During a T-dependent immune response, activated B cells enter structures called germinal centers (GC) in the follicles of secondary lymphoid organs. GC B cells proliferate and undergo diversification of their Ig through somatic hypermutation and class-switch recombination. These Ig diversifications require the activity of the enzyme activation-induced deaminase (AID). Clonal selection within GCs selects GC B cells with the highest affinities for antigen to mature into plasma cells and memory B cells. GCs are underpinned by stromal cells called follicular dendritic cells (FDC). FDC functions include secretion of B cell chemokines and the retention of Ag complexes that allow GC B cells to test the affinity of their Ig. FDCs require constitutive signaling through lymphotoxin beta receptor (LTβR) from lymphotoxin alpha-beta (LTαβ) on the surface of B cells to maintain their functions. In these studies, I investigated the properties of GCs using two primary experimental models. First, I employed genetic and pharmacological ablation of LTβR signaling to investigate the expression of AID and the function of GCs in the absence of FDCs. I determined that FDCs are not required for GC formation or the expression of AID in lymph nodes, but that FDCs are crucial for affinity maturation. Second, I examined the competition between AID−/− and WT B cells in the GCs of mixed BM chimeras to investigate the role of affinity maturation during clonal selection. I found that AID increases GC B cell apoptosis, likely by inducing DNA damage, and that this is important in regulating GC size. I also found that AID−/− B cells accumulate at the centrocyte stage of the GC reaction and that this is due to a partial block in plasma cell maturation.
The production of this thesis and the information therein through a long period of hard work is hereby completed in large part by the grace of, for, and with the aid of, though not in a capacity that is conceptual or technical but rather that is peripheral yet comprehensive and pervasive,

Libby
Acknowledgments

I could not bring myself
To catalog the names of those
To whom I owe my gratitude
As if to reduce my friends and family
Into such a bare thing as a list
Nor could I bring myself to do such a thing
As summarize their gifts to me
In a spare smattering of prose
And what a mistake it would be to leave one out
Or to overlook some dear gesture
And to have that mistake recorded here
For as long as these pages can be turned

Much rather, I would do this

I would thank each and everyone
Every time that we should meet
For all that you have done
And I would show to you how
You have made me a richer man
And I would do this, I hope,
With a smile and an open hand
And in everything I do
With all things being brighter
Thanks to you
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<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AID</td>
<td>Activation-induced Deaminase</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor belonging to the tumor necrosis factor family</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s Adjuvant</td>
</tr>
<tr>
<td>CSR</td>
<td>Class-switch recombination</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Break</td>
</tr>
<tr>
<td>DZ</td>
<td>Dark Zone</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular Dendritic Cell</td>
</tr>
<tr>
<td>FRC</td>
<td>Fibroblastic Reticular Cell</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal Center</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HEL</td>
<td>Hen egg lysozyme</td>
</tr>
<tr>
<td>IC</td>
<td>Immune complex</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgSF</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td>iLN</td>
<td>Inguinal lymph node</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneum</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
</tr>
<tr>
<td>LT</td>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>LTα</td>
<td>Lymphotoxin α</td>
</tr>
<tr>
<td>LTβ</td>
<td>Lymphotoxin β</td>
</tr>
<tr>
<td>LTβR</td>
<td>Lymphotoxin β Receptor</td>
</tr>
<tr>
<td>LZ</td>
<td>Light Zone</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>NP-CGG</td>
<td>4-Hydroxy-3-nitrophenylacetyl – Chicken gamma globulin</td>
</tr>
<tr>
<td>OVA</td>
<td>Chicken ovalbumin</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal Antibody</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>R-PE</td>
<td>R-phycoerythrin</td>
</tr>
<tr>
<td>RSS</td>
<td>Recombination signal</td>
</tr>
<tr>
<td>SCS</td>
<td>Subcapcular sinus</td>
</tr>
<tr>
<td>SCS Mφ</td>
<td>Subcapsular sinus</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
Chapter 1: Introduction

1.1 Adaptive Immunity

Immunity is the ability of an organism to resist pathogenic infection, colonization, or parasitism by another organism. The ability to detect molecules from foreign organisms is central to immunity. Innate immunity relies upon highly conserved receptors that have evolved to recognize molecules common to entire taxonomic groups of pathogens known as pathogen-associated molecular patterns (PAMPs). For this reason, innate receptors are known as pattern recognition receptors (PRRs). PAMPs that are recognized by PRRs include highly conserved molecules such as bacterial lipopolysaccharide and viral double-strand RNA. The ligand specificities of PRRs are fixed within an individual organism and change over evolutionary timescales. Adaptive immunity, on the other hand, is characterized by the ability to generate new receptors within short periods of time and within an individual organism. These new receptors can not only bind to conserved molecules like those targeted by PRRs (i.e. peptidoglycan, flagellin) but also to variable molecules unique to single species of pathogens. In most vertebrates, adaptive immune receptors are members of the immunoglobulin superfamily (IgSF) of proteins. New adaptive immune receptors are generated in direct response to stimuli and are subject to selection, hence the term ‘adaptive’. While innate immunity relies upon the inheritance of finite sets of immutable PRR genes, adaptive immunity generates receptors with new specificities through the recombination of gene segments and the deliberate mutation of recombined genes. These recombinations and mutations occur somatically and are non-heritable, allowing each individual within a species to generate unique adaptive immune responses based on encounters with pathogens.

1.1.1 The Origins of Adaptive Immunity

Innate immunity is common to nearly all metazoans and conserved sets of PRRs have been observed in a diverse array of organisms from numerous phyla\(^1\). An individual PRR gene encodes for certain ligand specificities. Although PRRs recognize PAMPs that are conserved among numerous pathogens, selective pressures on pathogens will inevitably lead to diversification or masking of PAMPs in order to evade immunity. In response, there is selective pressure on organisms to evolve a diverse array of receptors. In the case of the sea urchin genome, this has led to the generation of hundreds of homologous PRRs\(^2\). By creating a duplicate of a receptor, selective pressures are relaxed on one of the copies, allowing for the
accumulation of mutations that alter ligand specificity. In the case of the adaptive immune system of gnathostomes, it is believed that a series of genomic duplications in common gnathostome ancestors created homologous copies of IgSF genes\textsuperscript{3-7}. This could have resulted in the generation of distinct groups of Ig receptors (Ig, TCR, and MHC) as well as in the generation of tandem gene repeats.

Although receptor diversification has been observed in certain invertebrates, these invertebrate systems are far simpler and less comprehensive than the adaptive immunity observed in vertebrates\textsuperscript{8}. Interestingly, while adaptive immunity appears to be a characteristic of all vertebrates, there was an early divergence between the jawed vertebrates (gnathostomes), using the IgSF proteins, and jawless vertebrates (agnathans/cyclostomes), using VLR proteins, that resulted in the emergence of two analogous adaptive immune receptors\textsuperscript{9}.

Adaptive immunity in gnathostomes is based upon IgSF proteins\textsuperscript{10}. The ability to recombine and rewrite segments of IgSF genes into new and diverse receptors is an important feature of adaptive immunity. Adaptive immunity is therefore characterized by arrays of varied, interchangeable domains and by the mechanisms which rearrange these domains to produce functional receptors\textsuperscript{11}.

The emergence of adaptive immunity required a mechanism to recombine these genes. The individual segments of recombinatorial IgSF genes are flanked by recombination signal sequences (RSS). Because these RSS resemble sequences found in transposons, it is theorized that a transposable element initially inserted into an early IgSF receptor to create the adaptive receptor loci present in gnathostomes\textsuperscript{12}. By inserting itself into an exon of a receptor gene and then removing itself, a transposon could interrupt an exon with RSS that could be later be used for recombination\textsuperscript{10, 11}. The enzymes RAG1 and RAG2 are transposases that recombine genes flanked by RSS. RAG1 and RAG2 are common to all gnathostomes and it is believed that they were originally contained within a transposon that inserted into Ig receptor gene(s) and gave rise to the recombinatorial segments of present IgSF adaptive immune receptors\textsuperscript{13, 14}.

### 1.1.2 The Immunoglobulin Superfamily

The IgSF comprises a functionally diverse array of proteins all based on a structure known as the Ig domain. Fundamentally, the Ig domain is composed of two anti-parallel \( \beta \)-sheets folded together by a disulphide bond. The two most basic types of Ig domain are termed V-type and C-type domains. Though diversity has arisen in the Ig domains of the many IgSF proteins, they maintain the same fundamental Ig-fold structure as well as limited DNA sequence
conservation\textsuperscript{15,16}. In the context of adaptive immunity, IgSF proteins constitute three types of receptors (Ig, TCR, and MHC) as well as numerous co-receptors (CD4, CD8, etc.)\textsuperscript{17} and co-stimulators (CD28, CD80, etc.)\textsuperscript{18}.

The basic structure of the Ig domain was first observed in an Ig receptor, thereby lending its name to the entire superfamily. The Ig molecules exist as membrane receptors on B cells or as soluble antibody (Ab) secreted by plasma cells. As B cell membrane receptors, Ig allows for ligand-specific binding by B cells to induce activation and the internalization of Ag. As secreted Ab, Ig molecules effect host protection by binding to pathogens and targeting them for destruction. Ligands bound by Ig are known as antigens (antibody-generators, Ag). On the B cell surface, Ig molecules associate with other signal-transducing molecules to form a B cell receptor (BCR).

T cell receptors (TCR) exist solely as surface receptors. While Ig receptors bind to exposed, tertiary structures of Ag (conformational epitopes), TCRs bind to short peptide sequences that have been digested from internalized Ag and displayed on MHC molecules on the surfaces of other immune cells. TCRs are heterodimers composed of an α- and β-chain or a γ- and δ-chain. Each TCR chain is composed of two Ig domains\textsuperscript{19}.

The MHC molecules are surface receptors that are unique among the three receptor types. While Ig and TCR generate ligand specificities \textit{de novo} through recombination, MHC genes do not recombine and their ligand specificities are fixed in the germline. As well, MHC does not bind extracellular ligands; their ligands, short peptides, are inserted into the MHC binding region inside the cell and the MHC-peptide complex is then exported to the cell surface. The two main classes of MHC, MHC I and MHC II, differ in their structure. MHC I is composed of one membrane-bound polypeptide with a single Ig domain that is bound non-covalently to a single Ig domain called β2 microglobulin. MHC II is a heterodimer of membrane-bound α- and β-chains each containing a single Ig domain\textsuperscript{20,21}.

\textbf{1.1.3 The Role of Adaptive Immunity}

Although it was initially believed that adaptive immunity acted independently of innate immunity, comprehensive immunological research has since revealed that adaptive immunity relies on communication with the innate immune system in order to function. In some cases, molecules of the adaptive immune system have been co-opted by innate pathways, such as the activation of the complement cascade by Ab\textsuperscript{22}. It is thus important to study adaptive immunity in the context of innate immunity in order to discern its role\textsuperscript{23}. 


Adaptive immune responses in vivo can only begin once innate immunity has been first activated. Cells of the innate immune system are activated when they bind PAMPs through innate receptors such as toll-like receptors (TLR) or nod-like receptors (NLR). By contrast, stimulation of immune cells through adaptive receptors alone is not enough to induce a response. B cells binding antigen through their BCRs will only become fully activated if they also receive a signal through a PRR, such as TLR4, or if they receive help from a T cell. Likewise, T cells that bind MHC-peptide with TCR will only become functionally active if they receive co-stimulation from an activated innate cell. Thus, adaptive immunity can be seen as the second step of an immune response which is first triggered by innate immunity24-26.

In this light, it is clear that adaptive immunity did not evolve to replace innate immunity, but rather to complement it. The advantage of innate immunity is that it can act quickly and boldly to counter pathogens while the adaptive response is being initiated. Innate immunity can be quick because it begins a response with millions of cells whose receptors already recognize its targets and it can be bold because evolutionary pressures have ensured that innate receptors will not target friendly cells. Adaptive immunity, on the other hand, requires the time to expand clones of Ag-binding cells, refine the Ag-affinity of those cells, and ensure that receptors generated de novo will not target friendly cells. Innate immunity therefore possesses traits which adaptive immunity cannot replace. What adaptive immunity does offer is the ability to generate potent receptors to molecules that have never been encountered. Adaptive immunity can therefore counter pathogens which diversify or mask their PAMPs in order to evade clearance by innate immunity.
1.2 Immunoglobulin Diversification

Immunoglobulins (Ig) are the adaptive immune receptors of B cells. They are present on the surface of B cells as Ag receptors, allowing the cell to recognize and respond to Ag, and as secreted Ab, which can bind and neutralize pathogens. Within an individual organism there is a large diversity of Ig molecules exhibiting immense variation in their Ag-binding regions. The unique Ag-binding property of a single Ig is termed idiotype. As well, Igs exist as one of a small number of isotypes that differ in the structure of the non-Ag-binding region. Even further, any idiotype can be paired with any isotype. Such staggering diversity of protein structure is more than could practically be coded in the germline genome. Rather, this diversity results from recombining, rewriting, and mutating the genes that encode for the Ig peptides. Though the mechanisms that diversify Ig are complex and potentially deleterious, they enable an organism to develop an immune response to any possible Ag it may encounter in its lifetime.

Among the gnathostomes there is diversity in the structure of the Ig loci and in the mechanisms of Ig diversification. Even among species of mammals there are different arrangements of the Ig loci and different mechanisms used to generate Ig diversity. In this section I will limit discussion to the structures and processes found in mice and humans.

1.2.1 Immunoglobulin Structure

Of the three types of adaptive immune receptors, Igs are the largest and most complex. An Ig molecule is composed of four polypeptides: two identical light chains and two identical heavy chains all linked by disulphide bonds. Heavy chains are composed of an N-terminal V-type Ig domain (V_H) followed by 3-4 C-type Ig domains (C_H1-C_H3/4). The smaller light chains comprise an N-terminal V-type Ig domain (V_L) and a single C-type domain (C_L). One light chain is linked with one heavy chain by a C_H–C_L disulphide bond to form a heterodimer. Disulphide bonds between the heavy chains link the two dimers together to form a complete Ig. The resultant structure is roughly Y-shaped with two arms, the Fab regions, comprising the light chain and the heavy chain V_H-C_H1 and a base, termed the Fc region, comprising the heavy chain C_H2-C_H3/4 (Figure 1.1)\(^{15}\).

The Ag-binding sites of Ig are located at the N-termini of the Fab regions (Fragment antigen-binding) where the V_H and V_L domains meet. The V_H and V_L domains are the variable portion of the Ig and thus exhibit a high degree of diversity. It is the amino acid sequences of the
**Figure 1.1. Structure of IgM and IgG.** A simplified representation of the Ig-domain structure of monomeric IgM and IgG.
Figure 1.1

IgM

IgG

Ag Binding Region

Fc Region

Hinge Region
V\textsubscript{H} and V\textsubscript{L} domains as well as their 3-dimensional structure when paired that determine the Ag-binding properties of the Ig. The portion of an Ag that is bound by Ig is termed an epitope.

The Fc (Fragment crystallizable) region of Ig is composed of conserved C\textsubscript{H} domains and interacts with molecules and receptors of the host immune system to mediate processes such as complement cascade and phagocytosis. There are five types of Fc regions termed \(\mu\), \(\delta\), \(\gamma\), \(\varepsilon\), and \(\alpha\) that are used in the five Ig isotypes: IgM, IgD, IgG, IgE, and IgA, respectively\(^{27}\). The Ig isotype is determined by the heavy chain. Each Ig isotype possesses unique properties that are advantageous in different types of immune responses. In IgD, IgG, and IgA molecules the Fab and Fc regions are linked by a flexible hinge region that facilitates Ag-binding by allowing the Fab to move to accommodate Ags of different sizes and valencies. In IgM and IgE molecules the hinge region is replaced with an extra C\textsubscript{H} domain resulting in a stiffer structure. Each Ig isotype can exist in a membrane-bound or a soluble form due to alternate splicing of the 3’ end of the IgH mRNA. The C\textsubscript{H3/4} exon is followed by a sequence encoding a short hydrophilic domain called the S sequence. Downstream of the S sequence are two exons, M1 and M2, that encode a transmembrane and a cytoplasmic domain. Removal of either the S or M1/M2 regions during mRNA splicing will produce membrane-bound or soluble Ig, respectively\(^{28}\).

1.2.2 The Immunoglobulin Loci

The Ig genes are found at three separate loci all located on different chromosomes. There is one locus for the heavy chain and one locus for each of the two types of light chain: lambda (\(\lambda\)) and kappa (\(\kappa\)). Each Ig locus contains the information to code for a complete Ig polypeptide. This requires the transcription of a V-type exon, or V region, for the V\textsubscript{L} and V\textsubscript{H} and one or more C-type exons for the C regions of the light and heavy chains. C-type exons exist in complete form at all Ig loci. V regions, however, are split into fragments that require recombination to produce complete exons.

At the light chain loci, the V region is split into V (variable) segments and J (joining) segments. There are numerous tandem copies of V and J segments. Some copies of these segments have been rendered non-functional as a result of mutation and are present as pseudogenes. At the mouse \(\kappa\) light chain locus, for example, there are 85 functional V segments followed by 4 functional J segments. To produce a functional V region exon, one V segment must be recombined adjacent to a J segment. The new V-J exon is then recombined adjacent to a downstream C region to remove unused, intervening J segments and produce a complete gene ready for transcription. While the \(\kappa\) light chain locus contains a single C exon, the \(\lambda\) locus
contains 3 functional C exons in mice and 4 functional C exons in humans, any of which may be paired with the V-J.

The heavy chain locus is more complex. In addition to V and J segments, the heavy chain locus includes D (diversity) gene segments located between the V and J segments. The heavy chain locus thus requires an extra recombination event to produce a complete exon; a D segment must be paired with a J segment and the resulting D-J segment is then paired with a V segment. The heavy chain locus also contains numerous different C regions that encode for the different Ig isotypes listed above. Initially, a complete V-D-J exon is recombined adjacent to the Cμ region. The Cδ is paired with the Cμ and both C regions are transcribed together; alternate splicing of the Ig mRNA allows for simultaneous expression of both IgD and IgM. Later events may induce a B cell to recombine the Ig locus by moving the V-D-J exon to another downstream C region, excising the Cδ and Cμ from the locus in the process (Figure 1.2)27. Individual V, D, and J segments are flanked by RSS that allow for their recombination. As well, each C region is downstream of a conserved sequence known as a switch region that allows different C regions to be translocated to the V-D-J exon.

1.2.3 V(D)J Recombination

The process of producing a complete V region exon is called V(D)J recombination. Because V(D)J recombination is required to produce Ig, it occurs early in B cell development. V(D)J recombination also allows for the immense diversity of Ag-specificity seen among Ig molecules. V(D)J recombination generates diversity in two ways. Firstly, it generates combinatorial diversity by mixing V(D)J gene segments. At the human heavy chain locus the approximately 40 functional V segments, 25 D segments, and 6 J segments make for 6000 possible combinations. Secondly, the insertion of random nucleotides at the joining sites during recombination creates additional junctional diversity.

All V region segments are flanked on one or both sides by RSS that targets the segment for recombination. RSS contain two conserved sequences: a 7bp heptamer and a 9bp nonamer. Between the heptamer and nonamer is a non-conserved spacer region of either 12bp or 23bp in length creating what are called the 12-RSS and 23-RSS, respectively. A segment flanked by a 12-RSS can be joined to another segment flanked by a 23-RSS and vice-versa. At the heavy chain locus, both V segments and J segments are flanked by 23-RSS, preventing them from being joined directly. The D segments in between are flanked on both sides by 12-RSS, allowing them to be joined to the V and J segments on either side.
Figure 1.2. V(D)J Recombination and Class Switch Recombination. A simplified representation of the IgH locus. (1-4) Sequential recombination of the IgH locus to generate a complete IgM/IgD gene. (5-6) AID creates uracils in the switch regions (S) to initiate CSR to IgG.
Figure 1.2

1: \( V_1 \ V_2 \ V_n \ \text{ } \begin{array}{c} D_1 \ D_2 \ D_n \ \text{ } J_1 \ J_2 \ J_n \ \text{ } \begin{array}{c} S \ C_{\mu} \end{array} \end{array} \)  

D-J Joining

2: \( V_1 \ V_2 \ V_n \ \begin{array}{c} D_1 \ D_2 \ D_n \ J_2 \ J_n \ \text{ } \begin{array}{c} S \ C_{\mu} \ C_{\delta} \end{array} \end{array} \)  

V-DJ Joining

3: \( V_1 \ D_n \ J_2 \ J_n \ \begin{array}{c} S \ C_{\mu} \ C_{\delta} \ \text{ } S \ C_{\gamma_3} \ \text{ } S \ C_{\gamma_1} \end{array} \)  

VDJ-\( C_{\mu/\delta} \) Joining

4: \( V_1 \ D_n \ J_2 \ \begin{array}{c} S \ C_{\mu} \ C_{\delta} \ \text{ } S \ C_{\gamma_3} \ \text{ } S \ C_{\gamma_1} \end{array} \)  

5: \( V_1 \ D_n \ J_2 \ \begin{array}{c} AID \ C_{\mu} \ C_{\delta} \ \text{ } S \ C_{\gamma_3} \ \text{ } AID \ C_{\gamma_1} \end{array} \)  

Class Switch Recombination

6: \( V_1 \ D_n \ J_2 \ \begin{array}{c} S \ C_{\gamma_1} \end{array} \)  
V(D)J recombination is effected by a complex of enzymes called V(D)J recombinase. Integral to this complex are the products of the RAG genes, RAG-1 and RAG-2. The RAG1/2 complex recognizes and binds to the conserved sequence of RSS. A RAG1/2 pair at a 23-RSS can complex with a RAG1/2 pair at a 12-RSS to bring the segments into proximity. RAG1/2 then makes single-strand DNA breaks in the RSS spacers. This creates a free 3’ end that attacks the opposing strand and binds to the 5’ of the opposing nucleotide, creating closed DNA hairpins. A Ku70/80 protein complex stabilizes the hairpins together while a DNA-PK/Artemis complex creates a break in each hairpin. A break in the DNA hairpin can occur at a different site than the initial ligation. This results in DNA overhangs at the end of the gene segment that encode for palindromic sequences that can be incorporated into the sequence of the gene segment joint. This generates the addition of palindromic (P) nucleotides into the joint sequence. Furthermore, once the hairpin has been opened the enzyme TdT can add non-template (N) nucleotides to the 3’ ends of the overhangs. The addition of P and N nucleotides at the junctions of V region gene segments creates junctional diversity. V-(D)J junctions comprise Ag-binding sites in the Ig molecule, highlighting the importance of junctional diversity. After the opening of the two opposing hairpins and the optional addition of N nucleotides, the ends are joined by DNA ligase IV and gaps are filled by DNA synthesis. This completes the recombination process and generates a complete Ig locus allowing for the assembly of Ig molecules.

1.2.4 AID, SHM, and CSR

The process of V(D)J recombination generates IgM and IgD molecules with unique Ag-binding specificities. Following activation, B cells can further diversify their Ig molecules by the processes of somatic hypermutation (SHM) and class-switch recombination (CSR). Both of these processes require the activity of the enzyme activation-induced deaminase (AID). AID was first discovered in a murine B cell lymphoma line as a potential RNA-editing enzyme. Subsequent studies have since demonstrated that AID acts on DNA. Since its initial discovery, versions of AID have been discovered in nearly all vertebrates, highlighting its importance in adaptive immunity. AID acts by binding to single-strand DNA in the Ig-V regions and, through its catalytic domain, it removes the amine group from cytosines thereby converting them into uracils. This generates U:G mismatches in the genomic DNA that serve as the starting points for SHM and CSR. Although the catalytic domain of AID performs deamination to initiate both SHM and CSR, it has been discovered that AID also contains one domain required for SHM and another for CSR. This possibly explains the ability of AID to
initiate only CSR or SHM in B cells responding to certain stimuli\textsuperscript{39,40}. AID is strongly up-regulated in B cells following stimulation with CD40L or TLR agonists. It was initially believed that AID was only expressed by activated B cells, but recent evidence suggests that small amounts of AID are expressed by developing B cells in the bone marrow\textsuperscript{41,42}. Because AID can generate potentially dangerous DNA damage, its activity must be tightly regulated to limit mutations to the Ig-V regions\textsuperscript{43,44}. AID can only bind to single-strand DNA, and it has shown a preference for targeting cytosines in WRCY sequences, so-called ‘hotspots’\textsuperscript{45}. Although it was initially thought that the activity of AID was restricted to Ig-V regions by targeting specific DNA sequences, WRCY hotspots are found in abundance outside of the Ig loci and AID has been shown to induce deamination throughout the genome\textsuperscript{46,47}. Despite AID’s genome-wide activity, mutations are indeed highly targeted to the Ig-V regions. It has been suggested that the specific recruitment of error-prone DNA repair mechanisms to the Ig-V regions is responsible for the targeting of SHM and CSR\textsuperscript{46,48}. AID activity is also regulated by its level of transcription and its rate of entry into the nucleus\textsuperscript{49}.

The process of SHM induces mutations in the Ig-V regions at a frequency of up to $10^{-3}$ mutations per base pair per generation, nearly $10^6$ fold higher than the normal background rate. The goal of SHM is to induce mutations that increase the affinity of Ig for Ag. During SHM, AID generates deoxyuridines in the Ig-V region. These U:G mismatches can result in mutations by multiple mechanisms. Uracils that are not removed prior to cell division will serve as templates during replication resulting in C:G$\rightarrow$A:T mutation in one of the daughter cells. If the uracil is removed by the enzyme UNG it will generate an abasic site in the DNA and initiate the base excision repair (BER) pathway. By this mechanism, the uracil is removed to create an abasic site, APE endonuclease creates a single-strand nick in the DNA backbone, the gap is filled in by a replicative or error-prone DNA polymerase, and the ends are joined by DNA ligase. Alternatively, the U:G mismatch can be recognized by the mismatch repair pathway (MMR). During MMR, an Msh2/Msh6 complex binds to the mismatch. A single strand break is created in the DNA and EXO-1 acts on the break to generate a larger single-strand segment that is then filled in by an error-prone DNA polymerase. While BER and MMR act to properly repair DNA damage in the rest of the genome, BER and MMR at the Ig loci induce mutation. This is thought to occur by the targeted recruitment of the error-prone DNA polymerase polη at the Ig loci\textsuperscript{50-52}.

During CSR, AID generates deoxyuridines in the switch sequences directly upstream of the C regions. Induction of BER and/or MMR simultaneously on both strands in the switch
region generates double-strand breaks (DSB). DSBs in switch regions can then be brought together and re-ligated by the non-homologous end joining (NHEJ) or alternative end joining (AEJ) pathways. If a break in the Cμ switch region is ligated to a break in the Cγ switch region then the Cμ and Cδ constant regions are excised from the genome and Cγ is joined to the V region. This results in class-switching from IgM to IgG. Because AID can only act on single-strand DNA, transcription of the switch regions is required for SHM. Each switch region is accompanied by an upstream promoter, and different stimuli will initiate transcription of specific switch region promoters. In this manner, specific cytokines, when coupled with co-stimulation, will induce switching to specific Ig isotypes. IL-4 promotes transcription of the Cγ and Cε switch regions to promote CSR to IgG and IgE, respectively. Likewise, TGFβ promotes CSR to IgA\textsuperscript{39, 53}.

### 1.2.5 Antibody Functions

Membrane-bound Ig on the surface of B cells serves as a signaling receptor within BCRs. When secreted as Ab, Ig binds to target Ag to effect humoral immunity. The most basic function of Ab is simply to coat pathogens by binding to surface epitopes. This is called neutralization. Coating pathogens may block the function of their surface proteins or, in the case of intracellular pathogens, prevent their entry into cells. Neutralization is mediated by the Ag-binding properties of the Ab molecule. IgM Ab generated early in primary immune responses usually has lower affinity for Ag. However, IgM is secreted as pentamers and therefore possess a higher avidity that compensates for this. Ig that has undergone affinity maturation is secreted as monomeric Ab that binds pathogens due to increased affinity for Ag. IgA is secreted into the gut lumen and onto the surfaces of mucosa to prevent bacterial adhesion and colonization.

Ab mediates other immune functions through interactions with the Fc region of the heavy chain. IgD is not secreted and its exact function is still a matter of investigation. The Fc regions of the other four isotypes differ in their structures and effect different functions. Ab coating the surface of a pathogenic cell can initiate the complement pathway to generate membrane pores that kill the target cell. In this pathway, the Fc regions extending outwards from the target cell bind the C1 complex. Once fixed to the target, C1 triggers the complement cascade resulting in the formation of membrane attack complexes and the death of the target. The Fc regions of the Ab isotypes have differing affinities for binding to C1 and therefore differ in their abilities to fix complement. IgM and the IgG subclasses IgG1 and IgG3 are all strong
inducers of complement recruitment. IgA and IgG2 fix complement only weakly, and IgE is unable to induce complement attack.

The fixation of complement by Ab can enhance the phagocytosis of its targets in a process called opsonisation. In this process, a pathogenic cell is coated with Ab which may then fix complement to the cell surface. Receptors for complement components C3d (such as CD21 and CD35) or isotype-specific Fc receptors on the surfaces of phagocytic cells can then bind to the target cell and begin phagocytosis.

Secreted Ab can also modulate immune responses by binding to inhibitory or stimulatory Fc receptors found on immune cells. For example, binding of IgG to the Fc receptor CD16 on NK cells can promote cytotoxicity, whereas binding of CD32 on B cells inhibits activation and creates a negative feedback loop to regulate humoral responses54-56.
1.3 B cells

B cells are the effectors of humoral immunity. Every B cell expresses a unique Ig molecule on its surface within a BCR complex. This provides organisms with a vast polyclonal repertoire of adaptive immune receptors that can respond to any new pathogen encounter. B cells whose Ig molecules bind to Ag will transduce a signal through their BCR and become activated. Activated B cells can then increase the affinity of their Ig for Ag by undergoing SHM and change the effector function of their Ig by CSR. B cells can then secrete their Ig molecules as soluble Ab to effect host protection to pathogen. B cells are critical contributors to the immune system and B cell deficiencies can severely compromise host immunity.

1.3.1 B cell Development

Early observations in chickens that an avian organ called the bursa of Fabricius was required for Ab production and that the Ab-producing cells developed in this organ led to the name ‘B’ cells\(^{57}\). In mammals, B cells develop in the also appropriately-named bone marrow (BM). B cells initially develop from the hematopoietic stem cells (HSC) that are the common progenitors of all blood cells. Specific signals provided by the unique stromal environment of the BM gradually direct HSCs to mature into more specific progenitor cell types. HSCs can develop into multipotent progenitor (MPP) cells that are the common progenitors of myeloid cells (e.g. monocytes, neutrophils) and lymphocytes (e.g. B cells, T cells). Signaling through FLT3 on the surface of MPPs induces differentiation into common lymphoid progenitors (CLP). From there, signals including IL-7 and cell adhesion molecule (CAM) interactions with stromal cells can induce CLPs to become pro-B cells and thereby commit to the B cell lineage. As progenitor cells become pro-B cells they up-regulate Pax5, a transcription factor that is a master regulator of B cell development and phenotype.

Beginning at the pro-B stage, B cell development is largely characterized by the sequential recombination and testing of the Ig molecule. V(D)J recombination is a complex process during which much can go wrong. As well, recombination of the Ig locus can produce Ig molecules with auto-reactivity. Developing B cells must therefore proceed through a series of checkpoints to test their Ig molecules. Early pro-B cells initiate recombination of the D→J segments at the heavy chain loci. Late pro-B cells then undergo a V→DJ recombination at one of the heavy chain loci and mature into pre-B cells. Pre-B cells must then test for successful recombination of the heavy chain. This is done by expressing the heavy chain on the cell surface...
and testing its ability to bind Ag, thereby producing a BCR signal. Because the light chain has not yet been rearranged, the heavy chain is paired with an invariant surrogate light chain. This forms the pre-B receptor. If the pre-B receptor is able to bind an extracellular Ag and generate a signal, then successful rearrangement is confirmed and the pre-B cell can then begin rearranging a light chain. If the pre-B receptor fails then the B cell may recombine the other heavy chain allele. Pre-B cells that fail to produce a successful heavy chain undergo apoptosis. Successful signaling by the pre-B receptor also induces a process of allelic exclusion that allows for the expression of only one heavy chain and one light chain allele, ensuring that the B cell expresses only one type of Ig molecule. Following successful rearrangement of a light chain, pre-B cells become immature B cells and express fully-formed IgM molecules on their cell surfaces.

At the immature B cell stage, signaling through BCRs can induce different cell fates depending on the strength of signaling. Typically, the BM niche is devoid of foreign Ag. An IgM that binds Ag at this stage is therefore self-reactive and potentially dangerous to the organism. Strong IgM binding will generate a strong BCR signal that will either kill the immature B cell or induce it to rearrange another light chain. Intermediate signaling can also suggest autoreactivity and induces the immature B cell into a state of anergy in which it will mature and exit the BM but will be unable to enter an active state. Low or absent IgM binding indicates that the immature B cell is not dangerously self-reactive and allows the B cell to exit the BM as a mature B cell. This process is termed central tolerance. From there, the mature B cell may migrate to one of many B cell niches in the body. These mature B cells have never encountered foreign Ag and are therefore called naïve B cells. Naïve B cells remain in an inactive state until they are activated by binding to Ag and begin to enter an immune response.58-61.

1.3.2 B cell Niches

Upon emigration from the BM, B cells may circulate through the bloodstream or reside within a tissue. Most B cells reside in the spleen or in the lymph nodes which together comprise the secondary lymphoid organs. The spleen is a multifunctional organ that filters the bloodstream. Much of the parenchyma of the spleen is composed of red pulp in which old red blood cells are removed from circulation and the iron from their hemoglobin is recycled. Within the red pulp are pockets of lymphoid cells that are termed white pulp. White pulp is composed of a central arteriole surrounded by T cells in what is termed a T cell zone. The T cell zone is in turn surrounded by follicles comprised of mature naïve B cells. At the outer edge of the white
pulp is a marginal zone (MZ). Blood filters into the marginal zone which allows resident MZ macrophages to sample for Ag. The MZ also contains specialized MZ B cells that may have a special role in the transportation of Ag into the B cell follicle.

Lymph nodes (LNs) are specialized organs that serve to gather Ag and orchestrate immune responses (Figure 1.3). LNs are connected to the lymphatic system and are able to filter antigen from the lymph. The structure of LNs in some ways mirrors that of the splenic white pulp. The inner area of the LN is termed the medulla and contains vasculature that connects to the bloodstream and medullary chords that connect to the lymphatic system. The medulla is surrounded by the cortex. The inner area of the cortex, the paracortex, is populated by T cells. The T cell zone is defined by the chemokines CCL19 and CCL21 that are produced by resident paracortical stromal cells. These chemokines attract T cells through the chemokine receptor CCR7. The outer cortex is composed of B cell follicles. B cell follicles are established by the chemokine CXCL13 which is produced by outer cortex stromal cells such as FDC. CXCL13 attracts B cells through CXCR5. The entire LN is enclosed by an outer capsule. There is a luminal space between the capsule and the cortex called the subcapsular sinus (SCS). Lymph enters the SCS through afferent lymphatic vessels and moves through the SCS to the medullary chords where it then flows out through the efferent lymphatic vessel. Plasma cells in the LN migrate to the medullary chords and secrete Ab into the lymphatics. SCS macrophages embedded in the subcapsular wall can sample the lymph for Ag as it flows through the LN. Ag captured in this way can then be shuttled into the B cell follicles to initiate humoral responses. LNs are found throughout the body in order to provide comprehensive sensing of Ag. Peripheral LNs found beneath the skin and can collect Ag introduced by dermal wounds. Mesenteric LNs are associated with the gut and respond to intestinal Ag\textsuperscript{60, 62-65}.

The peritoneal cavity is home to a special lineage of B cell known as B-1 cells. B-1 cells are relatively few in number and express a semi-conserved repertoire of IgM molecules with limited V(D)J rearrangements that encode specificity for conserved bacterial Ag. Lymphoid tissues associated with mucosal surfaces (MALT) and with the gut (GALT) also contain B and T lymphocytes in order to confer protection to pathogens at these vulnerable surfaces\textsuperscript{66, 67}.\textsuperscript{18}
**Figure 1.3. Lymphoid Architecture in a Lymph Node.** Cross-section of a murine inguinal lymph node stained by immunofluorescence. Staining for B220 (blue) shows the B cell follicles of the cortex. GCs within the follicles express a reporter for AID activity (green). The paracortex (unstained) contains T cells. Plasma cells occupy the medulla and secrete Ab (red). The lymph node is surrounded by a small luminal space, the subcapsular sinus (SCS).
Figure 1.3

GC = Germinal Center
C = Cortex
PC = Paracortex
M = Medulla
SCS = Subcapsular Sinus
1.3.3 B cell Activation

By binding to Ag, Ig molecules on the surface of B cells sense pathogen and initiate activation. However, membrane-bound Ig molecules have short intracellular sequences that do not transduce signals. In order to generate an activation signal, Ig molecules associate with other membrane proteins to form the B cell receptor (BCR) complex. As mentioned previously, when Ig binds to Ag it transduces a signal to the cell through the activity of the BCR. Within a BCR, an Ig molecule is closely associated with an Igα/β heterodimer. Igα/β is a member of the IgSF and is composed of two extracellular Ig domains, Igα and Igβ, each possessing a transmembrane domain and a cytoplasmic tail. The cytoplasmic tails of Igα/β contain conserved amino acid sequences called immunoreceptor tyrosine-based activation motifs (ITAM). ITAMs are important signaling motifs found in many immune receptors, including BCRs. Signaling is initiated when the tyrosine residue in an ITAM is phosphorylated by a tyrosine kinase.

Phosphorylation of individual ITAMs can occur spontaneously at low levels that are not enough to signal. Clustering of ITAM-bearing proteins promotes the recruitment of kinases and initiates signaling. Larger clusters induce stronger signals. Each Ig molecule has two Ag binding sites and two or more Ig molecules can bind the same Ag if it has multiple epitopes. Large, polyvalent Ags are able to cross-link multiple BCRs, bringing their ITAMs together and promoting the recruitment of Src family kinases. Phosphorylation of the ITAMs recruits and activates another kinase, Syk. Syk then phosphorylates other signaling molecules including BLNK, PLC-γ, and GEFs resulting in further amplification and propagation of the BCR signal. Ultimately, this leads to the activation of the transcription factor NFκB that induces a plethora of immune functions. Other components of the BCR include CD19, CD21, and CD81. The binding of CD21 to complement component C3d allows phosphorylation of ITAMs on CD19 resulting in the further recruitment of Syk and PI3 kinases to enhance the BCR signal. Signaling through the BCR is regulated by the activity of phosphatases such as CD22 that deactivate ITAMs. A balance between the activity of kinases and phosphatases ensures proper levels of BCR signaling. Binding of Ag by the BCR results in internalization so that Ag can be digested and presented on MHCII.

For full activation into a T-dependent immune response a B cell must receive help in addition to BCR signaling. B cells receive help from CD4+ T cells. In order to receive T cell help, B cells must present peptides from internalized Ag on MHCII. If a T cell recognizes the peptide-MHCII with its TCR, it up-regulates surface molecules to engage with the B cell. One key help signal is the triggering of CD40 on the B cells by CD40L on the surface of an antigen-
activated CD4\(^+\) T cell. CD40 engagement induces intracellular signaling through the TRAF proteins to transduce multiple signals including the activation of NFκB. Engagement of CD40 fully activates the B cell, promoting proliferation, CSR, and SHM. The process of co-stimulation involves cross-talk between the B cell and the T cell that is mutually stimulatory. Other factors involved in B-T cross-talk include CD80/86-CD28 and ICOSL-ICOS, respectively. Furthermore, CD4\(^+\) T cells release cytokines including IL-2, IL-4, IL-6, IL-21 and IFN-γ to modulate the B cell response and induce CSR to specific isotypes\(^{68-71}\).
1.4 Germinal Centers

Following stimulation and recruitment into a T-dependent immune response, activated B cells enter structures known as germinal centers (GC). Within GCs, B cells proliferate rapidly and up-regulate AID to undergo CSR and SHM. GC B cells compete for limited resources and they are selected to survive and proliferate based on the affinity of their Ig molecules for Ag. In this process, termed clonal selection, B cells with the highest affinities for Ag are selected while those with lower affinities die by apoptosis. This is the basis for the affinity maturation of the humoral response. GCs are a rich source of memory B cells and plasma cells and play an important role in host protection.

1.4.1 Germinal Center Structure and Location

GCs are clusters of activated B cells located in the follicles of secondary lymphoid organs. By histology, GCs appear as circular areas of activated IgD- B cells that are surrounded by the naïve IgD+ B cells that compose the B cell follicle. Also present in GCs are CD4+ T follicular helper (Tfh) cells, tingible body macrophages that clear apoptotic debris, and a type of stromal cell termed the follicular dendritic cell (FDC). GCs are observed in the follicles of the spleen and LNs as well as in GALT and MALT. Ectopic GCs have also been observed in some tertiary lymphoid tissues at sites of inflammation. GCs therefore have the ability to form at sites of local T-dependent Ag-stimulation.

GCs are polarized into a light zone (LZ) and a dark zone (DZ) (Figure 1.4). The DZ is typically proximal to the T cell zone and contains densely-packed, proliferating cells known as centroblasts and is enriched in the chemokine CXCL12. Adjacent to the DZ, distal to the T cell zone, is the LZ. The LZ is less dense and is composed of centrocyte B cells. Centrocytes are non-proliferating and have higher expression of BCR, MHC, and co-stimulatory molecules than centroblasts. Also located in the light zone are CD4+ Tfh cells that provide co-stimulation to centrocytes. While FDCs are found throughout the GC, they are observed in higher densities in the LZ. All GC B cells and CD4+ Tfh cells express CXCR5 which allows them to home to the B cell follicle and the GC by binding CXCL13 which is particularly enriched in the LZ. The LZ-DZ polarity of GCs is established through a gradient of the chemokine CXCL12, which attracts cells through CXCR4. Centroblasts have a higher expression of CXCR4 allowing them to position themselves in the DZ.72-78
Figure 1.4. Splenic White Pulp and GC Structure. (A) Cross section of a murine spleen showing white pulp structure. IgD (blue) stains naive follicular B cells. GL7 (red) stains GC B cells. (B) A simplified representation of canonical GC structure. Centroblast B cells in the dark zone are attracted to CXCL12 through CXCR4. Centrocyte B cells in the light zone are attracted to CXCL13 through CXCR5. Centrocyte B cells interact with follicular dendritic cells (FDC) and CD4+ T follicular helper cells (Tfh).
Figure 1.4

A

- **GL7**
- **IgD**

B = B cell Follicle
T = T cell Zone
GC = Germinal Center
RP = Red Pulp

B

**CXCL13**

Light Zone

- Tfh
- FDC
- B cells

Dark Zone

- CXCL12
- B cells
1.4.2 The Germinal Center Reaction

Naïve B cells that are stimulated by Ag will migrate to the border of the T and B cell zones. There, Ag-stimulated B cells seek out co-stimulation from cognate CD4+ T cells. Successful co-stimulation prompts B cells to either differentiate immediately into short-lived plasma cells or to migrate back into the follicle to enter a GC response. B cells can either initiate a new GC or enter an existing GC. By tracking the unique IgV sequences of individual B cells, studies have been able to determine that GCs are typically seeded by as few as 1-3 B cell clones. GCs appear to be open structures that can recruit new B cell clones later, provided that they have sufficiently high affinity for Ag.79-81

Activation of B cells induces phenotypic changes that allow them to be distinguished from naïve B cells. Naïve B cells express both IgM and IgD on their surfaces, while GC B cells cease expression of IgD and can begin to express class-switched isotypes. GC B cells have higher expression of MHCII and the co-stimulatory molecules CD80 and CD86. GC B cells up-regulate the death receptor Fas (CD95) and as a consequence become more sensitive to apoptosis. GC B cells also bind the lectin PNA and the monoclonal Ab GL.82

In the current model of the GC reaction, B cells first enter GC DZs as centroblasts and begin rapid proliferation. This enables the immediate expansion of potentially useful B cell clones. Centroblasts also express AID and undergo SHM and CSR. In this manner centroblasts have the potential to induce mutations in their IgV regions that increase the affinity of Ig for Ag. Centroblasts can then migrate to the LZ by down-regulating CXCR4 and become centrocytes. In the LZ, centrocytes interact with FDCs and resident CD4+ Tfh cells and compete with each other in a process termed clonal selection. Centrocytes bind and internalize Ag through their BCRs. Ag in the LZ can be present in soluble form or as immune complexes (IC). ICs are large particles of complexed Ab, complement, and Ag. FDCs trap ICs using complement (CD21/CD35) and Fc receptors (CD32). Centrocytes that internalize Ag can then present Ag-derived peptides in the context of MHCII to CD4+ Tfh cells. CD4+ Tfh cells provide B cells with help, promoting GC B cell survival and counteracting signaling through Fas. CD4+ Tfh cells are relatively rare in the GC and only form lasting contacts with a single B cell at a time. Tfh display a preference for B cells with the most MHCII-peptide complexes on their surfaces. B cell clones with the highest affinities for Ag are able to bind more Ag than others and therefore secure for themselves more peptide to present to CD4+ Tfh. B cell clones that cannot secure T cell co-stimulation do not receive survival signals and succumb to Fas-mediated
apoptosis through ligation of FasL on CD4+ T cells. The GC reaction therefore selects B cell clones with the highest affinities for Ag by forcing them to compete for T cell help.

While GC B cells that fail during clonal selection die by apoptosis, successful centrocytes can commit to one of three fates. Centrocytes may differentiate back into centroblasts and return to the DZ. This allows for the clonal expansion and further affinity maturation of a useful Ig molecule. Alternatively, centrocytes may exit the GC and mature into either memory B cells or plasma cells. Plasma cells emigrate to the BM marrow where they can continually secrete protective Ab for prolonged periods of time. Memory B cells are extremely long-lived and can last the lifetime of an organism. They continually circulate and monitor for the reappearance of pathogen. When stimulated, memory B cells can migrate to secondary lymphoid organs and re-initiate GC reactions or they can turn into plasma cells to immediately begin the production of neutralizing Ab. In this manner, GCs initiated during a primary immune response sow the seeds for robust secondary responses.

1.4.3 Apoptosis in Germinal Centers

An important part of the GC response is the apoptosis of GC B cells. Clonal selection requires the culling of GC B cell clones with low affinity for Ag. Apoptosis is also important in removing GC B cell clones that develop autoreactivity following SHM. Apoptosis can be induced through the extrinsic pathway or the intrinsic pathway.

Apoptosis through the intrinsic pathway can be triggered by mitochondrial or DNA damage. In the case of GC B cells, aberrant DNA damage induced during SHM or CSR can be sensed by ATM, leading to the activation of pro-apoptotic factors such as Bak and Bax. This in turn leads to the release of mitochondrial membrane factors that signal for apoptosis by activating caspase 9 and downstream caspase 3. This process could be important in deleting potentially cancerous GC B cells.

Apoptosis in GC B cells through the extrinsic pathway can be triggered through the ligation of the Fas receptor (CD95) that is highly expressed on the surface of GC B cells. Fas ligation leads to the recruitment of fas-associated protein with death domain (FADD) that is able to activate caspase 8. Caspase 8 in turn activates the “executioner” caspase 3 that begins to effect apoptosis. Fas deficiency results in impaired selection of high affinity clones and the emergence of self-reactive GC B cell clones due to reduced negative selection. As well, the conditional deletion of Fas in GC B cells results in hyper-lymphoproliferation, indicating that Fas is also important in regulating the growth of GC B cells and maintaining homeostasis.
1.4.4 Follicular Dendritic Cells

As mentioned above, GCs are populated by stromal cells called FDCs. Despite their name, FDCs are not related to dendritic cells and are not derived from the hematopoietic lineage. FDCs do, however, display a dendritic morphology with long, thin processes and a high surface area. The origins of FDCs had long been a mystery, though experiments with BM chimeras demonstrated that they were radio-resistant. A recent study has shown compelling evidence that FDCs are derived from perivascular precursor cells in the secondary lymphoid organs\(^97\). These precursor cells are stimulated to mature into FDCs \textit{in situ} by lymphotoxin (LT) signaling. LT is present on the surface of various lymphocytes as a LT\(\alpha_1\)-LT\(\beta_2\) heterotrimer. The presence of LT\(\alpha\beta\) during organogenesis signals through the LT\(\beta\)R on FDC precursor cells to induce their maturation. Constitutive LT\(\alpha\beta\)-LT\(\beta\)R signaling is required to maintain the FDC phenotype\(^98,99\).

FDCs are present throughout B cells follicles in a resting state termed primary FDCs. Primary FDCs help maintain the organization of distinct T and B cell zones by secreting the B cell chemoattractant CXCL13. Constitutive low-level signals from LT\(\alpha\beta\) on B cells maintains the presence of primary FDCs. GC B cells express higher levels of surface LT\(\alpha\beta\). Contact between GC B cells and primary FDCs results in augmented LT\(\beta\)R-signalling and induces FDCs to mature into secondary FDCs. Secondary FDCs display increased expression of complement receptors (CD21/35) and Fc receptor (CD32) allowing them to bind large amount of Ag in the form of ICs. Secondary FDCs also increase expression of adhesion molecules ICAM-1, VCAM-1, and MA\(\alpha\)CAM-1 that allow them to form prolonged contact with GC B cells. These changes in FDC phenotype help promote and maintain GCs. FDCs are therefore regarded as the stromal cells that underpin GCs and are thought to be crucial for their organization and function\(^100-102\). However, there is conflicting evidence over the exact functions of FDCs and this is discussed in more detail in Chapter 6.
1.5 Lymphotoxin

The lymphotoxin (LT) proteins are members of the tumour necrosis factor (TNF) family of proteins. Some LT ligands bind to TNF receptors and it was originally believed that LT played a redundant or supportive role in TNF functions. The discovery of a unique LT receptor, LTβR, led to the growing appreciation for the unique and profound roles of LT signaling in the development of secondary lymphoid tissues.

1.5.1 Lymphotoxin Ligands and Receptors

The LT ligands are comprised of LTα and LTβ subunits. LT can exist in soluble form as LTα3 homotrimers or as membrane-bound LTα1β2 heterotrimers. Secreted LTα binds to TNFR1 and TNFR2 receptors. Membrane LTαβ binds exclusively to LTβR. Another ligand, LIGHT, binds to LTβR as well as another TNF family receptor HVEM. Compared to LTαβ-LTβR signaling, the role of LIGHT is less understood. Studies in LIGHT-knockout or LIGHT-transgenic mice have indicated a role for LIGHT signaling in promoting CD8+ T cell activity and the development of autoimmunity. LTαβ is expressed by many subsets of T, B, and NK cells. LTβR, on the hand, is expressed by dendritic cells, endothelial cells, and stromal cells. LT signaling therefore allows for cross-talk between lymphocytes and their environments. Indeed, studies using genetic ablation of LTα, LTβ, and LTβR have revealed that LT signaling is extremely important in the development and organization of lymphoid tissues and in DC function.

1.5.2 Lymphotoxin in Organogenesis and Lymphoid Architecture

Mice in which LTβR signaling has been abrogated due to genetic ablation of LTα, LTβ, or LTβR exhibit abnormal development of their secondary lymphoid tissues, indicating that signaling through LTβR is critical during development. These mice lack peripheral LNs as well as lymphoid structures in the GALT known as Peyer’s patches. Interestingly, gut-associated mesenteric LNs still form in the absence of LTβ. LTα3 signaling through TNFR1 is sufficient for the ontogeny of mesenteric LNs suggesting that they have different stromal requirements than peripheral LNs. Critical to the development of secondary lymphoid organs during embryogenesis are interactions between LTαβ-expressing lymphoid tissue inducer (LTi) cells and LTβR-expressing lymphoid tissue organizer (LTo) cells. LTi cells begin to up-regulate LTαβ in response to low levels of CXCL13 in developing tissues. LTαβ-LTβR
interactions between LTi and LTo cells induces LTo cells to mature and secrete more CXCL13. This in turn recruits more LTi cells and up-regulates LTαβ, thereby inducing a positive feedback loop. Stimulation of LTo cells in this manner induces up-regulation of cell adhesion molecules required for the development of the nascent organ.112

A similar mechanism exists to maintain the lymphoid architecture of mature secondary lymphoid organs. In mature tissues LTi cells are rare, but LTαβ is provided by B cells.113 B cells provide LTαβ to LTβR on FDCs in splenic and LN follicles. These interactions are also reinforced by a LT-CXCL13 positive feedback loop. Abrogation of this signal results in disorganized splenic white pulp characterized by mixed, convoluted T-B cell zones. The T cell zones themselves are defined by the attraction of T cells by the chemokines CCL19 and CCL21. These chemokines are secreted by T zone stromal cells called fibroblastic reticular cells (FRC). The emergence of FRCs is also dependent on LTβR signaling.114 Although the provision of LTαβ early in development by LTi cells is important for FRC development in LNs, it seems that FRCs are not heavily reliant on continued LTβR signaling in mature tissues.99, 112, 115

1.5.3 Lymphotoxin and Germinal Centers

The role of LT signaling in the GC response has been more difficult to determine. Studies using knockout mice have observed absent or abortive GC responses in the absence of LTβR signaling, clearly demonstrating the importance of this signaling pathway in the GC response.107, 116, 117 However, the pleiotropic effects of blocking LTβR signaling have made it difficult to determine the exact mechanisms by which GCs are LT-dependent.

As discussed previously, the presence of FDCs is dependent on LTβR signaling and their functions appear crucial to the proper functioning of GCs. The absence of FDCs could prevent CXCL13-mediated recruitment of activated B cells to GCs. FDC absence could also impair the accumulation of ICs in the GC that are needed for clonal selection.100

LTβR signaling affects the movement of Ag into B cell follicles. The MZ surrounding splenic white pulp is important for capturing Ag from the blood. MZ B cells may be able to shuttle Ag into B cell follicles. In the absence of LTβR signaling the MZ sinus lacks MadCAM-1. This correlates with an absence of MZ B cells and macrophages in the MZ and very likely impairs the transport of Ag into the follicle. Transport of Ag in LNs is also dependent on LTβR. FRCs generate a network of fibronectin/collagen conduits that are able to transport small soluble Ag from the SCS into the cortex. Larger Ag can be captured from the SCS by SCS macrophages that then initiate transport of the Ag into the B cell follicles. Blocking LTβR signaling results in...
a significant decrease in the number of SCS macrophages and likely impairs this mechanism of Ag transport. The presence of Ag in GCs is important for B cell stimulation and clonal selection and LTβR-dependent Ag transport could therefore be important in GC responses.\(^99,112,118\).
1.6 Thesis Overview

The investigations in this thesis stemmed from three main questions:

1. Where is AID expressed, and how is it regulated, in the absence of FDCs/GCs?
2. What is the importance of AID/SHM in clonal selection in GCs?
3. Is BAFF an important survival factor in positive clonal selection in GCs?

Chapter 3 deals with the first question through the use of LTβ<sup>−/−</sup> mice in which an absence of LTαβ-LTβR signaling abrogates FDCs and has been shown in the literature to result in impaired or absent GC formation. Specifically, I use LTβ<sup>−/−</sup> BM cells to reconstitute WT hosts thereby generating BM chimeras that are developmentally normal but lack LTαβ-LTβR signaling. AID is primarily expressed by B cells in GCs, and this system allowed me to observe where B cells expressed AID during a T-dependent response in the absence of GCs. Surprisingly, we observed B cells expressing AID and other canonical GC B cell markers forming GC-like clusters in peripheral LNs in the absence of FDCs. Though the peak of the response was normal, the absence of FDCs resulted in premature GC dissipation and reduced affinity maturation. These findings reveal that FDCs are not required for regulating the expression of AID or for initiating the formation of GCs as was previously believed.

Chapter 4 deals with questions 2 and 3 using mixed BM chimeras in which AID<sup>−/−</sup> and WT B cells compete among each other within the same GCs. By observing the number of AID<sup>−/−</sup> B cells within mixed GCs, it would be possible to infer how important SHM was in enhancing fitness during clonal selection. As well, by providing an excess of BAFF, it would be possible to test if it can rescue B cell clones of low fitness. Contrary to expectations, I observed that AID<sup>−/−</sup> B cells actually thrive in GCs despite their inability to undergo SHM. Providing excess BAFF did not affect the abundance of AID<sup>−/−</sup> or WT GC B cells. I observe that AID<sup>−/−</sup> GC B cells undergo lower rates of apoptosis, possibly due to a lack of AID-induced DNA damage. This enhanced survival may account for the abundance of AID<sup>−/−</sup> GC B cells. These results suggest that AID may regulate the GC response by inducing apoptosis in GC B cells.

Chapter 5 more closely investigates the findings of chapter 4 using similar BM chimeric models. I observed that AID<sup>−/−</sup> GC B cells were specifically accumulating as centrocytes, the
stage at which fate decisions are thought to occur. I also observed that the accumulation of AID⁻/⁻ GC B cells was independent of the affinity of the BCR for Ag using Ig-transgenic mice. AID⁻/⁻ GC B cells were observed to mature into plasma cells at a significantly reduced rate. This partial block in plasma cell formation may also contribute to the accumulation of AID⁻/⁻ GC B cells. Finally, I demonstrate that the partial block in plasma cell maturation is not due to an absence of DNA DSBs, which have been suggested to induce plasma cell maturation. These findings demonstrate that in the absence of AID, B cells accumulate as centrocytes and are deficient in plasma cell formation.
Chapter 2: Materials and Methods

2.1 Mice

C57Bl/6 mice were purchased from Charles River Laboratories. AID-GFP transgenic mice were obtained from Dr. Rafael Casellas (NIH, Bethesda, USA)\textsuperscript{41}. AID-GFP mice contain a transgenic insert of GFP fused to exon 5 of the full length AID sequence and under the control AID-locus cis-elements inserted into a BAC. LTβ\textsuperscript{−/−} mice\textsuperscript{106} were purchased from B&K Universal and crossed with AID-GFP mice to produce LTβ\textsuperscript{−/−} x AID-GFP mice. LTβR\textsuperscript{−/−} mice with the CD45.1 congenic allele were obtained from Rodney Newberry (Washington University School of Medicine, St. Louis, USA). Mice congenic for the CD45.1 allele and for the IgHa allotype on the C57Bl/6 background were obtained from the Jackson Laboratory (stocks 002014 and 001317, respectively). AID\textsuperscript{−/−} mice were obtained from Dr. Tasuku Honjo (Department of Immunology and Genomic Medicine, Kyoto University, Kyoto, Japan)\textsuperscript{31}. AID\textsuperscript{−/−} and CD45.1 mice were crossed to obtain AID\textsuperscript{+/−}CD45.1/2 mice for use as bone marrow hosts. BAFF transgenic (BAFF-Tg) mice were obtained from Dr. Fabienne Mackay (Garvan Institute, Darlinghurst, Australia)\textsuperscript{119}. BAFF-Tg mice contain the full length BAFF sequence along with a liver specific alpha-1-antitrypsin promoter and APO E enhancer resulting in transgenic BAFF being produced in the liver and spreading into the bloodstream. BAFF-Tg mice were crossed with AID\textsuperscript{−/−} mice to obtain BAFF-Tg\textsuperscript{+/−}AID\textsuperscript{+/−} mice for use as bone marrow hosts. OTII\textsuperscript{120} and MD4\textsuperscript{121} mice were obtained from the Jackson Laboratory (stocks 004194 and strain 002595, respectively). MD4 mice were crossed with AID\textsuperscript{−/−} and CD45.1 mice to obtain AID\textsuperscript{−/−}/MD4 and CD45.1/MD4 mice. Msh2\textsuperscript{−/−} mice were obtained from Tak Mak, Ontario Cancer Institute, Toronto, Canada\textsuperscript{122}. UNG\textsuperscript{−/−} mice were obtained from Hans Krokan, Department of Cancer Research and Molecular Medicine, Norwegian Univeristy of Science and Technology, Trondheim, Norway\textsuperscript{123}. UNG\textsuperscript{+/−} and Msh2\textsuperscript{+/−} mice were crossed to obtain Msh2\textsuperscript{−/−}UNG\textsuperscript{−/−} mice. All mice were housed in specific pathogen-free conditions. All animal experiments were performed in accordance with endpoints and standards of animal care approved by the University of Toronto, Faculty of Medicine Animal Ethics Committee (Protocols #20008480 and #20009022)

2.2 Bone Marrow Chimeras

To generate bone marrow (BM) chimeras, 6-8 week-old host mice were irradiated with two doses of 550eGy spaced 3-4hr apart using a Nordion Gammcell 40 Exactor. Following
irradiation, host mice were injected *intravenous* (i.v.) in the lateral tail vein with 2-3 x10^6 donor BM cells. Donor BM cells were prepared under sterile conditions by producing a single cell suspension in PBS. BM cells were isolated by centrifugation of end-snipped femurs and tibia in 1.5mL microtubes into PBS and then passed through a 70μm cell strainer. BM cells were then subjected to red blood cell (RBC) lysis by suspension in 1mL of 4°C ammonium chloride/potassium bicarbonate lysis buffer for 3min followed by immediate washing in PBS and re-suspension in PBS at 2-3 x10^7 cells/mL. For mixed BM chimeras, cell suspensions of the two donor types were mixed at ratios of 1:1, 1:3, or 3:1. BM chimeras were given water containing 2mg/mL Neomycin Sulfate (BioShop) for 1 week following irradiation. BM chimeras were then rested for 6-8 weeks prior to immunization or treatment. Bone marrow chimeras which received a secondary radiation dose following immunization received 500cGy.

### 2.3 Adoptive Cell Transfer

For adoptive transfer experiments, splenic B cells from AID^-/-/MD4 and CD45.1/MD4 mice and splenic T cells from OTII mice were isolated by magnetic negative selection with EasySep kits (STEMCELL Technologies). Purified cells were then strained and suspended in PBS. 2x10^6 of both genotypes of B cell and 3.5x10^5 OTII T cells were injected i.v. into 6-8 week-old host AID^-/-/CD45.2/- mice via the lateral tail vein. These mice were then rested for 24hr prior to immunization.

### 2.4 Immunizations and Treatments

AID^-/-/CD45.1, BAFF-Tg^-/-/AID^-/-, and UNG^-/-/Msh2^-/- mixed BM chimeras were immunized *intraperitoneum* (i.p.) with 100μL of a 1:1 mixture of 2mg/mL NP-CGG hapten-carrier conjugate (Biosearch Technologies) in PBS and alum adjuvant (Thermo Scientific) for a total of 100ug of NP-CGG protein injected per mouse. The ratio of hapten:carrier from lot to lot of NP-CGG varied from 24-35:1. For adoptive transfer experiments, host mice were immunized *subcutaneous* (s.c.) in each hind flank and i.p. with 50μg of HEL-OVA and 0.07μg LPS in alum adjuvant in a 50μL volume per injection. HEL-OVA protein was produced by conjugating Hen Egg Lysozyme with Chicken Ovalbumin (Sigma-Aldrich) using a S-HyNic/S-4FB Protein-Protein conjugation kit (SoluLink). AID-GFP→C57Bl/6 and AID-GFP x LTβ^-/-→C57Bl/6 BM chimeras were immunized s.c. in both hind flanks with 50μL of a 1:1 PBS:CFA mixture (CFA from Difco Laboratories) containing 0.4mg/mL (high dose) or 0.035mg/mL (low dose) R-PE
(Anaspec) or 0.4mg/mL of an irrelevant Ag (NP-CGG, Biosearch Technologies). For LTβR-Ig treatment, mice were injected i.p. on day -2 and day 5 p.i. with 100ug of a murine fusion protein comprised of LTβR and the Fc portion of the Ig (LTβR-Ig) (Biogen-Idec)\textsuperscript{108}. Control mice were injected i.p. on day -2 and day 5 p.i. with 100ug of MOPC21 mouse IgG1 isotype control.

2.5 Flow Cytometry

For flow cytometric analysis of splenic cells, spleens were gently ground between glass slides in PBS and passed through a 70μm cell strainer. BM cells were isolated by centrifugation of end-snipped femurs and tibia in 1.5mL microtubes into PBS and then passed through a 70μm cell strainer. Both splenic and BM cells were then suspended in 1mL of 4°C ammonium chloride/potassium bicarbonate lysis buffer for 3min to lyse red blood cells and then washed and re-suspended in PBS. For lymph node cells, lymph nodes were gently ground between glass slides and suspended in 3mL HBSS with 1mg/mL collagenase D and 60μg/mL DNase I (Roche Diagnostics) for 30min at 37°C followed by the addition of 6μL 1M EDTA. Cell suspensions were then filtered through a 70μm strainer and re-suspended in PBS. Prior to staining, cells were blocked with normal mouse serum (Jackson ImmunoResearch) and 2.4G2 mAb. For certain experiments cells were labeled with Live/Dead Aqua (Life Technologies) prior to staining or with 7-AAD (BD) after staining to label dead and dying cells. Cells were stained with antibodies against B220 (mAb RA3-6B2), CD1d (mAb 1B1), CD23 (mAb B3B4), CD45.1 (mAb A20), CD11b (mAb RA3-6B2), F4/80 (mAb BM8), and CD45.2 (mAb 104) (eBioscience); against CD169 (mAb MOMA-1) (GeneTex); against GL7 Ag, Fas (mAb Jo2), CXCR4 (mAb 2B11/CXCR4), IgM (mAb R6-60.2), CD21 (mAb 7G6), and CD138 (mAb 281-2) (BD); against phospho-H2A.X (mAb 20E3) (Cell Signalling); and against IgMa (mAb MA-69) and IgMb (mAb AF6-78) (Biolegend). Biotinylated Abs were labeled with streptavidin conjugated to PE-TexasRed (BD), APC, or APC-eFluor780 (eBioscience). Cells were labeled for antigen-specificity with NP-PE (Biosearch Technologies) or R-PE (Anaspec). For caspase 3 staining, cells were stained with CaspGLOW Fluorescein Active Caspase-3 staining kit (BioVision) following surface staining. Intracellular stains were performed using Cytofix/Cytoperm (BD), except for stains involving anti-phospho-H2A.X in which cells were permeabilized in -20°C methanol prior to staining. Fluorescent-labeled cells were analyzed on a BD FACSCalibur, FACSAria, or FACSCanto II flow cytometer.
2.6 Histology

Lymph nodes and spleens were gently blotted with paper tissue to remove excess moisture, immersed in OCT compound, and flash frozen in -80°C 2-methyl-butane. Frozen tissues were then sectioned at -20°C using a Leica cryostat to produce 6-8μm thick sections. Tissue sections were adhered to glass slides and fixed in -20°C acetone for 7min and allowed to dry before staining. Prior to staining, tissue sections were blocked with normal mouse serum (Jackson ImmunoResearch) and 2.4G2 mAb, except for sections which were to be stained with an anti-Rat secondary probe in which case only normal mouse serum was used. Lymph node sections were stained fluorescently with APC-conjugated Abs against B220 (mAb RA3-6B2) (eBioscience) and Mfg-e8 (mAb FDC-M1), CD35 (mAb 8C12), CD138 (mAb 281-2) (BD) as well as biotin-conjugated PNA (Vector Labs), anti-IgD (mAb 11-26) (eBioscience), and anti-fibronectin (Sigma). Biotin-conjugated reagents were then stained with streptavidin-APC (eBioscience). Unlabeled rat anti-mouse ER-TR7 (BMA Biomedical) was labeled with anti-rat-FITC (Southern Biotech). Ag-specific Ig was detected by applying R-PE directly to the tissue (Anaspec). The AID-GFP reporter transgene was too dim for direct histological visualization and so was amplified by staining with chicken anti-GFP pAb (Aves Labs) followed by anti-chicken-AlexaFluor488 (Life Technologies). Spleen sections were stained immunohistochemically with FITC- or biotin-conjugated antibodies against GL7 Ag (BD), IgD (mAb 11-26c, Southern Biotech), CD45.1 (mAb, A20), and CD45.2 (mAb 104) (eBioscience). These primary probes were then labeled with anti-FITC-AP (alkaline phosphatase) (Roche) or streptavidin-HRP (horseradish peroxidase) (Prozyme) and developed with AP and HRP substrate kits (Vector Laboratories). Sections were mounted with Dako Fluorescent Mounting Medium or Clarion mounting medium and visualized on a Leica DMRA2 microscope. Pictures were taken at 10x and 20x objective magnification on a QImaging Retiga EXi camera.

2.7 ELISA

Blood was collected from immunized mice and centrifuged to isolate the serum fraction. To measure Ag-specific Ab, wells in NUNC MaxiSorp plates (Thermo Scientific) were coated with 10μg/mL R-PE (Anaspec) overnight at 4°C and then blocked with 2%BSA/PBS for 1hr at 37°C. To measure relative Ab titers, R-PE-coated wells were incubated with a dilution series of serum from 1/100*2^0 to 1/100*2^13 for 2hrs at 37°C and then probed using biotinylated Abs against IgG (pAb Poly4053) or IgM (mAb RMM-1) (BioLegend). Wells were then incubated
with streptavidin-HRP enzyme, developed with liquid TMB substrate (BioShop), stopped with 1M H₂SO₄, and read at 450nm. Relative titers were calculated as the serum dilution at 1OD relative to a serum control. To measure relative Ag-affinity, we modified a protocol adapted from the literature. R-PE coated wells were incubated with a quantity of serum such that each well for each sample contained the same amount of anti-R-PE Ab (as calculated by relative titer ELISA). Wells were then incubated for 15min with a concentration range of ammonium thiocyanate from 0M to 1.2M in 0.1M phosphate buffer at pH7.15 before being washed. Wells were then probed and developed as described above for quantitative ELISA. Each sample was then plotted on a graph of ammonium thiocyanate concentration versus the logarithm of the OD as a percentage of the OD at 0M ammonium thiocyanate and fitted with a third-order polynomial regression line. The affinity index of each sample was calculated from the regression line as the molar concentration of ammonium thiocyanate required to reduce OD by 50% from the OD recorded at 0M ammonium thiocyanate.

2.8 ELISPOT

MultiScreen-HTS-HA filter plates (Millipore) were coated with 50μg/mL NP₃₀-BSA (Biosearch Technologies) in 1%BSA/PBS o/n at 4°C and blocked with 2%BSA/PBS for 4hrs at 37°C. RBC-lysed splenic or bone marrow cells were suspended in RPMI1640 with 50uM 2-ME, 10%FBS, and 1x Pen/Strep (Sigma-Aldrich), plated in wells at 5x10⁴-5x10⁵ cells/well, and incubated o/n at 37°C. Plates were washed thoroughly with 0.05% Tween20/PBS and probed with FITC-conjugated or biotinylated mAbs against IgMa, IgMb, and IgG (Biolegend). Anti-FITC (pAb, Roche Diagnostics) or streptavidin (ProZyme) conjugated to alkaline phosphatase enzyme was then used as a secondary probe. Wells were then developed with 50μL SigmaFast BCIP/NBT substrate (Sigma-Aldrich) for 20min and washed thoroughly. Spots were counted manually on a dissection microscope.

2.9 rtPCR

Fluorescently-labeled GC B cells were sorted using a FACSaria and RNA was isolated using a RNEasy Kit (Qiagen). Reverse transcription was performed on RNA samples with oligo-dT primers and Superscript III reverse transcriptase (Invitrogen) to generate cDNA. Real time PCR was performed on cDNA samples with primers against Bcl6 (F 5-ACTAGCGTGCCGGGTAAACT-3, R 5-AGTTTCTAGGAAAGGGCGGA-3),
Blimp1 (F 5-TAGACTTCACCGATGAGGGG-3, R 5-ACCAAGGAACCTGCTTTTCA-3),
and HPRT (F 5-CCCAGCGTCGTGATTAGC-3, R 5-GGAATAAACACTTTTTCCAAAT-3)
in Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific/Fermentas). Reactions
were performed in triplicate. The average Ct of triplicates and reaction efficiency were analyzed
using the Pfaffl\textsuperscript{126} method to determine the relative expression of Bcl6 and Blimp1 using HPRT
expression as the endogenous control\textsuperscript{127}.

2.10 Analysis

All data collected by flow cytometry using CellQuest or FACSDiva software (BD) was
analyzed using FlowJo software. Statistical analyses were performed using GraphPad Prism
software. Differences in group means were tested for statistical significance by student’s t-test.
Differences in group means whose p-values were lower than 0.05 were considered significant.
For ELISA analysis, third order polynomial regression lines were fitted using MS Excel
software. Histological photographs were taken in OpenLab software and saved in .tiff format.
Tiff files were analyzed in Volocity software to measure surface area. Images were color-
balanced, cropped, and re-sized using Adobe Photoshop CS software.
Chapter 3: AID-expressing germinal center B cells cluster normally within peripheral lymph nodes in the absence of follicular dendritic cells but dissipate prematurely


Michael Le assisted with histology and ELISA. Lingjin Meng assisted with histology. Lesley Ward assisted with ELISA. Dennis Ng assisted in the generation of BM chimeras. LTβR-Ig reagent was provided by Biogen-Idec.

Upon activation with T-dependent Ag, B cells enter germinal centers (GC) and up-regulate Activation-induced Deaminase (AID). AID⁺ GC B cells then undergo class-switch recombination and somatic hypermutation. Follicular dendritic cells (FDC) are stromal cells that underpin GCs and require constitutive signaling through the lymphotoxin beta receptor (LTβR). Although it has been shown that FDCs can be dispensable for the generation of high affinity Ab, in the absence of FDCs it is unclear where AID expression occurs. In a mouse model that lacks FDCs as well as other LT-sensitive cells I show that clusters of AID⁺PNA⁺GL7⁺ Ag-specific GC B cells cluster within the B cell follicle of the draining lymph node, suggesting that FDC are not strictly required for GC formation. However, later in the primary response FDC-less GCs dissipated prematurely, correlating with impaired affinity maturation. I examined whether GC dissipation was due to a lack of FDCs or of Ag-bearing subcapsular sinus macrophages (SCS MΦ), which are also LTβR-dependent, and found that in response to non-replicating protein Ag FDCs proved to be more critical for long term GC maintenance. These findings provide a spatial-temporal analysis of Ag-specific B cell activation and AID-expression in the context of a peripheral lymph node that lacks FDC and other LT-sensitive cell types, and reveals that FDCs are not strictly required for the induction of AID within an organized GC-like environment.
3.1 Introduction

T-dependent immune responses take place within germinal centers (GC) located in the follicles of secondary lymphoid organs. GCs are clusters of activated B cells that undergo proliferation, class-switch recombination (CSR), somatic hypermutation (SHM), and clonal selection. Together, these processes effect a robust humoral immune response for clearing infections\textsuperscript{86, 128}.

Activation-induced deaminase (AID) is a DNA-editing enzyme that is expressed by B cells following activation and is required to initiate CSR and SHM\textsuperscript{31, 129}. B cells up-regulate AID as they enter GCs to undergo Ab diversification. GC B cells subsequently down-regulate AID as they exit GCs to become memory or plasma cells\textsuperscript{41}.

GCs are underpinned by a type of non-hematopoietic stromal cell known as a follicular dendritic cell (FDC). FDCs secrete chemokines and survival factors and are thought to be crucial for GC structure and function\textsuperscript{100}. Both the development of FDCs and the maintenance of their phenotype require constitutive signaling through the lymphotoxin (LT) beta receptor (LT\(\beta\)R); FDCs are induced to mature \textit{in situ} from a perivascular precursor through engagement of LT\(\beta\)R on FDCs by membrane-bound LT\(\alpha\beta\) expressed on lymphocytes\textsuperscript{97, 103}. Activated B cells within GCs express even higher levels of LT\(\alpha\beta\) than their naive counterparts which results in higher LT\(\beta\)R-signaling to GC FDCs\textsuperscript{130, 131}. This increased level of LT\(\beta\)R signaling induces a secondary maturation of FDCs into a phenotype which is important in supporting the maturation of the humoral immune response. In mice in which LT signaling has been ablated, such as by genetic removal of LT\(\beta\), peripheral lymph nodes do not develop and the architecture of secondary lymphoid follicles in the spleen is disrupted. Notably, ablation of LT\(\beta\)R signaling results in the absence of identifiable FDCs\textsuperscript{106} and impaired formation of splenic GCs\textsuperscript{107, 116, 117}. As well, selective ablation of FDCs using diphtheria toxin has been shown to abruptly eliminate GCs\textsuperscript{132}. For these reasons, FDCs are thought to be crucial in the formation and structure of GCs.

The initiation of GCs requires B cells to become activated by binding Ag and then receiving co-stimulation from CD4\(^+\) T cells. The infiltration of Ag into B cell follicles is therefore crucial. Small soluble Ag of less than 70kDa in size can enter LN follicles through conduits\textsuperscript{133}, while larger Ag can be transported into follicles by subcapsular sinus macrophages (SCS M\(\Phi\))\textsuperscript{134, 135}. Ag that has infiltrated into the follicles is deposited on FDCs that retain Ag complexes through Fc and complement receptors on their surfaces.
GCs are the primary location of AID expression during T-dependent immune responses\textsuperscript{32, 41}. Though the mutagenic activity of AID is important for a robust humoral response, it can also generate autoreactive B cell clones or produce oncogenic mutations\textsuperscript{43}. It would be expected that the expression of AID would therefore be limited to microenvironments such as GCs, which contain FDCs and CD4\textsuperscript{+} T cells, where B cells are regulated and subjected to tolerance checks. However, B cells in LT\textalpha\textsuperscript{−/−} mice, which lack FDCs and GCs, are able to undergo CSR and SHM in response to immunization with high doses of Ag\textsuperscript{136}. This suggests that AID can be expressed outside of a canonical GC microenvironment during a T-dependent immune response. If AID can indeed be expressed outside of GCs, it is important to understand the location of AID expression and which microenvironments allow B cells to express AID. This question has clinical relevance to autoimmunity and ectopic tertiary lymphoid tissues that may be able to support AID expression\textsuperscript{137}.

In this chapter I sought to determine the location of AID-expressing Ag-specific B cells in the absence of FDCs within the LNs of LT\textbeta\textsuperscript{−/−} bone marrow (BM) chimeric mice. While the splenic lymphoid environment is noticeably disrupted in the absence of LT signaling, the architecture of the LNs remains mostly unaffected and allowed me to look for normal niches that could support AID-expressing B cells in a secondary lymphoid organ\textsuperscript{98, 107}. In the LNs of LT\textbeta\textsuperscript{−/−} BM chimeras, I observed that the initiation of GC responses is surprisingly normal and, at the peak of the primary response, AID\textsuperscript{+} GC B cells were able to form GC-like clusters in the absence of FDCs. Although the initiation and the peak of the GC response was normal in the absence of FDCs and LT\textbeta R signaling, these GCs disappeared prematurely and such abortive GCs correlated with impaired affinity maturation. Ablation of LT\textbeta R signaling not only eliminates FDCs, but also results in a significant reduction in the number of SCS M\Phi. Using a mixture of genetic and pharmacological inhibition of LT\textbeta R signaling to ablate SCS M\Phi and/or FDCs I observed only a weak correlation between the number of SCS M\Phi and the number of GC B cells, whereas a significant drop in the number of GC B cells late in the response occurred only when FDCs were ablated. These findings suggest that FDC are not required to create a permissive niche for the expression of AID in GC B cells. Surprisingly, these findings also demonstrate that FDCs are not required for GC B cells to form clusters in peripheral LNs although FDCs do appear to be important in the longer-term maintenance of GCs and subsequent high-affinity Ab responses.
3.2 Results

3.2.1 GC B cells develop in the absence of LTβ

In this study I employed a system for studying the humoral immune response within LNs using tools that allowed me to track Ag-specific B cells that express AID. Specifically, I tracked the GC response in the draining inguinal lymph node (iLN) following s.c. immunization with R-PE Ag in CFA adjuvant. Because LTβ−/− mice lack peripheral LNs106, 138 I created BM chimeric mice by reconstituting irradiated C57Bl/6 mice with LTβ−/− BM, thus producing iLN-sufficient LTβ-deficient mice (Figure 3.1A).

To track Ag-specific B cells I used the fluorochrome R-PE as a model protein Ag. B cells activated by R-PE in vivo can later be stained with R-PE ex vivo139, 140. To track AID expression in Ag-specific B cells, I used mice with an AID-GFP fusion protein transgene under the control of AID cis-elements41. The AID-GFP reporter induces fluorescence in AID-expressing GC B cells which is then quenched upon down-regulation of AID. LTβ−/− mice were crossed with AID-GFP mice to track AID expression in the absence of LTβ. Both AID-GFP x LTβ−/− and AID-GFP (WT) mice were used as BM donors to transfer into C57Bl/6 hosts. This allowed me to induce GCs within iLNs in the presence or absence of LTβ. AID-GFP→C57Bl/6 BM chimeras are hereafter referred to as AID-GFP (WT) BM chimeras and LTβ−/−/AID-GFP→C57Bl/6 BM chimeras are referred to as AID-GFP x LTβ−/− BM chimeras (Figure 3.1A). I confirmed the ablation of LTAβ-induced LTβR signaling in AID-GFP x LTβ−/− BM chimeras based on the reduced cellularity of iLNs (Figure 3.1B), consistent with the role of LTβR signaling in regulating the maturation status of high endothelial venules which are the portals for lymphocytes141. AID-GFP x LTβ−/− BM chimeras also had significantly reduced populations of splenic marginal zone (MZ) B cells, consistent with observations that the MZ of the spleen dissipates in the absence of LT signaling (Figure 3.1C)142. Chimeras contained less than 1% host-derived B cells but up to 5% host T cells (data not shown). Although residual host T cells can express LTAβ, it has been demonstrated that it is B cell-derived LTAβ that is required to generate mature FDC networks98, 143.

I next tested the GC response to R-PE in the draining iLN of AID-GFP (WT) and AID-GFP x LTβ−/− BM chimeras. I observed that mice immunized with an irrelevant Ag (NP-CGG) in adjuvant generate AID-GFP+ B cells that do not stain with R-PE, and B cells from mice immunized with R-PE do not fluoresce in the PE channel when R-PE is not added to the flow cytometry stain panel (Figure 3.2A). I then immunized AID-GFP (WT) and AID-GFP x LTβ−/−
**Figure 3.1. Confirmation of ablation of LTβR signaling in BM chimeric mice.** (A) A diagram illustrating the generation AID-GFP (WT) and AID-GFP x LTβ−/− BM chimeric mice. (B) The number of total cells and B cells in the iLNs of BM chimeras day 5 p.i. with R-PE/CFA, prior to the appearance of GCs. AID-GFP→WT n=8; AID-GFP x LTβ−/−→WT. Student’s unpaired t-test; *, P<0.05. Columns depict mean ± SEM, n=7 for each genotype. Data from two independent experiments is shown. (C) Representative flow cytometry of splenocytes from BM chimeras. The left column depicts total B220+ cells. The right column depicts cells from the IgM+CD23− gate. In the right column a gate is drawn around CD1dhiCD21+ marginal zone (MZ) B cells.

**Figure 3.2. Flow cytometry of AID-GFP+R-PE+ GC B cells.** (A) AID-GFP (WT) BM chimeric mice were immunized s.c. with R-PE/CFA or NP-CGG/CFA and B cells (B220+) from the draining iLN were analyzed by flow cytometry on day 7 post-immunization (p.i.) to establish the parameters of background R-PE staining of AID-GFP+ B cells. In the case of R-PE immunized mice, samples were stained with all fluorescent markers except R-PE (R-PE FMO). In the case of NP-CGG-immunized mice, samples were stained with R-PE. (B) B220+ B cells from the iLNs of R-PE/CFA immunized AID-GFP (WT) and AID-GFP x LTβ−/− BM chimeras on day 7 p.i. were analyzed for R-PE Ag-specificity and for AID-GFP expression. (C) The percentage of B220+AID-GFP+R-PE+ GC B cells from 7 AID-GFP (WT) and 7 AID-GFP x LTβ−/− BM chimeras on day 7 post-immunization. Columns depict mean ± SEM, n=7 for each genotype. (D) Representative histograms of the levels of Fas, GL7, and AID-GFP on B220+AID-GFP+R-PE+ GC B cells from iLNs of AID-GFP (WT) and AID-GFP x LTβ−/− BM chimeras on day 7 p.i. Representative data from three independent experiments is shown.
Figure 3.1

A

AID-GFP or AID-GFP x LTβ-/-

2x10^6 BM cells

2 x 550cGy

C57BL/6

C57BL/6

B

* * *

Cell# (x10^7)

<table>
<thead>
<tr>
<th></th>
<th>Total Cells</th>
<th>B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AID-GFP → WT</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>AID-GFP x LTβ-/- → WT</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

C

AID-GFP → WT

8.65

AID-GFP x LTβ-/- → WT

2.98

MZ B

40.8

4.36

CD23

CD21
Figure 3.2

A

B

C

D

B220^+ AID-GFP^+ R-PE^+ GC B cells

Fas — GL7 — AID-GFP

AID-GFP Follicular B cells
AID-GFP GC B cells
AID-GFP x LTβ^+ GC B cells
BM chimeras s.c. with R-PE in each hind flank to generate humoral responses in both iLNs. At day 7 of the response I observed a significant population of AID-GFP⁺R-PE⁺ B cells in AID-GFP (WT) and AID-GFP x LTβ⁻⁻ BM chimeras by flow cytometry (Figure 3.2B). Surprisingly, the percentage of AID-GFP⁺R-PE⁺ B cells in the iLN of AID-GFP x LTβ⁻⁻ BM chimeras was roughly equal to that in the iLN of AID-GFP (WT) chimeric mice (Figure 3.2C). I analyzed the expression of other GC B cell markers on AID-GFP⁺R-PE⁺ B cells from both types of BM chimeric mice and found that they displayed the same levels of Fas, GL7, and AID-GFP (Figure 3.2D). Taken together, these observations indicate that significant numbers of normal AID-expressing GC B cells are produced in the draining iLN in the absence of LTβ.

3.2.2 AID-GFP⁺ GC B cell clusters form in the absence of LTβ and FDCs

Given that AID-GFP⁺ GC B cells are readily detected in the draining iLN of immunized AID-GFP x LTβ⁻⁻ BM chimeric mice, I sought to determine the location of AID expression by immunofluorescence microscopy. At day 7 post-immunization, serial cross sections of iLNs from AID-GFP (WT) BM chimeric mice revealed discrete clusters of AID-GFP⁺ B cells located within B cell follicles (Figure 3.3A). AID-GFP fluorescence around the edges of LN sections are non-specific and are observed in transgene negative mice (not shown). These clusters of AID-GFP⁺ cells stained with PNA, indicating that they contained activated GC B cells. I also found that these GCs were populated with FDCs by staining for FDC-M1 and CD35. Staining sections with R-PE visualized R-PE-specific Ab. This revealed that GCs were bordered on the T cell zone-proximal side by clusters of CD138⁺ plasma cells secreting anti-R-PE Ab and the medullary cords were filled with anti-R-PE CD138⁺ plasma cells. R-PE staining did not reveal R-PE-immune complexes on FDCs; Ig in FDC-bound immune complexes likely have their Fab occupied by the R-PE immunogen and so would not react with R-PE added later as a staining reagent. Surprisingly, iLNs from AID-GFP x LTβ⁻⁻ BM chimeras also contained clusters of AID-GFP⁺ PNA⁺ B cells within B cell follicles as well as anti-R-PE CD138⁺ cells in clusters adjacent to GCs and in the medullary cords (Figure 3.3B). However, as expected, these clusters of AID-GFP⁺ PNA⁺ B cells did not contain any FDCs as verified by the absence of FDC-M1 and CD35 staining (Figure 3.3B, Figure 3.4). Therefore these observations show that clusters of AID⁺PNA⁺ GC B cells form in the iLNs of AID-GFP x LTβ⁻⁻ BM chimeras in the absence of mature FDCs. Because these B cells form clusters situated in the follicle and because they express numerous markers of the GC B cell phenotype (AID, GL7, Fas, PNA) I refer to these clusters as GCs in spite of their lack of a secondary FDC network.
Figure 3.3. Histological examination of the anti-R-PE response in iLN. Representative serial cross sections of iLN GCs. All tissues were stained with anti-GFP (green) and with R-PE (red), which stains secreted and membrane-bound anti-R-PE Ig. (A) Serial cross sections of an iLN from an AID-GFP (WT) BM chimera on day 7 p.i. with R-PE/CFA. The center panel is stained for B220 (blue) and depicts the entire LN as a composite of images taken at 10x objective magnification. Yellow boxes denote areas displayed to either side at 20x magnification. The upper-left yellow box denotes a GC which is shown at 20x magnification stained in blue for each PNA, Mfge8 (FDC-M1), CD35, and CD138. The lower-right yellow box denotes the LN medullary cord and is stained for CD138 (blue). (B) Serial cross section of an iLN from an AID-GFP x LTβ−/− BM chimera on day 7 p.i. with R-PE/CFA. Stains and magnifications are the same as described above. The left yellow box highlights a GC and the right yellow bow highlights the medullary cord. Scale bars represent 100µm (10x) and 50µm (20x).

Figure 3.4. AID-GFP+ GC B cell clusters in the absence of FDCs in AID-GFP x LTβ−/− BM chimeras.

Representative serial cross sections of four GCs from four AID-GFP x LTβ−/− →WT mice at (A,B) day 7 and (C,D) day 9 p.i. from two independent experiments. All tissues are stained with anti-GFP (green), R-PE (red), which stains secreted and membrane-bound anti-R-PE Ig; and with PNA, Mfge8 (FDC-M1), or CD35 (blue). Columns depict serial sections of iLN GCs. Pictures are taken at 20x objective magnification. Scale bars represent 50µm.
Figure 3.3

AID-GFP → WT, Day 7 p.i.

AID-GFP × LTβ−/− → WT, Day 7 p.i.
Figure 3.4

A  AID-GFP x LTβ⁺ → WT, Day 7 p.i.

B  AID-GFP x LTβ⁺ → WT, Day 7 p.i.

C  AID-GFP x LTβ⁺ → WT, Day 9 p.i.

D  AID-GFP x LTβ⁺ → WT, Day 9 p.i.
FDCs are but one of a number of stromal cell types found in secondary lymphoid organs. Another type of stromal cell, fibroblastic reticular cells (FRC), are found primarily in T cell zones but are also found in lower densities in B cell follicles. They form conduits comprised of a collagen core that is ensheathed by fibronectin and ERTR7 Ag. These conduits have been shown to transport small Ag and chemokines. As well, FRCs or MRCs are a possible source of CXCL13 chemokine, which is important for attracting B cells and T follicular helper cells. By histology, I observed that Fibronectin/ERTR7 networks are intact in the iLNs of AID-GFP x LTβ-/- BM chimeras and extend into GCs (Figure 3.5). Though not conclusive, this observation, in the context of what is known about FRCs, highlights a possible redundancy that allows for the formation of GCs in the absence of FDCs.

### 3.2.3 LTβ is required for the maintenance but not the initiation of GCs

I next investigated the kinetics of the GC response in FDC-less BM chimeras to look for differences. I immunized AID-GFP (WT) and AID-GFP x LTβ-/- BM chimeric mice as before and quantified AID-GFP^+R-PE^+ B cells by flow cytometry. GC B cells were not present in iLNs at day 5 post-immunization and then suddenly appeared at day 6 (Figure 3.6A). The initial response in AID-GFP x LTβ-/- BM chimeras was slightly delayed; there were fewer AID-GFP^+R-PE^+ B cells in AID-GFP x LTβ-/- BM chimeras at day 6, however that number had caught up to or exceeded the number found in AID-GFP (WT) BM chimeras by day 7 (Figure 3.6A). While the numbers of GC B cells slowly declined in AID-GFP (WT) BM chimeras after day 7, they were still present by day 12 (Figure 3.6B). In contrast, GC B cells in AID-GFP x LTβ-/- BM chimeras had completely vanished by day 12 (Figure 3.6B). Similar results were observed using immunofluorescence microscopy (Figure 3.7). Because FDC are thought to trap Ag through complement and Fc receptors for protracted presentation to Ag-specific B cells, I considered that high doses of Ag may alleviate the need for FDC-mediated Ag capture. To test this, I repeated the kinetic analysis by immunizing with a low dose of Ag (1.75µg R-PE per injection, as opposed to 20µg for the high dose). The low dose immunization resulted in much weaker GC responses (Figure 3.6C). Similar to the high dose response, the number of GC B cells in the draining iLN at the beginning (day 7) was slightly delayed in AID-GFP x LTβ-/- BM chimeras compared to AID-GFP (WT) chimeras, before catching up at the peak (day 9) (Figure 3.6C). As well, the response was sustained until day 12 in AID-GFP (WT) BM chimeras while it had completely dissipated by day 12 in the AID-GFP x LTβ-/- BM chimeric mice (Figure 3.6D). Therefore, LTβR signaling and FDCs are
Figure 3.5. Histological observation of ERTR7⁺Fibronectin⁺ networks within GC structures independent of LTβR-signaling. Cross sections of draining iLN from PE-immunized (A) AID-GFP (WT) and (B) AID-GFP x LTβ⁻/⁻ BM chimeric mice. Staining for fibronectin (Fn) networks throughout the draining LN (i) and within IgD⁻ GCs (ii). Serial sections of a single GC reveal Fn and ERTR7⁺ networks within an AID⁺PNA⁺ GC regardless of the presence (A, iii) or absence (B, iii) of CD35⁺FDC-M1⁺ FDCs (iii). (A, i) and (B, i) are composites of images taken at 10x objective magnification. (A, ii and iii) and (B, ii and iii) were taken at 20x objective magnification. Scale bars represent 100µm (10x) and 50µm (20x).

Figure 3.6. Kinetics of the R-PE-specific GC B cell response by flow cytometry. (A, C) Kinetic of B220⁺AID-GFP⁺R-PE⁺ GC B cells in the iLN of AID-GFP (WT) and AID-GFP x LTβ⁻/⁻ BM chimeras after s.c. immunization with (A) a high dose (20µg) or (C) a low dose (1.75µg) of R-PE in CFA, measured by flow cytometry. (B, D) Representative flow cytometry of B220⁺AID-GFP⁺R-PE⁺ GC B cells on day 12 post-immunization with (B) a high dose or (D) a low dose of R-PE in CFA. Student’s unpaired t-test performed for all statistical tests; *, P<0.05; **, P<0.01; ***, P<0.001. Line points (A, C) depict mean ± SEM, n=3-12 for each genotype at each timepoint. Data from (A) five and (C) four independent experiments are shown.
**Figure 3.5**

**A**

(i) AID-GFP → WT, Day 7 p.i.

(ii) [Imagery]

(iii) [Imagery]

**B**

(i) AID-GFP x LTβ^+ → WT, Day 7 p.i.

(ii) [Imagery]

(iii) [Imagery]
Figure 3.6

**A**
High Dose Immunization

- % of B220 Cells
- Cell # (x10^6)

**B**
Day 12 p.i., High Dose
- AID-GFP → WT
- AID-GFP x LTβ−/− → WT

**C**
Low Dose Immunization

- % of B220 Cells
- Cell # (x10^6)

**D**
Day 12 p.i., Low Dose
- AID-GFP → WT
- AID-GFP x LTβ−/− → WT
dispensable for the initiation of Ag-specific GC B cell expansion and clustering in the draining iLN, but LTβR signaling is important for the long-term maintenance of GC B cell numbers both at low and high doses of Ag.

### 3.2.4 LTβ is required for optimal affinity maturation

Given that GC B cells were not sustained in the draining iLN of AID-GFP x LTβ−/− BM chimeras following immunization, I assessed the effect of early GC dispersal on the affinity of the anti-R-PE humoral response after high dose immunization. I used ELISA to measure the amount of anti-R-PE Ab generated. AID-GFP (WT) BM chimeras had slightly more serum anti-R-PE IgM at day 6 (Figure 3.8A), in agreement with my previous observation that AID-GFP+R-PE+ GC B cells appear in slightly higher numbers at day 6 in AID-GFP (WT) than in AID-GFP x LTβ−/− BM chimeras (Figure 3.6). Also, there was a significant drop in the levels of serum anti-R-PE IgG in AID-GFP x LTβ−/− BM chimeras at day 20 that did not occur in AID-GFP (WT) BM chimeras, likely due to the earlier termination of the GC response in AID-GFP x LTβ−/− BM chimeras (Figure 3.8B). However, the titers of serum anti-R-PE IgM and IgG were the same in both types of chimeras at the peak of the response as were the longer-term (day 48) levels of serum anti-R-PE IgG, indicating that there was no significant defect in the production of antigen-specific Ab in AID-GFP x LTβ−/− BM chimeras (Figure 3.8A,B).

Although I observed no major defects in PE-specific IgM and IgG titres, AID-GFP x LTβ−/− BM chimeras produced serum IgG with significantly lower affinity for R-PE, indicating a defect in affinity maturation (Figure 3.8C). JH sequences from LTβ−/− B cells exhibited AID-induced mutations, indicating that SHM is indeed occurring in AID-GFP x LTβ−/− BM chimeras (data not shown). Therefore, the inability to sustain the GC response in AID-GFP x LTβ−/− BM chimeric mice corresponds with poor affinity maturation.

### 3.2.5 Premature GC dissipation is primarily due to the absence of FDCs

Although the early dissipation of GCs and the impaired affinity maturation in AID-GFP x LTβ−/− BM chimeras correlate with the absence of FDCs, I could not definitively conclude that they were caused by the absence of FDCs. This is because the absence of LTβR signaling has effects other than ablating FDCs, most importantly it results in a significant reduction in the number of SCS MΦ135. SCS MΦ trap immune complexes in the SCS and transfer them to non-cognate B cells which in turn deliver the Ag complexes to FDCs134. The interactions between
Figure 3.7. Kinetics of the R-PE-specific GC B cell response by histology. All tissues were stained with anti-GFP (green), with R-PE (red), which stains secreted and membrane-bound anti-R-PE Ig, and with anti-B220 (blue). Representative cross-sections of iLNs from (A) AID-GFP (WT) and (B) AID-GFP x LTβ−/− BM chimeric mice at days 5, 7, and 12 p.i. with R-PE/CFA. Images are composites of pictures taken at 10x objective magnification. Scale bars represent 100µm.

Figure 3.8. Relative titer kinetics and relative affinity for Ag of serum Ab specific for R-PE. The kinetics of the appearance of serum anti-R-PE (A) IgM and (B) IgG in BM chimeras immunized with a high dose of R-PE, measured by ELISA. Each type of BM chimera is represented at each time point by 4-9 individual mice. All columns depict mean ± SEM, n=4-9 for each genotype at each timepoint. (C) The measurement of the affinity of serum IgG for R-PE by denaturing ELISA. Higher values for affinity index (y-axis) denote higher affinity for Ag. Student’s unpaired t-test performed for all statistical tests; ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001. All columns depict mean ± SEM, n=6-9 for each genotype at each timepoint. Data from five (A,B) and two (C) independent experiments are shown.
Figure 3.7

AID-GFP → WT

AID-GFP x LTβ−/− → WT
Figure 3.8

A

α-R-PE IgM Titers

- AID-GFP → WT
- AID-GFP x LTβ± → WT

Relative Units

Day 5 Day 6 Day 7 Day 9 Day 12 Day 20 Day 48

B

α-R-PE IgG Titers

- AID-GFP → WT
- AID-GFP x LTβ± → WT

Relative Units

Day 5 Day 6 Day 7 Day 9 Day 12 Day 20 Day 48

C

α-R-PE IgG Ag-Affinity

- AID-GFP → WT
- AID-GFP x LTβ± → WT

Affinity Index

Day 12 Day 20 Day 48

* ns *** *** ***
FDCs and SCS MΦ may therefore be important to the deposition of Ag complexes within GCs that are required to sustain a GC response.

To determine whether the absence of FDCs, SCS MΦ, or both was the cause of early GC dissipation, I generated mixed BM chimeras by transferring LTβR−/− BM into C57Bl/6 hosts. In these LTβR−/− BM chimeras, the lack of LTβR on hematopoietic cells results in a significant deficiency of SCS MΦ135 while FDCs, which are radio-resistant, are unaffected. Treatment with LTβR-Ig, a soluble decoy receptor that blocks LTβ-LTβR interactions108,150, ablates both FDCs and SCS MΦ. By treating WT and LTβR−/− BM chimeras with or without LTβR-Ig I was able to ablate FDCs and/or SCS MΦ in order to determine the contribution of each cell type to sustaining GCs.

I immunized LTβR−/− and WT control BM chimeras with R-PE as before and treated them with LTβR-Ig or control mAb. iLNs from control-treated LTβR−/− BM chimeras had significantly fewer SCS MΦ than WT BM chimeras (Figure 3.9A) but still contained mature FDCs (Figure 3.10). An average of 97% of SCS MΦ in both WT and LTβR−/− BM chimeras were donor-derived (CD45.1+, data not shown). Consistent with a previous report135, treatment with LTβR-Ig resulted in a nearly complete loss of iLN SCS MΦ (Figure 3.9A) as well as ablating FDC. In LTβR−/− BM chimeras treated with control Ab, the number of GC B cells at day 12 p.i. was slightly lower than in WT BM chimeras, though this difference was not statistically significant (Figure 3.9B). A significant drop in the number of GC B cells at day 12 p.i. was only observed when BM chimeras were treated with LTβR-Ig (Figure 3.9B). As well, there was only a weak correlation between the number of SCS MΦ and the number of GC B cells in the iLN at day 12 p.i. (Figure 3.9C). Together, these results suggest that SCS MΦ play a partial role in sustaining GCs but that FDCs, or the cooperation between FDCs and SCS MΦ, are required for GCs to persist beyond the peak of the response.
**Figure 3.9. iLN GC response in LTβR<sup>-/-</sup> BM chimeras.** WT→WT and LTβR<sup>-/-</sup> BM chimeras were immunized with R-PE/CFA and treated at day -2 and day 5 p.i. with LTβR-Ig or MOPC21 control mAb. Cells from iLN were analyzed by flow cytometry at day 12 p.i. (A) The number of CD11b<sup>+</sup>CD11c<sub>low</sub>CD169<sup>-</sup>F4/80<sup>-</sup> SCS MΦ in the draining iLN. (B) The number of B220<sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup>R-PE<sup>+</sup> GC B cells in the draining iLN. Student’s unpaired t-test performed for all statistical tests; ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001. All columns depict mean ± SEM, n=8-13 for each group. (C) Plot of the number of SCS MΦ versus the number of GC B cells in the draining iLN at day 12 p.i. Each dot represents one iLN from one BM chimera. Plot includes LTβR<sup>-/-</sup> and WT BM chimeras from both LTBR-Ig and MOPC21 treated groups. Plot is fitted with a second order polynomial regression line. Data from two independent experiments is shown.

**Figure 3.10. GCs with FDCs in LTβR<sup>-/-</sup> BM chimeras.** Representative serial cross sections of GCs from a (A) WT→WT BM chimera and a (B) LTβR<sup>-/-</sup>→WT BM chimera treated with MOPC21 control mAb and stained with anti-B220 (Green) and PNA, anti-CD35, or FDC-M1 (blue). Columns depict serial sections of iLN GCs. Pictures are taken at 20x objective magnification. Scale bars represent 50µm.
Figure 3.9

A

SCS Macrophages

Cell# ($\times 10^3$)

WT $\rightarrow$ WT

LTβR$^-$$^- \rightarrow$ WT

MOPC21 (ctrl)

LTβR-Ig

B

GC B cells

Cell# ($\times 10^6$)

WT $\rightarrow$ WT

LTβR$^-$$^- \rightarrow$ WT

MOPC21 (ctrl)

LTβR-Ig

C

GC B cell# ($\times 10^3$)

SCS Mφ# ($\times 10^3$)

$R^2 = 0.3834$
Figure 3.10

A  WT → WT

B  LTβR−/− → WT
3.3 Discussion

A number of previous studies examining the spleens of LT-deficient mice and non-human primates treated with LT pathway inhibitors have consistently demonstrated poor or absent GC formation correlating with a lack of FDCs\(^{107, 116, 117, 151}\). FDCs also appear to be important in regulating GC responses in Peyer's patches in response to gut antigens\(^{152}\). A more recent paper has used ablation of FDCs by diphtheria toxin to demonstrate that FDC are required for the clustering and survival of GC B cells\(^{132}\). Together, these studies suggest that FDCs are required for the formation or function of GCs. Furthermore, the presence of secondary FDCs in ectopic lymphoid structures during autoimmunity is strongly associated with AID expression in B cells in the salivary glands of Sjögren's syndrome patients\(^{137}\) and in the synovium of Rheumatoid Arthritis patients\(^{153}\). These further observations suggest that FDCs are important in creating a niche that promotes AID expression.

I was therefore very surprised to observe normal expression of AID and a normal peak GC response in the draining iLNs of mice lacking both LT\(\alpha\beta\) and FDCs. Because of previous observations that SHM and CSR occur in FDC-deficient LT\(\alpha^-\) mice\(^{136}\), I initially expected my experiments to identify abnormal niches that supported AID-expressing B cells. However, not only did my experiments reveal AID-expressing B cells to be located in LN follicles, but they were also found in GC-like clusters devoid of FDCs. This suggests that FDCs are not in fact required to foster AID expression or to initiate GCs. What can account for the apparent discrepancy between my findings and those of previous papers? I believe a closer look at the nature of previous studies reveals two primary points that show my results are not necessarily in disagreement once context is taken into account.

The first point to consider is that previous studies have investigated the splenic lymphoid environment, whereas my experiments have looked at GC formation in a peripheral LN. Indeed, my work here is the first to show the formation of GCs in the absence of LT\(\beta\)R signaling in a peripheral LN. Because the LT\(\alpha^-\), LT\(\beta^-\), and LT\(\beta\)R\(^{-}\) mice used in previous studies all developed without peripheral LNs those studies focused on splenic GCs. Though the presence of GCs in the mesenteric LNs of LT\(\beta^-\) mice has previously been reported\(^{154}\), that finding has not made a significant impact on the general view of the role of FDCs in GC formation. This is perhaps due to the unique nature of mesenteric LNs. Firstly, mesenteric LNs are sites of constitutive lymphocyte activation and GC formation due to stimulation from gut Ag and lack periods of quiescence punctuated by de novo GC formation found in peripheral LNs\(^{66}\).
Secondly, the ontogeny of mesenteric LNs is different from peripheral LNs; whereas peripheral LNs require LTβR signaling in order to develop, mesenteric LNs are able to form in LTβR-/- and LTβR-/- mice due to signaling through TNFR1 by soluble LTα3. This has perhaps led to the perception of mesenteric LNs as a unique case. My findings support previous observations of mesenteric LN GCs, extend them to peripheral LNs, and further demonstrate that FDCs are not required to permit AID expression in GCs. My findings are therefore not in conflict with previous reports but rather serve as further evidence that GCs do not develop and function identically in all tissues. This may be due to differences in the stromal cell composition of LNs that compensate for FDCs.

The second point to consider is that previous studies have demonstrated a correlation between the absence of FDCs and the impairment of GC formation, but they did not establish causation. This is because previous work relied on the genetic ablation of LTβR signaling that not only ablates FDCs but also has effects on dendritic cell function, high endothelial venule maturation, the integrity of the splenic marginal zone, and the presence of LN SCS MΦ. Previous reports that GCs failed to form in the spleens of LT-deficient mice could not discount the disruption of the B-T zones and of the marginal zone as major factors. By contrast, the morphology of iLNs has remained largely intact in my BM chimeric model and this has allowed the observation of clusters of GC B cells in the absence of FDCs. In this light, my results do not conflict with previous reports but again highlight the differences between secondary lymphoid organs.

Though other studies have shown a correlation between the absence of FDCs and the absence of GCs, a recent paper by Wang et al. sought to prove causation by specifically targeting FDCs using a CD21-Cre x ROSA-DTR system. In their model expression of CD21 by FDCs induced expression of Cre recombinase which then removed a floxed stop element from a diphtheria toxin (DTx) receptor (DTR) insert, thereby rendering these FDCs sensitive to DTx-induced apoptosis. When FDCs were ablated at the peak of the GC response by treatment with DTx, Wang et al. observed that GCs disappeared from both splenic and peripheral LN follicles. Their study concluded that FDCs are required for the clustering of GC B cells in primary follicles, including in peripheral LNs. What could account for the difference between my conclusions and theirs? Firstly, due to the limitations of the system employed by Wang et al., they were only able to ablate FDCs 6-8 days p.i. and then wait 2 days to observe the effects. This means that in their model FDCs were present as Ag began entering the follicle and as GCs were forming. Therefore, the question of whether or not GCs can initiate without FDCs could...
not be addressed by their approach. In the system I have employed, FDCs were absent from the beginning of the response; the fact that clusters of GC B cells still formed in the complete absence of FDCs in my system strongly indicates that FDCs are not required for initial GC formation in LNs. However, Wang et al. do demonstrate a disappearance of GCs later in the response that fits with my observation that GCs disappear earlier in AID-GFP x LTβ−/− than in AID-GFP (WT) BM chimeras. Secondly, GC B cells express high levels of Fas and are primed for apoptosis; it is possible that DTx-mediated killing of FDCs inadvertently killed GC B cells through a bystander effect in their system156,157, accounting for the abrupt disappearance of GC B cells.

Because my LTβ−/− BM chimeras not only lacked FDCs but also had a significant reduction in the number of LN SCS MΦ, I was mindful that this model did not prove that it was the lack of FDCs that was responsible for the early dissipation of GCs. By generating LTβR−/− BM chimeras, which disrupts SCS MΦ but not FDCs, and combining this with pharmacological inhibition of LT signaling I was able to selectively ablate SCS MΦ and/or FDCs in order to determine which cell type was most important in sustaining GCs. I observed only a weak correlation between the number of SCS MΦ and the number of GC B cells late in the primary response and significantly reducing the number of SCS MΦ did not result in a significant reduction in the number of GC B cells at day 12 p.i. I therefore concluded that SCS MΦ play a secondary role, or cooperate with FDCs, in sustaining GCs. Only the ablation of FDCs by treatment with LTβR-Ig resulted in a significant decrease in the number of post-peak GC B cells.

Though I have observed normal initiation of GCs in the absence of FDCs in iLNs, this is not to say that FDCs do not play a role in organizing GCs. LNs are organized structures designed specifically for the orchestration of immune responses. As such, they may contain redundancies in the elements required to attract, organize, and properly stimulate lymphocytes. It has been observed that ablation of FDCs does not result in a decrease of CXCL13 transcripts in peripheral LNs132. This suggests that CXCL13, required for attracting GC B cells and T follicular helper cells, can be produced by cells other than FDCs in a lymph node. I have observed that ERTR7+ Fibronectin+ FRC networks, which can facilitate the transport of CXCL13133, are still intact in the follicles and GCs of AID-GFP x LTβ−/− BM chimeric mice, and a recent transcriptome analysis has shown that FRCs produce CXCL13 transcripts148. Marginal reticular cells are another possible source for CXCL13 in the absence of FDCs158. Outside of lymph nodes, however, FDCs may prove more critical to the formation of GCs. For example,
FDCs may be required for the organization of AID-expressing B cell clusters in salivary glands in Sjogren’s Syndrome\textsuperscript{137}. As well, FDCs have been observed in lymphoid infiltrates in the CNS during multiple sclerosis\textsuperscript{159}. While FDCs appear to be redundant in initiating GCs in iLNs, they appear to be critical in the longer-term maintenance of GCs required for robust affinity maturation.

In this chapter I have demonstrated that normal numbers of AID-expressing GC B cells are able to form clusters in the absence of FDCs and LT\textbeta R signaling. These GCs dissipate early, however, and result in a significant drop in the affinity for Ag of circulating Ab. My results show that FDCs are not strictly required for the formation of GCs or for allowing the expression of AID in B cells, but that they likely play a role in optimizing affinity maturation of the antibody response. I believe that my results, within the wider context of published literature, portray FDCs as facilitators rather than initiators; though their presence is not strictly required for GC formation, AID-expression, or affinity maturation, they are able to enhance and sustain these processes in order to augment to potency of humoral immune responses.
Chapter 4: AID\(^{-/-}\) B cells out-populate WT B cells in the GCs of mixed bone marrow chimeras due to reduced apoptosis


*A.Z. and B.B. contributed equally to this work.

Frances Vu, Leslie Summers-DeLuca, and Doug McCarthy assisted in the generation of BM chimeras. Ahmad Zaheen assisted in staining cells for active caspase 3.

Germinal centers (GC) are clusters of activated B cells that form in secondary lymphoid organs during a T-dependent immune response. GC B cells express the enzyme activation-induced deaminase to undergo somatic hypermutation (SHM) and class-switch recombination. By inducing mutations in their IgV regions, GC B cells can potentially increase their affinity for Ag. Within GCs, the B cell clones with the highest affinities for Ag are positively selected to continue the GC response and to mature into memory or plasma cells while clones with lower affinity for Ag die by apoptosis. This process is called clonal selection. During clonal selection, B cells compete for survival signals. One possible survival factor is B cell activating factor belonging to the TNF family (BAFF). To test the roles of SHM and BAFF in clonal selection, I generated mixed BM chimeras in which WT B cells competed with AID\(^{-/-}\) B cells, which cannot undergo SHM, in the same GCs to test is a lack of SHM resulted in reduced fitness during clonal selection. As well, I tested if an excess of BAFF in these mixed BM chimeras could lessen selective pressures and rescue low-affinity B cells during clonal selection. Surprisingly, AID\(^{-/-}\) B cells are overabundant in GCs despite their inability to undergo SHM. Excess BAFF did not affect the abundance of AID\(^{-/-}\) GC B cells. AID\(^{+/-}\) GC B cells are less apoptotic than WT GC B cells, possibly due to a lack of AID-induced DNA damage. These findings suggest that AID may regulate the GC response by inducing apoptosis in GC B cells.
4.1 Introduction

T-dependent humoral immune responses are characterized by the generation of large amounts of class-switched Ab with gradually increasing affinity for Ag. This process of affinity maturation relies upon the process of clonal selection that occurs within germinal centers (GCs)\textsuperscript{128}. GCs are clusters of activated B cells located in the primary follicles of secondary lymphoid organs. In response to T-dependent Ag, newly activated B cells enter germinal centers and express the enzyme activation-induced cytidine deaminase (AID) in order to undergo somatic hypermutation (SHM) and class-switch recombination (CSR).

Studies on the T-dependent response to the protein Ag NP-CGG have demonstrated that GC B cells develop mutations in the variable regions of their Ig molecules that increase their affinity for Ag, and that these high-affinity clones are consistently selected for clonal expansion and maturation into plasma cells\textsuperscript{79-81, 160, 161}. The process of clonal selection therefore selects for those B cells which have accumulated mutations that improve their affinity for Ag.

The precise mechanism by which high affinity GC B cells are selected is not entirely clear. It has been postulated that B cells compete for stimulation of the BCR by antigen, for help from GC-resident CD4\textsuperscript{+} T follicular helper cells, for survival factors such as BAFF secreted by follicular dendritic cells (FDCs), or for any combination of these stimuli\textsuperscript{74, 86, 128, 162}. GC B cells are sensitive to apoptosis, evident in their elevated surface expression of Fas, and B cell clones that do not access positive stimuli can undergo apoptosis induced by FasL on CD4\textsuperscript{+} T cells\textsuperscript{78, 94}.

B cell activating factor belonging to the TNF family (BAFF) is a soluble factor secreted by certain myeloid cells (e.g. monocytes, DCs) and there is evidence that BAFF can also be produced by follicular dendritic cells (FDC) within GCs\textsuperscript{163-167}. BAFF can bind to BAFF-receptor on B cells and promote their survival\textsuperscript{168}. Although BAFF is an important survival factor in B cell homeostasis, it remains to be determined if it is important in clonal selection as well.

B cells that lack AID are unable to initiate SHM and should be at a disadvantage during clonal selection because they are unable to generate affinity-increasing mutations. This provides an opportunity to test the role of BAFF in positive selection; if BAFF is a significant promoter of positive selection then elevated levels of BAFF should loosen selective pressures within GCs and rescue AID\textsuperscript{-/-} B cell clones which would normally be at a disadvantage.

In the experiments presented in this chapter, I initially sought to test if excess BAFF could rescue AID\textsuperscript{-/-} GC B cells from a hypothesized disadvantage during clonal selection. However, I found that contrary to my initial hypothesis AID\textsuperscript{-/-} B cells were actually
outnumbering WT B cells in the GCs of mixed BM chimeras. As well, I was unable to detect any effect of the transgenic over-expression of BAFF on the ratio of AID⁻/⁻ : WT GC B cells. I therefore changed my line of investigation to characterize the abundance of AID⁻/⁻ GC B cells. I found that AID⁻/⁻ GC B cells were present in disproportionately large numbers in splenic GCs, and that GCs populated solely by AID⁻/⁻ B cells were on average larger than those occupied solely by WT B cells. I determined that this was likely a result of the reduced rate of apoptosis of AID⁻/⁻ B cells, which do not undergo AID-induced DNA damage and therefore have lower rates of apoptotic death. Together, these experiments show that the activity of AID has a significant effect on the viability of GC B cells.
4.2 Results

4.2.1 AID<sup>−/−</sup> B cells disproportionately outnumber WT B cells in splenic GCs of mixed BM chimeras

GC responses select for B cells with the highest affinities for Ag<sup>81, 86, 169</sup>. As well, affinity maturation in GCs requires AID to initiate SHM<sup>31, 129</sup>. It is possible that B cells that cannot express AID, and therefore do not undergo SHM, would be at a selective disadvantage during clonal selection. In order to test this basic principle of clonal selection, I generated mixed BM chimeras by reconstituting host mice with a 1:1 mixture of AID<sup>−/−</sup> and WT BM (Figure 4.1A). In these mixed BM chimeras, the two donor B cell types were tracked using the congenic markers CD45.2 (AID<sup>−/−</sup>) and CD45.1 (WT). The host mice were either WT (AID<sup>+</sup>/<sup>−/−</sup>CD45.1/.2) or BAFF-Tg (AID<sup>+</sup>/<sup>−/−</sup>BAFF-Tg<sup>+</sup>/). Chimeras using AID<sup>−/−/</sup>CD45.1/.2 or AID<sup>−/−/</sup>BAFF-Tg<sup>+</sup>/ hosts are hereafter referred to as WT or BAFF-Tg BM chimeras, respectively.

In order to assess if either population of B cell has a selective advantage, I assessed the ratio of AID<sup>−/−</sup> : WT B cells. Within each individual BM chimera I first calculated this ratio among naïve follicular B cells (B220<sup>+</sup>GL7<sup>+</sup>Fas<sup>−</sup>) (Figure 4.1B). Follicular B cells have not yet undergone SHM and thus have not yet undergone any clonal selection based on the effects of AID. The ratio of AID<sup>−/−</sup> : WT B cells in the follicular population therefore serves as the background ratio. If the ratio of AID<sup>−/−</sup> : WT B cells among GC B cells (B220<sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup>) is different than the background ratio among follicular B cells, however, it indicates that clonal selection in the GC is resulting in one population out-competing the other. By dividing the GC ratio by the follicular ratio, I generate a normalized ratio that indicates selective advantage and that is normalized for any variation in the background ratio between individual mice (Figure 4.1C). In this study, ratios above 1 indicate a skew towards AID<sup>−/−</sup> GC B cells and ratios below 1 indicate a skew towards WT GC B cells.

To test this analytical approach, I first generated control mixed BM chimeras in which both types of donor BM were WT: C57Bl/6 (CD45.2<sup>+</sup>) and CD45.1-congenic on the C57Bl/6 background. Because there is no selectable difference between these two types of B cells, the ratio of CD45.2 : CD45.1 in the GC and in the follicle should be the same and thus result in a normalized ratio of 1. Indeed, the mean normalized ratio in splenic GCs from control chimeras at day 14 p.i. was nearly 1 (Figure 4.2A, Control), indicating no selectable advantage for either population in GCs. This verified the validity of my approach and established a control against which I could test for statistical significance.
Figure 4.1. Experimental approach and analysis. (A) A diagram illustrating the generation of mixed BM chimeric mice. (B) Flow cytometric gates used to analyze AID^{−/−} and WT B cells from B220^{+}Fas^{−}GL7^{−} follicular and B220^{−}Fas^{+}GL7^{+} GC populations. (C) An example of the formula used to calculate a normalized ratio to measure chimerism among GC B cells.
Figure 4.1

A

$\text{AID}^{+/-} (\text{CD45.2}^{+/-})$ and $\text{WT} (\text{CD45.1}^{+/-})$

$2 \times 10^6$ BM cells

$2 \times 550 \text{cGy}$

$\text{AID}^{+/-} \text{ CD45.1/.2}^{+/-}$ or BAFF-Tg$^{+/-}$ AID$^{+/-}$

$\text{AID}^{+/-} \text{ CD45.1/.2}^{+/-}$ or BAFF-Tg$^{+/-}$ AID$^{+/-}$

B

Follicular B cells

GC B cells

Fas

GL7

CD45.2

CD45.1

68.8

29.9

76.6

22.7

3.63

65.3

64

C

$\frac{76.6\% (\text{AID}^{+/-})}{22.7\% (\text{WT})} = 1.47$ [Normalized Ratio]

$\frac{68.8\% (\text{AID}^{+/-})}{29.9\% (\text{WT})}$ [Follicle Ratio]
I next immunized AID<sup>−/−</sup> versus WT mixed BM chimeras i.p. with NP-CGG Ag in alum adjuvant and analyzed the ratios in splenic GCs at various timepoints. Surprisingly, at all timepoints p.i. the mean normalized ratio was significantly above the control group, indicating a higher proportion of AID<sup>−/−</sup> B cells than WT B cells in the GCs of WT BM chimeras (Figure 4.2A). This skew towards AID<sup>−/−</sup> GC B cells was consistent in all of my experiments, even when I varied the ratio of AID<sup>−/−</sup>:WT BM cells that were used to generate the BM chimeras (Figure 4.2B).

The finding that AID<sup>−/−</sup> B cells were overabundant in GCs, undermined my original experimental goal to test whether or not BAFF can rescue B cell clones that are competitively disadvantaged during clonal selection because, surprisingly, the AID<sup>−/−</sup> GC B cells were not disadvantaged. Nevertheless, I immunized BAFF-Tg mixed BM chimeras in order to see if an excess of BAFF would alter the ratio of AID<sup>−/−</sup>:WT GC B. I found that the mean ratio of AID<sup>−/−</sup>:WT GC B cells among the BAFF-Tg chimeras was the same as among the WT chimeras at day 14 and day 21 p.i. (Figure 4.2C).

Together, these results indicate that AID<sup>−/−</sup> B cells are present in disproportionally higher numbers compared to WT B cells in the splenic GCs of mixed BM chimeras. As well, removing BAFF as a limiting factor does not preferentially affect either AID<sup>−/−</sup> or WT GC B cells.

### 4.2.2 Splenic GCs occupied by AID<sup>−/−</sup> B cells are more numerous and larger than WT GCs in mixed BM chimeras

I next sought to further explore the unexpected finding that AID<sup>−/−</sup> GC B cells seemed to thrive when competing against WT GC B cells. I therefore proceeded to investigate the physical makeup and dimensions of the GCs in WT mixed BM chimeras by histology. Serial sections of spleens were all stained for IgD, to discern IgD<sup>+</sup> B cell follicles from IgD<sup>−</sup> GCs, and either GL7 (GCs), CD45.1 (WT cells), or CD45.2 (AID<sup>−/−</sup> cells) in order to visualize the chimeric composition of individual GCs. I was able to observe GCs that contained both CD45.2<sup>+</sup> (AID<sup>−/−</sup>) and CD45.1<sup>+</sup> (WT) cells, as well as GCs that contained almost exclusively one cell type or the other (Figure 4.3). Though the majority of GCs were mixed, I counted the number of GCs that contained exclusively AID<sup>−/−</sup> or WT cells and found that GCs occupied exclusively by AID<sup>−/−</sup> GC B cells significantly outnumber GCs occupied exclusively by WT GC B cells (Figure 4.4 A). As well, the average size of exclusive AID<sup>−/−</sup> GCs was slightly larger than exclusive WT GCs in spleens at day 21 p.i. (Figure 4.4 B). Together with the ratios ascertained by flow cytometry,
Figure 4.2. Chimeric ratios of AID\textsuperscript{+} : WT GC B cells. (A) Control mixed BM chimeras (CD45.1\textsuperscript{+} WT and CD45.2\textsuperscript{+} WT BM donors) were immunized with NP-CGG/Alum and the normalized splenic GC ratio was calculated at day 14 p.i. WT BM chimeras were immunized in the same manner and the normalized splenic GC ratio was calculated at various times p.i. Dots depict individual BM chimeras and bars indicate group mean. The dotted line denotes a normalized ratio of 1. The mean ratio from WT mixed BM chimeras were compared to the mean ratio from the control group by student’s unpaired t-test; *, P<0.05; **, P<0.01; ***, P<0.001. (B) WT BM chimeras were generated using three different ratios of AID\textsuperscript{+} : WT donor BM cells and the normalized ratios of splenic GC B cells were calculated at day 14 p.i. (C) WT and BAFF-Tg BM chimeras were generated and the normalized ratios of splenic GC B cells were calculated at day 14 p.i. Data from (A) nine, (B) six, and (C) four independent experiments are shown.
Figure 4.2

A

Normalized Ratio (AID⁺⁺ : WT)

Control  Day7  Day9  Day11  Day14  Day21

***  *  *  ***  ***  **

B

Normalized Ratio (AID⁺⁺ : WT)

Donor BM Ratio

3 : 1  1 : 1  1 : 3

C

Normalized Ratio (AID⁺⁺ : WT)

WT  BAFF-Tg  WT  BAFF-Tg

Day 14  Day 21
these histological observations suggest that AID\textsuperscript{−/−} B cells are more prevalent in splenic GCs than WT B cells.

**4.2.3 WT GC B cells have a higher rate of apoptosis than AID\textsuperscript{−/−} GC B cells**

Because AID\textsuperscript{−/−} GC B cells cannot undergo SHM, and because increases in affinity for antigen due to SHM are the basis of clonal selection, I initially expected AID\textsuperscript{−/−} B cells to be under-represented in GCs. My findings that AID\textsuperscript{−/−} GC B cells are instead over-represented required an alternate explanation.

In order to initiate SHM and CSR, AID converts genomic cytosines into uracils\textsuperscript{170}. This creates DNA mismatches that can be converted into abasic sites and single- or double-strand DNA breaks\textsuperscript{50}. AID therefore induces DNA damage which can negatively affect the viability of the cell. It is therefore possible that AID\textsuperscript{−/−} GC B cells are less susceptible to DNA damage-induced apoptosis. To test this, I immunized mixed BM chimeras as before and stained splenic B cells for the presence of activated caspase 3, an “executioner” caspase that effects apoptosis\textsuperscript{93}. I then compared the proportions of AID\textsuperscript{−/−} and WT GC B cells that stained positive for active caspase 3 by flow cytometry. At day 21 p.i., a significantly lower proportion of AID\textsuperscript{−/−} GC B cells stained positive for caspase 3 compared to WT GC B cells (Figure 4.5). This suggests that AID\textsuperscript{−/−} GC B cells undergo lower rates of attrition due to apoptosis and this could account for their over-representation in the GCs of mixed BM chimeras.
**Figure 4.3. Histology of splenic GCs from mixed BM chimeras.** Serial sections of splenic GCs from mixed BM chimeras at day 14 p.i. were stained by immunohistochemistry. Rows display three consecutive serial sections of a single GC. Columns depict three separate samples with the same staining scheme. All sections were stained for IgD (blue). Serial sections were labeled with GL7 (brown) to show GCs, CD45.1 (brown) to show WT cells, or CD45.2 (brown) to show AID<sup>−/−</sup> cells. GCs were composed of (A) a mixture of both cell types, (B) predominantly WT cells, or (C) predominantly AID<sup>−/−</sup> cells. Pictures were taken at 20x objective magnification. Scale bars represent 50µm.

**Figure 4.4. Quantification of GC numbers and surface areas.** (A) GCs from splenic serial sections at day 14 and day 21 p.i. were categorized as containing predominantly WT or AID<sup>−/−</sup> cells and quantified. Columns depict mean ± SEM, n=6-7 for each genotype at each timepoint. (B) The surface area measurements of GCs categorized as predominantly WT or AID<sup>−/−</sup> from single splenic sections. Bars denote the group mean. Student’s unpaired t-test performed for all statistical tests; *, P<0.05; **, P<0.01; ***, P<0.001.

**Figure 4.5. Measurement of Caspase 3 activity in GC B cells.** (A) Representative histogram of AID<sup>−/−</sup> and WT B cells from mixed BM chimeras stained to detect active caspase 3 and analyzed by flow cytometry. (B) Comparison of the % of B cells staining for active Caspase 3 among WT and AID<sup>−/−</sup> Follicular and GC B cells. Columns depict mean ± SEM, n=7 for each group. Student’s unpaired t-test performed for statistical tests *; P<0.05.
Figure 4.3

A  
GC  
GL7  IgD  
WT Cells  
CD45.1  IgD  
AID\(^{-}\) Cells  
CD45.2  IgD

B

C
Figure 4.4

A. Number of GCs

- WT GC
- AID<sup>−/−</sup> GC

<table>
<thead>
<tr>
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<th>Day 14</th>
<th>Day 21</th>
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<tr>
<td>WT GC</td>
<td></td>
<td></td>
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<tr>
<td>AID&lt;sup&gt;−/−&lt;/sup&gt; GC</td>
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**p-values:** *** (p < 0.001), ** (p < 0.01)

B. GC Surface Area

- WT GC
- AID<sup>−/−</sup> GC

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<th>Day 14</th>
<th>Day 21</th>
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<tr>
<td>WT GC</td>
<td></td>
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<tr>
<td>AID&lt;sup&gt;−/−&lt;/sup&gt; GC</td>
<td></td>
<td></td>
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</table>

**p-value:** * (p < 0.05)
Figure 4.5

A

WT GC B cells

AID⁺ GC B cells

Caspase 3

B

Caspase 3⁺ B cells

WT B cells

AID⁺ B cells

0%
4%
8%
12%

Follicle
GC
Follicle
GC
4.3 Discussion

Clonal selection in GCs is a process whereby B cell clones with the highest affinities for Ag are selected to continue in the GC reaction or to mature into plasma and memory cells. Clonal selection therefore requires mechanisms that can (a) promote survival and proliferation in high affinity clones or (b) induce or allow low affinity clones to die. GC B cells can be cleared by being denied access to positive survival stimuli or by direct induction of apoptosis, such as by signaling through Fas. It has been demonstrated that B cells of low affinity for Ag have higher death rates than those of high affinity during competition within GCs. The clearance of undesirable GC B cells by apoptosis is important for affinity maturation, tolerance, and homeostasis. My results here demonstrate that AID-/- GC B cells have lower rates of apoptosis than WT GC B cells when competing within the same GCs. The viability of AID-/- GC B cells evidently allows them to overcome the disadvantage posed by the inability to undergo SHM and to heavily populate GCs.

The experiments and results I show here were published in combination with results generated by colleague pursuing a similar line of investigation in non-chimeric AID-/- mice (Appendix I). My experiments demonstrate that AID+/- GC B cells have lower rates of caspase 3 activity in mixed BM chimeras in vivo. In addition, further experiments from our joint publication show that naive AID-/- B cells have lower rates of caspase 3, 8, and 9 activity and higher rates of proliferation when stimulated in vitro. As well, our publication showed a correlation between the amount of AID being expressed and the levels of apoptosis in GC B cell lines in vitro. Together, these results suggest that AID has a negative effect on GC B cell viability. This finding is not unexpected, considering that AID acts by creating lesions in genomic DNA. Not only does AID induce targeted mutations and DNA breaks at the Ig loci, but it is able to deaminate deoxycytidines genome-wide. It is likely that any B cell having to cope with such widespread DNA damage would be at a higher risk for DNA damage-induced apoptosis. Indeed, AID-induced lesions have been shown to activate DNA damage response pathways. In addition, B cells that manage to repair AID-induced DNA lesions will have paused at cell cycle checkpoints, resulting in delayed cell division. Together, our results are the first to demonstrate an increase in apoptosis and a decrease in proliferation in GC B cells lacking AID.

GC B cells are rapidly proliferating cells that are at an increased risk of developing auto-reactivity or oncogenic mutations. Therefore, it is possible that the constraints placed on cell
viability by AID play a protective role. Indeed, studies have implicated AID-deficiency in autoimmunity\textsuperscript{179-181}. It has also been suggested that low-level expression of AID in developing and transitional B cells is important for maintaining central tolerance\textsuperscript{42}. However, other studies have implicated AID in the generation of autoimmune diseases\textsuperscript{182, 183}, thus the destructive/protective nature of AID in tolerance appears to be complex. Because AID may constrain GC B cell viability but may also induce deleterious mutations, it is likely that the wider effects of AID activity beyond affinity maturation and CSR constitute a double-edged sword, so to speak.

Though my observation that AID\textsuperscript{−/−} B cells thrive in GCs were of great interest, it undermined my ability to test the role of BAFF in positive clonal selection. In order to test my initial experimental question, a better experimental approach would be to adoptively transfer populations of low and high affinity B cells into WT or BAFF-Tg hosts and measure skews in the GC population. This could be achieved by using B cells containing a pre-arranged IgH knock-in that confers low (B1-8\textsubscript{lo}) or high affinity (B1-8\textsubscript{hi}) to 4-hydroxy-3-nitrophenylacetyl (NP) Ag\textsuperscript{184}. It would then be possible to see if an over-abundance of BAFF enhances the ability of low-affinity B cells to populate GCs.

What is clear is that GC B cells that lack AID thrive to a degree that is counter-intuitive in light of their inability to undergo SHM and affinity maturation. However, the abundance of AID\textsuperscript{−/−} GC B cells is not due to any clonally-selective advantage, but likely due to a lack of AID-induced DNA damage that can lead to cell cycle arrest or apoptosis. Though other mechanisms exist to clear GC B cells by apoptosis, the abundance of AID\textsuperscript{−/−} GC B cells suggests that the induction of DNA damage by AID may have been evolutionarily co-opted into constraining the size of the GC B cell population. Another possibility is that the low levels of DNA damage induced by AID create a baseline amount of apoptotic conditioning in which the stoichiometry of the primary survival/apoptotic signaling pathways evolved. Either way, the evidence suggests that the GC reaction seems tuned to function properly in the context of AID activity.
Chapter 5: AID⁻/⁻ GC B cells accumulate as centrocytes and mature into plasma cells inefficiently, though not due to deficiencies in Ag-affinity or in the generation of double-strand DNA breaks

At the time this thesis was completed, some of the data contained in this chapter was under revision at in The Journal of Immunology. Bryant Boulianne*, Olga L. Rojas*, Dania Haddad*, Ahmad Zaheen, Anat Kapelnikov, Thanh Nguyen, Conglei Li, Razq Hakem, Jennifer L. Gommerman, Alberto Martin. AID and Caspase 8 shape the Germinal Center response through Apoptosis. J. Immunol. In revision. Copyright © 2013 The American Association of Immunologists, Inc.
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Frances Vu, Leslie Summers-DeLuca, and Doug McCarthy assisted in the generation of BM chimeras.

Germinal centers (GC) are clusters of activated B cells that form in secondary lymphoid organs during a T-dependent immune response. B cells enter GCs and become rapidly proliferating centroblasts. Centroblasts express the enzyme activation-induced deaminase (AID) to undergo somatic hypermutation and class-switch recombination. Centroblasts then mature into centrocytes to undergo clonal selection. GCs select the highest affinity clones to mature into memory or plasma cells. Low affinity clones undergo apoptosis. I have previously shown that AID⁺⁻ GC B cells have lower rates of apoptosis and accumulate in GCs. Using MD4 Ig-transgenic mice I observed that this accumulation of AID⁺⁻ GC B cells is not due to the affinity of the BCR for Ag. Using mixed bone marrow chimeras I observed that AID⁺⁻ GC B cells accumulate as centrocytes and exhibit a partial block in plasma cell maturation. This reduced rate of plasma cell formation is not due to an absence of AID-induced DNA lesions that have been suggested to promote maturation. These findings demonstrate that in the absence of AID B cells accumulate as centrocytes and are deficient in plasma cell maturation.
5.1 Introduction

During a T-dependent immune response, germinal centers (GC) form in the follicles of secondary lymphoid organs. GCs are composed of clusters of activated B cells that rapidly proliferate. As well, GC B cells undergo somatic hypermutation (SHM), class-switch recombination (CSR), and clonal selection for those GC B cells with the highest affinities for antigen (Ag). The processes of SHM and CSR require the activity of the B cell-specific enzyme activation-induced deaminase (AID)\textsuperscript{31, 129}. AID is up-regulated by B cells following activation to initiate SHM and CSR, and is subsequently down-regulated as GC B cells exit the GC to become memory or plasma cells\textsuperscript{41}.

GCs are classically divided into two zones: the dark zone and the light zone\textsuperscript{72, 73}. B cells entering GCs first enter the dark zone and become rapidly proliferating centroblasts. Centroblasts also express AID to undergo CSR and SHM and then migrate to the light zone to become centrocytes. Centrocyte B cells compete for Ag and T cell help, and those centrocytes with the highest affinities for Ag receive positive survival signals in a process termed clonal selection. Positively selected centrocytes may recycle to the centroblast stage in order to expand a successful clone and to further improve their affinity for Ag, or they may exit the GC and mature into memory or plasma cells. The exact combinations of signals that determine specific GC B cell fates are still a matter of investigation\textsuperscript{85}. These signals can include binding of the BCR by Ag, co-stimulation from T cells, cytokines, and TNF family molecules. GC B cells that are out-competed during clonal selection and fail to receive the proper signals die by apoptosis. Recently, double-strand breaks (DSB) in genomic DNA have been implicated in plasma cell differentiation\textsuperscript{185}.

In the previous chapter, I demonstrated that AID\textsuperscript{-/-} GC B cells accumulate in the GCs of mixed BM chimeras in spite of their inability to undergo SHM. AID\textsuperscript{-/-} GC B cells undergo lower rates of apoptosis and higher rates of proliferation and that this is likely due to a lack of AID-induced DNA damage\textsuperscript{174}. The survival of AID\textsuperscript{-/-} GC B cells is likely the cause of their overabundance in the GCs of mixed BM chimeras. However, the behavior and the fate of these AID\textsuperscript{-/-} GC B cells remained unclear.

In this chapter I investigate AID\textsuperscript{-/-} GC B cells in more detail by examining their abundance in the centrocyte and centroblast populations within GCs and among plasma cells in the BM. I demonstrate that AID\textsuperscript{-/-} GC B cells accumulate specifically as CXCR4\textsuperscript{low} centrocytes in GCs. While I have reported previously that AID\textsuperscript{-/-} B cells are less apoptotic than WT B cells,
I show here that AID−/− GC B cells also fail to mature efficiently into plasma cells. Thus, a partial block in plasma cell maturation could also contribute to the high numbers of AID−/− B cells accumulating in GCs. I also demonstrate that the accumulation of AID−/− GC B cells is not due to differences in the affinity for Ag of the BCR and I discount the role of DNA double-strand breaks in causing the partial block in AID−/− plasma cell formation.
5.2 Results

5.2.1 AID<sup>−/−</sup> B cells accumulate as CXCR4<sup>low</sup> centrocytes in the GCs of mixed BM chimeras

I have previously described a mixed bone marrow (BM) chimeric system in which donor BM cells from AID<sup>−/−</sup> (CD45.2) and WT (CD45.1) mice are used to reconstitute lethally-irradiated AID<sup>+/−</sup>CD45.1/.2 hosts. This system allowed me to generate splenic GCs in which AID<sup>−/−</sup> and WT GC B cells proliferate and compete among each other and in which I could track B cells from either genotype using CD45.1/.2 as a congenic marker. Using this system, I have reported that AID<sup>−/−</sup> B cells are present in greater numbers than WT B cells in mixed GCs<sup>174</sup>.

I next wanted to determine if there was a specific stage of the GC cycle at which AID<sup>−/−</sup> B cells were accumulating. GC B cell subtypes can be discriminated as CXCR4<sup>high</sup> centroblasts and CXCR4<sup>low</sup> centrocytes<sup>72</sup>. I immunized mixed BM chimeras as described before and evaluated the mean fluorescent intensity (MFI) of CXCR4 surface staining on AID<sup>−/−</sup> and WT GC B cells at various timepoints p.i. (Figure 5.1A). In all BM chimeras, at all timepoints, the CXCR4 MFI of the AID<sup>−/−</sup> GC B cell population as a whole was significantly lower than that of WT GC B cells. Taking another analytical approach, I re-gated all B220<sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup> GC B cells into CXCR4<sup>high</sup> and CXCR4<sup>low</sup> populations (Figure 5.1B). Among CXCR4<sup>high</sup> centroblasts, there is a small skew towards AID<sup>−/−</sup> cells at day 7 which is not present at any other timepoints. In fact, from day 7 to day 11 p.i. the mean normalized ratio of AID<sup>−/−</sup> : WT CXCR4<sup>high</sup> GC B cells was nearly 1 and was not significantly different from control BM chimeras (WT : WT) (Figure 5.1C). This was in stark contrast to the CXCR4<sup>low</sup> compartment, where there was significantly more AID<sup>−/−</sup> than WT GC B cells (Figure 5.1D). This indicates that within splenic GCs, AID<sup>−/−</sup> cells were accumulating specifically as CXCR4<sup>low</sup> centrocytes.

5.2.2 AID<sup>−/−</sup> GC B cell accumulation is not due to the affinity of the BCR for Ag

AID<sup>−/−</sup> GC B cells accumulate at the centrocyte stage, which is when GC B cells are undergoing affinity-based clonal selection. Although AID is responsible for initiating the DNA lesions which lead to SHM and affinity maturation, mutations caused by AID are random and have a greater probability of either not affecting or lowering affinity for Ag than increasing it. It is possible that affinity-lowering mutations might result in a competitive disadvantage for WT GC B cells at the early stages of the GC response which would allow for AID<sup>−/−</sup> GC B cells to
Figure 5.1. Analysis of WT and AID<sup>−/−</sup> GC B cells by levels of CXCR4 expression. Mixed BM chimeras were immunized and their splenic B<sup>220</sup><sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup> GC B cells were analyzed at various time points p.i. (A) Surface expression of CXCR4 on AID<sup>−/−</sup> and WT GC B cells measured by flow cytometry. (B) Representative histogram of CXCR4 staining on B<sup>220</sup>− non-B cells, B<sup>220</sup><sup>−</sup>GL7<sup>−</sup>Fas<sup>−</sup> follicular B cells, and B<sup>220</sup><sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup> GC B cells. Bisecting the population of B<sup>220</sup><sup>−</sup>GL7<sup>+</sup>Fas<sup>+</sup> GC B cells into (C) CXCR4<sub>high</sub> and (D) CXCR4<sub>low</sub> fractions to investigate the ratio of AID<sup>−/−</sup> : WT B cells among (C) centroblast and (D) centrocyte B cells. Student’s unpaired t-test; ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001.
Figure 5.1
take over the GC niche. To test this possibility, I used MD4 mice which contain a pre-arranged
IgH transgene that encodes for an IgM with high affinity for the protein hen egg lysozyme
(HEL)\textsuperscript{121}. I crossed MD4 mice with CD45.1 congenic mice, as well as with AID\textsuperscript{-/-} mice and
adoptively transferred 2x10\textsuperscript{6} of each genotype of MD4 B cells, along with 3.5x10\textsuperscript{5} OTII T cells
as a source of T cell help into AID\textsuperscript{+/-}CD45.1/2 hosts.

GC B cells generated in this model all possess the same high affinity for Ag throughout
the immune response. I immunized these mice s.c. with HEL-OVA protein in alum adjuvant and
to enumerate both WT and AID\textsuperscript{-/-} HEL-specific GC B cells in the draining inguinal lymph nodes
(iLN) (Figure 5.2A). Though AID\textsuperscript{-/-} and WT MD4 B cells were introduced at a 1 : 1 ratio,
there was again a significant skew towards AID\textsuperscript{-/-} MD4 GC B cells at day 7 p.i. (Figure 5.2B).
This indicates that the Ag-affinity of the BCR is not playing a role in the relative abundance of
AID\textsuperscript{-/-} GC B cells.

\textbf{5.2.3 AID\textsuperscript{-/-} GC B cells do not efficiently form plasma cells in mixed BM chimeras}

Within GCs, it is at the centrocyte stage that clonal selection occurs. Centrocytes that
successfully survive clonal selection are able to recycle back into centroblasts or emigrate from
the GC as memory or plasma cells\textsuperscript{85, 128}. The fact that AID\textsuperscript{-/-} GC B cells were accumulating as
centrocytes suggested that they were experiencing a block in fate decisions. Because AID\textsuperscript{-/-} B
cells were present in normal numbers in the centroblast compartment, it was clear that they were
not recycling in mass. The next question was whether or not they were maturing and emigrating
from GCs in normal numbers. To test this, I produced mixed BM chimeras in which the WT B
cells possess the IgH\textsuperscript{a} allotype as a congenic marker to differentiate them from the IgH\textsuperscript{b} allotype
on AID\textsuperscript{-/-} B cells. I then immunized these chimeras as before and measured the number of BM
marrow plasma cells at 21 days p.i. by ELISPOT (Figure 5.3A). WT plasma cells produced
IgM\textsuperscript{a} or IgG spots and AID\textsuperscript{-/-} plasma cells produced IgM\textsuperscript{b} spots. Although there were
significantly more splenic AID\textsuperscript{-/-} GC B cells, as measured by flow cytometry, ELISPOT analysis
revealed that there were equal numbers of AID\textsuperscript{-/-} and WT plasma cells in the BM (Figure 5.3A).
Although AID\textsuperscript{-/-} GC B cells were developing into long-lived BM plasma cells, they were doing
so inefficiently enough that the skew in the splenic GC compartment did not carry over into the
BM plasma cell compartment.

The transcriptional programs that determine GC B cell and plasma cell phenotype are
suppressive of one another and are controlled to large degrees by the transcription factors Bcl6
and Blimp1, respectively\textsuperscript{186}. Thus, the expression of Bcl6 is an indicator of retention in the GC
**Figure 5.2. Accumulation of AID−/− MD4 GC B cells with fixed BCR affinity.** AID−/− MD4 and CD45.1+ MD4 B cells were transferred into host mice along with OTII T cells and immunized s.c. with HEL-OVA. iLN GC B cells were analyzed by flow cytometry day 7 p.i. (A) B220+GL7+Fas+ GC B cells can be distinguished as donor AID−/−MD4 (CD45.1+IgMa+) and CD45.1+MD4 (CD45.1+IgMa+) or as host polyclonal GC B cells (CD45.1−IgMa−). (B) AID−/− MD4 and CD45.1+MD4 B cells were transferred into hosts at a 1:1 ratio, but skewed towards AID−/−MD4 GC B cells at day 7 p.i.

**Figure 5.3. AID−/−/WT plasma cell chimerism.** Mixed BM chimeras were immunized and their splenic GCs and BM plasma cells were analyzed at day 21 p.i. by flow cytometry or Ag-specific ELISPOT, respectively. (A) Flow cytometric analysis of GC chimerism and Ag-specific ELISPOT analysis of BM plasma cell chimerism. (B) rtPCR of Bcl6 and Blimp1 transcripts in sorted AID−/− and WT GC B cells from mixed BM chimeras. Relative expression values denote the fold of the expression of the target gene in AID−/− GC B cells over that in WT GC B cells, normalized to an endogenous reference gene. Values higher than 1 indicate higher relative expression in AID−/− GC B cells, while values lower than 1 indicate higher relative expression in WT GC B cells. Bars depict mean ±SEM, n=5 for each gene target.
Figure 5.2

A

[Diagram showing Fas vs. GL7 and CD45.1 vs. IgM for GC B cells, WT MD4, WT Polyclonal, and AID-/- MD4]

B

[Graph showing ratio AID+ : WT vs. Cell Input and GC Chimerism]
Figure 5.3

A

![Graph A](image)

B

![Graph B](image)
while expression of Blimp1 indicates maturation into plasma cells. I sorted AID-/- and GC B cells from mixed BM chimeric spleens to isolate RNA and generate cDNA. I then performed rtPCR on the cDNA of each cell type to measure the relative expression of Bcl6 and Blimp1 (Figure 5.3B). rtPCR revealed slightly higher expression of Bcl6 and slightly lower expression of Blimp1 in total AID-/- GC B cells compared to WT GC B cells. This difference in transcripts represents a trend that does not demonstrate a significant difference in factor expression, though it does provide corroborating evidence of the impaired plasma cell maturation seen in AID-/- cells by ELISPOT. Together, these data indicate that AID-/- GC B cells do no efficiently form BM plasma cells.

5.2.4 Radiation-induced double-strand DNA breaks do not rescue AID-/- plasma cell formation in vivo

Recent evidence from in vitro experiments has suggested that the formation of DNA double-strand breaks (DSBs) initiates a signal that promotes the maturation of GC B cells into plasma cells\textsuperscript{185}. This suggests that the DSBs formed during CSR may promote plasma cell differentiation. Because AID-/- B cells cannot undergo CSR, or form the required DSBs in the IgH locus, they may be missing a signal required for normal rates of maturation. To test this, I immunized mixed BM chimeras and then dosed them with 500cGy whole-body ionizing irradiation near the peak of the GC response at day 10 p.i. This had the effect of inducing genomic DNA DSBs in a large number of AID-/- GC B cells. This radiation dose was chosen as it has been demonstrated that it is sufficient to promote plasma cell maturation in activated B cells in vitro\textsuperscript{185}. If DNA DSBs were the missing signal, then I would see an increase in the number of AID-/- plasma cells in the BM. Irradiation induced genomic DSBs equally in AID-/- and WT B cells as verified by staining for γH2AX, a phosphorylated histone component present at sites of DNA DSBs (Figure 5.4A)\textsuperscript{187}. Irradiation did result in a large reduction of total CXCR4\textsuperscript{high} centroblasts, which are proliferating and therefore radio-sensitive, but did not significantly affect CXCR4\textsuperscript{low} centrocytes (data not shown). I then looked at B220\textsuperscript{low}CD138\textsuperscript{+}NP\textsuperscript{+} antigen-specific BM plasma cells at day 21 p.i. by flow cytometry and measured the ratio of AID-/(CD45.2\textsuperscript{+}) : WT(CD45.1\textsuperscript{+}) plasma cells. Irradiation-induced DSBs did not increase the ratio of AID-/- : WT plasma cells compared to controls, suggesting that DSBs are not a sufficient driver of the maturation of AID-/- GC B cells into plasma cells in vivo (Figure 5.4C).
I further sought to investigate the role of DNA DSBs in plasma cell development by generating AID-expressing B cells which were unable to produce DSBs. UNG and Msh2 are DNA repair factors that initiate DSBs to execute their repair functions. Mice double-deficient for these factors are unable to produce DSBs in response to AID. If DSBs are required for plasma cell maturation, UNG\textsuperscript{−/−}Msh2\textsuperscript{−/−} B cells should recapitulate the plasma cell deficiency of AID\textsuperscript{−/−} B cells. I generated mixed BM chimeras with WT and UNG\textsuperscript{−/−}Msh2\textsuperscript{−/−} BM and immunized as before. UNG\textsuperscript{−/−}Msh2\textsuperscript{−/−} B cells are unable to properly handle DNA damage and undergo high rates of apoptosis, as is evident in the elevated levels of TUNEL\textsuperscript{+} UNG\textsuperscript{−/−}Msh2\textsuperscript{−/−} GC B cells (Figure 5.5A). This resulted in a low number of UNG\textsuperscript{−/−}Msh2\textsuperscript{−/−} B cells in the GC and a low ratio of UNG\textsuperscript{−/−}Msh2\textsuperscript{−/−} : WT GC B cells (Figure 5.5B). However low, the ratio among GC B cells was the same as the ratio among BM plasma cells which indicates that though the UNG\textsuperscript{−/−}Msh2\textsuperscript{−/−} B cells fare poorly in the GC, they do form plasma cells inefficiently in spite of their lack of DNA DSBs. Taken together, these results suggest that DNA DSBs, though potential contributors, are neither necessary nor sufficient for the in vivo maturation of GC B cells into plasma cells.
Figure 5.4. Induction of DNA DSBs in GC B cells and plasma cell chimerism. Mixed BM chimeras were immunized and then irradiated at day 10 p.i. to induce DSBs. (A) Representative histogram of γH2AX in both AID−/− and WT B cells from BM chimeras 3hrs after irradiation. (B) Flow cytometry of B220lowCD138+ BM plasma cells. (C) The ratio of AID−/− : WT B220lowCD138−NP− BM plasma cells in control and irradiated BM chimeras.

Figure 5.5. GC B cell and plasma cell chimerism in UNG−/−Msh2−/−/WT BM chimeras. Mixed BM chimeras of UNG−/−Msh2−/− and WT BM donor cells were immunized and their splenic GCs and BM plasma analyzed by flow cytometry. (A) Measuring apoptosis of UNG−/−Msh2−/− (DKO) and WT B220+GL7+Fas+ GC B cells by flow cytometric TUNEL staining. (B) Chimerism of splenic GC B cells and BM plasma cells in BM chimeras measured by flow cytometry. Student’s unpaired t-test; ns, not significant; **, P<0.01.
Figure 5.4

A

$\gamma$H2AX

B

CD138

B220

C

BM Plasma Cell Chimerism

Normalized Ratio

AID-/- : WT

Control

Irradiated
Figure 5.5

A

TUNEL\(^+\) GC B cells

\%

of GC B cells

WT DKO

Day 7

WT DKO

Day 14

WT DKO

Day 21

B

Normalized Ratio

UNG\(^{-}\)/Msh2\(^{-}\): WT

GC B cells

Day 7

Day 14

Day 21

Plasma Cells

Day 14

Day 21
5.3 Discussion

In the previous chapter I demonstrated that AID−/− B cells accumulate in large numbers in splenic GCs when in competition with WT B cells. That data was included in a publication with a colleague suggesting that the accumulation of AID−/− GC B cells was caused by a lack of AID-induced DNA damage. In this chapter I examined previous results in more detail and found that AID−/− B cells were specifically accumulating as CXCR4low centrocytes in the splenic GCs of mixed BM chimeras.

Because it is at the centrocyte stage that fate decisions occur, this raised the possibility that AID−/− centrocytes exhibited perturbed maturation. It was already evident that AID−/− GC B cells were not undergoing apoptosis in large numbers, which is the usual fate for clones with low affinity for Ag. As well, the mean normalized ratios of AID−/− : WT centroblasts were nearly 1, indicating that AID−/− centrocytes were not recycling into centroblasts in large numbers either. The next logical step was to investigate the emigration of AID−/− GC B cells into plasma cells. Human patients with hyper IgM syndrome 2, in which there is a loss of function mutation in the AID locus, present normal or elevated levels of serum IgM and AID−/− mice exhibit elevated levels of serum IgM compared to WT. As well, AID-deficiency has been linked to autoimmunity through the production of autoreactive Ab in murine autoimmune disease models. These observations indicated that AID−/− B cells are indeed able to mature into IgM-secreting plasma cells. The question remained, however, whether or not AID−/− B cells matured into plasma cells at a normal frequency when competed against WT B cells. In analyzing BM plasma cells by flow cytometry and ELISPOT, I observed equal numbers of AID−/− and WT BM plasma cells. Because AID−/− B cells outnumbered WT B cells so significantly in the centrocyte population it would be expected, if all things were equal, that this skew would carry over into the plasma cell population. Evidently, AID−/− centrocytes are able to mature into IgM-secreting BM plasma cells, but at a much reduced rate. This is supported by my rtPCR data: Bcl6 promotes the GC B cell program while Blimp1 controls the plasma cell program and the two factors are antagonists of one another. The fact that AID−/− GC B cells tended to express higher levels of Bcl6 and lower levels of Blimp1 than WT GC B cells suggested that fewer AID−/− GC B cells were initiating the plasma cell transcriptional program. Although the expression difference between AID−/− and WT was small, it represents the difference among all GC B cells. It is likely that rtPCR analysis of centrocytes alone would reveal a larger difference, but the low frequency of these cells makes sorting and isolating RNA from them very difficult.
Based on my observations, a partial block at the maturation stage is a likely contributor to the accumulation of AID−/− B cells in GCs. However, GC B cells should not remain static in the centrocyte stage; they are primed for apoptosis and must either commit to a fate decision or die by neglect73, 85. While AID−/− B cells may be piling up as centrocytes, their lower rate of apoptosis is likely what allows them to remain in large numbers in GCs. Thus, it is likely that a combination of lower DNA damage due to AID-deficiency and a low frequency of plasma cell maturation combine to account for the accumulation of AID−/− B cells in GCs.

Having determined that AID−/− GC B cells were maturing inefficiently into plasma cells, I then proceeded to investigate the cause. GC B cells can receive a number of different signals including BCR-stimulation, CD40/CD80/CD86 costimulation, and stimulation from cytokines including IL-4 and IL-21131, 190, 191 although the exact combinations of signals that determine maturation into plasma cells in vivo is still being investigated85. A series of in vitro experiments on Ramos cells and human tonsil-derived B cells demonstrated that DNA DSBs can initiate a signal that suppresses Bcl6, thereby supporting the Blimp1 program and plasma cell maturation185. That study showed that DNA DSBs created by ionizing radiation or etoposide activate the DNA damage sensor ATM. ATM in turn activates LKB1 which then suppresses the activity of a factor called CRTC2. Because CRTC2 promotes Bcl6, suppressing the activity of CRTC2 decreases Bcl6 levels and can tilt the balance in favor of its antagonist, Blimp1. Interference with this signaling axis reduced the number of Blimp1+ plasma cells in an in vitro mock GC cell assay. The study also stimulated murine AID−/− B cells in vitro and found that they expressed higher levels of CRTC2 in the nucleus than WT B cells, suggesting that a lack of AID-induced DNA DSBs results in elevated CRTC2 activity. These observations constitute convincing evidence that the creation of DNA DSBs promote the maturation of GC B cells into plasma cells, and along with other studies suggest that DNA DSBs are important signals in a number of cell processes192-194. The lack of AID-induced DNA DSBs in AID−/− GC B cells therefore seemed to be a likely cause for the low number of AID−/− plasma cells that I observed. However, when I induced DNA DSBs in AID−/− GC B cells it failed to increase the ratio of AID−/− plasma cells in mixed BM chimeras. Furthermore, by interfering with AID-induced DSBs using the Ung−/−Msh2−/− BM chimeras, PC formation seemed unaffected.

In assessing why I was unable to corroborate previous findings in my model, it is important to consider the differences between the signaling environments of in vivo GC B cells and in vitro-stimulated B cells. In the in vitro studies, naïve B cells were initially stimulated with IL-4, IL-10, and an agonistic anti-CD40 mAb. IL-4 and IL-10 were continually provided to
the cultured B cells while anti-CD40, which shuts down plasma cell maturation\textsuperscript{195}, was withdrawn partway through the mock GC reaction. This means that the cells in their study were not receiving signals through their BCRs and stopped receiving co-stimulatory signals at a defined timepoint. The GC B cells in my \textit{in vivo} experiments, however, would have had constant access to antigen, CD4\textsuperscript{+} T follicular helper cells, and other cytokines. My evidence therefore suggests that DNA DSBs are not a critical or sufficient signal to restore normal rates of plasma cell maturation due to the complex signaling environment of \textit{in vivo} GCs.

Alternatively, it is possible that DSBs must be present within the Ig loci in order to initiate the appropriate maturation signals and that my experiments, which created genome-wide DSBs, was not sufficient. However, as highlighted above, interfering with DSB formation at the Ig loci using Ung\textsuperscript{−/−}Msh2\textsuperscript{−/−} BM chimeras seemed to corroborate the findings of the irradiation experiment. Nevertheless, this possibility could be further tested by inserting loxP sites into the IgH loci of AID\textsuperscript{−/−} mice to see if Cre-mediated DNA DSBs can rescue plasma cell maturation.

However, it still remains determine the missing signal(s) that AID\textsuperscript{−/−} GC B cells require to undergo a normal rate of plasma cell maturation. I believe that the next most likely candidate is signaling through class-switched BCRs; whereas IgM possesses a non-signaling 3-amino acid cytoplasmic tail, IgG possesses a longer cytoplasmic tail containing a conserved YXXM amino acid motif that confers a qualitatively and quantitatively different signal that enhances plasma cell maturation\textsuperscript{196,197}. As well, IgG\textsuperscript{+} memory B cells are more likely to mature into plasma cells than IgM\textsuperscript{+} memory B cells upon stimulation with Ag\textsuperscript{198}. These observations suggest that signaling through an IgG\textsuperscript{+} BCR promotes the plasma cell program. AID\textsuperscript{−/−} B cells, which can only express IgM\textsuperscript{+} BCRs, lack this signal. In order to test this, a modified version of my MD4 adoptive transfer experiment (section 5.2.2) could be performed in which the AID\textsuperscript{−/−} B cells contain a modified version of the MD4 transgene in which the MD4 IgM cytoplasmic tail has been replaced by the IgG cytoplasmic tail\textsuperscript{196}. The number of AID\textsuperscript{−/−} plasma cells that are generated in this new model could be compared to the older one. Alternatively, the IgG cytoplasmic tail could be knocked-in to the IgM tail loci of AID\textsuperscript{−/−} mice. AID\textsuperscript{−/−} BM cells that are homozygous, heterozygous, or negative for this knock-in could be used to generate AID\textsuperscript{−/−} : WT mixed BM chimeras and the ratio of AID\textsuperscript{−/−} BM plasma could be assessed in each condition. These experiments could determine if class switched BCRs are crucial for plasma cell maturation in a competitive GC environment.

In this chapter I have observed that as a consequence of AID-deficiency, B cells will accumulate as CXCR4\textsubscript{low} centrocytes in competitive GCs and will exhibit a low frequency of
plasma cell formation. This behavior is not due to the affinity of the BCR for antigen nor is it
due, at least primarily, to a lack of DNA DSBs. The behavior of B cells within GCs and the
signaling requirements for these behaviors is a complex matter that is still an area of
investigation. My results here further our understanding of the GC reaction and help in bringing
us closer to a full understanding of this process.
Chapter 6: Discussions

6.1 Perspectives on the Purpose of Germinal Centers

Although adaptive immunity is found in all vertebrates, GCs are only observed in higher vertebrates (birds/mammals)\(^{199}\). Even then, affinity matured Ab responses can still occur in the absence of GCs after genetic abrogation of LT\(^{136}\). These observations raise the question: what is the purpose of GCs?

Taking an evolutionary perspective can sometimes lend insight to these questions of biological purpose. GCs primarily form in the secondary lymphoid organs. Spleens with white pulp consisting of B cell zones enclosing T cell zones are apparent in cartilaginous and bony fish\(^{200, 201}\) and the splenic white pulp displays increasing stromal complexity from amphibians to reptiles\(^{202}\), though they still lack recognizable GCs. Lymph nodes emerged more recently and are newer than the lymphatic systems from which they develop. Lymph nodes are present in certain species of birds and are ubiquitous in mammals\(^{203}\). It is in the organisms with lymph nodes, which unlike the spleen are organs devoted exclusively to lymphoid responses, that we observe GCs. The fact that GCs are present in birds and mammals, but not in amphibians or reptiles, suggests that perhaps GCs arose to solve a problem posed by endothermy, or warm-bloodedness. Temperature affects the metabolic rates of bacteria, and it is tempting to speculate that increased rates of bacterial growth in the tissues of endothermic organisms required the evolution of better adaptive immune responses. Endothermic organisms do indeed exhibit more robust affinity maturation than ectotherms, and this difference correlates with the presence of GCs\(^{204-206}\). Do GCs therefore help to cope with the danger of quicker, more dangerous primary infections?

Mathematical modeling of affinity maturation suggests that the features of GCs, including the spatial coupling of mutation and selection\(^{207}\) and the cyclical nature of mutation and selection\(^{208, 209}\) could enhance the efficiency and potency of adaptive immune responses. The ability of GCs to enhance affinity maturation is important when we consider that the emergence of a single affinity-increasing mutation is so statistically rare that it cannot account for the consistent and timely emergence of large numbers of high affinity B cell clones that we observe. However, the enhancements granted by GCs seem more important in generating secondary responses and may have little impact on the primary clearance of pathogens\(^{207, 210}\). GCs don’t peak until 1-2 weeks after immunization and they persist until long after Ag has been cleared. It is entirely possible that Ag can be effectively cleared by the time that the GC
response has only just reached its peak. Significant numbers of Ag-specific Ab-secreting cells are generated early in a primary response before the formation of GCs\textsuperscript{79,80,211}. As well, blocking the formation of GCs with soluble co-stimulation antagonists results in only a 50% decrease in early serum Ab levels, although it does result in impaired long-term affinity maturation\textsuperscript{212}. Similarly, in my studies with LTβ\textsuperscript{−/−} BM chimeras, mice with short-lived GCs generated normal titers of serum Ab with the same affinity for Ag as WT mice in the first 2 weeks of the response. In those chimeras, the consequence of early GC dissipation didn’t manifest itself until later in the form of significantly reduced affinity maturation. Indeed, infection of mice with attenuated \textit{Salmonella} can generate a robust extra-follicular Ab response that is effective at containing primary infection\textsuperscript{213}. A study using poxvirus infections found that while mice deficient in CD8\textsuperscript{+} T cell responses died early during infection, B cell deficient mice did not die until much later\textsuperscript{214}. These observations suggest that GCs are more concerned with generating memory and long-term affinity maturation than with clearing primary infections. In this case, innate immunity, CD8\textsuperscript{+} T cells, and early bursts of Ab with low-to-moderate affinity for Ag could deal with the primary infection while GCs work to handle chronic infections and secondary challenges.

The generation of affinity matured Ab and memory B cells is not unique to GC responses, however. LTα\textsuperscript{−/−} mice lacking GCs generate affinity matured Ab that has undergone SHM\textsuperscript{136}. Ag-stimulated B cells that receive sufficient co-stimulation can turn into memory B cells without entering GCs\textsuperscript{140}. As well, GC-less TNFR1\textsuperscript{−/−} mice generate large quantities of neutralizing Ab and memory B cells in response to VSV infection\textsuperscript{215}. If even these processes can occur without GCs, what then is their purpose? The answer, it seems, is enhancement. Though GCs do not add a unique weapon to the immune arsenal, they augment existing mechanisms of humoral immunity. LTα\textsuperscript{−/−} mice only generated Ag-specific Ab in response to high doses of Ag and even then it was of lower affinity with fewer mutations than in WT mice\textsuperscript{136,216}. Similarly, TNFR1\textsuperscript{−/−} mice generated weak responses to low doses of Ag or non-replicating VSV, and persisting serum Ab in response to live VSV was of lower affinity and declined prematurely when compared to WT mice\textsuperscript{215}. My experiments from Chapter 3 similarly demonstrate that higher levels of affinity maturation only occur when GCs persist for longer. This suggests that for proper affinity maturation it is not sufficient for GCs to initiate and reach peak numbers. Rather, they must also persist for longer periods of time in order to ensure continual cycles of mutation, selection, and proliferation. Key to sustaining affinity maturation is the ability to collect and maintain deposits of Ag for clonal selection. Indeed, Ag complexes can be observed sustaining small GCs for months after initial challenge\textsuperscript{217,218}. The ability of GCs to focus Ag
along with B and T cells into one location may even account for the ability to respond to low doses of non-replicating Ag. The prolonged persistence of Ag might also be important in maintaining long-term memory B cells, although this is still a matter of debate\textsuperscript{219}.

Returning to the original question of evolutionary purpose, the ability of GCs to augment affinity maturation and secondary responses still presents clear advantages. If, hypothetically, a GC-less organism has a 50% chance of surviving a primary infection and a 60% chance of surviving secondary infections, whereas an organism with GCs has a 50% chance of surviving primary infections and an 80% chance of surviving a secondary infection, then the evolutionary benefits are clear. As well, the ability to generate memory in response to small amounts of Ag means that limited exposure to a pathogen could allow an organism to mount a successful secondary response to a larger infection later on that would overcome a primary response. Such a robust secondary response could also limit collateral damage from overwhelming inflammation induced by the innate immune system. Whether GCs evolved in endotherms in response to the threat of faster bacterial metabolism or whether their appearance was a random emergence remains uncertain, though fascinating to contemplate nonetheless.
6.2 The Functions of Follicular Dendritic Cells

FDCs exhibit many prominent features. They can capture large amounts of Ag on their surfaces using Fc and complement receptors, produce gradients of attractant chemokines, and secrete cell survival factors. Based on their phenotype alone, the functions they serve in supporting humoral immunity should be obvious. However, numerous studies have shown that various functions attributed to FDCs can still be carried out in their absence. A series of puzzling observations that questioned the role of FDCs were summed up in a 2003 opinion article entitled *Are follicular dendritic cells really good for nothing?* Despite FDCs being assuredly useful in humoral responses, the publication’s odd title evoked the emerging picture that the nature of FDCs is more nuanced than was originally hypothesized.

The question of whether or not FDCs were “good for nothing” centered around a debate over the role of immune complexes (IC) that are consistently observed to be deposited on FDCs during primary immune responses. The general belief had been that GC B cells competed for Ag in the form of ICs on the surface of FDCs and that this formed the basis of clonal selection. In this model, B cells received BCR stimulation as well as Ag-non-specific signals from the FDCs as a reward. As well, because ICs on FDCs were multivalent and contained complement components, Ag presented in this form to B cells was more stimulatory than soluble Ag. Indeed, Ag-stimulation obtained by B cells from the surface of FDCs is more potent than soluble Ag in vitro and this enhancement of B cell activation from FDCs is contact-dependent. While this may be due to the formation of immunological synapses between the surfaces of B cells and FDCs, in vitro experiments also showed that providing FDC-derived, Ag-bearing iccosomes alongside Ag-irrelevant FDCs enhanced stimulation more than iccosomes alone. This suggests that FDCs also provide Ag-independent signals that enhance B cell activation. However, the emergence of conflicting evidence began to question the importance of ICs deposited on FDCs. First, there were studies using genetic ablation of complement receptors to prevent FDCs from retaining ICs. Immunization of CR2−/− mice, in which both complement receptors 1&2 are absent, was able to generate normal numbers of splenic GCs with no defect in affinity maturation. Experiments reconstituting CR2−/− mice with WT BM so that only radio-resistant cells lacked complement receptors demonstrated that CR2−/− BM chimeras immunized with bacteriophage also exhibited normal numbers of splenic GCs and Ab titers. Another experimental approach knocked out the ability of B cells to secrete Ab, thereby preventing the formation of ICs. This demonstrated that during a primary
response in the absence of ICs there was no defect in GC formation and that GC B cells exhibited normal SHM. All of this evidence was summed up in another 2003 opinion article that suggested ICs deposited on FDCs were largely irrelevant in promoting GC responses\textsuperscript{223}. What, then, is the role of FDCs trapping Ag? Looking back more closely at the data, we can see that the issue is more nuanced than it seemed. Firstly, we must take note of the doses of immunizing Ag used in these studies. Some experiments found that low doses of Ag generated weaker primary responses\textsuperscript{228, 231} while others only used high/repeated doses of Ag\textsuperscript{229, 230}. As well, even responses to high doses of Ag showed defects. While some studies did not investigate very long after the primary response to high-dose immunization, those that did noted reduced numbers of BM plasma cells and more rapid declines in Ag-specific serum IgG than in controls. Other studies noted slightly delayed affinity maturation\textsuperscript{232} or a severe impairment in response to large, particulate Ag such as SRBC\textsuperscript{233} in the absence of FDC ICs. In some of these studies, the focus was on proving whether or not it was Ag deposited on FDCs that stimulated GC B cells. The observations that GC B cells could mount robust responses in the absence of FDC ICs therefore seemed to prove that they did not drive GCs. Looking more closely at the data, however, we see that the question of whether or not FDC ICs are required for GC responses is misleading. Rather, the question should be whether or not FDC ICs enhance GC responses. To this latter question, the answer seems quite clearly to be "yes". In the previous section, I discussed how GCs enhance humoral responses rather than being required for them. Similarly, the retention of ICs by FDCs enhances GC responses rather than being an absolute requirement for their function. The ability of FDCs to focus Ag in a small area amplifies the response to low concentrations of Ag, allowing the generation of memory to minor initial exposures to pathogen. Furthermore, the ability to retain pockets of Ag for extended periods of time allows for sustained affinity maturation and maintains circulating levels of Ab at higher levels\textsuperscript{234}. My experiments in LT\(\beta\)\textsuperscript{-/-} BM chimeras further support this notion by showing that GCs dissipate early in the absence of FDCs, resulting in impaired affinity maturation. Looking at the variety of immunization protocols used in these studies, it seems that Ag-capture by FDCs imparts adaptability to the wide variety of conditions in which an organism may encounter pathogens. Finally, it is important to note that there is little data on the role FDC ICs play in conferring host protection to live infections. This approach is deserving of more investigation and could further define the role of Ag-retention by FDCs.

The role of FDCs in the formation of GCs has also proven to be a slightly confusing issue. FDCs produce the chemokine CXCL13 that is crucial for attracting B cells and producing
By signaling through its receptor CXCR4, CXCL13 attracts not only B cells but also CD4+ T follicular helper cells that are crucial in the GC response. Therefore, it would appear that FDCs are crucial for organizing follicles and GCs. Again, however, this picture has been clouded by conflicting observations. As discussed in Chapter 3, numerous studies demonstrated that mice lacking FDCs also failed to form GCs. However, the methods used to ablate FDCs in these studies had pleiotropic effects and a causal relationship could therefore not be established. One study did note that mice lacking FDCs generate GCs in mesenteric LNs. This observation failed to significantly influence the perception of FDCs as being required for GC formation, perhaps due to the unique properties of mesenteric LNs that I have previously discussed. A more recent paper that specifically ablated FDCs again suggested that they are required for GC B cell clustering, but it carries some caveats that I have mentioned already. My work is the first to investigate a GC response in a peripheral LN during the absence of FDCs. My experimental approach using a combination of genetic ablation, BM chimerism, and pharmacological inhibition has corroborated earlier observations in mesenteric LNs, extended their implications to peripheral immunity, and produced strong evidence that FDCs are not an absolute requirement for follicle and GC organization. As with the role of FDC ICs, I believe that the role of FDC-derived chemokines is more nuanced than a simple question of strict requirement. The evidence from LNs suggests that FDCs may be redundant in this role. Ablation of FDCs does not result in a significant decrease in the amount of CXCL13 transcripts in peripheral LNs. Since FRCs and possibly MRCs can produce CXCL13, it is likely that other stromal cell types are able to fill this role in the absence of FDCs. LNs are structures optimized to orchestrate lymphoid responses, and it makes sense that they would have functional redundancies. In a multi-functional organ like the spleen, however, the organizational role of FDCs may be more important. Ablation of FDCs results in a noticeable decrease of CXCL13 transcripts in the spleen, and the severe disorganization of splenic white pulp in LT-deficient mice may indeed be due to the absence of FDCs. Where FDC-derived cytokine may be most relevant is in ectopic follicles formed during inflammation. Many studies of autoimmune diseases suggest that FDCs are crucial in generating ectopic follicles in the CNS, gut, synovium, and salivary glands. FDC-derived CXCL13 may also be important for the organization and maintenance of inducible broncus-associated lymphoid tissues (iBALT) in response to respiratory infection.

In addition to the functions discussed above, FDCs can also secrete a variety of cytokines and cell factors to enhance or modulate GC responses. FDCs secrete IL-6 following...
stimulation by ICs and interference in FDC-derived IL-6 results in severely impaired GC responses\textsuperscript{241, 242}. The canonical FDC marker FDC-M1 was recently shown to be the same molecule as the FDC-secreted factor Mfeg8 which is suggested to have a role in mediating phagocytosis of apoptotic GC B cells and maintaining tolerance\textsuperscript{144}. In addition to stimulation, FDCs can also serve a regulatory role. A recently described molecule, FDC-secreted protein (FDC-SP), can reduce the magnitude of the GC response\textsuperscript{243}. The influence of FDCs on humoral responses is evidently complex and multi-faceted. That some of their purported functions can be carried out in their absence is not necessarily indication of their significance. Further investigations into the many roles of FDCs must be mindful to isolate FDC-sourced signals and to mine their results not just for the presence or absence of effects, but for their magnitude and quality as well.
6.3 B cell Fate Decisions

There are four primary outcomes for a GC B cell: death by apoptosis, maintenance of GC phenotype, or emigration as a plasma or memory cell. Apoptosis is the negative outcome for a GC B cell, although one that is crucial for proper affinity maturation and homeostasis. Apoptosis can be induced extrinsically through engagement of Fas (CD95) on the surface of the B cell, or it can be induced intrinsically such as by DNA damage. My work in Chapter 4, which was included with complementary data in a 2009 publication, suggests that DNA damage as a result of AID activity increases the amount of apoptosis occurring in GCs. This side-effect of AID appears to be important in constraining GC size, as AID-/+ mice display constitutive, enlarged GCs. This constraint on the viability of GC B cells may help to protect against autoimmunity. However, the apoptotic fate of GC B cells is largely determined by the pro-apoptotic signal of Fas and the pro-survival signal of CD40. FasL is expressed by CD4+ T cells and can initiate apoptosis of low affinity B cells. In Fas-deficient lpr mice there is recruitment of lower-affinity B cell clones into the memory and plasma cell populations, presumably due to the inability to cull them in the GC. As well, conditional deletion of Fas on GC B cells results in B cell hyper-proliferation, indicating that Fas-mediated apoptosis is also crucial for homeostasis. GC B cells can be rescued through CD40-CD40L interactions with CD4+ T cells, but must present sufficient Ag to cognate T cells in order to receive this signal. B cells with the highest affinity for Ag can acquire more of it to present on MHCII and out-compete other B cells for T cell help. In this prevailing model of GC selection, B cells that do not acquire sufficient Ag to compete for co-stimulation succumb to stimulation from abundant FasL.

B cells receiving positive signals can choose to retain their GC phenotype and undergo further cycles of proliferation, mutation, and selection or they can choose to mature into plasma cells and exit the GC. These two B cell fates are primarily determined by the activity of the transcription factors Bcl6 and Blimp1. Bcl6 promotes the GC B cell phenotype. Deficiency in Bcl6 results in absent GC formation and affinity maturation and the transgenic over-expression of Bcl6 results in enlarged GCs, hyper-lymphoproliferation, and a reduction in plasma cells. Bcl6 promotes AID, allowing for SHM and CSR, and suppresses the activity of the DNA-damage sensor ATR. Expression of Bcl6 is also required for CD4+ T cells to adopt a T follicular helper cell phenotype, up-regulating CXCR5, and migrating into GCs.
In contrast, Blimp1 promotes maturation into plasma cells. Deletion of Blimp1 results in a near complete absence of plasma cells and increasing expression of Blimp1 in B cells correlates with development of the plasma cell phenotype. Blimp1 promotes the plasma cell phenotype by inhibiting proliferation and CSR. As well, Blimp1 cooperates with IRF4 to promote XBP1, which is critical for the development of the secretory machinery necessary to produce large quantities of soluble Ab. Bcl6 and Blimp1 appear to promote their respective transcriptional programs in large part by suppressing each other. Bcl6 and Blimp1 can bind directly to their opposing locus to induce direct suppression. Bcl6 can also indirectly suppress Blimp1 by promoting other Blimp1-suppressive factors. While the roles of Bcl6 and Blimp1 in promoting GC B and plasma cell phenotypes are well understood, the external stimuli that initiate these programs are still being determined. Naïve B cells stimulated by Ag subsequently seek out co-stimulation from CD4+ T cells before entering GCs, and signaling through CD40 is required for GC responses. Paradoxically, however, CD40 stimulation has been shown to suppress Bcl6. Though some evidence suggests signaling through CD40 supports plasma cell differentiation, in vitro stimulation experiments have demonstrated that CD40 signaling can be a very strong suppressor of Blimp1 and plasma cell phenotype. Another study suggests that stimulation with IL-21 can override CD40-mediated suppression of Blimp1 and induce plasma cell formation. However, there is also evidence that IL-21 promotes both Blimp1 and Bcl6, and that IL-21 from co-stimulatory T cells are required for optimal Bcl6 expression. As discussed in Chapter 5, DNA damage can initiate a signal that ultimately suppresses the activity of Bcl6. Though I demonstrated that this signal was not sufficient to restore normal plasma cell maturation in AID-/- GC B cells in vivo, this signal could cooperate with others to suppress Bcl6 enough to allow the Blimp1 program to gain control. In light of the confusing, seemingly contradictory, observations described above, it is most likely that the induction of Bcl6/Blimp1 activity is extremely context-dependent. The behaviour of naïve B cells stimulated by anti-Ig and CD40L can vary depending on the combination of TLR agonists that are provided concurrently. It is also possible that while CD40 stimulation suppresses both Bcl6 and Blimp1, simultaneous stimulation through CD40 and IL-21R partially rescues Blimp1 activity while keeping Bcl6 suppressed. Also of note, the activity of Bcl6 as a transcriptional repressor requires binding with co-repressors. Bcl6 can partner with many different co-repressors and different combinations might elicit different functions. In this light, the amount of Bcl6 present in a cell might not be the sole determinant of its commitment to the GC B cell phenotype. There is much further investigation required to untangle the maze of
signals that promote either Blimp1- or Bcl6-mediated fate decisions. Further studies will need to examine various combinations of signals, various timings of these signals, and to demonstrate their effects both in vitro and in vivo. As well, because Bcl6/Blimp1 can be post-transcriptionally regulated, investigations must be sure to measure protein levels in addition to mRNA transcripts.

In Chapter 5 I also discussed the possibility that signaling through class-switched BCRs could promote plasma cell maturation. One study observed that IgM+ memory B cells overwhelmingly return to GCs following re-stimulation with Ag, whereas IgG+ memory B cells almost exclusively generated plasma cells in the same conditions. IgM+ memory B cells generally have fewer mutations and lower affinity for Ag than class-switched IgG+ memory B cells. These observations suggest a model wherein high-affinity memory is directed to the immediate production of neutralizing Ab while low-affinity memory is sent back to GCs to undergo affinity maturation. The decision between these two fates could be mediated by the difference in the signals transduced by IgM- versus IgG-containing BCRs. Membrane IgM has a short cytoplasmic tail composed of 3 amino acids that has no activity. Membrane IgG and IgE, however, possess a tail that is 28 amino acids long and contains a conserved YXXM motif that can modulate BCR signaling. In a model using B cells with IgM or IgG transgenes with prearranged affinity for Ag, IgG-transgenic B cells generate more plasma cells and higher titers of circulating Ab than IgM-transgenic B cells in both primary and secondary responses. However, inserting the IgG cytoplasmic tail onto the IgM transgene dramatically increased the number of plasma cells and Ab. Studies of the IgG cytoplasmic tail have suggested that it prevents CD22/SHP-1 from inhibiting BCR signaling and results in increased proliferation and calcium flux in response to Ag-stimulation. Whether or not these specific modulations of BCR signaling are responsible for the enhanced plasma cell formation seen in IgG+ B cells remains to be determined. However, in light of these observations it is very possible that the inability of AID−/− B cells to class-switch and receive signals through an IgG+ BCR is responsible for their partial block in plasma cell formation that I observe. Adapting the IgM/G hybrid transgene to the models used in Chapter 5 could answer this question and improve our insight into GC B cell fate decisions.

Finally, GC B cells can also differentiate into long-lived memory B cells. The commitment to the memory B cell fate is the least understood of the four fates described here. Whereas it is known that the GC B and plasma cell fates are largely governed by Bcl6 and Blimp1, respectively, no canonical memory B cell transcription factor has been identified. One
possibility is that memory B cell maturation occurs in the absence of Bcl6 and Blimp1. Stimulation through CD40 can suppress both Bcl6 and Blimp1 and may induce memory commitment in the proper context. It has been shown that early access to co-stimulation can generate memory B cells prior to GC formation. It has also been observed that modifications that produce defects in the selection of GC B cells affect memory B cells in similar fashions.
Chapter 7: References


AID constrains germinal center size by rendering B cells susceptible to apoptosis

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The germinal center (GC) is a transient lymphoid tissue microenvironment that fosters T cell–dependent humoral immunity. Within the GC, the B cell–specific enzyme, activation-induced cytidine deaminase (AID), mutates the immunoglobulin locus, thereby altering binding affinity for antigen. In the absence of AID, larger GC structures are observed in both humans and mice, but the reason for this phenomenon is unclear. Because significant apoptosis occurs within the GC niche to cull cells that have acquired nonproductive mutations, we have examined whether a defect in apoptosis could account for the larger GC structures in the absence of AID. In this report, we reveal significantly reduced death of B cells in AID−/− mice as well as in B cells derived from AID−/− bone marrow in mixed bone marrow chimeric mice. Furthermore, AID-expressing B cells show decreased proliferation and survival compared with AID+/+ B cells, indicating an AID-mediated effect on cellular viability. The GC is an etiologic site for B-cell autoimmunity and lymphomagenesis, both of which have been linked to aberrant AID activity. We report a link between AID-induced DNA damage and B-cell apoptosis that has implications for the development of B-cell disorders. (Blood. 2009;114:547-554)

Introduction

A fundamental hallmark of humoral immunity is the ability to produce class-switched antibodies that have enhanced affinity for antigen. This process, termed affinity maturation, allows clonally selected B cells to refine their response to theoretically target any antigen with high specificity. After activation, B cells mutate the immunoglobulin (Ig) locus in a process known as somatic hypermutation (SHM).1 Mutated clones that have acquired increased affinity for antigen preferentially expand over their low-affinity counterparts. The selection process occurs in the germinal center (GC), a transient microenvironment that forms in secondary lymphoid tissue shortly after antigen exposure.2 The GC provides a competitive setting for B cells whereby ineffectual clones are actively cleared from the system via apoptosis, although the processes that govern this selection still remain vague.

SHM and class switch recombination (CSR) are initiated by the enzyme activation-induced cytidine deaminase (AID),3,4 which deaminates cytidines to uridines specifically at the Ig locus.5-10 AID-generated uridines are then engaged by various DNA repair pathways that either lead to the generation of point mutations in the antibody variable region or recombiningogenic events that lead to CSR. AID−/− mice lack mutations at their Ig locus and are incapable of producing class-switched antibodies.4 Interestingly, these mice were previously shown to harbor an abnormally high proportion of splenic GC B cells.11 This phenotype is also recapitulated in humans with AID deficiencies.3 Although a link between reduced gut immunity (resulting from an inability to produce mucosal IgA) and peripheral GC formation was hypothesized to account for these abnormalities, this remains to be conclusively proven, and the possibility of a B cell–intrinsic effect that could explain the profound expansion of GC B cells has not been examined.

GC B cells represent a unique lymphoid compartment of actively proliferating cells where numerous apoptotic factors must synergize to induce the elimination of nonproductive clones. Factors contributing to the intrinsic pathway of apoptosis, such as Bcl-2, Bcl-xL, and Bim,12-17 and the extrinsic pathway, such as Fas,18-22 have been implicated in GC selection. The unique physiology of these cells makes them highly susceptible to disease progression, often serving as etiologic sites for autoimmune and malignant B cells.13,23-26 Recent evidence has implicated AID as a fundamental contributor to the genetic aberrations that lead to these disease phenotypes.24,27-29 Given the importance of apoptosis as a parameter for both GC B-cell selection and lymphomagenesis, we investigated the relationship between AID-induced DNA mutation and cell death to understand how this enzyme may impinge on survival and death within the GC niche. Here we report that many of the potentially harmful AID-induced genetic alterations may indeed lead to the death of these cells within the GC.

Methods

Mice and immunizations

Wild-type (WT) and AID−/− mice bred on the C57BL/6 background (obtained from Charles River Laboratories and Tasuku Honjo, Kyoto University, Kyoto, Japan, respectively) used for antibiotic and apoptotic experiments were 10 to 12 weeks of age before immunizations. Mice were immunized once intraperitoneally with 200 μg of the hapten 4-hydroxy-3-nitrophenyl (NP) conjugated to chicken gamma globulin (CGG; Biosearch...
mixed at a ratio of 3:1, 1:1, or 1:3. Irradiated mice were reconstituted with B6.SJL-Ptprc<sup>+/-</sup> mice (C57BL/6 mice congenic for the SJL CD45.1 allele) were purchased from The Jackson Laboratory. B6.SJL-Ptprc<sup>+/-</sup> mice were crossed with AID<sup>+/−</sup> mice to produce an F1 generation positive for both the 1 and 2 alleles of CD45. These F1 mice were used as recipients for B6.SJL-Ptprc<sup>+/-</sup> and AID<sup>+/−</sup> bone marrow (BM); F1 mice were irradiated twice with 550 cGy using a Gammacell 40 Exactor (MDS Nordion). BM cells were isolated from both AID<sup>+/−</sup> and B6.SJL-Ptprc<sup>−/−</sup> (WT) mice, suspended in phosphate-buffered saline (PBS) at 2 × 10<sup>6</sup> cells/mL, and mixed at a ratio of 3:1, 1:1, or 1:3. Irradiated mice were reconstituted with 2 × 10<sup>6</sup> cells by injecting 100 µL of the BM cell suspension into the lateral tail vein. Control BM chimeric mice were produced in the same manner as mixed BM chimeric mice but were reconstituted with a mixture of C57BL/6 and B6.SJL-Ptprc<sup>−/−</sup> BM cells. BM chimeric mice were kept on antibiotic water containing 2 µL of neomycin for 1 week after irradiation. After 6 to 8 weeks, BM chimeric mice were micrinated intraperitoneally with 100 µg NP-CGG precipitated 1:1 in alum. All animal study protocols were approved by the University of Toronto’s Division of Comparative Medicine Committee.

Flow cytometric analyses

Spleens of nonchimeric mice were harvested 11 days after initial immunization. Splenocytes were obtained by mashing tissue through a 70-µm cell strainer and lysing red blood cells in 1 mL ammonium chloride/potassium bicarbonate lysis buffer for 1 minute at room temperature. Cells were then blocked in PBS with 2% fetal calf serum (HyClone) and anti-FcyIII/II antibody (clone 2.4G2). To visualize the GC by flow cytometry, combination of the following antibodies were used: anti-mouse B220 (eBioscience; clone RA3-6B2), anti–mouse IgD (eBioscience; clone 11-26c), peanut agglutinin (Biomedicala), anti–mouse Fas (eBioscience; clone 15A7), and anti–mouse GL7 (BD Biosciences; clone GL7).

Spleens of mixed BM chimeric mice were harvested 7, 11, 14, and 21 days after immunization. Splenocytes were isolated as described above and stained with anti–mouse B220 or anti–mouse CD19 (eBioscience; clone eBioI3D3), anti–mouse GL7, anti–mouse Fas, anti–mouse CD45.1 (eBioscience; clone A20), and anti–mouse CD45.2 (eBioscience; clone J04). All stained cells were analyzed using a FACSCalibur or FACSaria flow cytometer (BD Biosciences) and FlowJo software (TreeStar Inc) plotting with biexponential x- and y-axes.

Immunofluorescent microscopy and immunohistochemistry

Extracted spleens of mice were flash frozen in Tissue-Tek OCT (QIAGEN) and stored at −80°C. Thawed tissue samples were cryosectioned on a 5-µm thickness and stained for GCs using fluorescently labeled anti–mouse IgD and peanut agglutinin. Alternatively, spleen sections were labeled with fluorescein isothiocyanate (FITC)-GL7 and IgD-biotin followed by anti–FITC-alkaline phosphatase plus streptavidin-conjugated horseradish peroxidase (both Roche) and developed using horseradish peroxidase and alkaline phosphatase–specific kits according to the manufacturer’s instructions (Vector Laboratories). These sections were compared with a serial section stained with anti-CD45.1 and anti-CD45.1 to identify GC occupied by AID<sup>+/−</sup> versus WT B cells. Stained sections were visualized using a Leica confocal fluorescent microscope and analyzed using Velocity software (Improvision) and Photoshop 7.0 (Adobe).

Primary B-cell stimulation

Spleens from 10- to 12-week-old mice were harvested and pure B cells isolated using a magnetic negative selection B-cell enrichment kit (Stem-Cell Technologies) according to the manufacturer’s instructions. B-cell purity was assessed by flow cytometry and found to be at least more than 95% B220 positive. Purified cells were seeded at 4 × 10<sup>5</sup> cells/well of a 96-well plate and exposed to 25 µM lipopolysaccharide (Escherichia coli) serotype 055:B5; Sigma-Aldrich) or 10 µg/mL anti–mouse IgM f(ab‘)<sub>2</sub> (Jackson ImmunoResearch Laboratories; 115-006-020) and anti–mouse CD154 (CD40L; eBioscience; clone MR1). Cultured cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (HyClone) and 50 µM β-mercaptoethanol (Invitrogen) at 37°C and 5% CO<sub>2</sub>. To assess proliferation, cultured cells were stained with the dye carboxyfluorescein diacetate succinimidyl ester (Invitrogen) and monitored every 24 hours for dilution of the dye corresponding to cell proliferation. To assess apoptosis, cultured cells were stained with annexin V (eBioscience) at room temperature for 15 minutes and a caspase 3–specific substrate (BioVision) at 37°C for 30 minutes and assessed by flow cytometry.

In vivo apoptosis assays

To assess apoptosis levels in the GC in vivo, harvested splenocytes were first surface stained for GC markers as mentioned in “Flow cytometric analyses.” For caspase activity, cells were treated with the fluorescently conjugated CaspGLO® reagents specific to caspases 3, 8, and 9 (BioVision) according to the manufacturer’s instructions with minor alterations. Briefly, 10<sup>6</sup> cells were surface stained and then treated with CaspGLO® reagent for 30 minutes at 37°C in PBS and assessed immediately via flow cytometry. To assess late-stage apoptosis in vivo, the APO-BRDU kit (BD Biosciences) was used according to the manufacturer’s instructions. Briefly, after surface staining, 1 to 2 × 10<sup>6</sup> cells were fixed in 1% paraformaldehyde. Cells were then incubated with terminal deoxynucleotidyl transferase and 5-bromo-2-deoxyuridine, stained with FITC-labeled anti-bromodeoxyuridine antibody, and assessed immediately via flow cytometry.

In vitro cell culture

Ramos is an Epstein-Barr virus–negative human Burkitt lymphoma cell line. Cells were grown in Iscove modified Dulbecco medium (Invitrogen) and 10% bovine calf serum (HyClone). Western blots for AID expression were performed as previously described<sup>10</sup> with mouse anti-human AID (Cell Signaling Technology) and rabbit polyclonal anti-β actin (Abcam) as per the manufacturer’s instructions. A naturally low-expressing AID clone of Ramos (R1) was electrophoretically with 10 µg linearized pCEP4-AID in Iscove modified Dulbecco medium at 250 V, 960 µF, plated into 96-well plates, and selected for transfectants using 0.8 mg/mL hygromycin B to express human AID in this cell line. Apoptosis was measured in Ramos cells by annexin V staining as in “Primary B-cell stimulation.”

dsDNA break assays

Double-stranded DNA (dsDNA) breaks were assayed using a plasmid integration assay as previously described<sup>10</sup> using electroporation conditions as previously described.<sup>6</sup> Transient transfection efficiencies were calculated by electroporation of Ramos cells with the pmaxGFP plasmid (Amaza) and analyzed by flow cytometry 24 hours later.

Statistical analysis

All analyses were performed using GraphPad Prism. For Student t tests, 2-way analysis of variance, and Mann-Whitney tests, P values of .05 or less were considered significant.

Results

Splenic GCs are larger in the absence of AID

To examine the size and structure of GCs that form in the absence of AID, mice were immunized with the T-dependent antigen NP-CGG. The cellular response was examined 11 days after immunization by staining splenocytes for GC-specific cell surface markers. In WT mice, we observed an increase in the number of GC B cells (B220<sup>−</sup>, GL7<sup>−</sup>, Fas<sup>−</sup>) upon immunization with...
NP-CGG (Figure 1A). Confirming earlier reports,11 we found that AID−/− mice had larger numbers of GC B cells compared with WT controls both in the presence and absence of immunization (Figure 1A). We repeated these analyses using an alternate GC-specific surface stain (B220+, PNAhigh, IgDlow) and obtained similar results (data not shown).

The increased number of GC B cells in AID−/− mice might be the result of enlarged GC size and/or more numerous GC seeding in the spleen. Thus, we performed immunofluorescent staining of frozen spleen sections to discriminate between these 2 possibilities. We found the average area of GCs (PNAhighIgDlow clusters located within IgD+ follicles) measured in both unimmunized and immunized AID−/− spleens to be significantly larger than the average area of GCs measured in WT spleens (Figure 1B). Furthermore, GCs in the AID−/− spleen formed at a significantly higher frequency than in the WT spleen. Together, these data indicate that an AID deficiency leads to larger and more numerous GCs both before and after immunization.

Administration of broad-spectrum antibiotics to AID−/− mice does not abrogate GC expansion

It was previously shown that AID deficiency may contribute to a reduced ability to retain intestinal commensal bacteria growth ostensibly because of the inability to produce mucosal IgA.11 Subsequent bacterial escape into the periphery has been hypothesized as a potential explanation for the spontaneous GC formation observed in these mice. Furthermore, short-term treatment of these mice with broad-spectrum antibiotics was able to ablate spontaneous GC formation in the peripheral tissue of 5-week-old mice.11 However, GC B cells are known to accumulate over time in the spleen of AID−/− mice,12 and the long-term effects of antibiotics on this process remain unknown. In 2 independent experiments, we administered broad-spectrum antibiotics to mice over a period of 1 or 2 months to detect their effects on GC B-cell populations after immunization. Consistent with previous reports,11 antibiotic treatment had a dampening effect on the spontaneous GC B-cell population in unimmunized AID−/− mice (supplemental Figure 1, available on the Blood website; see the Supplemental Materials link at the top of the online article), although GC B cells in these mice were still present in high numbers in the spleen. Importantly, antibiotic-treated AID−/− mice still had increased numbers of GC B cells compared with their WT counterparts, suggesting that this treatment did not affect the abnormal retention of these cells in the AID−/− mouse.

Preferential accumulation of AID−/− GC B cells in mixed BM chimeric mice

To further discount the possibility that GC expansion in AID−/− mice is the result of uncontrolled regulation of intestinal microflora, we examined the GC response of AID−/− B cells in a mixed BM chimeric system. Host mice (CD45.1+, CD45.2+) were irradiated and reconstituted with a mixture of BM cells from CD45.1-congenic WT and CD45.2 AID−/− donor mice. After immunization, we tracked the populations of WT (CD45.1+, CD45.2+) and AID−/− (CD45.1−, CD45.2−) B cells in both the follicle (B220+ GL7− Fas−) and in the GC (B220+, GL7+, Fas+; Figure 2A). The relative accumulation of AID−/− versus WT GC B cells was calculated by tabulating the ratio of AID−/− B cells to WT B cells in the GC compared with the same ratio in the follicle where B cells are quiescent and selection is not a factor. In control chimeras injected with WT CD45.1 and WT CD45.2 BM, B cells derived from both WT congenic donors were equally represented within the GC niche (the median normalized ratio was ~1), thus
validating our approach (Figure 2B; control). In contrast, normalized ratios for all time points after immunization in the AID−/−/WT mixed BM chimeric mice were significantly higher than the control, indicating a preferential survival/accumulation of AID−/− GC B cells compared with WT GC B cells (Figure 2B). Importantly, this skew toward AID−/− GC B cells was evident even when chimeric mice were reconstituted with 3-fold more WT BM versus AID−/− BM cells (Figure 2C). These data are consistent with the observation of an expanded GC compartment in nonchimeric AID−/− mice and conclusively demonstrate that this phenomenon is not entirely dependent on a breach in gut immunity and the loss of IgA.

**GCs seeded by AID−/− B cells are more numerous and larger than those seeded by WT B cells**

To better describe the abnormal accumulation of AID−/− GC B cells in mixed BM chimeric mice, we studied GC size via histologic examination of serial spleen sections. We observed 3 different types of GC: those that were occupied by a mixture of WT and AID−/− B cells (~60% of all GCs), those that were occupied predominantly by WT B cells, and those that were occupied predominantly by AID−/− B cells. In Figure 3A, the top panel represents an example of a GC that exclusively contains AID−/− B cells, whereas the bottom panel represents an example of a GC that is occupied predominantly by WT B cells. Evaluating only GCs that are primarily occupied by WT or AID−/− B cells, we found a statistically significant increase in the number of GCs occupied by AID−/− B cells at both 14 and 21 days after immunization (Figure 3B). Furthermore, the average size of AID−/− GCs was significantly greater than WT GCs at day 21. However, because of the skew toward AID−/− GCs, data from day 14 lacked enough WT GCs to obtain statistical significance (Figure 3C). Thus, in a mixed BM chimeric system where WT and AID−/− GC B cells are in direct competition, AID−/− GCs are, on average, larger and more frequent than WT GCs.

**Reduced apoptosis in AID−/− GC B cells**

Given the importance of apoptosis as a selective measure within the GC, we examined cellular markers for both early and late stages of cell death to determine whether defects in these pathways were contributing to GC B-cell accumulation in the AID−/− mouse. We focused on caspase 3, the major apoptotic effector enzyme that acts in conjunction with initiator caspases 8 and 9, which are involved in alternate apoptotic pathways and in some cases cell activation. To quantify caspase activity, we used a fluorescently conjugated caspase-specific substrate that can be combined with cell-surface staining to compare apoptotic activity between the GC and

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**Figure 2. AID−/− GC B cells outnumber WT GC B cells in mixed BM chimeric mice.** (A) Mixed BM chimeric mice were immunized with NP-CGG, and splenocytes were analyzed by flow cytometry 7, 11, 14, and 21 days later. B220+ B cells were divided into follicular B cells (GL7− Fas+) or GC B cells (GL7− Fas+). The level of chimerism among splenic follicular and GC B cells was determined by taking the ratio of the percentage of AID−/− B cells (CD45.1− CD45.2+) to WT B cells (CD45.1− CD45.2−). The chimerism among GC B cells was then divided by the chimerism among follicular B cells to produce a normalized ratio whereby values < 1 indicate a skew toward AID−/− B cells. (B) Normalized ratios of splenic GC B cells from mixed BM chimeric mice from various time points after immunization were compared with those of control BM chimeric mice harvested at day 14 after immunization. Each dot represents an individual mouse, solid lines represent median values, and the dotted line represents a normalized ratio of 1. Statistics represent comparisons between experimental mice and the control chimeras. Data are pooled from 6 independent experiments. *P ≤ .05, **P ≤ .01, and ***P ≤ .001. (C) Normalized ratios of splenic GC B cells from mixed BM chimeric mice reconstituted with different ratios of AID−/− WT BM cells and harvested at day 14 after immunization are compared with one another.

**Figure 3. GCs occupied by AID−/− B cells are more numerous and larger in immunized mixed BM chimeras.** (A) Mixed BM chimeras were generated where irradiated hosts were reconstituted with a 1:1 mixture of WT (CD45.1) and AID−/− (CD45.2) donor BM. Most GCs were occupied by both WT and AID−/− B cells. However, some GCs were occupied by either WT or AID−/− B cells exclusively as evidenced by staining for CD45.1 in the absence of CD45.2 in the GL7+ GC, or vice versa. GCs were enumerated (B) and measured for surface area (C) at day 7 and day 14 after immunization. Median values are shown in panel C. Eight mixed BM chimeric mice were evaluated at each time point. Statistics represent comparisons between WT GC versus AID−/− GC. *P ≤ .05, ***P ≤ .001.
folicular (ie, background) populations. Figure 4A shows a representative example of caspase 3 staining in folicular (gray filled histogram) versus GC B cells (empty histogram), and a gating strategy is shown for enumerating caspase 3–positive events. Compared with folicular B cells, we found increased activity of all 3 caspases in WT GC B cells, which is expected because of the intense apoptotic selection occurring in this cellular compartment (Figure 4B). Consistent with the observed accumulation of AID−/− GC B cells, however, caspase activity in AID−/− GC B cells remained at background levels. This suggests that reduced apoptosis may be contributing to the phenotypic differences observed between WT and AID−/− GCs.

Because antibiotic treatment revealed an immunization-dependent increase in the GC compartment of AID−/− mice, we tested caspase activity in these mice as well. We found that the reduced apoptotic levels found in AID−/− B cells were not affected by antibiotic treatment (supplemental Figure 1B). We extended this study to our mixed BM chimeric system using host mice reconstituted with a 1:1 mixture of WT and AID−/− BM. Data are mean ± SD of n = 6 chimeric mice. (D) Mice were immunized with NP-CGG and 11 days later were harvested and stained to mark GC populations and then incubated with fluorescently labeled substrates specific for caspasases 8, 9, or 3. Plots were gated either on the folicular B-cell population (B220+, Fas−, PNA−) or the GC B-cell population (B220−, Fas+, PNA+) and assessed for caspase activity (shown is a representative WT sample). (B) Data were accumulated for immunized mice for caspasases 8, 9, and 3. Statistics represent comparisons between WT GC versus AID−/− GC. Data are mean ± SD of n = 6 to 9 mice per genotype. (C) Caspase 3 activity for AID−/− and WT GC B cells is shown for immunized mice reconstituted with a 1:1 mixture of WT and AID−/− BM. Data are mean ± SD of n = 6 chimeric mice. (D) Mice were immunized with NP-CGG and 11 days later were harvested and stained to mark GC populations and fixed for use in a flow cytometry–based TdT-mediated dUTP nick-end labeling assay. Gated cells were assessed for apoptotic activity (shown is a representative WT sample). (E) Accumulated data were expressed as a percentage of positive cells. Statistics represent comparisons between WT GC versus AID−/− GC. Data are mean ± SD of n = 6 to 9 mice per genotype. **P ≤ 0.01. ***P ≤ 0.001.

AID levels correlate with apoptosis and dsDNA-break formation in Ramos cells

We further explored the association between AID expression and cell death using the GC-derived Burkitt lymphoma cell line Ramos. We found that apoptotic levels, as measured by annexin V binding, were elevated in the Ramos clone R7 that expresses AID (Figure 6A) compared with the AID-negative Ramos clone R1. Furthermore, transgenic expression of AID in the Ramos R1 clone increased the level of apoptosis to the levels seen in the Ramos R7 clone, despite the fact that expression level of AID was approximately 3-fold higher in the Ramos clone R1-AID than the Ramos clone R7 (Figure 6A). This is probably the result of the fact that the mutation rates in both clones are similar,8 which suggests the presence of a factor that is required for AID activity but is present in a limiting amount, as previously discussed.52 These results indicate that the observed changes in apoptotic levels in Ramos cells are an AID-dependent effect.

Because of the ability of AID to generate dsDNA breaks, we hypothesized that these events may be responsible for inducing cell-stress pathways. We previously showed that exogenous plasmid DNA incorporates into sites of dsDNA breaks caused by AID in hybridomas.58 Using this same assay, we found approximately 7-fold higher levels of incorporation of the neomycin resistance plasmid in AID-high Ramos 7 and 80 clones33 compared with the AID-negative Ramos 1 clone (Figure 6C), suggesting that the AID-high clones have more dsDNA breaks in their genome. In
addition, the uptake of DNA by the AID-negative Ramos 1 clone was approximately 3-fold higher than the AID-high Ramos 7 and 80 cells using a transient transfection assay (Figure 6C), showing that the lower level of incorporation in the AID-negative Ramos 1 clone was not the result of the inherent inability of these cells to take up DNA. These data demonstrate that AID expression leads to an approximately 21-fold increase in dsDNA breaks in Ramos cells (ie, 7-fold higher stable transfection efficiency × 3 to correct for reduced transient transfection efficiency), which is consistent with the associated apoptotic phenotype of GC B cells that express AID.

**Discussion**

Programmed cell death within the GC plays a critical role in the selection of B-cell clones that have modified their Ig locus through SHM.34,35 The importance of apoptosis as it pertains to affinity maturation has been shown previously through the genetic manipulation of apoptotic genes. Transgenic expression of prosurvival factors of the Bcl-2 family, such as Bcl-2 and Bcl-xL,14,15,36 leads to a variety of abnormalities in GC formation and function, such as a reduction in apoptosis within the GC and a subsequent defect in affinity maturation. Studies using mice with either deleted or defective Fas receptors have implicated a similar role for this death receptor in clonal selection.21,22 Furthermore, studies in Bim-defective mice have reported an abnormal accumulation of antigen-specific GC B cells in the spleen.

AID-induced DNA damage in GC B cells, such as that which occurs during CSR, activates DNA damage response pathways as has been previously shown.37 In contrast, AID−/− GC B cells would undergo expansion within the GC without the associated DNA-damaging events. Our study provides several lines of evidence that a B cell–intrinsic defect in apoptosis is contributing to GC size in the absence of AID. Specifically, we found that BCR/CD40-stimulated AID−/− B cells (1) have a higher proliferative capacity and (2) undergo reduced apoptosis compared with WT B cells (Figure 5). This view was further supported by experiments using Ramos cells (Figure 6) where we conclusively showed a correlation between AID levels and induction of apoptosis. AID-induced strand breaks are crucial to the process of CSR; however, when unresolved, they ultimately lead to cell-cycle arrest and death. Indeed, we observed that AID-induced dsDNA break levels correlated with apoptosis levels in GC B cells and propose that a side effect of the mutagenic activity of AID is the generation of apoptosis-prone B cells that must compete for survival signals within the GC. This is illustrated well in mixed BM chimeric mice where AID−/− B cells are subjected to the normal competitive constraints of the GC milieu and still show evidence of unrestrained accumulation. Taken together, our study is the first to demonstrate an intrinsic apoptosis defect in AID−/− GC B cells that explains their abnormal accumulation in the GC of mice and in humans.

Upon ex vivo stimulation of primary B cells through the BCR and CD40, we observed a proliferative and apoptotic defect stemming from AID expression. We could not, however, recapitulate this result through lipopolysaccharide stimulation of primary cells via the Toll-like receptor 4 (TLR-4; data not shown), confirming earlier reports.4 This finding adds to the growing evidence for unique signaling pathways regulating AID expression through the BCR and other surface receptors.38,39 These studies have shown that signaling through the BCR delays AID expression and down-regulates certain AID-induced processes, such as class-switching, while having no effect on total transcript and protein levels. Furthermore, costimulation of both the BCR and TLR-4 was enough to dampen CSR in a phosphatidylinositol 3-kinase–dependent manner.39 Phosphatidylinositol 3-kinase–signaling interferes with AID activity only when signaled through the BCR, therefore implying a distinct signaling cascade compared with TLR-4. Unlike the BCR and CD40, however, TLR-4 signaling is not known to induce GC formation in vivo; thus, the ex vivo defect reported here might explain why AID deficiency has a GC-specific phenotype regarding apoptosis.40
the enlarged GC compartment observed in the AID−/− mouse because, in both BM chimeric mice as well as antibiotic-treated mice (supplemental Figure 1), AID−/− GC B cells accumulate to a greater degree than WT GC B cells. These data are also corroborated by previous studies in which AID−/− mice were shown to produce tertiary lymphoid organs even when raised in a germ-free facility.42

The GC is often linked to the development of autoimmune B cells, a process associated with abnormal persistence of B cells within this structure. Spontaneous GC formation, such as that found in AID−/− GCs, has also been linked to progression of humoral autoimmunity.43 In humans, a significant portion of patients with inactivating mutations in AID develop autoimmune syndromes by as yet unknown mechanisms.44 Furthermore, aged AID−/− mice were recently reported to develop a fatal autoimmune gastritis linked to activation of peripheral B cells and the development of tertiary lymphoid organs.42 We suggest that the development of autoimmune diseases in AID−/− mice may be linked to improper maintenance of the GC and potential escape of hazardous B-cell clones. These data emphasize the necessity for a fine-tuned balance of AID expression under physiologic conditions that is required for protection against B-cell disease.

In conclusion, we have shown that GC B cells from AID−/− mice have reduced levels of apoptosis contributing to the expansion of these lymphoid structures. Our findings provide clues to the mechanism of B cell–mediated disease development, including humoral autoimmunity and lymphoma. We emphasize the usefulness of the AID−/− mouse as a tool for the study of apoptotic factors that play a role in clonal selection within the GC.

Acknowledgments

The authors thank Drs M. Ratcliffe and M. D. Scharff for comments on this manuscript and the Martin laboratory for helpful discussions.

This work was supported by the Canadian Institutes of Health Research (Ottawa, ON; grant 183815; J.L.G., A.M.). J.L.G. was supported by a Canadian Institutes of Health Research New Investigator Award. A.M. was supported by a Canada Research Chair award.

Authorship

Contribution: A.Z. planned and performed the experiments and wrote the manuscript; B.B. and J.-Y.P. planned and performed crucial experiments; S.R. helped with experiments and contributed essential input into experimental design; and J.L.G. and A.M. conceived the experimental approach and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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