analyzed for % effector (left) and central (right) memory. Data are from 2-4 mice in 1 experiment.

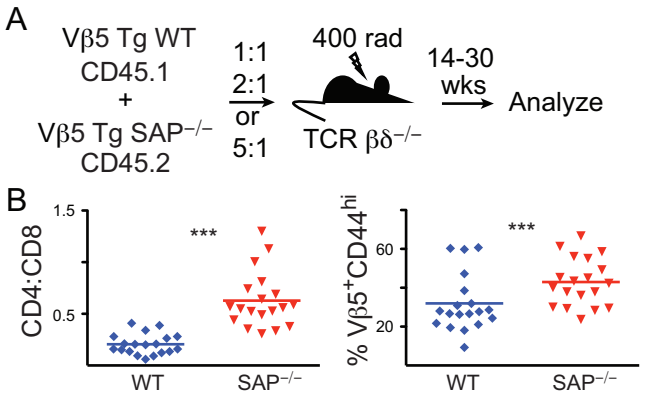


Figure 20. SAP is not required for the activation of Vβ5^{+} CD4 T cells.
A) Vβ5 Tg WT (blue diamonds) and SAP$^{-/-}$ (red triangles) T cells were analyzed from the mLN of mixed bone marrow chimeras 14-30 wks post reconstitution. B) Ratio of CD4:CD8 T cells and % Vβ5^{+}CD44^{hi} of CD4 T cells were analyzed in mLN of mixed bone marrow chimeras. Data are from 4 independent groups of chimeras totaling 19 mice in 7 independent experiments.

phenotypes of SAP null mice, these data indicate that there is a slight defect in deletion of Vβ5 Tg CD4 T cells in the absence of SAP, which is amplified in a competitive environment. In addition, SAP deficient T cells activate normally, but T cell extrinsic SAP is required for activation. These activation data are consistent with the fact that revising T cells are not affected by the absence of SAP.

One possible concern raised by the revising T cell phenotype is that if there is a defect in positive selection in $SAP^{-/-}$ mice, then dual-TCR expressing T cells could develop in the thymus, erroneously accounting for the appearance of a normal revising T cell population. To address this concern, we analyzed Vβ5 Tg Rag2p-GFP Tg WT and $SAP^{-/-}$ mice as well as TCR nonTg Rag2p-GFP Tg mice for $V\beta8^+$ TCR expression in GFP^- and GFP^+ CD4 SP thymocytes (Fig. 21A). Vβ8 expression was found on both populations from TCR nonTg mice, but only in the GFP^- (recirculating CD4 T cell) population, in Vβ5 Tg WT and $SAP^{-/-}$ mice (Fig. 21B). The absence of $V\beta8^+$ CD4 T cells in the GFP^+ CD4 SP thymocyte population indicates that positive selection occurs normally in $SAP^{-/-}$ mice.

The existence of post-revision T cells in $SAP^{-/-}$ mice raises the question of whether cells that revise in the absence of SAP have the same phenotype as their WT counterparts. TCRβ expression may be higher on a per cell basis in both revising and post-revision T cells from Vβ5 Tg $SAP^{-/-}$ compared to WT mice (Fig. 22A, B). However, this phenotype was not statistically significant, and its biological significance is unclear. $SAP^{-/-}$ post-revision T cells express reduced CCR7 and elevated CXCR5 levels (Fig. 23A), indicating that they are likely to remain in the B cell follicle. However, the chemokine receptor phenotype (Fig. 23A), PD-1, ICOS, and OX40 expression (Fig. 23B), and Bcl-6 and Blimp-1 RNA levels (Fig. 23C) of $SAP^{-/-}$ revising T cells mirror those of their WT counterparts. $SAP^{-/-}$ revising T cells do have slightly reduced

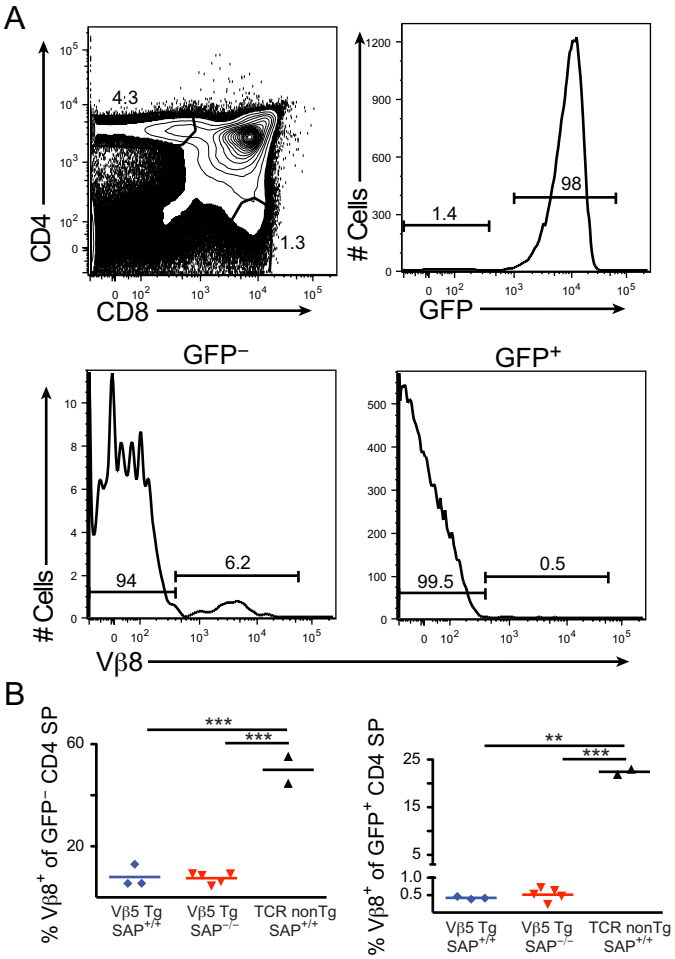


Figure 21. Positive selection is not impaired in SAP$^{-/-}$ mice.
A) Representative gating of (top left) CD4 and CD8 SP thymocytes, (top right) GFP expression by CD4 SP thymocytes and (bottom) Vβ8 expression by GFP^{-} and GFP^{+} CD4 SP thymocytes from a 12 wk old Vβ5 Tg SAP$^{-/-}$ mouse. Numbers next to gates represent the % of cells in that gate. B) % Vβ8^{+} of GFP^{-} (left) and GFP^{+} (right) CD4 SP thymocytes in Vβ5 Tg WT (blue diamonds), Vβ5 Tg SAP$^{-/-}$ (red triangles), and TCR nonTg WT (black triangles) mice. Data are from 2-5 11-24 wk old mice in 4 independent experiments.

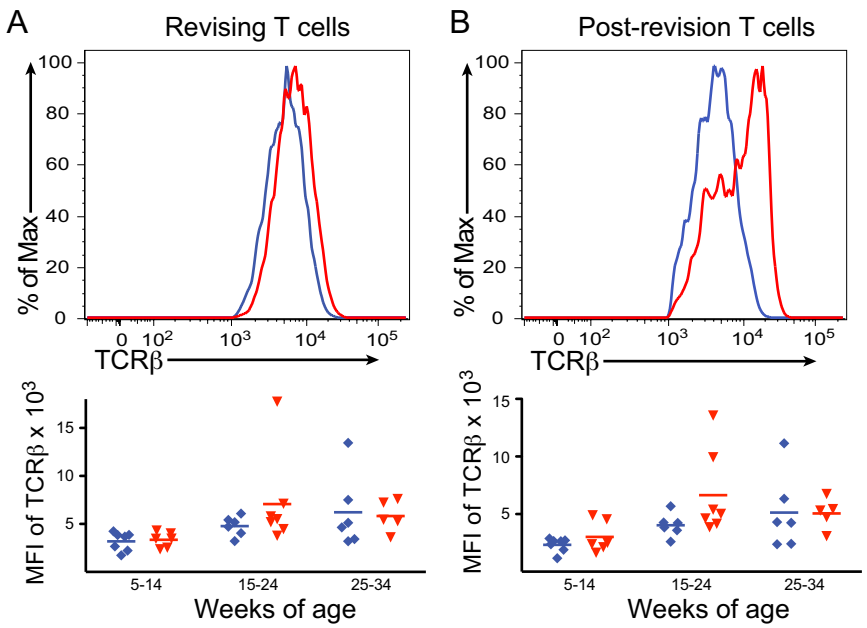


Figure 22. TCRβ expression may be elevated on SAP$^{-/-}$ revising and post-revision T cells. Top: representative histograms and bottom: graphs depicting MFI of TCRβ on A) revising and B) post-revision T cells from mLN of Vβ5 Tg WT (blue diamonds) and SAP$^{-/-}$ (red triangles) mice 5-34 wks of age. Data are from 5-7 mice per group in 5-9 independent experiments.

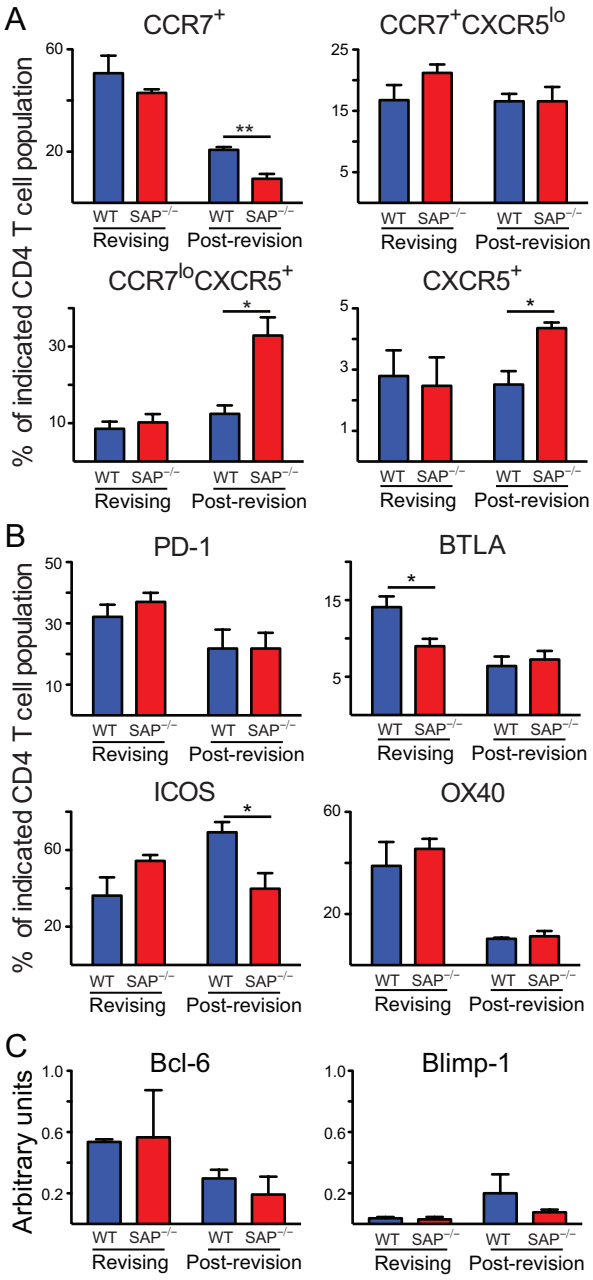


Figure 23. Revising T cells maintain a Tfh-like phenotype in the absence of SAP.
Vβ5 Tg WT (blue) and SAP$^{-/-}$ (red) revising and post-revision T cells were isolated as described in Fig. 6 and analyzed for A) % expressing the indicated chemokine receptors, B) % expressing PD-1, BTLA, ICOS, and OX40, and C) levels of RNA specific for Bcl-6 and Blimp-1. Bars indicate mean +/- SEM from (A and B) 3-7 mice per group in 2-3 independent experiments or C) 2-3 pools of 5-8 mice per group in 2 independent experiments.

BTLA expression (Fig. 23B), but this is the only exception to that trend. These data indicate that revising T cells acquire a pre-Tfh phenotype and localize normally in the absence of SAP, but that homing of cells after revision is likely affected.

Discussion

The data in this chapter indicate that GCs are required for efficient revision, suggesting that the GC provides a microenvironment allowing for control of *Rag* expression and selection for the newly generated and expressed TCR. Such a regulatory microenvironment could explain how revision can occur safely.

An alternate explanation for the GC requirement is that TCR revision is induced selectively by activated B cells, given that expression of Mtvs is increased upon LPS stimulation, along with MHC class II expression (46). However, our results indicate that activating B cells does not enhance TCR revision, demonstrating that the GC localization we observe is not simply the result of a requirement for activated B cells. The data on injection of B-1 B cells, which have elevated MHC class II expression, corroborate this point in that increasing the frequency of B cells with high MHC class II expression does not increase the frequency of post-revision T cells. Our findings that LPS stimulation does not influence Mtv-8 superAg RNA expression are surprising in light of evidence that LPS stimulation enhances expression of Mtv-8 envelope RNA (105). However, superAg and envelope are encoded by different portions of the Mtv-8 locus and are differentially regulated (47, 106).

Overall our efforts at increasing GC formation or MHC class II expression show no correlated enhancement of revision. This was surprising in light of previous results showing that revision occurs in the GC and requires Ag presentation by a B cell. However, GC induction by SRBC or anti-CD40 is acute, whereas TCR revision occurs over the long term. It is possible that even with multiple injections to induce GC formation, GCs were transient enough not to have an effect on revision. The Blimp-1^{gfp} data corroborate this possibility; in these mice, the GC enhancement is chronic, and here we see the expected increase in revision.

When taken together, the GC and Mtv-8 requirements for TCR revision could suggest that revision is induced only by B cell-expressed Ag. However, while superAg-induced revision models predominate (28, 31), revision can be driven by conventional self Ag (34) and immunization with peptide from self Ag (35) as well. The demonstration that TCR revision can be induced by peptide Ag indicates that this process is not limited to Ag expressed by B cells or to superAg presented outside the peptide-binding groove of MHC class II (45).

The requirement of Bcl-6 for both initiation and completion of revision and SAP only for completion is consistent with previous research on the differentiation of Tfh. Bcl-6 expression is upregulated during the initial stages of Tfh differentiation (70), whereas SAP is only required for GC Tfh formation (72). Analysis of revision in $SAP^{-/-}$ mice is complicated by the fact that Mtv-8-dependent deletion of CD4 T cells (40) is reduced in the absence of SAP (Fig. 20B), therefore it is difficult to directly compare the proportion of WT and $SAP^{-/-}$ post-revision T cells in the same mouse. Additionally, CD44 expression by $V\beta5^{+}$ CD4 T cells appears to be decreased in $SAP^{-/-}$ mice but increased in $SAP^{-/-}$ T cells in chimeras. This contrast indicates that in a $SAP^{-/-}$ mouse, activation of CD4 T cells is reduced in a T cell-extrinsic manner. These results indicate that Mtv-8-induced T cell activation occurs normally in the absence of SAP, confirming the lack of defect in revising T cells in $SAP^{-/-}$ mice. The largely normal Tfh phenotype of SAP null revising T cells further corroborates this point. Overall, our data suggest that $Bcl\text{-}6^{+}$ pre-Tfh initiate revision, and that the transition from revising to post-revision T cell requires sustained SAP-mediated interactions in the GC.

It is surprising that Bcl-6 impacts both initiation and completion of revision, but Blimp-1 appears to only affect completion. However, given that analysis of the effect of Blimp-1 on

revision was completed in heterozygotes, it is possible that the heterozygote phenotype is simply too subtle to clarify whether or not Blimp-1 influences the initiation of revision.

Another surprising result was the apparent enhancement of revision in the absence of CXCR5, despite decreased GC populations. As extrafollicular GCs are known to form in the absence of CXCR5 (104), these results indicate that revision can occur in extrafollicular GCs, and suggest that eliminating migration into the B cell follicle may increase the efficiency of revision.

The TCRβ expression pattern of revising and post-revision T cells in the absence of SAP is intriguing. Analysis of Ag-induced TCR downregulation in AND TCR Tg WT and $SAP^{-/-}$ CD4 T cells indicates that there is no defect in TCR downregulation in the absence of SAP (107). However, SAP associates with the cytoplasmic tail of CD3, and there is reduced TCR signaling in the absence of SAP (108). The most likely explanation for the TCRβ phenotype in Vβ5 Tg $SAP^{-/-}$ mice is that SAP deficient T cells compensate for decreased TCR signaling with increased TCR expression. The AND TCR Tg results indicate that the signaling defect is likely independent of TCR downregulation, which could explain why the phenotype is so subtle.

Revision has previously been understood to occur in two steps: first, TCR downregulation and *Rag* expression, and second, expression of the new TCRβ leading to formation of a post-revision T cell (109). Our data on the chemokine expression of SAP null post-revision T cells suggest that there is actually a third step to this process: SAP-dependent exit from the GC. GC defects in the absence of SAP are T cell intrinsic (110), and SAP contributes to signaling within T cells (111), raising the possibility that this homing phenotype may result from changes in T cell signaling in the absence of SAP.

Overall these data indicate that GCs are required for revision to complete efficiently, although experimental induction of GC formation does not enhance revision. Additionally, the initiation and completion of the revision process require distinct types of cell interactions.

Portions of this chapter and Figs. 9, 11-13, 16, 18, 20, and 23 were previously published (93).

Chapter Five: Concluding Remarks

The data in this thesis demonstrate that revising T cells congregate at the T cell – B cell boundary of B cell follicles, adopt a Tfh-like phenotype, and can function as Tfh. Efficient revision requires Bcl-6 and SAP, but is inhibited by Blimp-1. These data localize TCR revision to the GC, a microenvironment in which this tolerance process can be regulated, preventing DNA damage and promoting the generation of cells expressing TCRs that are both useful and safe.

TCR revision is not the only example of Ag receptor modification after initial receptor gene rearrangement. Both B cells (25) and T cells (26) undergo RAG-mediated secondary receptor editing in generative compartments. The BCR evolves further through somatic hypermutation, which is precisely regulated in the GC through AID expression (27). These are examples of Ag receptor alteration that can reduce autoreactivity or fine-tune a response. The potential dangers of Ag receptor manipulation can be diminished by limiting the expression of RAG, TdT, and AID to a controlled microenvironment. Therefore, revision is likely regulated by similar means.

Understanding the regulation of revision is important for establishing whether or not this process is useful and physiologically relevant. Most models of revision focus on mice carrying TCR transgenes or knock-in loci, leading to the possibility that the process is aberrant and only appears in highly unusual circumstances. However, the data in this thesis indicate that this is not likely to be the case. The Tfh phenotype and function of revising T cells place these cells into the context of known T cell biology, indicating that they are not an aberrant population. Also, the GC localization and Bcl-6 and SAP requirements of TCR revision suggest revision is induced by

GC-specific B cell – T cell interactions, providing a means for regulating *Rag* expression and imposing selection for the newly expressed TCR. These data do not address the frequency of revision in TCR nonTg mice or healthy humans, but by addressing the regulation of the process, they do indicate a means by which revision could occur in those contexts.

To investigate revision in the context of TCR nonTg mice, we will analyze the rate of revision using reporters of recombination. Constructs provided by the Cook lab include genes encoding fluorescent proteins flanked by RSS sequences such that expression of a functional RAG complex results in rearrangement of the constructs and a transition from DsRed to GFP expression by the cell (112). By transducing CD4 T cells with these constructs, we hope to measure the rate of revision, first in Vβ5 Tg and then in TCR nonTg T cells. We have cloned the constructs into a lentiviral vector (113) to promote stable expression in T cells. While control experiments optimizing the transduction protocol (114) and analysis are ongoing, we have determined that activation of Vβ5 Tg T cells to increase transduction frequency does not impair their survival in adoptive hosts or their tendency to undergo TCR revision. We have thus laid the groundwork for the analysis of revision in a more physiologically relevant system.

TCR revision has been depicted as a two-step process. First, interaction of TCRβ with Mtv-8 causes downregulation of the TCR and upregulation of *Rag* expression. Then, transcription and translation of the new endogenous TCRβ leads to surface expression of the TCR and transition into a post-revision T cell (109). This is consistent with in vitro data (115) and studies on TCR editing (26) showing that surface TCR downregulation promotes *Rag* expression. The data in this thesis indicate that the first step occurs at the T cell – B cell boundary of the B cell follicle and is Bcl-6-dependent but SAP-independent (Fig. 24A). The second step, occurring in the B cell follicle, is dependent on both Bcl-6 and SAP, and inhibited

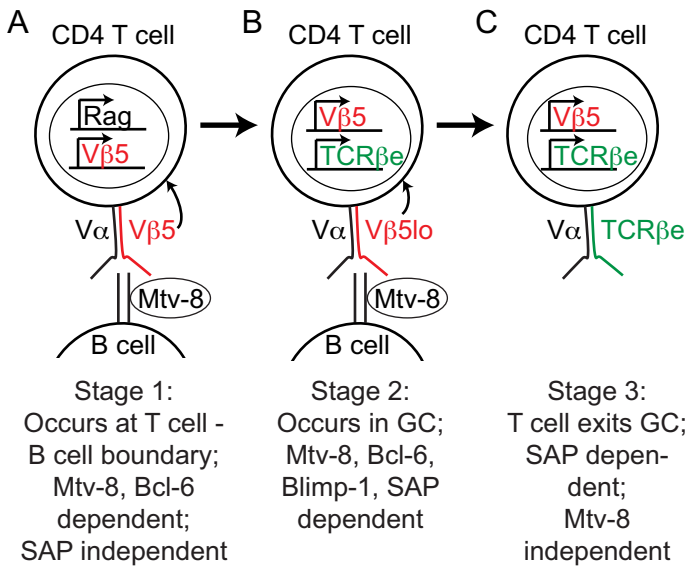


Figure 24. Model of the multistep pathway of TCR revision.
Revision occurs in three distinctly localized steps, A) initiation at the T cell – B cell boundary, B) completion in the GC, and C) exit from the GC.

by Blimp-1 (Fig. 24B). Exit from the B cell follicle is a separate SAP-dependent step usually completed by post-revision T cells (Fig. 24C). Overall, these results indicate that revision is a three-step process affecting cells with a Tfh-like phenotype, with each stage occurring in a distinct microenvironment. This illuminates the GC as the site of Ag receptor modification for T cells as well as B cells. Overall, results from our lab and others demonstrate that receptor editing and revision processes have evolved to occur in confined microenvironments to preserve the potential benefits while minimizing the risks associated with Ag receptor modification.

Portions of this chapter and Fig. 24 were previously published (93).

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