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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Peripheral B cell Tolerance mediated by Follicular Dendritic Cell-Displayed Self-Antigen

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

in

Molecular Pathology

by

Irene Wu Yau

Committee in charge:

Professor Robert Rickert, Chair
Professor Jack Bui, Co-Chair
Professor Michael David
Professor Gen-Sheng Feng
Professor Guy Salvesen

2011

The Dissertation of Irene Wu Yau is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011

Dedication

I dedicate this work to my parents, Bill and Ai-Ling Wu, who have been an encouragement through the years. Their love and selflessness is inspiring. I love them and am thankful for their support in my pursuits.

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One of the top five things people regret in life, according to the writings of one palliative care nurse, is not having the courage to express their feelings. Since this dissertation will most likely be my one and only dissertation, I will be liberal with my acknowledgements and have no regrets.

I would like to thank my advisor Robert Rickert, who graciously allowed me to study in his laboratory and learn from him. I appreciate his support and belief in my success. His guidance has not only been integral to my research progress, but has made these past years of research enjoyable. I feel confident in my future endeavors from the training I received in his laboratory. I am grateful to have had a chance to study and conduct research in immunology, a subject I love and greatly respect.

I also thank my committee members who shared their time and thoughts concerning my research. I appreciated their timely responses, which helped me greatly in planning and coordinating.

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Chapter 2 includes modified work from the submitted manuscript, “Elimination of self-reactive B cells by follicular dendritic cell-displayed self-antigen.” The co-authors are as follows: Matthew H. Cato, Tatiana Hurtado de Mendoza, and Robert C. Rickert. The author of this dissertation is the first author of the manuscript.

Chapter 3 includes work from the manuscript that has been accepted for publication in *Nature Protocols*, 2011. The co-authors are as follows: Matthew H. Cato and Robert C. Rickert. The author of this dissertation is the co-first author of the manuscript. Also included in this chapter is work from the published manuscript in *Molecular and Cellular Biology*, 2011. The co-authors are as follows: Matthew H. Cato, Suresh K. Chintalapati, Sidne A. Omori, and Robert C. Rickert. The author of this dissertation is the third author of the manuscript.

Vita

Irene Wu Yau

Education

Doctor of Philosophy, 2011
University of California, San Diego
Molecular Pathology
Research: Peripheral B cell Tolerance
Advisor: Dr. Robert C. Rickert

Bachelor of Science, Molecular Biology, March 2004
University of California, Los Angeles

Conference Presentation

B cells and Autoimmunity International Conference 2010
Peripheral B cell tolerance in a novel mouse model

Poster Presentation

La Jolla Immunology Conference 2010
Defining Peripheral B cell Tolerance Mechanisms using a Novel Mouse Model

International Congress of Immunology 2010
Peripheral B cell tolerance in a novel mouse model

Arthritis Research Conference 2007
BAFF promotes B cell responses via induction of ICOSL

La Jolla Immunology Conference 2007
BAFF promotes B cell responses via induction of ICOSL

Teaching Experience

Undergraduate Immunology, Teaching Assistant
April – June 2008

Training Grant

Gastrointenstinal Predoctoral Training Grant recipient from the University of California, San Diego
June 2008 – June 2011

Related Experience

Molecular Pathology Retreat, UCSD
Journal Selection Committee

- Selection of published research papers from peers for award recognition

Research Experience

Laboratory researcher, Molecular Biology Institute UCLA
Dr. Harumi Kasamatsu Laboratory
2003-2004

UCLA Center for Human Nutrition
2003

Pathology and Laboratory Medicine UCLA
2001

Publications

Yau IW, Cato MH, Hurtado de Mendoza T, Rickert RC. Elimination of self-reactive B cells by follicular dendritic cell-displayed self-antigen. *Journal of Experimental Medicine*. In submission.

Cato MH, Yau IW*, Rickert RC. Magnetic-based purification of "untouched" murine germinal center B cells for ex- vivo manipulation and biochemical analysis. *Nature Protocols*. In Press. *Co-first author

Cato MH, Chintalapati SK, Yau IW, Omori SA, Rickert RC. Cyclin D3 is selectively required for proliferative expansion of germinal center B cells. *Molecular Cell Biology*. 31(1):127-37.

ABSTRACT OF THE DISSERTATION

Peripheral B cell Tolerance mediated by Follicular Dendritic Cell-Displayed Self-Antigen

by

Irene Wu Yau

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2011

Professor Robert Rickert, Chair

Professor Jack Bui, Co-Chair

The main focus of this dissertation is examining the mechanisms of peripheral B cell tolerance (Chapter 2). Generating a diverse repertoire of B cells reactive against foreign pathogens, yet tolerant to self-tissue, is imperative for an effective immune system. Random gene rearrangement at the immunoglobulin loci results in the majority of newly formed B cells being self-reactive. At an initial checkpoint in the

bone marrow, a large portion of self-reactive B cells are rendered unresponsive or are eliminated through apoptosis. A second, less well-defined checkpoint in B cell tolerance occurs in the periphery as developing transitional B cells mature in the spleen. Indeed, studies have shown that rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients have a defect at this second crucial checkpoint. Within the follicle, follicular dendritic cells (FDCs) retain immune complexes and opsonized foreign antigens by Fc and complement receptors, respectively, important for B cell selection during the germinal center response. However, the selection of self-reactive B cells by self-antigen on FDCs has not been addressed.

To this end, a mouse model (*Cd21*$^{\text{cre}}$mDEL^{loxp} mice) that expresses self-antigen membrane-bound Duck Egg Lysozyme (mDEL) on FDCs to study the fate of mDEL-binding B cells was generated. The results from this model show that self-antigen displayed on FDCs mediates effective elimination of self-reactive B cells at the transitional stage. A portion of Chapter 2 will be on the design and generation of the appropriate mouse model to address the question of peripheral B cell tolerance in late-stage transitional and follicular B cells in the spleen. The remaining half of the chapter will present the results of peripheral B cell tolerance studies in *Cd21*$^{\text{cre}}$mDEL^{loxp} mice.

Chapter 3 presents work on various aspects of the germinal center reaction including the tools used to study GC B cells, the proteins involved in forming a GC reaction, and exploring mechanisms of eliminating self-reactive B cells that may arise in a GC reaction.

Chapter 1: Introduction

Background

The immune system is a finely tuned system that distinguishes between foreign pathogen and self-antigen. A breach in this mechanism leads to immune deficiency or autoimmunity. The immune system can be broken down into two parts – the innate and adaptive immune system. While innate immunity recognizes foreign components that span a broad range of pathogens, adaptive immunity recognizes specific components unique to a specific pathogen. B cells are part of the latter system and have receptors, called B cell receptors (BCRs) or immunoglobulins (Igs), which bind pathogenic components.

B Cell Development in the Bone Marrow

Hematopoietic stem cells (HSCs) in the bone marrow give rise to Common lymphoid progenitors (CLPs), which give rise to lymphocytes including B cells. The goal in B cell development is to produce a functional B cell receptor (BCR) that can signal and activate the B cell in the presence of foreign pathogen, but not self-antigen. B cell maturation stages are defined by the stage of BCR assembly.

The BCR consists of two heavy chains and two light chains formed by random gene rearrangement at the immunoglobulin locus to generate a unique receptor on each B cell. The heavy chain rearrangement region is made up of three segments, V, D, and J segments. The light chain rearrangement region is made up of two segments, the V and J segments.

The pro-B cell stage is the earliest B cell developmental lineage in which the heavy chain undergoes VDJ rearrangement, but lack surface heavy chain expression. The pre-B cell expresses the rearranged heavy chain on its surface as a pre-B receptor to ensure competent signaling. Upon successful signaling, the B cell is allowed to progress and form a rearranged VJ light chain. The immature B cell expresses the intact BCR on the surface, which includes the rearranged heavy and light chains.

Transitional B cells

After a functional B cell receptor is formed during development in the bone marrow, B cells enter the periphery as transitional B cells and circulate to the spleen where they complete their maturation. Roughly 1-2X10^7 immature murine B cells are formed daily in the bone marrow, but only 10% of those actually enter the periphery (1).

Transitional B cells are progressing towards maturation and immunocompetancy, but can be divided into subsets that differ phenotypically by specific marker expression. T1 cells, the earliest transitional stage, are characterized by $IgM^{hi}CD24^{hi}$ $CD21^{neg}IgD^{neg}$ $CD23^{neg}$ $AA4.1^{hi}$ expression. T2 cells descend from T1 cells and are characterized by $IgM^{hi}CD24^{hi}$ $CD21^{pos}IgD^{pos}$ $CD23^{pos}$ $AA4.1^{hi}$ (2-5). Follicular B cells descend from T2 B cells. A third transitional subset that is phenotypically identical to T2 B cells except with lower IgM expression are the T3 B cells that may represent naturally occurring anergic B cells that do not develop into mature B cells (6). Flow cytometric analysis can be used to identify transitional cell subsets shown in Figure 1.

Transitional B cells are primarily found in the spleen but not in the lymph node due to lack of CD62L expression, an adhesion molecule that allows cells to enter the lymph node (4). Transfer studies of sorted transitional cells suggest that T1 B cells are located in the red pulp and outer PALS while T2 B cells can be found in the B cell follicle (3). It is unclear what types of splenic-resident cells transitional B cells encounter and the importance of those encounters as they complete their maturation in the spleen.

While T1 cells undergo apoptosis upon BCR engagement, there is disagreement as to whether T2 cells undergo apoptosis or proliferation upon antigen engagement as various groups have reported opposing results (1-3, 7). It is unclear why the results differ, but it could be due to the heterogeneity of transitional B cells since they are on a continuous path towards maturation.

Transitional B cells have been shown to be capable of processing and presenting antigen to CD4 T cells via MHCII:TCR interactions (8). Because transitional B cells do not optimally upregulate CD80 or CD86 (CD28 ligands) with BCR engagement, unlike mature follicular B cells, only in the presence of external CD28 ligand were T cells able to proliferate and produce the necessary cytokines for B cell activation (3, 8, 9). Although both T1 and T2 cells were rescued from apoptosis with external T cell-help factors, T2 B cells proliferated to a greater degree and had increased survival compared to T1 B cells. This indicates that although transitional cells may not be optimal as antigen presenting cells to T cells (lack of CD28 ligands), they may be able to survive in the presence of prior activated T cells in an ongoing immune response (1).

Like immature B cells in the bone marrow, any disruption of the transitional B cell BCR and downstream signaling factors lead to a developmental block (4). In general, the earlier the defect in BCR signaling, the earlier the block is in transitional B cell maturation. For example, mice lacking the cytoplasmic tail of Igα have a block at the T1 to T2 B cell transition (10), while mice with a disruption in B cell linker protein (BLNK or SLP-65) have a block in the T2 to mature follicular B cell stage (11).

BAFF

B-cell activating factor of the TNF family (BAFF), also known as Blys, is needed for the survival and maturation of most B cell subsets starting from the T2 transitional B cell subset (12). BAFF is able to bind to three different receptors: BAFF receptor, TACI, and BCMA. BAFF and another ligand APRIL binds to TACI with equal affinity, while BAFF binds to BCMA with lower affinity than APRIL. BAFFR is a B cell exclusive receptor and is expressed on B cells starting from T2 stage (13). BAFF is the sole ligand that binds to BAFFR (14). In BAFF or BAFFR knockout mice, B cells are arrested at the T1 stage and lack T2, marginal zone (MZ), and follicular (FO) B cells, demonstrating that BAFFR signaling is crucial for B cell maturation (15, 16). In contrast, BAFF transgenic mice have an increased number of B cells, especially marginal zone B cells, and develop autoimmunity that resembles systemic lupus erythamatosus (SLE) and Sjogren's syndrome (17). Coincidently, higher levels of BAFF are found in patients with various autoimmune diseases (12).

The predominate source of BAFF for B cells is unknown, although myeloid cells, follicular dendritic cells (FDCs), and stromal cells are capable of producing BAFF. In reconstitution experiments in which BAFF KO bone marrow were used to reconstitute wild type mice, B cells were able to mature and populate the spleen (18). Interestingly, in reciprocal experiments in which WT bone marrow were used to reconstitute BAFF KO mice, the B cells were unable to mature and very few B cells developed, which indicates that the radioresistant stromal cells are the predominate source of BAFF for B cell survival and maturation (18).

BIM

The pro apoptotic Bcl-2 family protein Bim is involved in B cell homeostasis (19). Bim is regulated both at the transcriptional and post-translational level. Bim mRNA levels are upregulated in B cells with the removal of growth factors and Bim can be phosphorylated and ubiquitinated. Anergic B cells have been shown to have elevated Bim levels and is believed to play a role in B cell selection as mice deficient in Bim do not efficiently eliminate autoreactive B cells (20, 21). Interestingly, mice deficient in Bim or mice that over express the pro-survival protein Bcl-2 show similar phenotypes including increased FO and T2 B cells (22). This points to the fact that Bcl-2 and Bim play major but opposing roles in B cell homeostasis. Bim is believed to affect B cell survival by binding directly to Bcl-2 or by competing with Bcl2 (23). Bim also interacts with Bax, which leads to the release of cytochrome c through permeabilization of the mitochondrial membrane (24). BAFF over expression leads to restoration of anergic B cells in some autoreactive mouse models. It has been shown

that BAFF antagonized Bim function through downregulating Bim protein levels and blocking it from binding to Bcl-2 (25).

B Cell Activation

In normal B cell development, B cells finish their development in the spleen and are immunocompetent. They circulate in the blood and between secondary lymphoid organs to maximize the probability of encountering their cognate antigen (26). The secondary lymphoid organs such as the spleen and lymph nodes are highly organized structures. Although the spleen and lymph node differ in their organization, they both consist of T cell rich areas and B cell rich follicles (Fig. 2).

Follicular Dendritic Cells

Within the follicle are resting naive B cells and a network of stromal cells and follicular dendritic cells (FDCs). It has been difficult to study FDCs because they make up less than 1% of all splenic cells, although some progress has been made in understanding FDC function and attributes. The origin of FDCs is unclear, although much of the evidence supports the idea of a mesenchymal origin (27). They are radioresistant and rely upon lymphotoxin production from B cells for survival (28). FDCs are long-lived cells with many dendritic processes that express complement receptors and Fc receptors. Complement and immunoglobulin binding to antigen can be “captured” by these receptors on FDCs as immune complexes (ICs) and presented to naive B cells for activation or contribute to an ongoing immune response (Fig. 3). It is unknown to what degree FDCs contribute to initial B cell activation and their role in

maintaining immune responses as FDCs can bind to antigen for years (29). In addition to binding antigen, FDCs express BAFF, but because B cells are still present at relatively normal numbers and substantial amounts of BAFF transcripts were found in lymphotoxin beta knockout mice, FDCs are unlikely to be the predominate source of BAFF for B cells. These findings do not rule out the fact that FDC production of BAFF may still be important for B cell development or immune response (28). FDCs also secrete CXCL13, a chemokine that attracts CXCR5-expressing B cells into the follicle and contribute to lymphoid organization in the spleen and lymph node (30).

Germinal Center

B cells can undergo T-independent (TI) or T-dependent (TD) activation once they bind antigen. In TD responses, B cells rapidly proliferate and can either differentiate immediately into antibody-secreting cells or form germinal centers in the follicle that is termed secondary follicle (Fig. 4). The GC is a highly specialized area where B cells divide and become memory cells or antibody-secreting plasma cells. This is also the site where random mutation events in the antigen-binding region of the BCR, called somatic hypermutation (SHM), can result in higher affinity antibodies (affinity maturation), and where isotype switching occurs (class switch recombination).

During the GC reaction, a large number of B cells undergo apoptosis as BCR binding affinity are altered through SHM. During this event, FDCs upregulate their Fc and complement receptors. It is thought that the immune complexes on FDCs allow for the selection of higher affinity BCRs while the lower affinity BCRs undergo

apoptosis. The role of FDCs in B cell immune responses remains controversial since experimental models have shown that GC reactions can occur without IC deposition on FDCs (28). The apoptotic cells are marked for engulgment by Mfge8 secreted by FDCs and quickly cleared in the GC through engulfment by tingible body macrophages (31, 32).

The GC B cells are made up of centroblasts and centrocytes. Centroblasts undergo SHM and proliferation but lack pro-survival protein Bcl-2, which make them highly sensitive to apoptosis. Isolated GC cells rapidly die in-vitro without proper simulation. Centrocytes are the progeny of centroblasts and become plasma cells or memory cells. The events that lead GC B cells to differentiate into plasma cells is partly dependent on the downregulation of transcription factor BCL-6, which leads to the transcription of BLIMP1. Mice that are deficient for BLIMP1 do not generate plasma cells. Less is known about the steps leading to memory B cell formation (33).

Central Tolerance

B cell development occurs in the bone marrow where a functional BCR is formed and tested for proper binding and signaling. Newly formed B cell receptors that do bind to self-antigen either undergo deletion by apoptosis, receptor editing to form a new BCR that is not self-reactive, or anergy, where these B cells enter into the periphery but remain in a state of unresponsiveness. The term tolerance refers to the mechanism by which B and T cells mount an immune response only to foreign pathogens and not to self-components. A break in tolerance can lead to autoimmunity,

which is why understanding these mechanisms is important in developing treatments for autoimmune diseases.

In the case of receptor editing, if the B cell receptor signals in the presence of self-antigen in the bone marrow, light chain gene rearrangement continues and a new BCR specificity is created. The first mouse model for receptor editing contained an immunoglobulin transgene specific for a certain MHCI subtype. When the mice were crossed to a non-self antigen bearing strain, the B cells all expressed the transgenic BCR, but when crossed to a self-antigen bearing strain, developing B cells would undergo continuous rearrangement at the endogenous immunoglobulin locus (34). Receptor editing predominates the central tolerance mechanisms (35), and a breach in receptor editing mechanisms in the bone marrow is suggested in SLE and type 1 diabetes patients (36).

With the creation of Goodnow's B cell transgenic mouse model, anergy could be studied in-vivo (37). This model, called MD4, contained the heavy and light chain B cell receptor transgene from the HyHEL10 hybridoma that binds with high affinity ($K_a = 1x10^{10}$) to hen egg lysozyme (HEL). In normal development, a diverse repertoire of receptors is generated but in MD4 mice, nearly 90% of B cells bind to HEL with the same affinity. When MD4 mice were crossed to the ML5 line (MD4XML5), which express soluble HEL, B cells were found in the periphery that had a decreased lifespan, decreased surface levels of IgM, and unresponsive BCRs (38).

Interestingly, anergic B cells compete very poorly in the presence of wild type B cells and are eliminated within three days as opposed to several weeks in mice in which the majority of B cells were HEL-binding. It was also found that these HEL-

binding B cells could not enter the follicle, a phenomenon called "follicular exclusion" (39). It was found that anergic B cells had an increased requirement for BAFF, which is a key survival factor for mature B cells. BIM was elevated in anergic HEL-binding B cells and it is thought that higher levels of BAFF are needed to overcome the pro-apoptotic signals (20).

In several anergic B cell models such as the MD4XML5 model and SW_{HEL} X ML5 model over-expression of BAFF partially restored B cell maturation and some B cell subsets. In the SW_{HEL} X ML5 model, in which only a fraction of B cells bind HEL (40), BAFF over-expression allowed the previous HEL-binding B cells blocked at the transitional stage to mature into follicular B cells and enter the B cell follicle, while MZ B cells failed to develop (20, 41). The HEL-binding B-cells that would normally become anergic in the presence of continuous soluble HEL exposure became responsive to BCR stimulation and had comparable anti-HEL IgG serum antibodies to that of SW_{HEL} or BAFFX SW_{HEL} mice. In this model, BAFF over expression prevented self-reactive B cells from becoming anergic. Interestingly though, in the presence of increased competition with non-HEL binding B cells by reconstituting mice with SW_{HEL} and WT BM at a 90:10 ratio, BAFF over expression did not restore self-reactive B cell maturation and these mice had similar anti-HEL serum IgG levels as SW_{HEL} X ML5 mice, indicating that BAFF did not prevent anergy in these HEL-binding B cells (41). As expected, increased BAFF did not restore immature HEL-binding B cell development in the bone marrow in the presence of membrane-bound HEL, which reinforces that fact that BAFF responsiveness in B cells does not occur until the T2 transitional stage.

Clonal deletion is another tolerogenic mechanism that occurs in B cell development as well. This phenomenon was seen in other mouse models. In one mouse model (KLK3), HEL expression was membrane-bound on most cells including ones in the bone marrow where B cell development occurs. The membrane-bound HEL has a higher avidity than soluble HEL. KLK3xMD4 double transgenic mice displayed a lack of peripheral B cells but normal numbers of immature B cells in the bone marrow indicating that deletion had occurred during development (42).

These three mechanisms employed during development make up B cell central tolerance. It is now known that a large proportion of B cells generated in the bone marrow are self-reactive. In fact, it was found that in the bone marrow of healthy humans, about 75% of early immature B cells are self-reactive, but many are removed at this early checkpoint in the BM (43) (Fig. 5, Checkpoint #1). Although the mechanisms in central tolerance are a stringent process, some autoreactive lymphocytes escape central tolerance and exit into the periphery. The percentage of autoreactive newly emigrated B cells from the bone marrow is around 40%. These newly emigrant transitional B cells go into circulation and finish their maturation in the white pulp of the spleen to become mature naïve B cells (1). The percentage of autoreactive mature naïve B cells is further reduced to 20% due to mechanisms in the periphery that prevent the maturation of these self-reactive B cells (Checkpoint #2).

Peripheral Tolerance

Peripheral tolerance refers to tolerogenic mechanisms that take place once B and T cells have left the bone marrow and thymus, respectively. Not as much is known

about the mechanisms and importance of B cell peripheral tolerance. Different reports have identified the existence of regulatory B cells in mice that serves to secrete anti-inflammatory cytokines or promote T regulatory cell induction (44, 45). It is also unclear whether any of the central B cell tolerance mechanisms also take place in the periphery. There is some evidence that receptor editing can occur outside of the bone marrow but it is unknown whether this occurrence is physiologic and most studies suggest that receptor editing no longer occurs in the periphery (46, 47).

In one mouse model of peripheral B cell tolerance, MD4 mice were mated with mice that expressed membrane HEL only on the thyroid (48). In this model, B cells were not deleted nor inactivated and had normal numbers in the periphery compared to non-transgenic mice. The authors speculated that the B cells might not have gained access to HEL self-antigen due to the endothelial lining that surrounds the thyroid follicles.

On the other hand, self-reactive B cell deletion in the periphery has been observed in another mouse model (49). In this model, immunoglobulin transgene that was specific for an MHCI subtype that was engineered to be expressed on the membrane of liver cells. This system was used to mimic the situation where developing B cells do not encounter tissue specific self-antigens that are not expressed in the bone marrow. In this model, there were normal numbers of transgenic B cells in the bone marrow while they were absent in lymph nodes. The authors concluded that the recirculating B cells were deleted and the B cells that were found in spleen and bone marrow were newly arrived and had not encountered their cognate antigen yet. The caveat to this model is that the promoter used for liver-specific antigen expression

is active in other tissues including bone marrow derived cells and may not reflect the situation of auto-reactive B cells encountering an organ specific antigen (49). A more recent study from the same group using another antigen expressed specifically in the liver concluded much of the same results as before, but also found that the remaining B cells in the spleen were anergic, semi-mature B cells and that the main mechanism of peripheral B cell tolerance is deletion as no evidence of receptor editing was found (50).

These reports seem contradictory to each other and may simply reflect where the pseudo-self-antigen is expressed. A shortcoming of these mouse models is that it may not accurately portray where B cells encounter self-antigen in the periphery. Furthermore, these previous models of peripheral B cell tolerance do not address late-stage B cell maturation in the spleen, where B cells naturally reside and complete their maturation.

FDCs, Peripheral Tolerance, and the Germinal Center

The main focus of this dissertation is examining the mechanisms of peripheral B cell tolerance. While other models have been created to study peripheral tolerance, they may not accurately portray where late stage transitional B cells and FO B cells may encounter self-antigen in the periphery. Given that FDCs are naturally located in the spleen and present antigen to B cells in the form of immune complexes and opsonized foreign antigens by Fc and complement receptors, respectively, we examined the consequences of B cell encounter with self antigen retained on FDCs to further understand peripheral B cell tolerance mechanisms.

This dissertation also presents work on various aspects of the germinal center reaction including the tools used to study GC B cells, the proteins involved in forming a GC reaction, and exploring mechanisms of eliminating self-reactive B cells that may arise in a GC reaction.

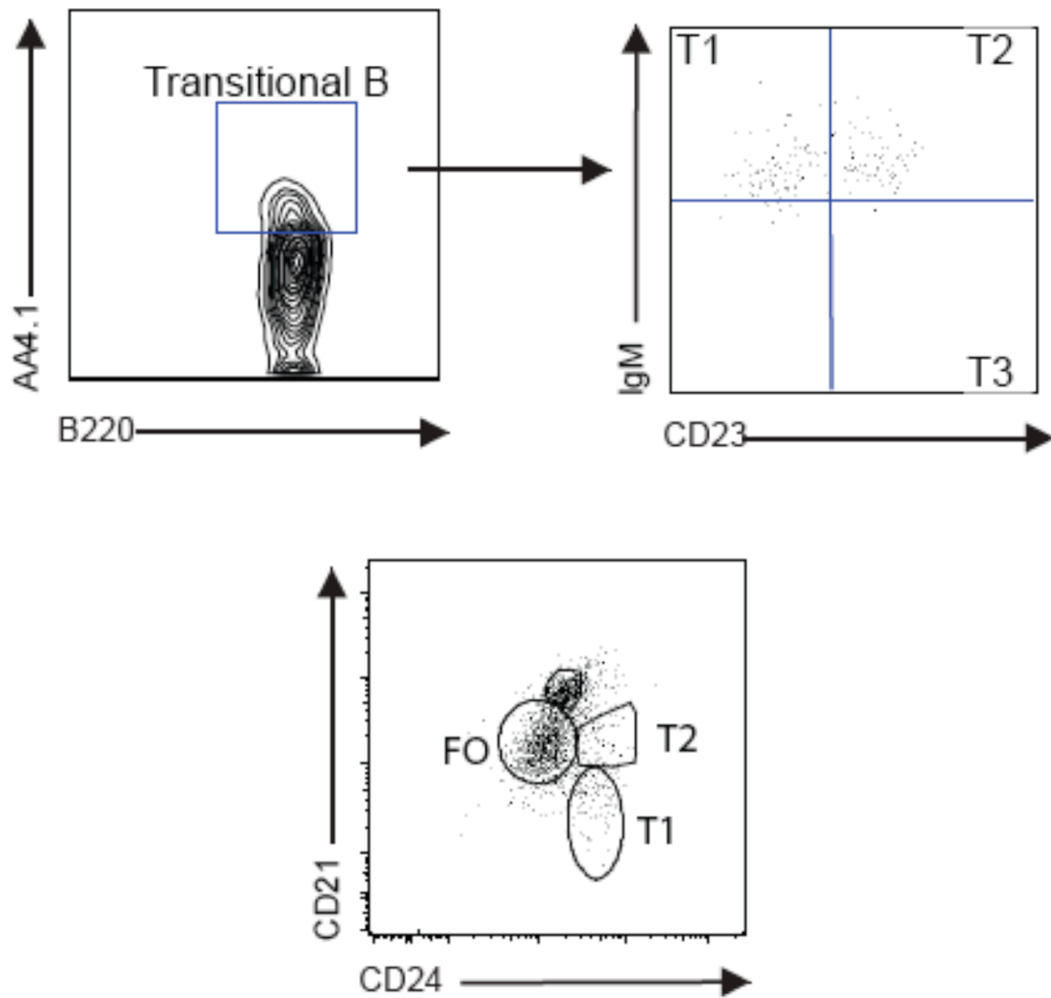


Figure 1. Flow cytometry plots to delineate transitional B cell subsets using two different sets of antibodies.

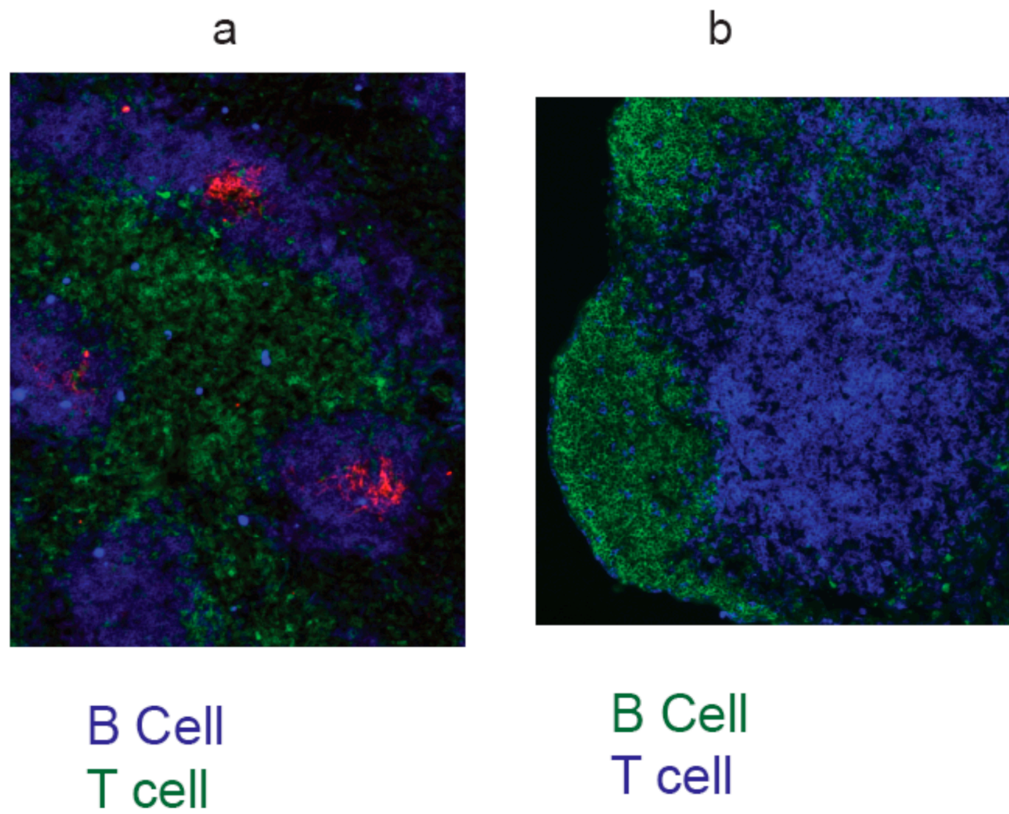


Figure 2. Immunohistochemistry. Spleen (a) and lymph node (b) sections showing B cell follicle and T cell zone.

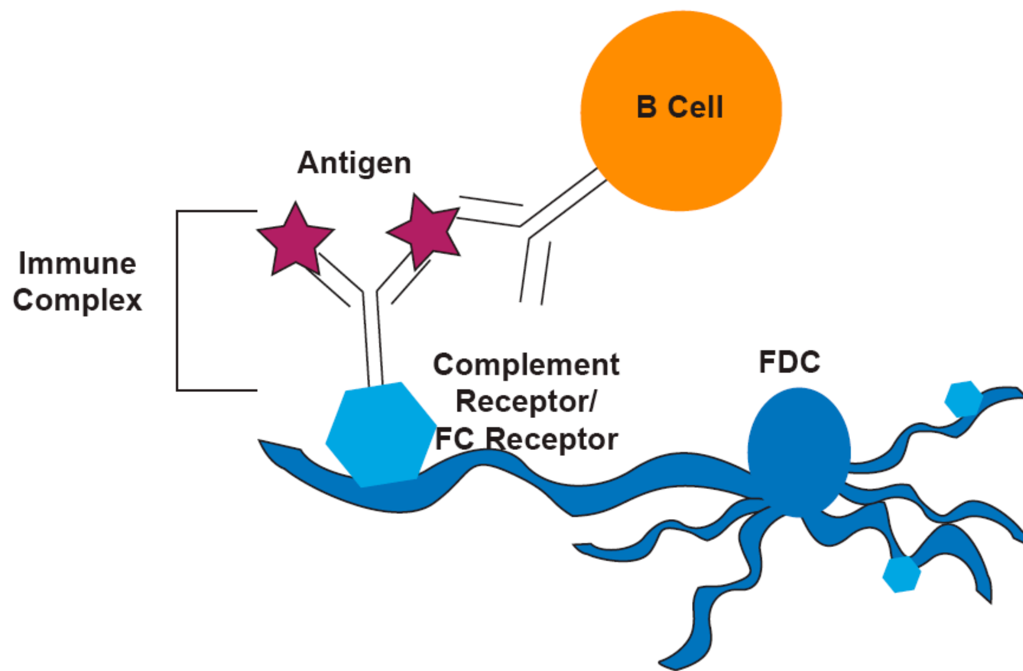
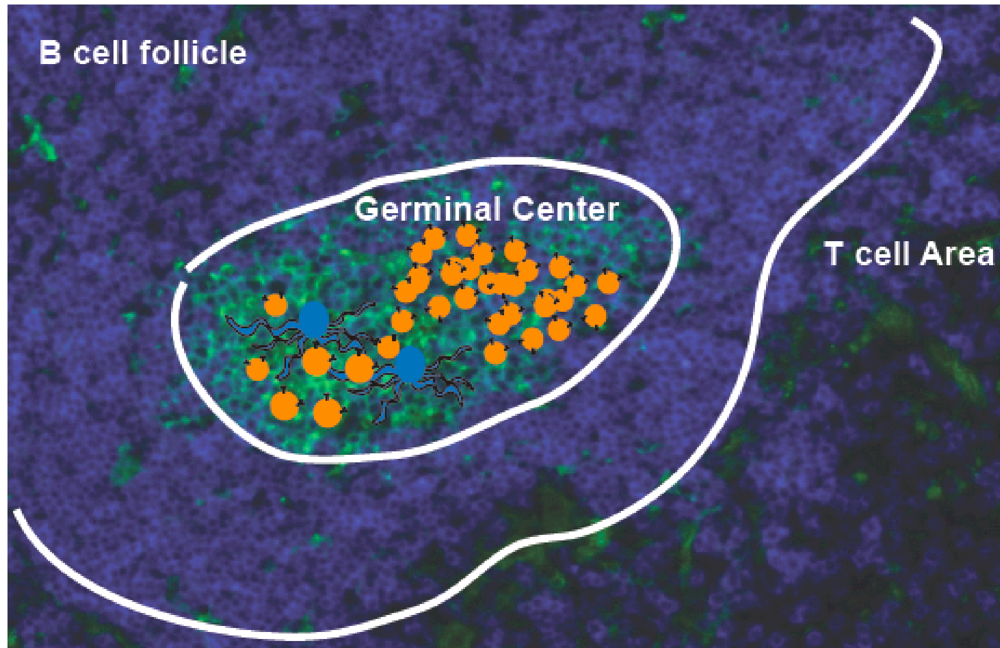
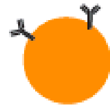


Figure 3. Follicular dendritic cell and B cell interaction diagram.





GC B cells can:
- undergo affinity maturation
- Isotype switch
- differentiate into memory B cells
- differentiate into plasma B cells

Figure 4. Spleen section showing the germinal center.

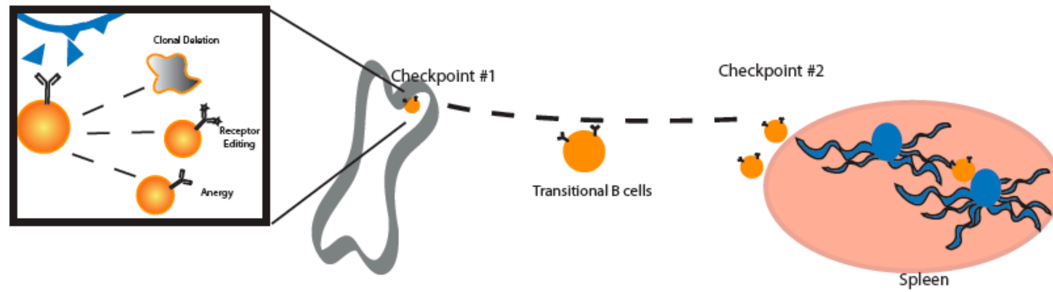


Figure 5. Schematic of B cell tolerance events in the bone marrow and in the periphery. The first tolerance checkpoint occurs in the bone and the second checkpoint occurs as transitional B cells mature to follicular B cells in the periphery.

Chapter 2. Peripheral B cell Tolerance mediated by follicular dendritic cell-displayed self-antigen

Introduction

The goal in early B cell development is the production of functional B cell receptors (BCR) that can signal properly and activate the B cell in the presence of foreign pathogen, but not self-antigen. Yet, the majority of human and mouse BCRs produced through random gene rearrangement at the immunoglobulin loci in the bone marrow are self-reactive (43). To restrain these potentially autoreactive B cells, tolerance mechanism are in place throughout B cell development and maturation. At an initial checkpoint in the bone marrow, a large portion of self-reactive B cells either undergo receptor editing, are rendered unresponsive, or are eliminated through apoptosis (37, 51). A second, less well-defined checkpoint in B cell tolerance occurs in the periphery as developing transitional B cells mature in the spleen (43). Indeed, studies have shown that rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients have a defect at this second critical checkpoint(52, 53).

Previous mouse models examining peripheral B cell tolerance have primarily focused on B cell autoreactivity against specific tissue antigen. An early study using a thyroid-specific self-antigen expressing mouse model did not indicate any selection mechanisms against autoreactive B cells, which the authors attributed to a lack of access to self-antigen (48). On the other hand, elimination and transitional B cell arrest was evident in liver-specific self-antigen mouse models.

Transitional B cells progress towards maturation and immunocompetancy in the spleen, and are divided into transitional 1 (T1) and the more mature transitional 2 (T2) subsets (2-4). In vitro studies indicate that while T2 B cells are more responsive than T1 B cells, transitional B cells of both subsets are sensitive to apoptosis upon BCR engagement (3). T2 B cells are able to survive and proliferate if provided with external T cell signals such as Il-4 and anti-CD40 antibody, but undergo apoptosis with $CD4^+$ T cells in culture due to their incapacity to upregulate T cell costimulatory molecules (1). The consequences of self-antigen binding by late stage transitional B cells have not been explored in vivo yet. Although there is evidence that T1 B cells remain in the red pulp while T2 B cells can enter the B cell follicle through histological analysis of transferred cells (3), it is also unclear what types of splenic-resident cells transitional B cells encounter as they complete their maturation in the spleen.

In the secondary lymphoid organs, >90% of B cells are in intimate contact with the vast networks of follicular dendritic cells (FDCs) (54). FDCs naturally present antigen to B cells in the form of immune complexes and opsonized foreign antigens by Fc and complement receptors, respectively. These interactions are important for B cell selection and contribute to affinity maturation during the germinal center response (28). However, the selection of self-reactive B cells by self-antigen on FDCs has not been addressed. For this purpose, I generated a mouse model that expresses self-antigen on FDCs and show that self-antigen displayed on FDCs mediates effective elimination of self-reactive B cells at the transitional stage. My results suggest that late

transitional B cells interact with FDCs and implicate FDCs as an important component of peripheral B cell tolerance.

Results

Creating a mouse model

Creating a solid mouse model is crucial to adequately address the role of follicular dendritic cells in peripheral B cell tolerance and autoimmunity since currently there are no mouse models available. To address the question of B cell peripheral tolerance, several aspects were considered: 1) the type of self-antigen expressed, 2) where the self-antigen will be expressed in the periphery, and 3) the type of B cells that will interact with the self-antigen.

In Goodnow's MD4 mouse models addressing B cell tolerance, the B cells expressed a BCR that binds strongly to HEL (K_a=1x10^{10}). Because the same BCR binds with only moderate affinity to Duck Egg Lysozyme (DEL) (K_a=1x10^{7}), I decided that expressing DEL as the self-antigen would be a more physiologic mouse model.

The location in the periphery where the self-antigen is expressed in our model is also crucial. Two previous mouse models that expressed self-antigen in different areas in the periphery to study B cell peripheral tolerance gave varying results. Neither organs that expressed self-antigen were in areas where B cells naturally reside. FDCs can be found only in the B cell follicle and come into contact with B cells through their interdigitating follicles that can bind intact antigen through Fc and complement receptors. In addition to macrophages, non-cognate B cells, and dendritic cells, FDCs can act as antigen presenting cells to naive B cells visualized by two-photon microscopy (55). Therefore, my mouse model will express DEL only on the surfaces of FDCs.

Although a relatively new technique, I decided to employ lentiviral vector transgenesis for transgenic mouse line generation. This method has several advantages: 1) it is less time consuming than traditional transgenic methods, 2) the C57Bl/6 strain can directly be used rather than crossbreeding for generations, 3) it is more efficient than traditional transgenic methods. A cre-lox lentiviral flip vector (LB2-FLIP) will ensure membrane-bound mDEL expression only when cre recombinase is also expressed (56) (Fig. 6).

The membrane-bound DEL (mDEL) transgenic mouse (mDEL^{loxp}) was crossed to *Cd21*$^{\text{cre}}$ mice (57), and designated as *Cd21*$^{\text{cre}}$mDEL^{loxp}. *Cd21*$^{\text{cre}}$ mice express cre recombinase primarily in B cells and FDCs under the control of the complement receptor 1/2 (Cr1/2 or CD21/35) promoter. Since FDCs are radioresistant, the mice will be lethally irradiated and reconstituted with BM from B cell receptor transgenic mice with affinity to DEL. Several mouse models were created with B cell receptor affinity to DEL with the first being Goodnow's model of egg lysozyme-binding B cells where all B cells express the HyHel10 IgM receptors. In recent years, SW_{HEL} mice have been created where 10-40% of B cells have affinity to various egg lysozymes and can undergo isotype switching. This model is more physiologic than previous models since B cells naturally undergo isotype switching during immunogenic reaction; moreover, pathogenic autoreactive antibodies have been found to be of various isotypes. Therefore, *Cd21*$^{\text{cre}}$mDEL^{loxp} mice will be irradiated and reconstituted with SW_{HEL} BM so that a portion of B cells will have an affinity for DEL that is expressed on FDCs. This sets up a model for examining tolerogenic mechanisms in the periphery.

Additionally, as this mouse model conditionally expresses mDEL, other spatial and temporal aspects of peripheral tolerance could be addressed in the future. For example, mDEL^{loxp} mice could be crossed to *Mx1*-cre mice in which DEL would only be expressed upon interferon induction. Immunizing these mice and inducing interferon production can be employed to study tolerance mechanisms in the GC. Additionally, osteoblast cre-expressing mice (OC-*Cre*) could be used to study developing, memory, or plasma cell encounter with self-antigen in the bone marrow. Breeding mDEL^{loxp} mice with villin-cre mice can be used to study B cell tolerance in the gut, which has not been well studied. *mDEL^{loxp}* mice are currently bred with col2a-cre mice, which secrete cre in chondrocytes, to use as a model for arthritis.

There were 23 pups that were born from the transduced and implanted embryos. Thirteen pups carried the transgene and after breeding the founders, eleven founders had germline transmission of the transgene. The Thy1.1 expression levels in the founder lines differed as well as the percentage of cells that expressed Thy1.1, which was between 5%-100% expression, indicating varying levels of transgene expression. The different expression levels can be due to different incorporation sites. One study examined whether there were any biases in lentiviral integration site and found more viral integration at protein-coding regions. Protein-coding regions are transcriptionally active sites where unwinding of DNA strands occur allowing for easier viral integration. Although lentiviral integrated more often at protein-coding regions, around 95% of integrations were in intron regions (58).

DEL expression was only detectable on B cells by flow cytometry in the lines where expression of Thy1.1 was at or nearly at 100%. It was also clear that in some

founder lines, multiple insertions of the transgene were detected since pups in the same litter had varying levels of Thy1.1 expression. Table 1 indicates the numbers and characteristics of each founder line.

Validation of Cd21^{cre}mDEL^{loxp} mice

Multiple validation techniques were used to ensure the *Cd21*^{cre}mDEL^{loxp} mouse model worked as designed summarized below.

1) To ensure that the LB2-FLIP vector works properly, Thy1.1 expression was assessed by flow cytometry analysis. All cells expressed Thy1.1 in mDEL^{loxp} mice. All cells except for a portion of B cells expressed Thy1.1 in *Cd21*^{cre}mDEL^{loxp} mice indicating that the Thy1.1 gene is deleted in CD21-expressing cells mediated by cre-recombinase. (Fig. 7a)
2) To ensure that DEL expression was membrane-bound, DEL expression was assessed by flow cytometry on primary B cells from *Cd21*^{cre}mDEL^{loxp} mice (Fig. 7b).
3) To ensure that DEL was not cleaved, activation markers CD86 and 69 on MD4 B cells was assessed after culturing in serum from *Cd21*^{cre}mDEL^{loxp} and mDEL^{loxp} mice showed that DEL was not at detectable levels in the serum (Fig. 8).
4) To ensure that mDEL expression was specific to CD21/35-expressing cells in the spleen, EYFP expression in EYFPmDEL^{loxp} mice were examined in the spleen and found to have EYFP expression coinciding with FDCs in the spleen (Fig. 9a), indicating that cre-recombinase is expressed in FDCs. EYFP expression was also examined in various tissues and found to have little expression in the kidney and

heart (Fig. 9b). By PCR, the recombined product was found in various tissues (Fig. 10).

5) To ensure that mDEL expression was not in other tissues, Thy1.1 expression was evaluated in various tissues from *Cd21*^{cre}mDEL^{loxp} and mDEL^{loxp} mice and found Thy1.1 deletion predominately in the spleen. Importantly, deletion of Thy1.1 was not observed in the bone marrow, indicating developing HEL-binding B cells in the bone marrow remained antigen inexperienced (Fig. 11).
6) To ensure that mDEL expression was not expressed at detectable levels in other tissues that HEL-binding B cells would come in contact with, CD86 expression levels was examined in various tissues and found elevated CD86 activation marker in the spleen, but not in other tissues (Fig. 12)
7) To ensure that the Cd21^{cre} and mDEL^{loxp} transgene does not affect B cell development, Cd21^{cre} and mDEL^{loxp} mice were used as controls in every experiment.
8) Blood samples were taken from every experimental mice and examined for normal Thy1.1 expression by flow cytometry analysis prior to irradiation.

Reduced self-reactive B cells in mice expressing mDEL

To study the effect of FDC-bound self-antigen on late-stage development of self-reactive B cells, a transgenic mouse line, mDEL^{loxp}, that conditionally expresses the 'self' protein duck egg lysozyme (DEL) in membrane-bound form (mDEL) after Cre-mediated recombination was generated (Fig. 6). The mDEL^{loxp} mice were bred to *Cd21*^{cre} mice (*Cd21*^{cre}mDEL^{loxp}) to induce expression of mDEL on FDCs (59). To eliminate CD21-expressing B cells, but preserve mDEL expression on radio-resistant FDCs, *Cd21*^{cre}mDEL^{loxp} and control mice (littermates carrying either the *Cd21*^{cre} or mDEL^{loxp} transgene) were lethally irradiated and reconstituted with bone marrow from SW_{HEL} mice in which 10-20% of B cells express a high affinity (4.5×10^{10} M^{-1}) B cell receptor (BCR) specific for hen egg lysozyme (HEL) that also binds DEL with moderate affinity (1.3×10^{7} M^{-1}) (40, 60) (Fig. 13). Analysis of *Cd21*^{cre}mDEL^{loxp} mice showed significantly decreased percentages of HEL-binding B cells in both the spleen (Fig. 14a) and lymph node (Fig. 14b), which corresponded to a greater than two-fold reduction in the absolute number of HEL-binding cells in the spleen (Fig. 14c) and five-fold reduction in the lymph node (Fig. 14d). The percentages of immature (IgD^-) HEL-binding B cells in the bone marrow of *Cd21*^{cre}mDEL^{loxp} and control mice (Fig. 14e) were similar, consistent with the fact that mature FDCs are only present in the peripheral lymphoid tissues. By comparison, mature (IgD^+) recirculating HEL-binding B cells in the bone marrow were decreased in *Cd21*^{cre}mDEL^{loxp} mice, reflecting a reduced percentage of mature B cells exiting the spleen and entering circulation. These primary and secondary lymphoid organ

analyses indicate that DEL self-antigen expression on FDCs caused the elimination of HEL-binding B cells.

Self-reactive B cells eliminated at the Transitional stage

The normal HEL-binding B cell frequency in the bone marrow, but reduced frequency in the spleen in *Cd21*^{cre}mDEL^{loxp} mice led us to examine the transitional B cell compartment encompassing late B cell maturation in the spleen (1). Using common surface markers to delineate Transitional 1 (T1), Transitional 2 (T2), and mature follicular (FO) B cell sub-populations (2, 4), we found no significant difference in the percentage (Fig. 15a) or absolute numbers (Fig. 15b) of T1 HEL-binding B cells (AA4.1^{+}, IgM^{+}, CD23^{-}, CD21^{low} CD24^{hi}) between *Cd21*^{cre}mDEL^{loxp} and control mice, but a more than 3-fold decrease in the frequency and a nearly seven-fold decrease in the absolute cell number of HEL-binding T2 B cells (AA4.1^{+}, IgM^{+}, CD23^{+}, CD21^{+}, CD24^{hi}) (Fig. 15a, c and Fig. 16). These results suggest that HEL-specific B cells are unable to complete the T2 maturation stage. The lack of an increase in HEL-binding T1 B cells despite a decrease in the T2 frequency suggests that autoreactive cells are eliminated upon encounter with FDC-displayed self-antigen.

Because B cell development at the transitional stage proceeds sequentially from the T1 to T2 to FO stages (1), the T1 to T2 transitional B cell impairment was confirmed by analyzing spleens from *Cd21*^{cre}mDEL^{loxp} and control mice at early time points after bone marrow reconstitution when the majority of peripheral B cells are at the transitional stage (61). Twelve days after reconstitution, similar frequencies of HEL-binding T1 cells were observed between control and *Cd21*^{cre}mDEL^{loxp} mice (Fig.

17, left panels) and only a slight reduction of T2 HEL-binding B cells was observed in *Cd21*^{cre}mDEL^{loxp} relative to control mice (Fig. 17d, left panels). By day 13, approximately half of the HEL binding B cells matured to T2 cells in control mice, indicating progression from the T1 to T2 stage. By comparison, the frequency of HEL-binding T2 cells was sharply reduced in *Cd21*^{cre}mDEL^{loxp} recipients (Fig. 17d, right panels), indicating elimination at the T1 to T2 stage imposed by DEL-expressing FDCs. Histological analysis shows that while non-HEL-binding B cells appear to be in contact with FDCs, the HEL-binding B cells appear to be near but not contacting FDCs on day 13 after reconstitution (Fig. 18). HEL-binding B cell surface Ig receptors may be engaged with mDEL on FDC surface, thereby masking the BCR during section staining. Another possibility for fewer HEL-binding B cells contacting FDCs in *Cd21*^{cre}mDEL^{loxp} mice may be that the transitional B cells have downregulated their BCRs or have undergone apoptosis after making contact with FDCs.

Partially activated phenotype in self-reactive B cells in mice expressing mDEL.

In addition to the alteration in development, flow cytometric analysis of B cells from *Cd21*^{cre}mDEL^{loxp} mice revealed two subsets characterized by high or low HEL-binding (Fig. 19a). The low-HEL-binding population is not present in control mice and reflected decreases in both surface IgM and IgD (Fig. 19a, b), indicative of antigen encounter (38). The reduced frequency of T2 and FO HEL-binding B cells in *Cd21*^{cre}mDEL^{loxp} mice suggests that the high-HEL-binding population is predominately transitional B cells that have yet to encounter FDCs. As predicted, a majority of the high-HEL-binding B cell population displayed high CD93 levels

(assessed by the AA4.1 antibody), characteristic of the transitional stage; whereas a minority expressed lower CD93 levels, indicating that a fraction of mature FO HEL-binding B cells remained antigen inexperienced (Fig. 19b, right histogram). The low-HEL-binding population also consists of both CD93 high- and low-expressing B cells (Fig. 19b, right histogram).

Assessing various B cell activation markers indicative of antigen experience revealed that low-HEL-binding B cells expressed elevated CD86, CD69, and MHCII relative to the high-HEL-binding cells in *Cd21*$^{\text{cre}}$mDEL^{loxp} and control mice (Fig. 19c). Although the low-HEL-binding B cells expressed higher levels of activation markers, no germinal center B cells were detected by flow cytometry or immunohistologic staining (data not shown). Splenic sections from *Cd21*$^{\text{cre}}$mDEL^{loxp} mice also showed that the majority of HEL-binding B cells were at the T-B border, a location consistent with the documented movement of antigen-activated B cells poised for T cell contact (62) (Fig. 19d). The population of low-HEL-binders and increased levels of activation markers were restricted to the spleen (Fig. 12), indicating that the HEL-binding B cells that encounter self-antigen bound to FDCs do not egress from the secondary lymphoid organs and are eliminated at the site of antigen encounter.

Self-reactive B cells have intact proximal BCR signaling but are short-lived in mice expressing mDEL.

Although a population of antigen-experienced HEL-binding B cells is present in *Cd21*$^{\text{cre}}$mDEL^{loxp} spleens, it was important to determine if BCR signaling remained intact (as measured by induced Akt phosphorylation (pAKT) and calcium flux). Both

non- and HEL-binding B cells in *Cd21*^{cre}mDEL^{loxp} mice showed increased pAKT amounts after stimulation with anti-IgM F(ab)'$_2$ (Fig. 20a). Gating on the low and high *Cd21*^{cre}mDEL^{loxp} HEL-binding populations showed similarly increased pAKT levels (20b). Calcium flux assay showed a similar degree of calcium release between control and *Cd21*^{cre}mDEL^{loxp} HEL-binding B cells (Fig. 20c). The fact that AKT was phosphorylated and calcium was mobilized indicates that BCR-proximal signaling events remain intact in self-reactive B cells.

A functional outcome mediated by intact BCR signaling is the upregulation of the T cell costimulatory molecule CD86 (B7.2), which allows for T cell activation and B cell survival and proliferation (38). To assess the ability of *Cd21*^{cre}mDEL^{loxp} HEL-binding B cells to fully upregulate and sustain expression of CD86, B cells from control and *Cd21*^{cre}mDEL^{loxp} mice were cultured in the presence of soluble HEL or DEL. While HEL-binding B cells from control mice upregulated CD86 in the presence of soluble HEL or DEL, B cells from *Cd21*^{cre}mDEL^{loxp} mice only weakly upregulated CD86 to levels similar to those observed in the low HEL-binding B cells directly *ex vivo* (Fig. 21a). I also found that these B cells were impaired in BCR-induced survival (Fig. 21b). It has been reported that the pro-apoptotic BH3-only Bcl-2 family member Bim is increased in anergic B cells and is a key factor in the elimination of self-reactive B cells (21, 41). Analysis of intracellular Bim expression in splenic B cells revealed that the low HEL-binding population in *Cd21*^{cre}mDEL^{loxp} mice expressed elevated Bim (Fig. 21c). In addition, our TUNEL assay revealed a higher percentage of HEL-binding B cells in *Cd21*^{cre}mDEL^{loxp} mice that incorporated labeled dUTP indicative of DNA fragmentation and apoptosis, while no differences were seen in the

non-HEL-binding B cell population between *Cd21*^{cre}mDEL^{loxp} and control mice (Fig. 21d). These findings suggest that although early BCR signaling events are intact in freshly isolated HEL-binding B cells, this antigen-experienced population is likely short-lived *in vivo* due to high Bim expression.

Studies in mouse models have shown that anergic B cells can be rescued from apoptosis with excessive BAFF and that BAFF signaling leads to Bim phosphorylation and degradation (25, 41). Moreover, elevated serum BAFF levels are present in patients with autoimmune diseases such as SLE, RA, and Sjogren's syndrome (63). Thus, it is possible that elevated BAFF could rescue these antigen-experienced HEL-binding B cells and relieve the censoring mechanism at the T1 to T2 transition.

Preliminary experiments to test the effects of elevated BAFF levels on self-reactive B cells in the periphery by daily injections of soluble BAFF for 6 days revealed slightly higher levels of T2 HEL-binding B cells in BAFF-injected verses PBS-injected *Cd21*^{cre}mDEL^{loxp} mice, but no difference in FO B cells (Fig. 22a). Interestingly, there was an increase in HEL-binding CD138+ plasma B cells in *Cd21*^{cre}mDEL^{loxp} mice (Fig. 22b).

Discussion and Analysis

In summary, I find that FDC-bearing self-antigen eliminates self-reactive B cells at the transitional stage, providing a context for peripheral B cell tolerance events in secondary lymphoid follicles. Indeed, transitional B cells have a greater sensitivity to BCR-induced apoptosis that subsides with maturation (1), presumably as a mechanism to eliminate self-reactive B cells in the periphery that have escaped deletion in the bone marrow.

Consistent with our finding of intact T1 but reduced T2 cells is the observation that T1 cells reside in the outer PALS and in the red pulp while T2 cells can be found in the follicles (3, 4). Although transitional B cells mature in the spleen, it is unclear what types of splenic-resident cells they encounter. Thus, ours is the first report of self-reactive transitional B cell elimination specifically upon encounter with FDC-displayed antigens in the spleen, where B cells naturally progress from transitional to FO B cells.

Although a large portion of T2 B cells is eliminated, some FO self-binding B cells were detected. FO cells in this antigen-experienced HEL-binding population could represent B cells that have matured before encountering DEL self-antigen or antigen-experienced late-stage transitional B cells that require multiple antigenic 'hits' before elimination (64).

Impaired CD86 upregulation, which is observed in some anergic B cell mouse models, leads to FAS-mediated elimination conferred by $CD4^+$ T cells (65). The weak CD86 expression on *Cd21*^{cre}mDEL^{loxp} HEL-binding B cells *in vitro* is reminiscent of

anergic as well as transitional B cells that are unable to upregulate T cell costimulatory molecules (3, 40).

I envision a model in which the T1 B cells maturing to T2 B cells enter the follicle and encounter self-antigen on FDCs to undergo negative selection (Fig. 23). A portion of the self-reactive B cells may develop into mature follicular B cells and encounter self-antigen, becoming partially activated and instructed to move to the outer PALS. Repetitive encounters with self-antigen on FDCs result in increased Bim protein in these partially activated self-reactive B cells thereby promoting their elimination by apoptosis.

FDCs are strategically located within the B cell follicle and present antigens along their long dendritic processes. Multiple roles have been attributed to FDCs, including selection for high affinity B cells during an immune response (66), chemokine and survival factor secretion allowing for proper lymphoid organization and B cell homeostasis (28), and secretion of splenic milk fat globule epidermal growth factor 8 (Mfge8) to mark apoptotic cells for engulfment (32). In addition, a recent study showed that dendritic cell presentation of shed placental material specifically retained on FDCs could tolerize antigen-specific T cells even in the presence of adjuvant. Although it is unclear exactly how this form of antigen presentation induced the deletion of T cells rather than activation, it was speculated that FDCs could produce an unknown factor that mediates tolerance (67). Our study suggests that FDCs can play an important and previously unknown role in peripheral B cell tolerance and likely contributes to the observed autoreactive B cell reduction that occurs at the juncture between transitional and mature follicular B cell

development.

Materials and Methods

mDEL^{loxp} mouse generation.

Only the protein sequence of DEL is known while the DNA sequence of DEL has not been published. While several papers have identified three different DEL amino acid sequences, DEL III (60) was the sequence used in the construction of our mDEL mouse. Using the known protein sequence of DEL and the known DNA sequence of HEL, the DNA sequence for DEL was constructed in such a way to keep as much of the HEL DNA sequence as possible except for the codons that encoded for different amino acids. Genescript generated recombinant DEL DNA. Table 2 shows the recombinant DNA sequence used to generate DEL expression. Letters in bold are HEL sequences that were changed to generate the appropriate DEL amino acid.

The MHCI H2-k1 transmembrane and cytoplasmic tail (exons 6-9) cDNA was conjugated to the DEL sequence to produce a DEL protein that is membrane-bound. The MHCI cDNA was cloned from a C57BL/6 mouse through a PCR reaction using C57/Bl6 cDNA with MHCI TM XmaI forward primer (CAC TCC CCG GGT AAA GAG CCT CCT C) and PmeI MHC ex8 reverse primer (GCG TTT AAA CTC ACG CTA GAG AAT GAG GGT C). The MHCI portion was ligated to DEL at the XmaI restriction site and the whole DEL-MHCI fragment (mDEL) was cloned into the MSCV-P2GM vector with restriction enzymes AgeI and PmeI. Membrane-bound DEL expression was verified by tranducing Bal17 cells (Fig 24a) with MSCV virus produced from MSCV-P2GM mDEL plasmid. Table 3 shows the protein sequence of recombinant mDEL. After verifying that mDEL was detectable on the surface, mDEL was cloned into the lentiviral vector LB2-FLIP and verified that the vector functions

as expected with mDEL expression present only in the presence of cre-recombinase (Fig. 24b).

DEL-MHCI lentivirus was generated by Tatiana Hurtado de Mendoza (Verma Laboratory, Salk Institute) with a 5.7 X 10^9 viral titer determined by p24 ELISA. The lentivirus was injected into ~200 single cell-staged C57BL/6 embryos and implanted into ten pseudopregnant ICR females. Of those implanted females, five survived and produced 23 pups. Of the 23 pups, 13 founders carried the transgene assessed by flow cytometry analysis of peripheral blood for surface Thy1.1 expression (Table 1).

These founders were bred to Cd21*cre* mice to produce the F1 generation of pups. Germline transgene transmission was assessed in the F1 generation by flow cytometry analysis of peripheral blood for surface Thy1.1 expression. Eleven founder lines were shown to have germline transgene transmission. Only one line, Founder 1, was found to have surface Thy1.1 expression on 100% of cells. Founder 1 was used for subsequent experiments.

PCR for the mDEL transgene (genotyping reaction): mDEL transgene can be genotyped using DEL Fwd primer (TGA GAT CAG ACA TAA CAG AGG CCG) and MHCI exon8 Rev primer (GCT AGA GAA TGA GGG TCA TGA ACC), which produces a 385 bp product (Fig. 25). The PCR cycle is as follows: 94°C, 3', (1 time); 94°C, 30s, 53.8°C 30s, 72°C 45s, (35 times); 72°C, 7', (1 time)

In brief**.** The DEL construct was synthesized according to the published amino acid sequence with one modification at Y3F, which prevents DEL from priming I-A^b-restricted T cells (60, 68). Synthesized DEL was fused to the MHC I transmembrane and cytoplasmic tail cDNA sequence region isolated from a C57BL/6 mouse and

cloned into the LB2-FLIP lentiviral vector (56) (Hynes Laboratory) (Supplemental Figure 1). High titer lentivirus ($5.7X10^9$ viral particles/ml) was injected into C57BL/6 embryos as described (69). Germline transmission of the transgene was confirmed by PCR of the mDEL transgene and expression of surface marker Thy1.1 by flow cytometry.

Mice

mDEL^{loxp} mice were bred to *Cd21*^{cre} mice (57) to generate *Cd21*^{cre}mDEL^{loxp} mice. *Cd21*^{cre}mDEL^{loxp} or control mice were lethally irradiated (10 gray) and reconstituted with bone marrow from SW_{HEL} mice by intravenous injection six hours post-irradiation and analyzed 7-12 weeks later. Some mice were reconstituted with lineage-depleted bone marrow isolated by negative MACs sorting of cells labeled with biotinylated anti-B220, -CD19, -IgM, -CD3, and -CD11b (eBioscience) antibody followed by anti-biotin MACS beads. *Cd21*^{cre}mDEL^{loxp} and SW_{HEL} mice were housed in pathogen-free environment in the Animal Facility at the Sanford-Burnham Medical Research Institute and were maintained on a C57BL/6 background. For the BAFF studies, 7 ug BAFF (eBioscience, huCD257, 14-8943) or PBS was injected daily for 6 days in *Cd21*^{cre}mDEL^{loxp} and control mice. Mice were sacrificed and analyzed on the 7th day.   All experiments conformed to the ethical principles and guidelines approved by the SBMRI Institutional Animal Care and Use Committee.

Flow cytometry.

Single cell suspensions from spleen, lymph node, and bone marrow were incubated with either biotinylated HEL (Genetex) or with soluble HEL followed by biotinylated anti-HEL antibody (Rockland). The following antibodies were used from eBioscience: anti-B220, -CD3, -Thy1.1, -CD21/35, -CD23, -IgM, -AA4.1, -IgD, -CD24, -CD86, -CD69, -MHCII, -CD138, -GL7, -FAS, -IgG1, -BAFFR, and Streptavidin-PercPcy5 and -Pecy7. Live cells were assessed by forward and side scatter profiles. For intracellular Bim stain, splenocytes were incubated with surface antibodies and fixed in BD Cytofix/Cytoperm (BD biosciences) for 15 minutes on ice, washed two times, and incubated overnight with Permeabilization Buffer (eBioscience). Splenocytes were subsequently incubated with Bim Rabbit mAb (C34C5, Cell Signaling) or isotype control (Rabbit DA1E mAb IgG, Cell Signaling) followed by secondary FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch). For intracellular pAKT staining, two million splenic cells were resuspended in 100 ul of 5% FBS in PBS. Cells were stimulated with anti-IgM $F(ab')_2$ (Jackson ImmunoResearch Laboratories) at a concentration of 10 ug/ml for the indicated time point. Stimulations were halted by adding 37°C BD fix/perm buffer for 10 minutes and washing two times with Permeabilization Buffer. Cells were subsequently incubated with surface antibodies in FACS buffer, washed, and incubated in 0.2% saponin buffer with phospho-AKT antibody (Ser473, D9E, Cell Signaling) for 1 hour and secondary anti-rabbit Fitc antibody for 1 hour. All cells were acquired on a FACS Canto using FACS DIVA software (BD Biosciences) and analyzed using FlowJo (Treestar). Data are displayed with logarithmic scale.

In vitro culture

B cells were isolated from splenic cells according to standard procedures and were cultured in a 96- or 48- well plate at 1.5 X 10^6 B cells/ml in complete RPMI media with or without soluble HEL or DEL (500 ng/ml) for sixteen hours.

Calcium flux.

Two million splenic B cells were resuspended in media. Cells were incubated at 37°C for 30-45 minutes in the dark, washed with media, and labeled with B220-PE for 20 minutes. Stimulations to induce calcium release were done with either anti-IgM $F(ab)'_2$ or with HEL-APC fusion protein generated by combining biotinylated HEL with streptavidin-conjugated APC at a 5:1 ratio by weight for five minutes.

Immunofluorescence microscopy

Splenic tissue was embedded in Tissue-TEK O.C.T compound (Sakura Finetek U.S.A., Torrance, CA) and frozen at -80°C. Frozen tissue were then sectioned, mounted on Superfrost/Plus microscope slides, fixed in cold acetone for 10 minutes, and blocked with 5% FBS and 1% BSA in PBS for 1 hour. Sections were first incubated with soluble HEL for 30 minutes. Tissues were then stained with the following antibodies: biotinylated anti-HEL, Streptavidin Cy3 (Jackson ImmunoResearch), CD5, and IgD. Tissue sections were imaged with a Zeiss Axio ImagerM1 using Slidebook software (Intelligent Imaging Innovations, Denver, CO).

Statistical Analysis

All statistical analysis was performed by using unpaired, two-tailed, Student's T-test. P-values less than 0.05 were considered significant.

Acknowledgements

The author of this dissertation would like to acknowledge the co-authors of this work: Matthew H. Cato, Tatiana Hurtado de Mendoza, and Robert C. Rickert. Their contributions have been noted throughout the text and figures of this chapter where applicable.

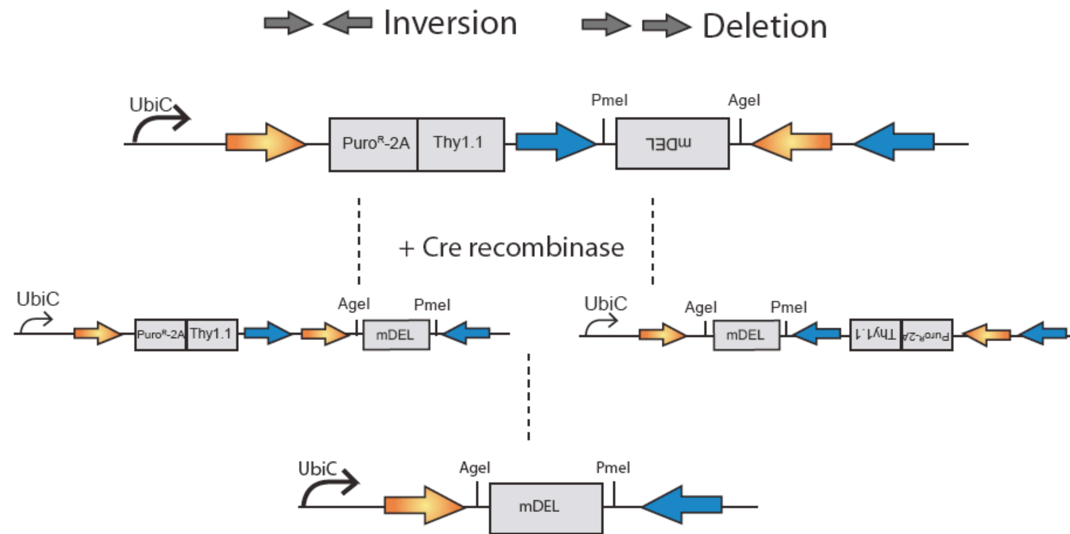


Figure 6. Schematics of the LB2-FLIP lentiviral vector used for mDEL^{loxp} mouse generation. Two distinct sets of *loxP* sequences (orange and blue arrows) flank the inverted mDEL sequence. Integration allows for ubiquitous expression of Puromycin and the Thy1.1 surface marker, while the inverted mDEL sequence prevents transcription. The introduction of cre-recombinase mediates inversion events of both sets of *loxP* recombination sequence sets leading to mDEL transcription. Subsequent recombination events lead to deletion of Thy1.1 as the final recombination event and the retention of heterologous *loxP* sites.

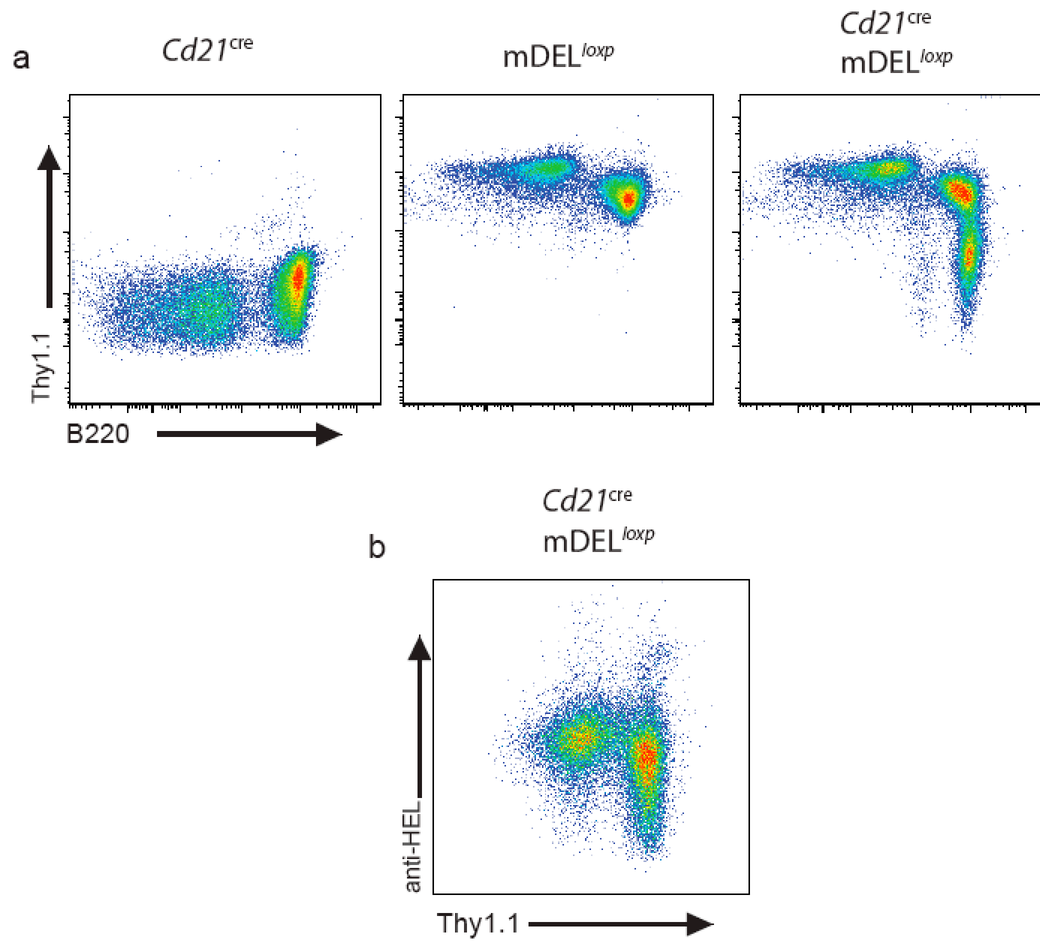


Figure 7. **a,** Flow cytometry plot of splenic cells from *Cd21*^{cre}mDEL^{loxp} and mDEL^{loxp} mice depicting Thy1.1 surface levels as an indication of transgene expression in the appropriate cell type. **b,** Flow cytometry plot of B cells from *Cd21*^{cre}mDEL^{loxp} mice depicting mDEL levels.

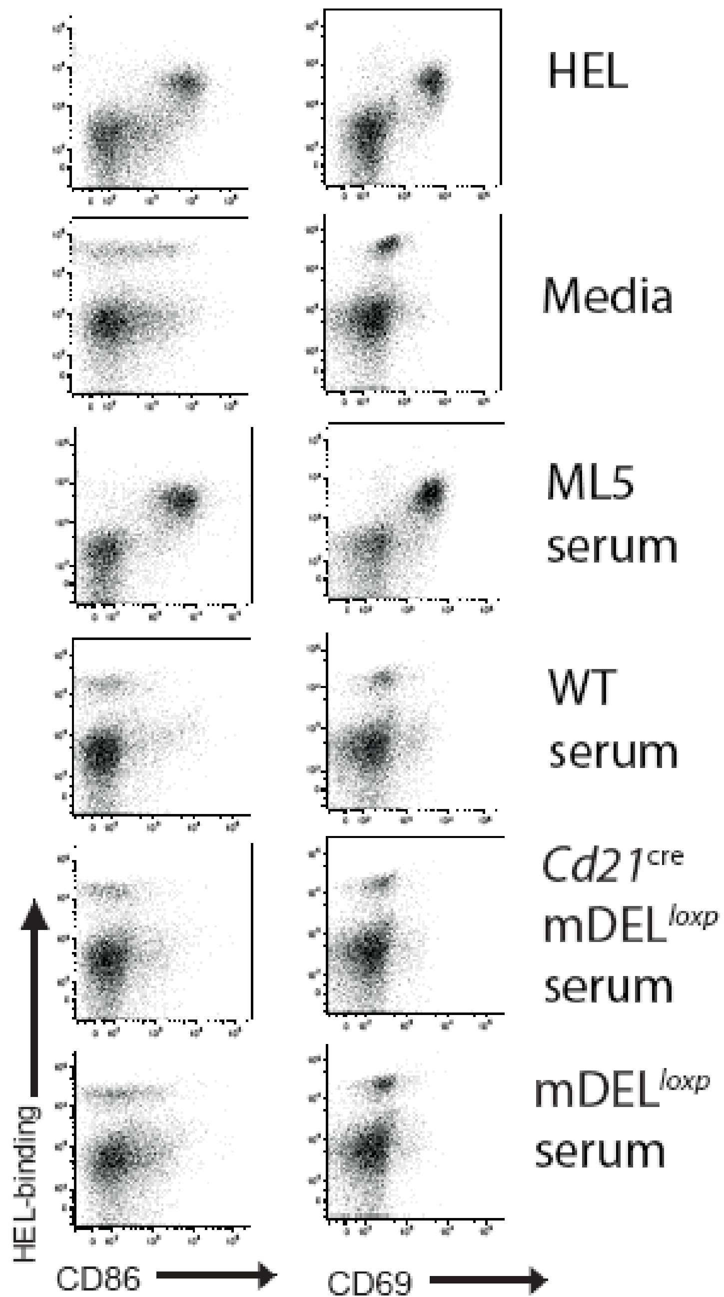


Figure 8. Flow cytometry plot of HEL-binding B cell activation status. B cells were placed in culture for 24 hours with soluble HEL or serum from various mice including *Cd21*^{cre}mDEL^{loxp} and mDEL^{loxp} mice to determine presence of lysozyme.

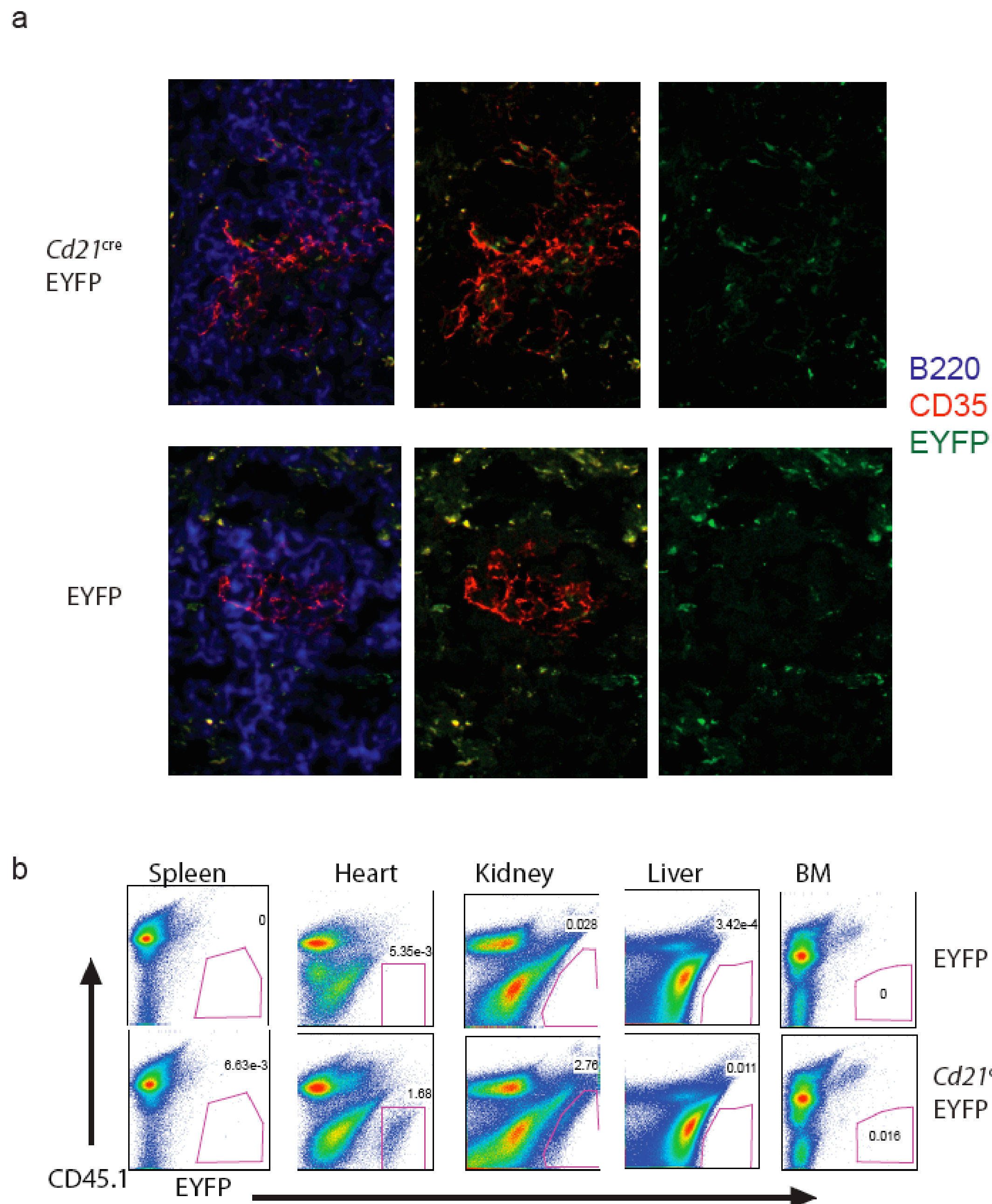


Figure 9. Verification of cre-recombinase expression in FDCs. *Cd21*^{cre} mice were bred to EYFP mice, which express EYFP fluorescent protein in the presence of cre recombinase. *Cd21*^{cre} EYFP mice were lethally irradiated and reconstituted with CD45.1 BM to eliminate EYFP^{+} B cells. **a,** Immunohistochemistry of splenic sections that show EYFP (green) expression coinciding with FDCs (red) in *Cd21*^{cre} EYFP mice. **b,** Flow cytometry plots showing gates on the EYFP-expressing cells from the labeled tissues.

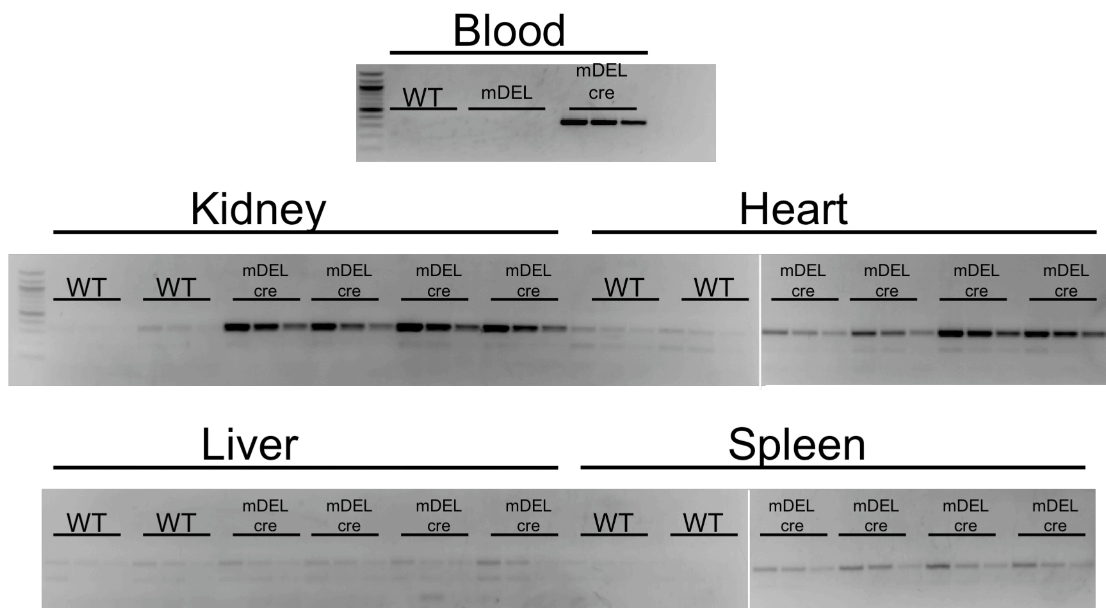


Figure 10. PCR of the recombined product (mDEL) in various tissues. (Matt Cato performed the DNA extraction and PCR reaction for this figure.)

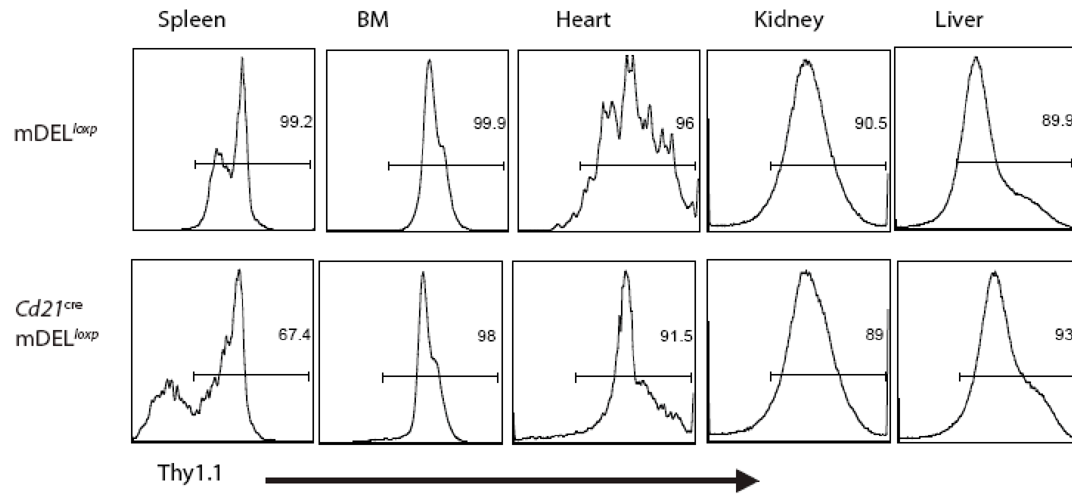


Figure 11. Flow cytometry plots of Thy1.1 expression on different tissues as an indication of cre-recombinase mediated expression of mDEL.

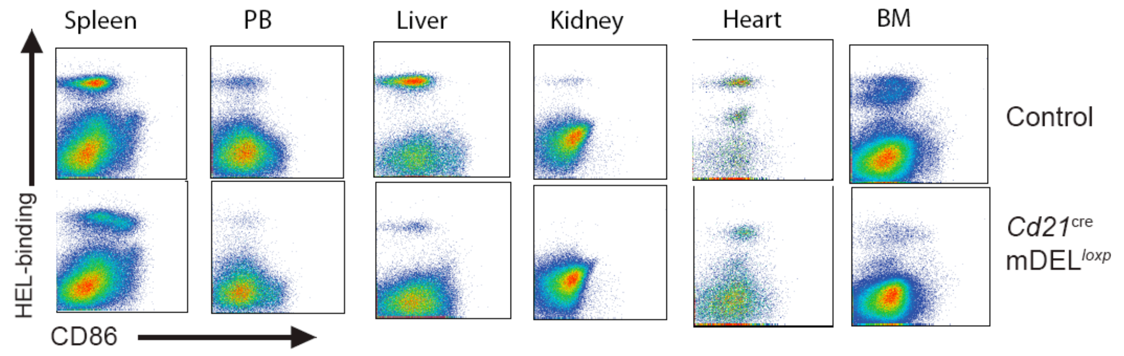


Figure 12. Flow cytometry plot of HEL-binding B cell activation status in various tissues as an indicator of antigen encounter in the tissue.

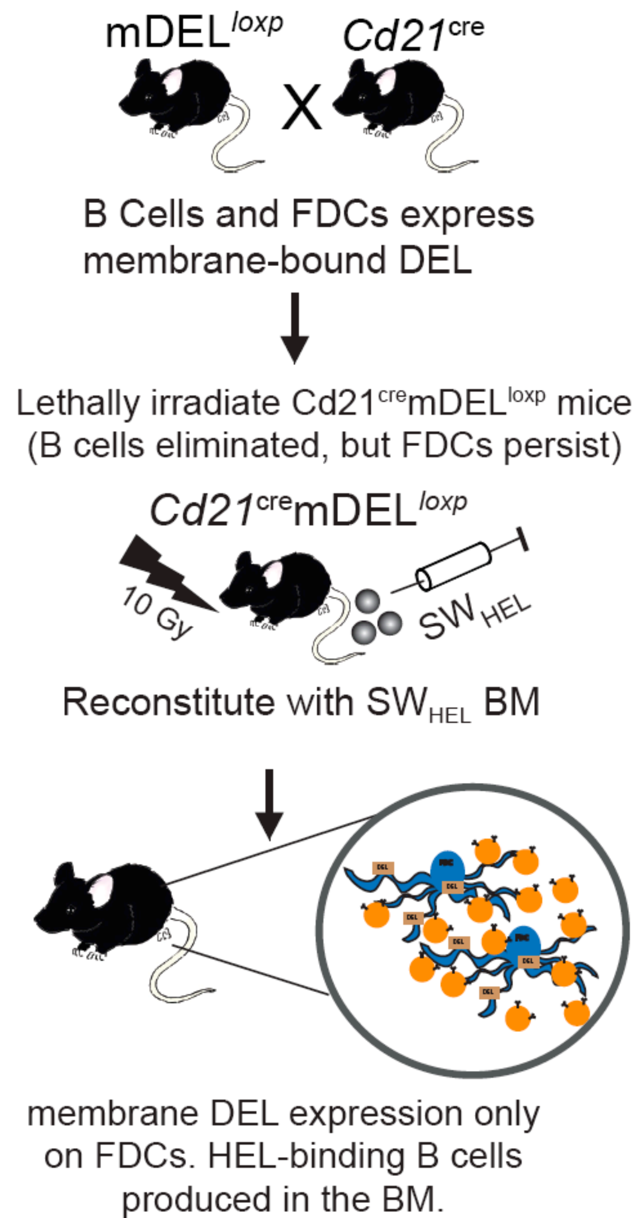


Figure 13. *Cd21*^{cre}mDEL^{loxp} mice generated by breeding mDEL^{loxp} mice to *Cd21*^{cre} mice to induce mDEL expression on FDCs. *Cd21*^{cre}mDEL^{loxp} and control mice were subsequently lethally irradiated and reconstituted with bone marrow from SW_{HEL} mice.

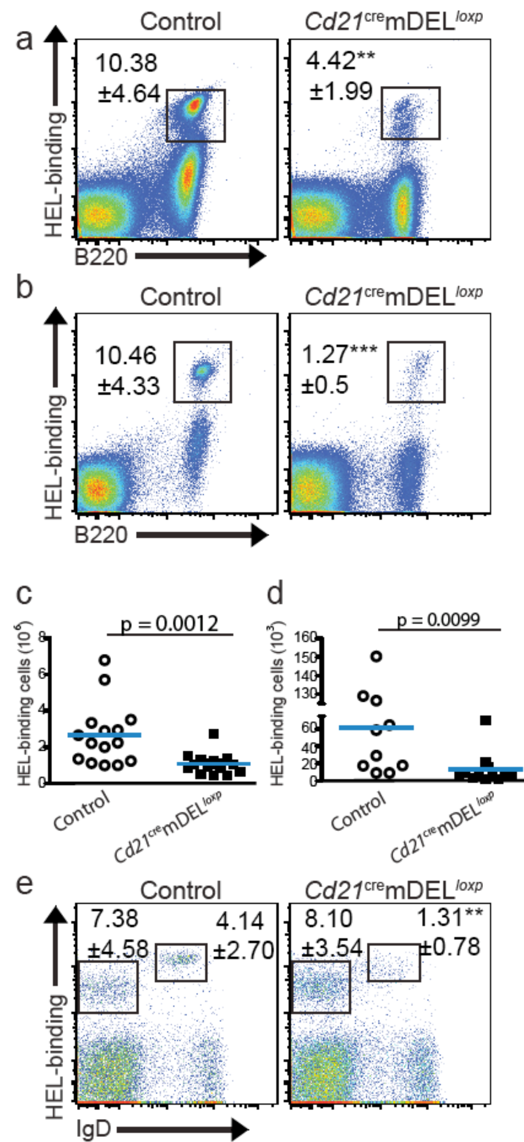


Figure 14. **a**, **b** Flow cytometry plots of HEL-binding B cells in the spleen (a) and lymph node (b). **c, d** Cell counts of total HEL-binding B cells in the spleen (c) and lymph node (d). **e,** Flow cytometry plots of the bone marrow show immature (IgD^-) and mature (IgD^+) HEL-binding B cell frequency. Representative plots are shown. Frequencies ($B220^+$ gated) showing standard deviation values are pooled from four independent experiments (n=3-5, error bars are SD, **P < 0.005, ***P < 0.0005).

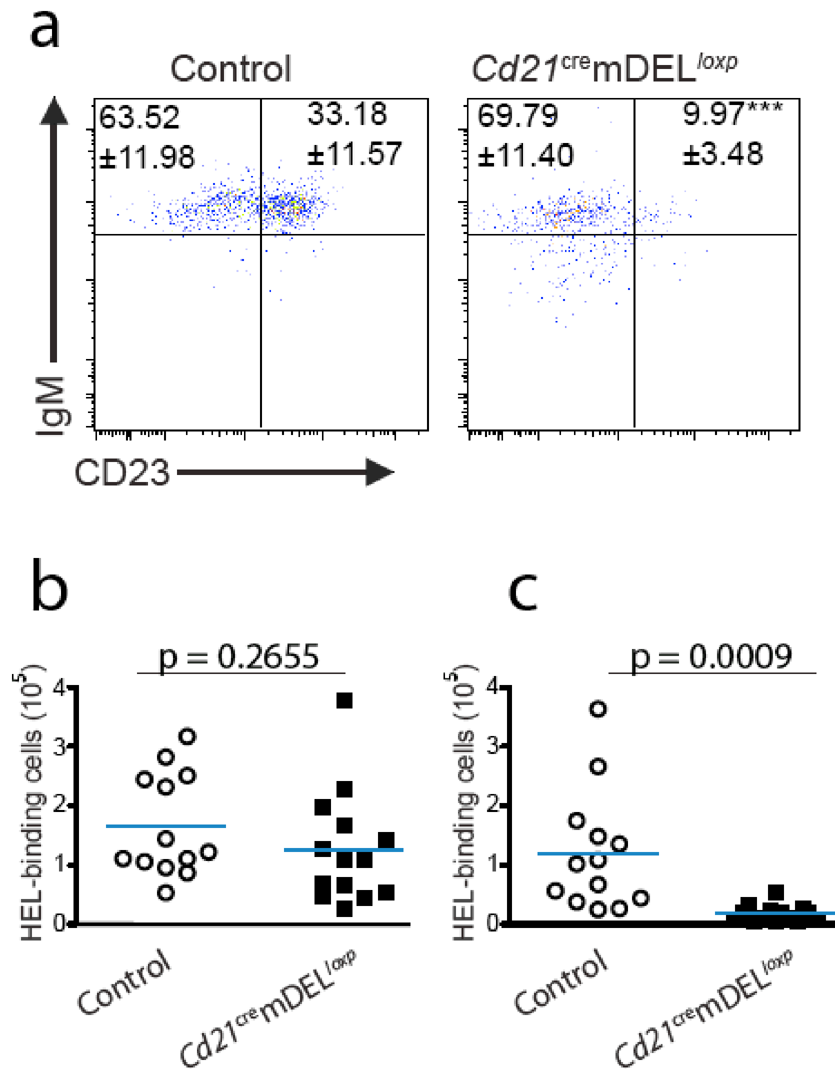


Figure 15. Lower frequency of T2 HEL-binding B cells, but intact T1population in mDEL expressing mice. **a**, Flow cytometry plots of *Cd21*^{cre}mDEL^{loxp} and control spleens show T1 (IgM$^+$CD23$^-$) and T2 (IgM$^+$CD23$^+$) B cell frequency (HEL-binding, AA4.1$^+$ gated). **b, c** Enumerated T1 (b) and T2 (c) HEL-binding B cell subsets. Frequencies and cell counts showing standard deviation values are pooled from three independent experiments (n=3-5, error bars are SD, ***P < 0.0005).

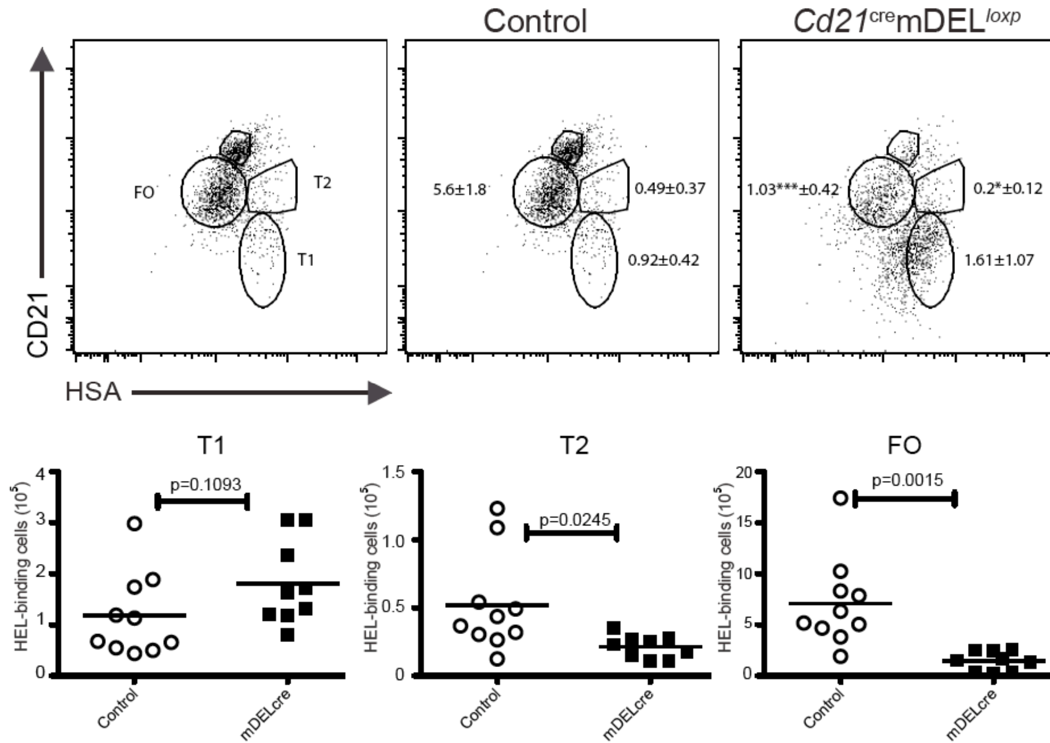


Figure 16. Transitional B cell subsets distinguished by HSA and CD21 expression. Flow cytometry analysis of the spleen show mature follicular (FO, CD24low, $CD21^+$), T-1 (CD24hi $CD21^-$), and T-2 ($CD24^{hi}CD21^+$) HEL-binding B cells (B220 gated). Representative plots are shown. B cell subsets were enumerated (bottom panels). (Frequencies and cell counts showing standard deviation values are pooled from three independent experiments (n=3-5, error bars are SD, *P < 0.05, **P < 0.005, ***P < 0.0005).

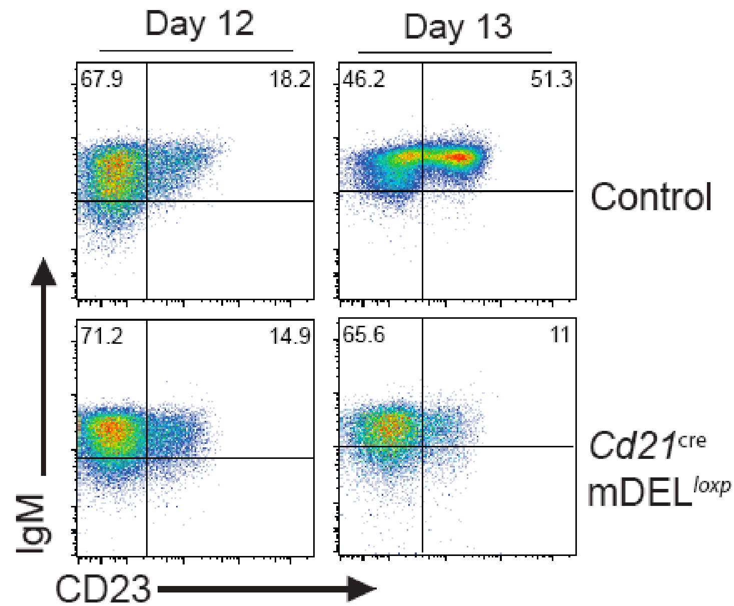


Figure 17. Spleens from *Cd21*^{cre}mDEL^{loxp} and control mice were analyzed day 12-13 after bone marrow reconstitution. Flow cytometry analysis showing T1 (IgM^{+}CD23^{-}) and T2 (IgM^{+}CD23^{+}) B cell frequency (HEL-binding, AA4.1^{+} gated). Representative plots are shown from two independent experiments (n=2-3).

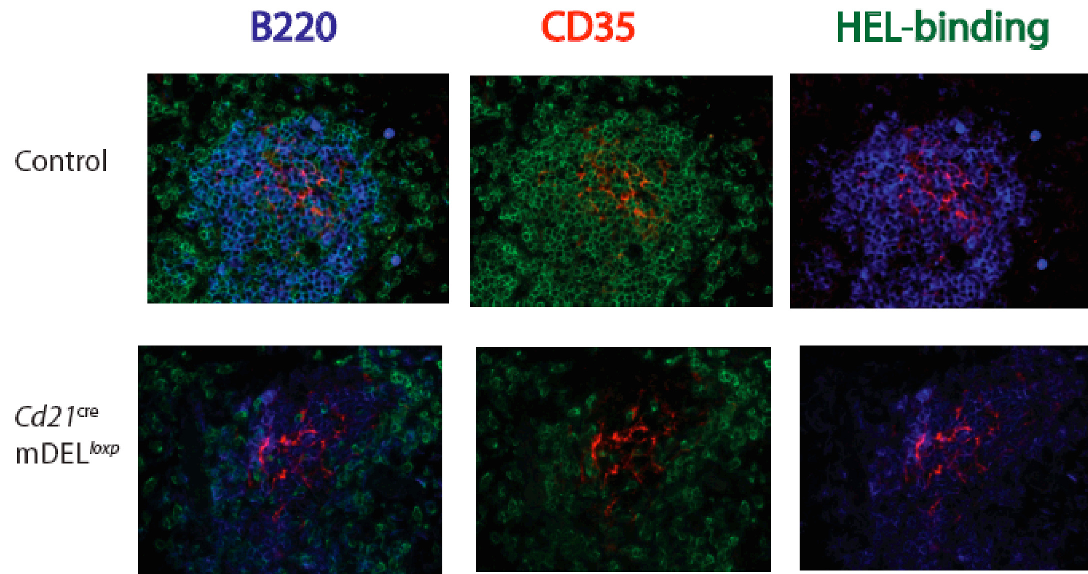


Figure 18. Immunohistochemistry of day 13 reconstituted spleen. HEL-binding B cells (green) do not appear to be closely contacted with FDCs (red), while non HEL-binding B cells (blue) are in *Cd21*^{cre}mDEL^{loxp} splenic sections at day 13 after reconstitution.

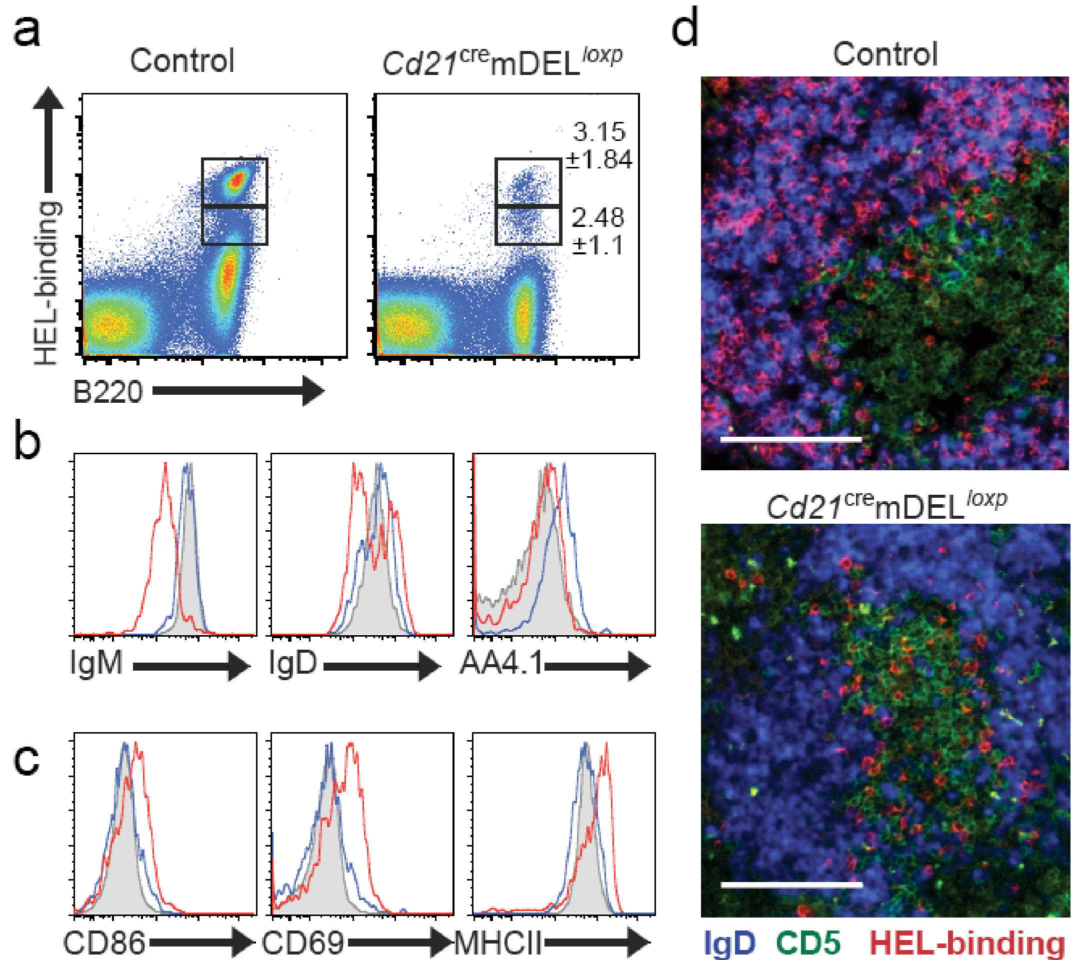


Figure 19. Analysis of the HEL-binding B cell subsets found in *Cd21*^{cre}mDEL^{loxp} and control mice by flow cytometry (a-c) and histology (d). **a,** Flow cytometry plots showing gates on the high and low HEL-binding B cell population from *Cd21*^{cre}mDEL^{loxp} and control spleens. **b,** Overlay histograms of IgM, IgD and AA4.1 expression levels of control (gray dashed line) and high- (blue) and low- (red) HEL-binding B cells in *Cd21*^{cre}mDEL^{loxp} B cells. **c,** Overlay histograms of CD86, CD69, and MHCII expression levels of control (gray dashed line) and high- (blue) and low- (red) HEL binding B cells in *Cd21*^{cre}mDEL^{loxp} B cells. **d,** T cells (CD5^{+}, green), B cells (B220^{+}, blue), and HEL-binding cells (red) in splenic sections. Scale bar, 100μm. Representative plots (a-c) and images (d) are shown from three independent experiments (n=3-5 per experimental group, frequencies showing SD values).

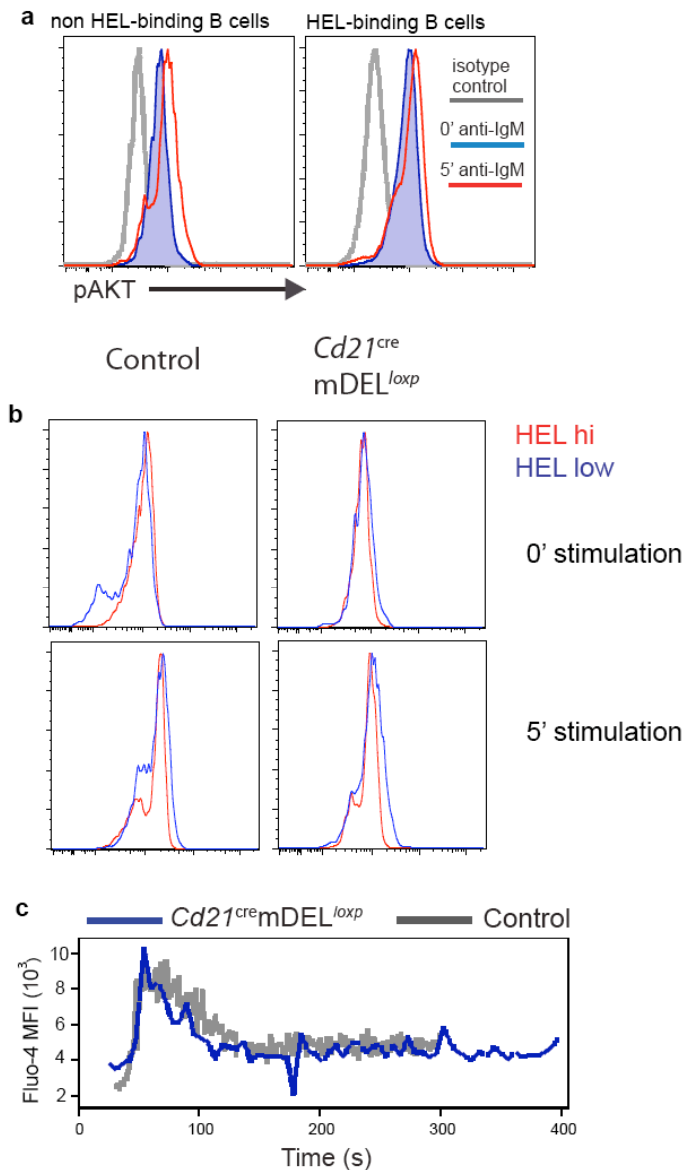


Figure 20. Intact proximal BCR signaling in HEL-binding B cells from mDEL-expressing mice. **a,** Overlay histograms of intracellular pAKT expression levels of unstimulated (blue, shaded) and IgM F(ab)'$_2$ stimulated (red) B cells and isotype control (gray) from *Cd21*^{cre}mDEL^{loxp} non- and HEL-binding cells in the spleen. A representative plot is shown from two independent experiments (n=2-4 per experimental group). **b,** Overlay histograms of intracellular pAKT expression levels of the hi and low HEL-binding B cell population. **c,** Calcium mobilization assay. B cells were stimulated with HEL-APC fusion protein. Overlay plots of fluo-4 levels of non- (gray) and HEL-binding cells (blue) are shown. A representative plot is shown from two independent experiments (n=2-3 per experimental group).

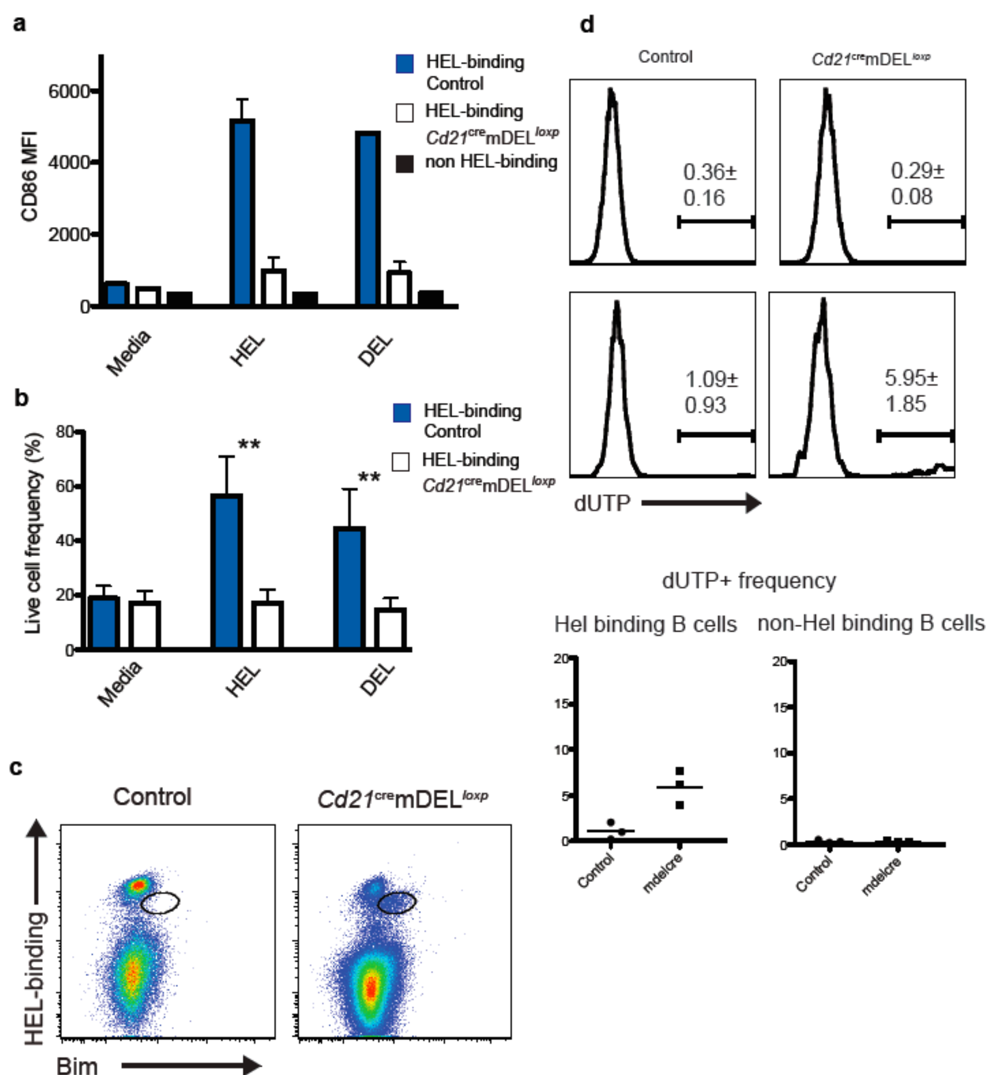


Figure 21. HEL-binding B cells are short-lived. **a,** Plot of relative CD86 levels calculated by m.f.i (median fluorescent intensity). A representative plot with SD bars is shown from three independent experiments (n=2-3 per experimental group). **b,** Live HEL-binding cells in culture from (a) based on forward side scatter flow cytometry analysis. Data is pooled from two independent experiments (n=2-3 per experimental group, error bars are SD, $^{**}P < 0.005$). **c,** Flow cytometry analysis of intracellular BIM levels in B cells from *Cd21*$^{\text{cre}}$mDEL^{loxp} and control spleens (B220^{+} gated). Bim-high HEL-binding cell population outlined in black. Representative plots shown from three independent experiments (n=2-5 per experimental group). **d,** Histogram (top) of dUTP-labeled HEL-binding B cells. Frequencies (bottom) of dUTP-labeled B cells from *Cd21*$^{\text{cre}}$mDEL^{loxp} and control mice (n=3 per experimental group, one independent experiment).

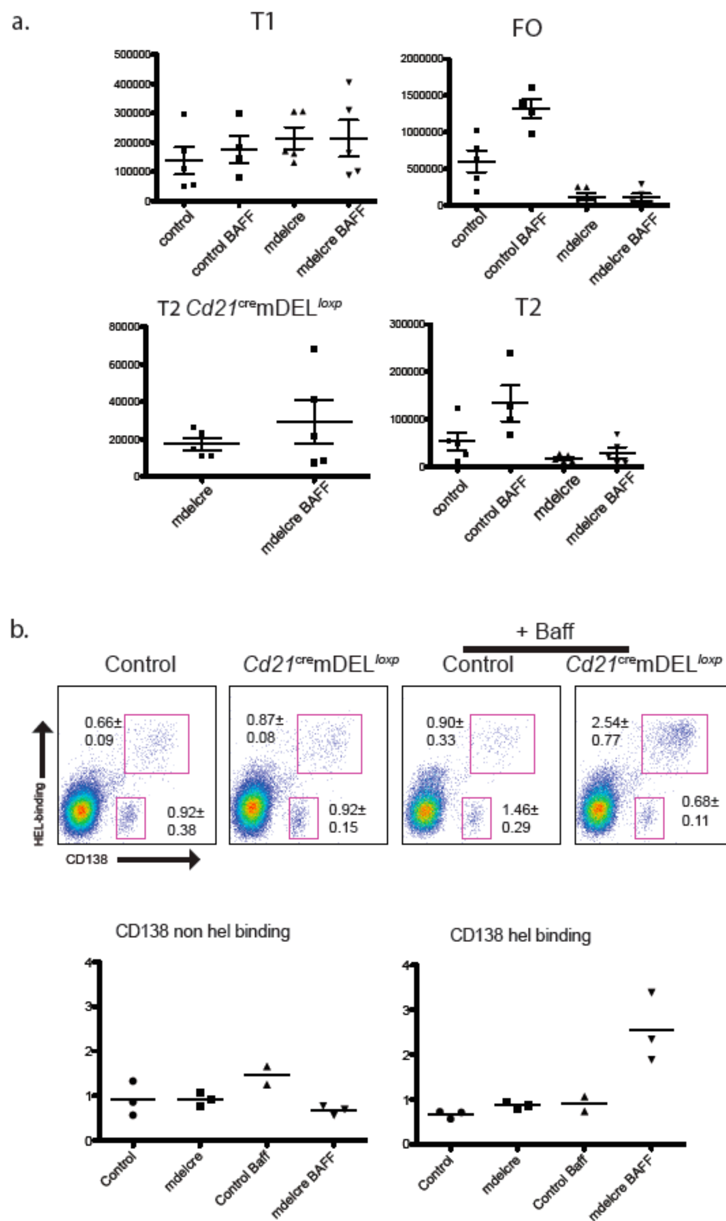


Figure 22. Elevated BAFF levels increase HEL-binding T2 and plasma cell population in mDEL-expressing mice. **a,** Frequencies of the B cell subsets as assessed by HAS and CD21 levels. **b,** Flow cytometry plots (top) gating on HEL-binding and non-HEL binding $CD138^+$ plasma cell population. Frequencies of each plasma cell population are plotted (bottom). Pooled data from two independent experiments (n = 2-3 mice per experimental group).

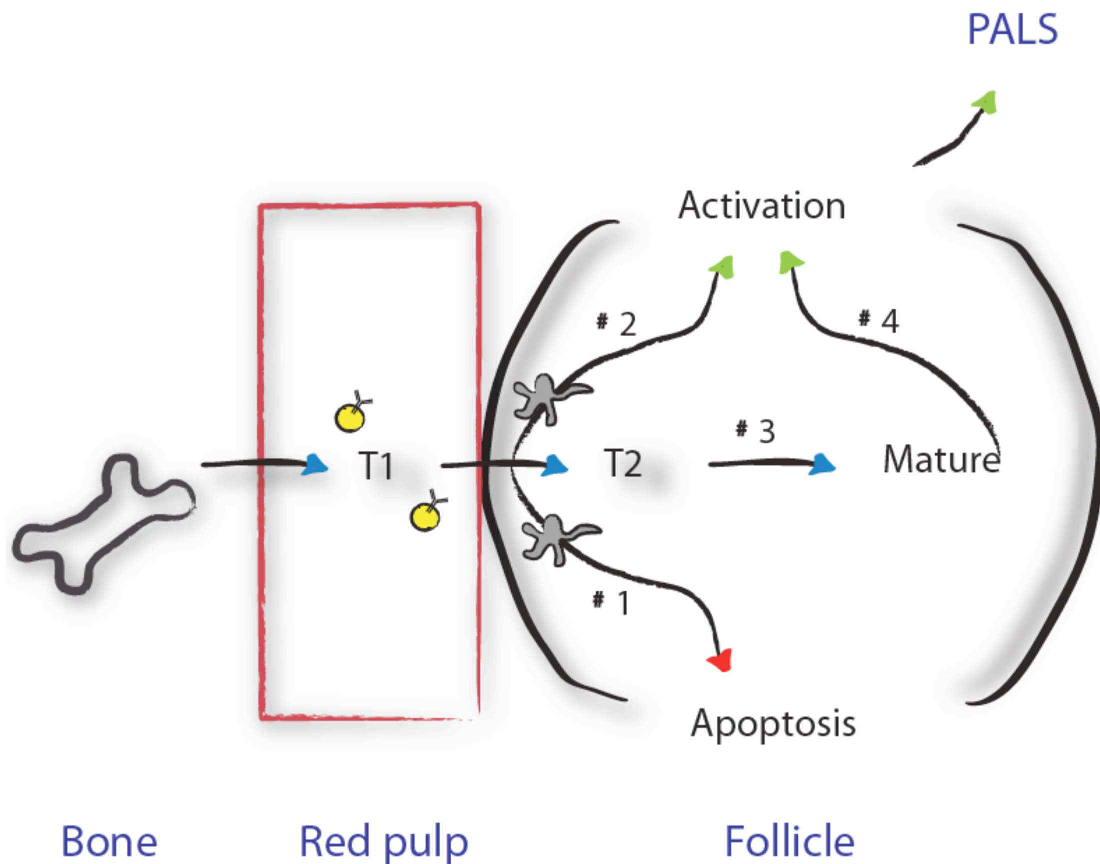


Figure 23. Schematic explaining self-reactive B cell elimination by FDC-bound self-antigen. Self-reactive B cells not eliminated in the bone marrow enter into the periphery as T1 B cells. In the spleen, T1 B cells are found in the red pulp and encounter self-antigen on FDCs as they develop into T2 B cells in the follicle and are eliminated (#1). A portion of self-reactive B cells are partially activated (#2) or evade antigen encounter (#3) until maturation (#4) and go to the outer PALS. With each successive antigen-encounter, self-reactive B cells sustain Bim levels and undergo apoptosis in the spleen. Figure courtesy of Robert C. Rickert.

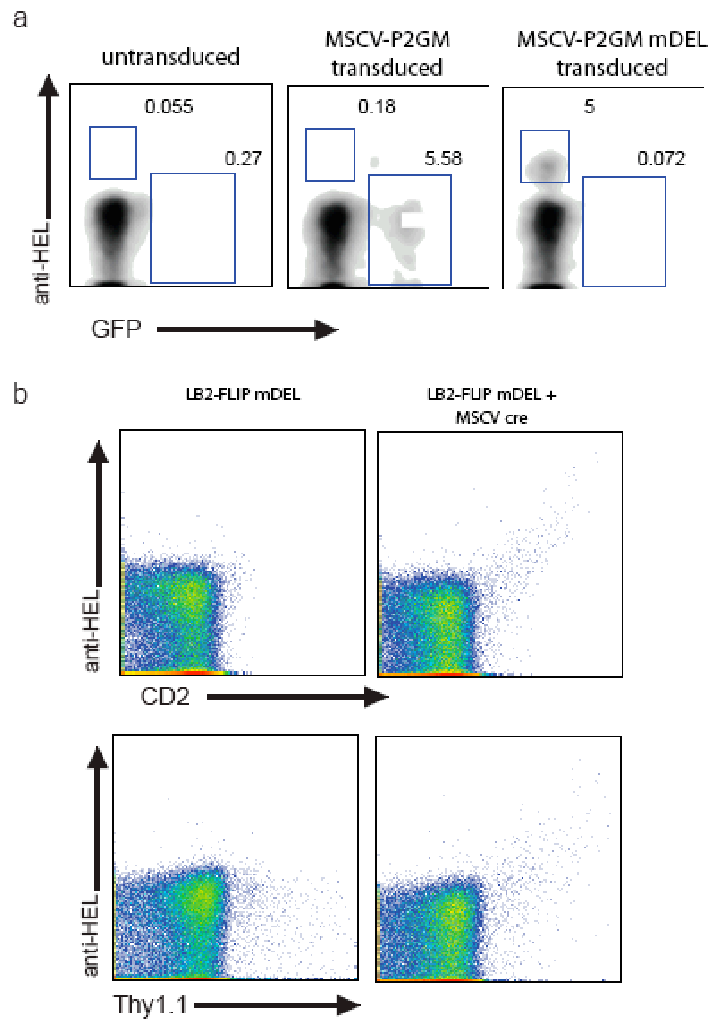


Figure 24. Membrane-bound DEL verification in Bal17 cell lines. **a,** Bal17 cells were transduced with MSCV-P2GM mDEL or empty vector along with GFP-expressing virus. **b,** Transfection of empty or mDEL LB2-FLIP lentiviral vector (Thy1.1 marker) along with transduction of an MSCVcre virus (CD2 marker) in phoenix cells.

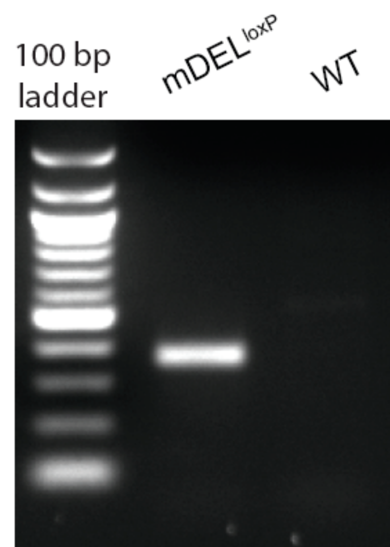


Figure 25. PCR product of the mDEL transgene that is 385 bp.

Table 1. mDEL^{loxp} founder line phenotype.

Founders	FO %Thy1.1 expression on B cells	Litters Analyzed	Transgene Genotype	Thy1.1 Expression (CD3+)	DEL detection by FACS	Thy1.1 deletion in B cells
FO1	48.60%	1	present	100%	yes	yes
FO3	43.50%	1	present	87%	yes	yes
FO6	39.40%	1	present	5-45%	no	yes
FO8	40.10%	1	present	5.70%	no	yes
FO12	19.30%	2	present	10%	no	yes
FO13	49.20%	1	present	50%	no	yes
FO14	10%	1	present	75%	no	yes
FO16	45.10%	1	present	42%, 80%	no	yes
FO17	31.30%	1	present	73%	no	yes
FO19	42.80%	1	present	45%	no	yes
FO21	29.90%	2	present - all silenced	0%	no	no
FO22	8.31%	1	present	yes	no	yes
FO23	24.70%	2	Not present	no	no	no

Table 2 recombinant DEL DNA sequence

ACTAGTGGCGCGCCTTAATTAAGGC**A**AC *ATG* *GCG* *AAG* *GCT* *TTG* *CTG* *ACC*
CTC *GTC* *TTT* *TGC* *CTC* *CTA* *CCC* *CTG* *GCT* *GCT* *CAG* *GGG* AAA GTC
TTT G**a**A CGA TGT GAG CTG GCA GCG GCT ATG AAG CGT C**t**C GGA
CTT GAT AAC TAT CGG GGA TAC AGC CTG GGA AAC TGG GTG TGT
GCT GCA AA**c** T**a**C GAG AGT A**g**C TTC AAC ACC CAG GCT ACA AAC
CGT AAC ACC GAT GGG AGT ACC GAC TAC GGA ATC CTA CAG ATC
AAC AGC CGC TGG TGG TGC **g**AC **a**AT GGC A**a**G ACC CCA GGC TCC
A**a**G AAC **gc**T TGC **gg**C ATC CCG TGC TCA G**t**C CTG CTG AG**a** TCA
GAC ATA ACA G**a**G **gc**C GTG A**gg** TGC GCG AAG A**g**G ATC GTC AGC
GAT GGA AAC GGC ATG AAC GCG TGG GTC GCC TGG CGC AAC CGC
TGC A**g**G GGC ACC GAC GTC **tc**G **aa**G TGG ATC AGA GGC TGC CGG
CTG GCCCCGGG

AAA Start of coding region for DEL
GCG added to give a G at the +4 position for kozak sequence
GC extra nucleotides to make sequence in frame with MHCI transmembrane sequence
TTT (F) is present in HEL, but not DEL, which decreases Ag processing in C57Bl/6 mice.
Italics is signal sequence
Lowercase bold letters are changes in the HEL sequence that gives the correct DEL amino acid
Uppercase bold letters are known sequence difference between HEL and DEL

Table 3 Protein sequence of mDEL

KVFERCELAAAMKRLGLDNYRGYSLGNWVCAANYESSFNTQATNRNTDGST DYGILQINSRWWCDNGKTPGSKNACGIPCSVLLRSDITEAVRCAKRIVSDGNG MNAWVAWRNRCRGTDVSKWIRGCRLAPGKEPPPSTVSNMATVAVLVVLGA AIVTGAVVAFVMKMRRRNTGGKGGDYALAPGSQTSDLSLPDCKVMVHDPHS LA

DEL sequence
MHCI transmembrane sequence
MHCI cytoplasmic tail exon 7
MHCI cytoplasmic tail exon 8
MHCI cytoplasmic tail exon 9

Chapter 3: Cyclin D3, ICOSL, and the Germinal Center

Introduction

B cells are activated upon BCR engagement with cognate antigen in soluble or immune-complexed form. Activated B cells are instructed to move to the T-B boundary through upregulation of the chemokine receptor CCR7, which engages CCL19 and CCL17 in the T-cell zone (62). It is at the T-B boundary where activated B cells receive the necessary survival signals through engagement of the CD40 receptor by CD40 ligand (CD40L) expressed on activated T helper (Th) cells (70). Th cells also provide necessary cytokines, such as IL-4, that lead to B cell proliferation and CSR by the enzyme activation-induced cytidine deaminase (AID). After T cell interaction, activated B cells rapidly proliferate extrafollicularly as plasmablasts or migrate into the central follicle to become GC B cells (71, 72).

It is unclear the extent which antigen affinity dictates extrafollicular or intrafollicular B cell fate. Initial experiments indicated that immunizing SW_{HEL} mice with low affinity antigen binding led to a reduction in the extrafollicular plasmablast response, but a strong germinal center response while immunizing with high affinity antigen led to a predominately extrafollicular response (73). A later study from the same group found that low affinity antigen engagement with the BCR resulted in both follicular and extrafollicular B cell fates (74).

In a normal immune response, the extrafollicular plasmablasts are the initial B cell producers of low affinity antibody but are short-lived, while the GC B cells are later producers of higher affinity antibodies and differentiate into long-lived plasma and memory B cells (27). These highly proliferative B cells seed the germinal center as centroblasts in the dark zone and undergo SHM. Not as much is known about the regulation of GC B cell proliferation. B cells express cyclin D2 and cyclin D3, which belong to the cyclin family involved in cell cycle. While cyclins have been shown to be important for B cell development (75), the role for cyclin D2 and cyclin D3 in peripheral B cells is unknown.

Centroblasts in the dark zone differentiate into more mature GC B cells called centrocytes and enter the light zone. The B cells that have generated higher affinity BCRs have the ability to bind to antigen complexes found on FDCs, while the lower affinity B cells undergo apoptosis (76). FDCs in the GC, called secondary FDCs, upregulate various surface proteins such as Fc receptor and integrin ligands and along with complement receptor expression, promote the GC response.

Self-reactive B cells can form as a result of SHM if germinal center tolerance mechanisms are not in place. In a mouse model of SLE, researchers found that ANAs were generated through SHM originally from nonautoreactive B cells (77). Interestingly, recent studies have detected non-pathogenic autoreactive IgG^{+} memory B cells in healthy humans. These memory B cells were determined to arise from SHM presumably from the germinal center (78).

A subset of T cells, called T follicular helper cells (Tfh), are also found in the B cell follicle and provide crucial survival signals to GC B cells in the form of CD40L

and perhaps through other cytokines (79-81). Tfh cells can be identified through specific makers ($ICOS^{+}$, $CXCR5^{+}$, $PD\text{-}1^{+}$, $Bcl6^{+}$). Tfh cells are crucial for proper germinal center responses exemplified by the absence of germinal centers and CSR impairment in ICOS deficient mice (82). ICOSL, the ligand for ICOS, is expressed on B cells, myeloid cells, and endothelial cells. A drastic reduction in the development of RA and lupus nephritis was seen after injecting mice with blocking anti-ICOSL antibodies, and points to a Tfh role in certain autoimmune diseases (83).

Taking the importance of a proper germinal center response in immunity against pathogens, I examined the role of cyclin D3 in GC B cells, the importance of ICOSL specifically on B cells in the germinal center response, as well as explored the mechanism of selecting against self-reactive GC B cells arising from SHM events during a germinal center response.

Results

GC B cell isolation

Germinal center B cells typically make up 5-10% of B cells in the spleen and can be identified by specific markers ($GL7^+$, Fas^+, IgD^-, PNA^+, $BCL6^+$). It has been difficult to isolate GC B cells in high enough quantity and purity because of their low frequency in the spleen and their short-lived nature. The initial germinal center protocol developed in the Rickert laboratory by Matthew Cato was further optimized (methods) to increase yield and purity by the author of this dissertation. Figure 26 shows typical GC B cell sorting purity by flow cytometry.

GC B cell culture

GC B cells are short-lived and undergo apoptosis unless proper signals are received. It has been of interest to determine what signals can keep isolated GCs alive in vitro. We find that anti-CD40 is the only stimuli that allow GC B cell survival (Fig 27a). Furthermore, stimulating the BCR lead to the rapid disappearance of GC B cells in culture after two days (Fig. 27b). While Il-4 and BAFF promotes the survival of naïve follicular B cells in culture, these stimuli alone do not promote GC B cell survival. This is surprising especially given the fact that IL-4 secreted by Th cells is involved in GC B cell expansion and class switch. GC B cells have lower BAFF R levels, but higher IL-4 R levels than naïve follicular B cells (Fig 28). The presence of IL4 with anti-CD40 results in only a slightly higher frequency of GC survival in vitro (Fig. 27).

Cyclin D3 required for GC B cells

Using the newly established GC B cell isolation technique, I examined the role of cyclin D2 and cyclin D3 in peripheral B cell cycle. While cyclin D2 and D3 protein were both absent in non-GC B cells, only cyclin D3 protein was expressed in GC B cells (Fig. 29a). Semi-quantitative RT-PCR on GC and non-GC B cells revealed the presence of both cyclin D2 and cyclin D3 transcripts (Fig. 29b). The results indicate that while cyclin D3 is regulated translationally or post-translationally in GC B cells, cyclin D2 is regulated at the level of transcription in non-GC and GC B cells.

Our lab also examined immunized $bCcnd2^{-/-}$ and $bCcnd3^{-/-}$ mice, which produce B cells deficient in cyclin D2 and cyclin D3, respectively, and found abrograted GC response in $bCcnd3^{-/-}$ but not $bCcnd2^{-/-}$ mice (84). Antigen-specific IgM and IgG were normal, but affinity maturation was impaired in $bCcnd3^{-/-}$ mice. Although $bCcnd3^{-/-}$ mice were unable to generate mature GC B cells assessed by $GL7^{+}FAS(CD93)^{+}$ cells, they were able to generate early GC PNA^{+} B cells. These data indicate that cyclin D3 is not required for initial CSR and plasma cell generation but is required for affinity maturation (84).

To further understand the post-translational regulation of cyclin D3, we focused our attention on GSK3α/β, which has been reported to phosphorylate and induce the proteosomal degration of D-type cyclins, but has reduced kinase activity when phosphorylated at serine resides 21 and 9. We examined phosphorylated GSK3β (p-GSK3β) levels in GC B cell and found increased p-GSK3α/β in GC B cells compared to non-GC B cells (Fig 30a), which suggests a mechanism for cyclin D3 regulation in GC B cells.

To further test the idea that the inactive form of GSK3α/β results in the accumulation of cyclin D3, LiCl was used to inhibit GSK3β activity in cultured GC B cells. While cyclin D3 levels were reduced in untreated GC B cells compared to freshly isolated cells, LiCl treatment prevented the loss of cyclin D3 protein (Fig. 30b). Additionally, LiCl treatment in non-GC B cells resulted in increased cyclin D3 levels (Fig. 30c).

To determine the kinase that phosphorylates GSK3α/β, GC B cells were cultured with various inhibitors and cyclin D3 protein levels were assessed. While phosphatidylinositol 3-kinase (PI3K) signaling regulates cyclin D3 stability in early B cells (75), treating GC B cells with LY294002, an inhibitor of PI3K, did not result in the reduction of cyclin D3 protein levels (Fig. 30b), suggesting that GSK3β phosphorylation is not mediated by PI3K. On the other hand, inhibiting PKA, a kinase found to promote AID activity (85), with H-89 treatment resulted in a sharp reduction of cyclin D3 protein in cultured GC B cells (Fig 30b). Cyclin D3 levels were restored when GC B cells were co-cultured with H-89 and LiCl (Fig 30b). These results strongly suggest that phosphorylation of GSK3α/β by PKA leads to cyclin D3 stability in GC B cells.

Importance of ICOSL expression in B cells

Productive T cell-B cell collaboration is needed for optimal germinal center reactions to occur, which involves the engagement of costimulatory molecules such as ICOS:ICOSL on T and B cells, respectively. The importance of this engagement is seen in ICOS and ICOSL KO mice, which have defective GC formation and antibody

production. ICOSL is expressed on B, myeloid, and non-hematopoietic cells, but the contribution of ICOSL expression on each cell type for germinal center reactions is unknown. Our lab was interested in studying the effects of ICOSL expression on B cells.

Reconstituting uMT mice, which lack B cells, with either WT or ICOSL KO BM to generate chimeric mice (uMT/WT and uMT/ICOSL KO) resulted in mice with B cells that are completely donor-derived, while the other hematopoeitic cells are a mix of both host and donor. Immunized uMT/ICOSL KO mice revealed a reduction in GC B cells compared to uMT/WT by flow cytometry and impaired germinal centers by histological analysis, although primary serum antibody production was intact (Mills, D. unpublished results).

Injections of anti-ICOSL blocking antibodies into WT immunized mice at d7 and day 9 resulted in impaired GCs while GCs remained intact in control Ig-injected mice (Mills, D. unpublished results), indicating that ICOSL is important for GC maintenance.

Because uMT/ICOSL KO mice have a myeloid compartment with mixed ICOSL expression levels, some of the GC impairment effects could be due to the myeloid cells. To remedy this, ICOSL KO B cells were adoptively transferred into uMT mice and immunized. GC formation did not seem to be impaired in mice that were adoptively transferred with ICOSL KO B cells assessed by histological analysis of the spleen (Fig. 31). However, the frequency of GC B cells was slightly lower and serum antigen-specific IgG1 antibodies were significantly lower in immunized ICOSL KO B cell- adoptively transferred uMT mice than in WT B cell-adoptively transferred

uMT mice (Fig. 32a, b). Taking these results together, ICOSL expression on B cells is important for normal GC responses but can be compensated with expression on other cell types such as myeloid cells.

While costimulatory molecules such as CD80 and CD86 is upregulated with CD40, BCR, TLR, and IL-4R engagement, ICOSL is constitutively expressed on naïve B cells but downregulated after IL-4 engagement. David Mills (Rickert Laboratory) found that BAFF, in addition to CD40 engagement (86), induces ICOSL expression in B cells, but BAFF's effect on ICOSL was independent of CD40 engagement, as the same effect was seen in CD40 deficient B cells (Fig. 33). ICOSL levels on naïve B cells from BAFF Tg mice were higher than wildtype, although levels on GC B cells were similar between WT and BAFF Tg mice (Fig. 34).

Since BAFF upregulates ICOSL expression on B cells and ICOS expression is restricted to T and NK cells, it was hypothesized that BAFF could promote T-B cell collaboration. To test this, B cells were treated with and without BAFF and subsequently co-cultured with naïve CFSE-labeled ova-restricted OT-II T cells for six days. There were more CFSE-low T cells, a measurement of proliferation, in the culture with BAFF-treated B cells than medium alone-treated WT B cells after six days (Mills, D. unpublished results). Furthermore, there was no differences in T cell proliferation with BAFF- or medium alone-treated ICOSL KO B cells, which indicate that BAFF could enhance T-B cell collaboration (Mills, D. unpublished results).

Elevated BAFF levels are association with various autoimmune diseases and BAFF transgenic mice develop lupus-like nephritis (12). Because BAFF can induce ICOSL expression and there was evidence that BAFF can enhance T-B collaboration,

our lab hypothesized that BAFF-mediated pathogenesis can be ameliorated when crossed to ICOSL KO mice. Indeed, BAFF Tg/ICOSLKO mice had reduction in MZ B cells, serum IgA levels, rheumatoid factor and anti-DNA serum antibody, and glomerular IC deposition when compared to BAFF Tg mice (Mills, D. unpublished results), which seems to indicate that that upregulated ICOSL expression in part, regulates BAFF-mediated autoimmunity (Mills, D. unpublished results).

Because BAFF Tg/ICOSL KO mice have reduced IgA levels, it would be hypothesized that increased BAFF levels would promote B-T interaction through ICOSL on B cells if ICOSL expression on B cells is important. Using an in vitro culturing system, WT or ICOSL KO B cells were cultured with OTII T cells and anti-IgM F(ab)'$_2$-OVA conjugate with or without BAFF to examine if ICOSL-mediates T-B collaboration to induced IgA isotype switch. Contrary to our hypothesis, greater frequencies of IgA B cells in cultures with BAFF-treated ICOSL KO B cells than WT B cells on days 4-6 was observed. Only on day 7 was the frequency of IgA-switched B cells higher among WT B cells (Fig 35).

These results indicate that although ICOSL expression may be important on B cells, it does not affect isotype switching, has minimal effects on T-B cell collaboration, and ICOSL on other cell types can compensate for the lack of expression on B cells for germinal center reactions.

AIRE expression is not detected in FDCs in the secondary lymphoid organs

AIRE is a protein expressed in mTECS in the thymus and is involved in the negative selection of developing T cells (87). It functions in part by promoting the

transcription of tissue specific antigen (TSA) on mTECS that eliminate developing T cells that bind to self-antigen in the thymus. Combining the fact that several groups have published the detection of AIRE transcripts in the peripheral lymphoid organs (88, 89), the fact that mTECS and FDCs are dependent on lymphotoxin for development (90), and the fact that mechanisms must be in place to negatively select GC B cells that become self-reactive during SHM events, we examined whether AIRE is expressed on FDCs as a means of regulating self-reactive GC B cells. While we found AIRE and transcripts in the stromal portion of the spleen (Fig. 36), no AIRE protein was detected in the GCs by immunohistochemistry (Fig. 37), indicating other mechanism that must be in place during a GC reaction that ensures the negative selection of newly formed self-reactive GC B cells.

Discussion and Analysis

In summary, I show that GC B cells can be isolated with high purity and quantity in a cost-effective manner using magnetic bead-based technology. FACS-based GC B cell sorting, which is currently the predominate sorting method, results in a pure GC B cell population, but it is time-consuming and does not yield enough cells within a reasonable amount of time for biochemical analysis. It would take about 8 hours of sorting time to sort $1X10^7$ GC B cells by FACS versus only 2.5 hours by magnetic beads. Furthermore, GC B cells undergo cell death within several hours of isolation, which eliminates FACS-based sorting for large quantities of GC B cells. Our GC B cell protocol is the first reported magnetic bead-based technology which can be used for biochemistry and gene expression profiling.

Experimental analysis showed that the sorted B cells can be cultured, but many undergo rapid cell death unless rescued by anti-CD40, but not IL-4, a cytokine that promotes the survival of naïve cells in culture. CD40 ligand is expressed on T cells and is the primary cell-type to provide the survival signal for GC B cells, which demonstrates the importance of cognate T-B collaboration for the GC reaction.

While T cells provide the necessary survival signals to GC B cells in the form of CD40L, activated $CD4^+$ T cells need engagement of the costimulatory molecule ICOS by ICOSL for Tfh generation and continued CD40L expression. Results show that ICOSL is needed on B cells for serum antibody and GC B cells production in a primary immune response, but may be dispensable for normal GC structures in adoptive transfer studies. On the other hand, BM chimera mice, in which all B cells were ICOSL deficient and myeloid cells were both ICOSL sufficient and deficient,

showed impaired GC B cell production, impaired GC structures, but intact antibody responses, indicating that myeloid ICOSL expression may be more important in GC reactions. It is worth mentioning that both WT and ICOSL KO adoptively transferred mouse spleens contained small GC structures presumably due to the low quantities of B cells inherent to the adoptive transfer method. Similarly, impaired antibody responses were reported previously in B cell adoptive transfer studies from immunized ICOSL KO mice (82). During our studies, another group published a study on the importance of ICOSL on B cells using cre-mediated conditionally deleted ICOSL$^{fl/fl}$ mice (80). This group found reduced GC B cells and Tfh formation as well as smaller GC structures in mice with ICOSL deficient-B cells.

I also show that BAFF upregulates the expression of ICOSL and that BAFF Tg B cells have higher levels of ICOSL expression. While the effects of BAFF on ICOSL expression on B cells were being examined in our lab, another group published the same findings. Furthermore, they found that ICOSL expression is downregulated after ICOS engagement even in the presence of anti-CD40 or BAFF, which indicates a negative feedback mechanism in the ICOS:ICOSL pathway (91).

Although the results in one in-vitro assay indicates that BAFF may promote T-B cell collaboration through B cell ICOSL expression (Mills, D. unpublished results), subsequent in-vitro assays did not show that BAFF-induced upregulation of ICOSL expression on B cells promoted increased IgA class switch and T cell activation. Around the same time as our studies, another group published the surprising result that BAFF Tg mice still developed autoimmunity despite the absence of T cells, indicating that BAFF-induced deregulation of B cell tolerance is independent of T cell help, but

needed intact TLR signaling (92). It is known that strong BCR signaling concurrent with signaling through the TLR pathway (93) can result in a T-independent B cell response. Although BAFF-induced autoimmunity can occur independently of T cells, it does not exclude the possibility that T cells still play a role in BAFF-mediated autoimmunity physiologically. Also, the fact that ICOSL levels on BAFF Tg GC B cells were not higher than WT GC B cells (Fig. 34) indicate that normal induction of ICOSL occurred in the GC even in the presence of elevated BAFF levels. This may indicate that intact downregulation of ICOSL may overcome the BAFF-induced upregulation of ICOSL in controlling the GC reaction.

Experimental analysis also indicate that while AIRE transcripts were detected in the stromal portion in the spleen by RT-PCR, we did not find AIRE expression in the GC of immunized mice. Therefore, the immunohistochemistry data do not point to AIRE expression on FDCs as a mechanism for selecting against GC B cells that have become self-reactive through SHM. Subsequent to our studies, a published study found AIRE expression in stromal cells of the T cell zone in the secondary lymphoid organs, which is attributed to mediating CD8+ T cell peripheral tolerance (94). This group also found AIRE-independent expression of TSA in the stromal population in the T cell zone. Perhaps there is also an unidentified stromal subset in the B cell follicle that expresses TSAs.

Finally, I find that cyclin D3, but not cyclin D2, is uniquely required for proper GC responses. While cyclin D2 and D3 transcripts were detected in non-GC B cells, only cyclin D3 transcripts were detected in isolated GC B cells. Cyclin D2 and cyclin D3 protein were both absent in non-GC B cells indicating translational or

posttranslational regulation. The transcriptional regulation of cyclin D2 in GC B cells is likely conferred by the dominant transcriptional repressor expressed in GC B cells, BCL6, which has been reported to target cyclin D2(95). Experimental analysis also show increased levels of phosphorylated GSK3β, the inactive form, in GC B cells, which would prevent cyclin D3 phosphorylation and degradation. Addition of the PKA but not PI3K inhibitor resulted in the loss of cyclin D3 in GC B cells indicating that GSK3β phosphorylation is mediated by PKA. Indeed, PKA has been found to regulate AID in B cells. These results show that PKA-mediated GSK3β phosphorylation leads to cyclin D3 protein accumulation in GC B cells.

In agreement with that fact that cyclin D3 protein, but not cyclin D2, was detected in GC B cells, chimeric mice with B cells deficient in cyclin D3, but not cyclin D2, are unable to mount a normal GC response. These results are surprising given that fact that B cell stimulation induces cyclin D2 and cyclin D3. In addition, loss of cyclin D3 results in only a partial block in early B cell development and the immature B cells that developed contained high levels of cyclin D2 protein indicating that cyclin D2 could in part compensate for D3 deficiency (75). Unlike early B cell development, cyclin D2 cannot compensate for the loss of cyclin D3 in mature GC B cells defining a unique role for cyclin D3 in the GC B cell response.

Materials and Methods

Mice

ICOSL$^{-/-}$, OT-II, uMT, and BAFF Tg mice were housed in pathogen-free environment in the Animal Facility at the Sanford-Burnham Medical Research Institute and were maintained on a C57BL/6 background. Chimeric mice were generated by sublethally (5 gray) irradiating uMT mice followed by intravenous injection of WT or ICOSL$^{-/-}$ bone marrow and allowing 6 weeks for complete reconstitution. Adoptive transfer studies were carried out by intravenous injection of WT or ICOSL$^{-/-}$ B cells. All experiments conformed to the ethical principles and guidelines approved by the SBMRI Institutional Animal Care and Use Committee.

Immunizations

For the ICOSL$^{-/-}$ experiments, chimeric mice were immunized intraperitoneally with 50 ug of NP-KLH precipitated in alum. For germinal center B cell isolation, mice were immunized with 0.2 ml of sheep red blood cell (SRBC) prepared by washing pelleted SRBCs twice with PBS and resuspending in PBS to a final concentration of 10% (vol/vol).

Germinal center and non-germinal center B cell isolation

Spleens were isolated from day 5-6 SRBC-immunized mice and disassociated between the frosted ends of two glass slides. Cells were treated with RBC-lysis buffer and resuspended in MACS buffer (0.5% BSA and 5 mM EDTA in PBS) at a final concentration of 10^8 cells/ml. 90% of total cells were allocated for GC B cell isolation

and incubated with the following biotinylated antibodies for 25 minutes on ice: CD43 (2.5 ug/ml), IgD (2 ug/ml), and CD11c (2 ug/ml). The remaining 10 of cells were allocated for non-GC B cell isolation and incubated with the following biotinylated antibodies for 25 minutes on ice: CD43 (2.5 ug/ml), GL7 (2 ug/ml), and CD11c (2 ug/ml). Cells were washed, resuspended in 0.8 ml MACS buffer for every 10^8 cells, and incubated with 20 ul of anti-biotin microbeads for every 10^8 cells for 20 minutes on ice. Cells were washed and loaded into LS columns. The GC and non-GC B cell population were collected as the flow-through population.

Flow cytometry

Single cell suspensions from spleen, lymph node, and bone marrow were incubated with either biotinylated HEL (Genetex) or with soluble HEL followed by biotinylated anti-HEL antibody (Rockland). The following antibodies were used from eBioscience: anti-B220, -CD3, -CD23, -IgM, -IgD, -IgA, -GL7, -FAS, -IgG1, and -BAFFR. Live cells were assessed by forward and side scatter profiles. All cells were acquired on a FACS Canto using FACS DIVA software (BD Biosciences) and analyzed using FlowJo (Treestar). Data are displayed with logarithmic scale.

Elisa

NP30- or NP3-BSA in 0.05% Azide in PBS was coated in 96-well high binding capacity plates overnight at 4°C and blocked with 0.25% BSA in PBS for 1 hour. Mouse sera was serially diluted in 0.5% BSA in PBS and incubated in 96-well high binding capacity plates for 2 hours at RT. Plates were washed three times and

incubated with the appropriate alkaline phosphatase-conjugated anti-mouse Ig secondary antibodies for 1 hour at RT. After washing three times, phosphatase substrate (Sigma, St. Louis, MO) was added to the samples and the A_{405} was measured. Data are presented as the A_{405} of individual wells within the linear range of the assay.

In vitro culture

B and T cells were isolated from splenic cells using magnetic MACS beads according to manufacturer's guidelines. Cells were cultured in a 96-well plate at 1.5 X 10^6 cells/ml in complete RPMI media. B cells were stimulated with the following reagents: anti-IgM F(ab)'$_2$ (10 ug/ml, Zymed), LPS (10 ug/ml), anti-CD40 (10 ug/ml), recombinant murine IL-4 (20ng/ml), and recombinant human BAFF (200 ng/ml). For T cell B cell collaboration assay, B cells were isolated by depleting $CD43^+$ cells. OTII $CD4^+$ T cells were isolated by depleting $B220^+$, $CD11b^+$, $CD11c^+$, and $GR1^+$ cells. Cells were cultured and stimulated with the following reagents: BAFF (200 ng/ml), TGFbeta (1 ng/ml), and F(ab)'$_2$-OVA conjugates (1 ug/ml). For germinal center B cell cultures, cells were stimulated with the following reagents: IL-4 (50 ng/ml), anti-CD40 (10 ug/ml), and BAFF (200 ng/ml).

Reverse transcription-PCR (RT-PCR)

Total RNA was purified from ~ 10^6 cells from the spleen, lymph node, or thymus using nucleospin RNA II columns, according to the manufacturer's protocol. For AIRE detection, splenic cells were further divided into lymphocyte and stromal

cells. cDNA synthesis was performed using an Advantage RT-for-PCR kit (Clontech) with oligo(dT) primer. Cyclin D2, cyclin D3, AIRE, and GAPDH primers were used in PCR reactions with serially diluted cDNA.

Inhibitor studies and Western blot analysis.

Freshly isolated cells were cultured at 10^6 cells/ml in RPMI 1640 containing 10% FBS and 10 uM H-89 dihydrochloride (Calbiochem), 10 uM LY294002 (Calbiochem), 10 mM LiCl (sigma), or 100 uM N6,2'-o-dibutyryladenosine 3',5'-cyclic monophophate (Sigma), as indicated. At the indicated time points, cells were collected, whole-cell lysates were prepared, and equivalent cell numbers were resolved by SDS-PAGE. Whole-cell lysates from 24-hour LPS-stimulated cells were used as a positive control. Western blotting and immunodetection were conducted with the following antibodies: cyclin D2 (M-20) (Santa Cruz Biotechnology), cyclin D2 (DCS22), phospho-GSK-3beta (5B3) (Cell Signaling Technology), and the appropriate horseradish peroxidase-labeled secondary (Jackson Immunoresearch). ERK, AKT, or beta-Actin was used as a loading control.

Immunofluorescence microscopy

Splenic tissue was embedded in Tissue-TEK O.C.T compound (Sakura Finetek U.S.A., Torrance, CA) and frozen at -80°C. Frozen tissue were then sectioned, mounted on Superfrost/Plus microscope slides, fixed in cold acetone for 10 minutes, and blocked with 5% FBS and 1% BSA in PBS for 1 hour. Sections were first incubated with soluble HEL for 30 minutes. Tissues were then stained with the

following antibodies: biotinylated PNA, Streptavidin Cy3 (Jackson ImmunoResearch), anti-IgM Cy5 (Jackson ImmunoResearch), anti-AIRE, rabbit anti-Rat, anti-CD3e APC, anti-MHCII fitc, and anti-B220 fitc and APC. Histology sections were imaged with a Zeiss Axio ImagerM1 using Slidebook software (Intelligent Imaging Innovations, Denver, CO).

Acknowledgements

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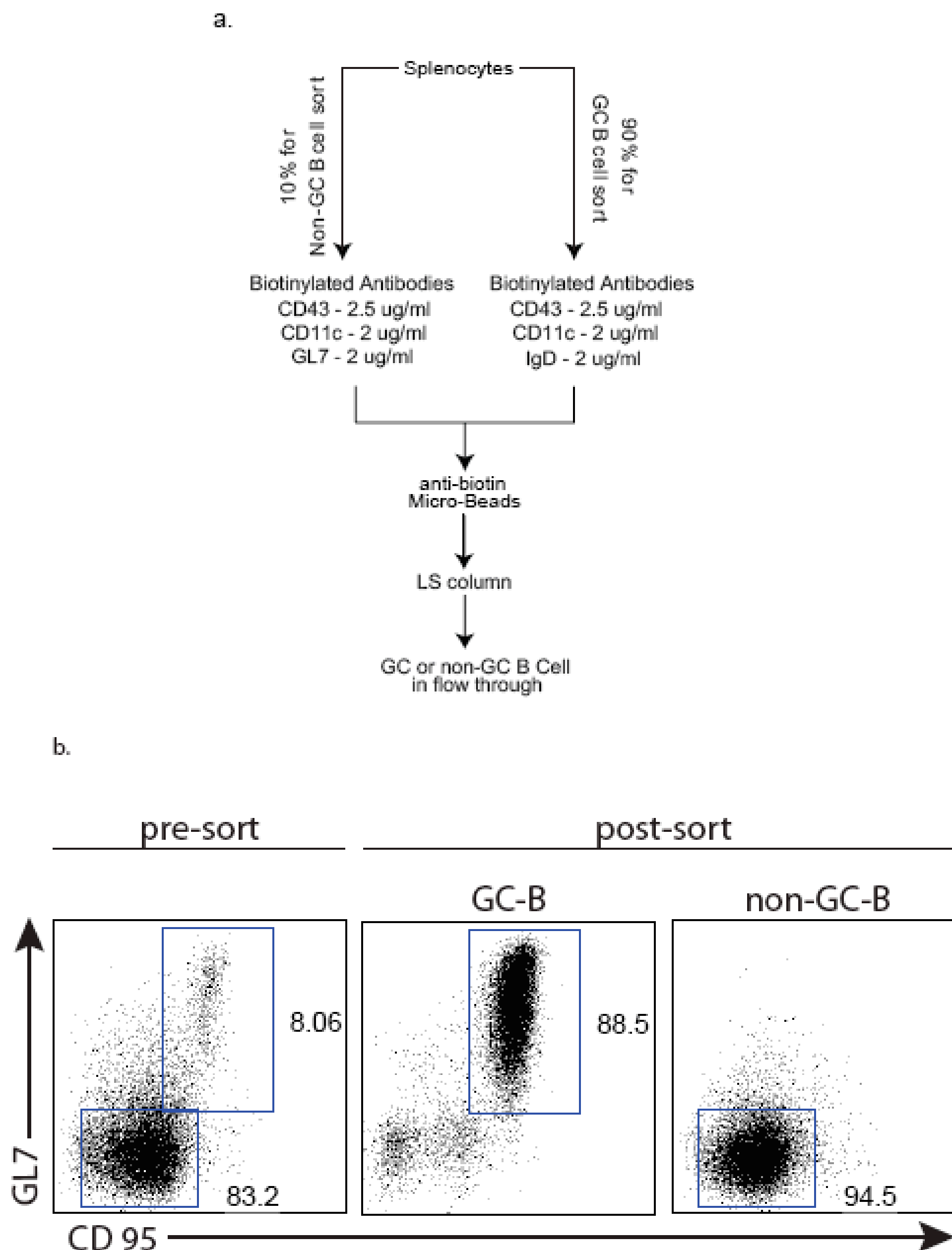


Figure 26 GC B cell sort. **a,** GC and non-GC B cell sorting scheme. **b,** Flow cytometry plots showing the GC and non-GC B cell purity.

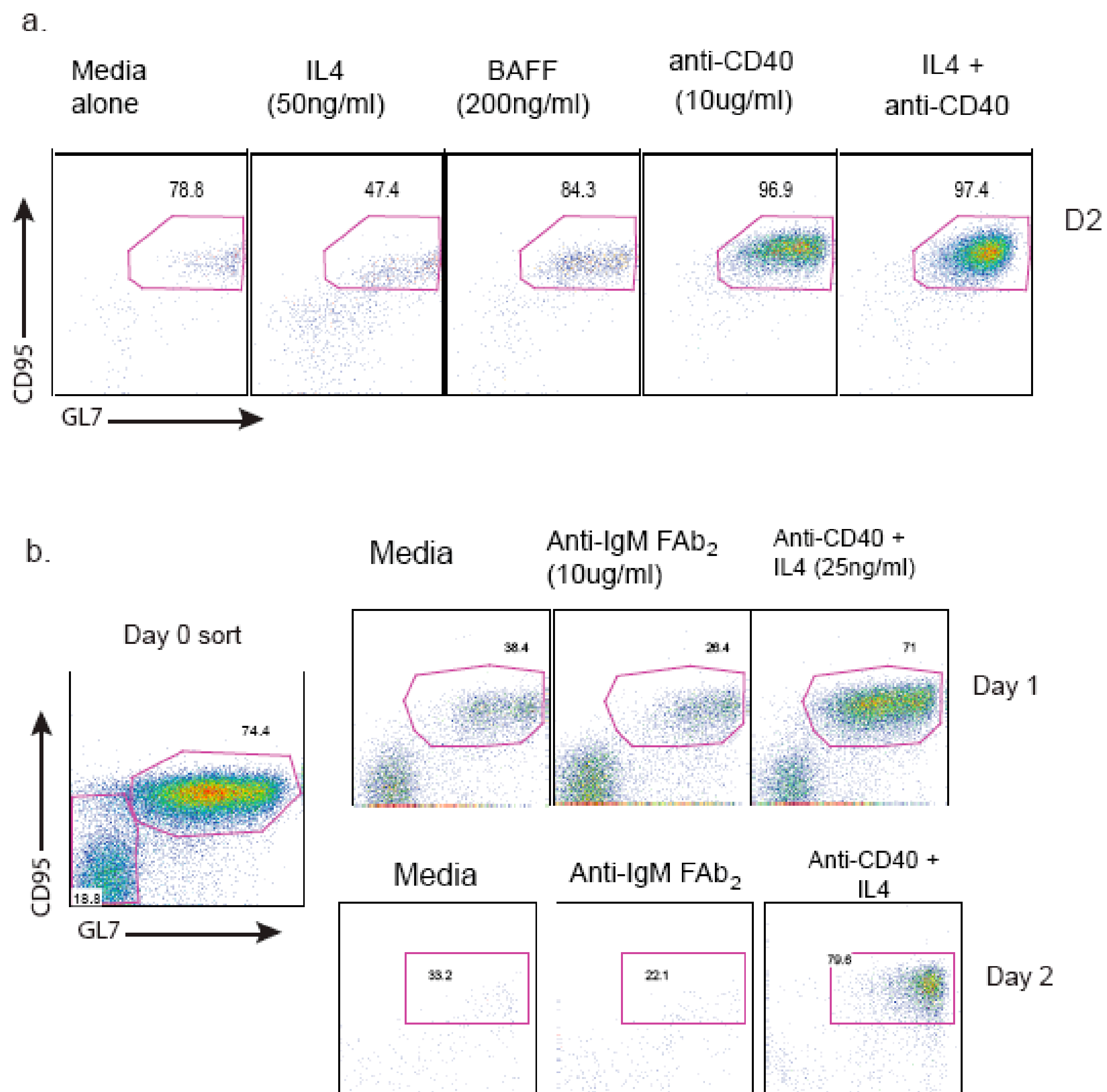
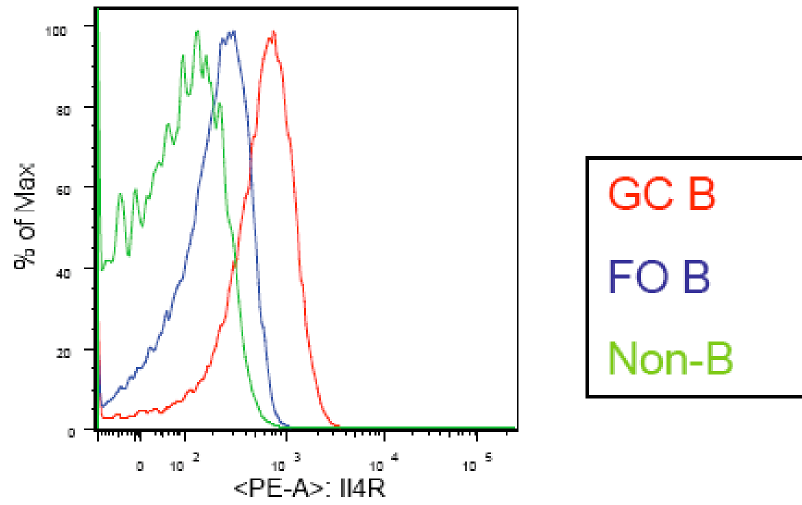


Figure 27. Cultured GC B cells require CD40 signaling for survival, but are rapidly eliminated upon BCR engagement. **a,** Flow cytometry plots of GC B cells were cultured with various stimulants stated above after 2 days. **b,** Flow cytometry plots of GC B cells cultured with the various stimulants stated above after 1 and 2 days.

a.



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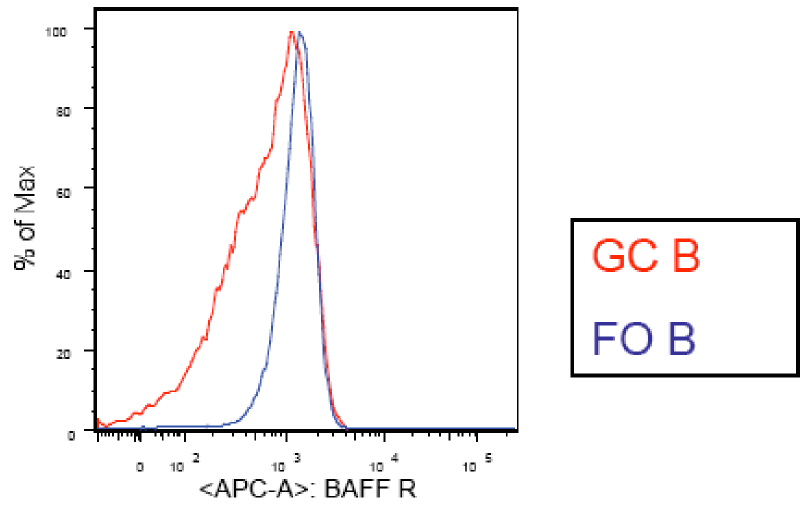


Figure 28. IL4 and BAFF receptor levels on GC B cells. GC and FO B cells were isolated from SRBC-immunized mice and analyzed for surface IL4R (a) and BAFFR (b) levels.

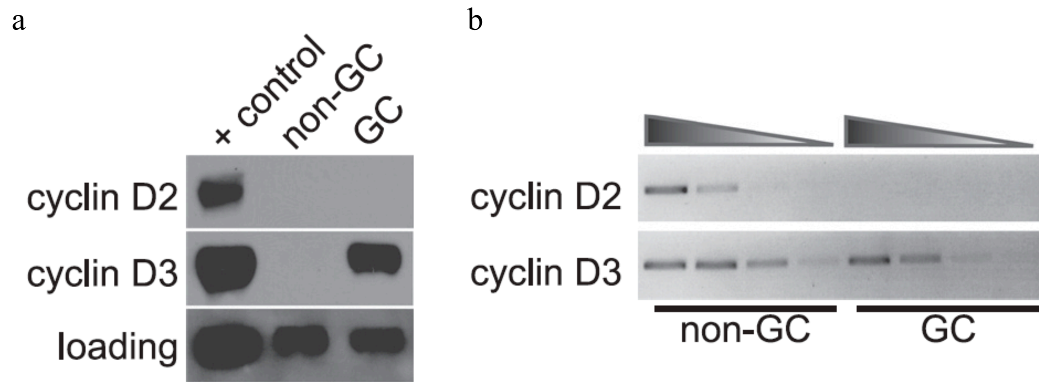


Figure 29. Cyclin D3 expression in GC B cells. Lysates and RNA was prepared from sorted GC and non-GC B cells and subjected to Western blot (a) or RT-PCR analysis. Suresh Chintalapati and Matthew Cato performed the western blot and RT-PCR reaction.

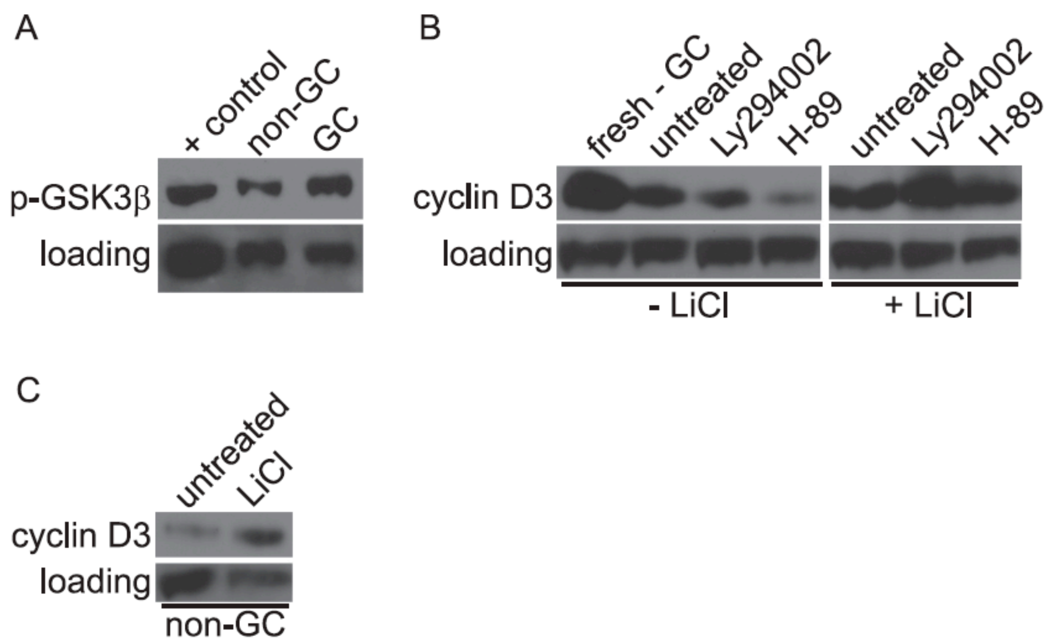


Figure 30. Cyclin D3 regulation. a, Lysates were prepared from sorted GC and non-GC B cells and probed for phosphorylated GSK3beta. b, Lysates prepared from sorted GC B cells cultured with the indicated reagents for 120 minutes were probed for cyclin D3 protein. c, Lysates prepared from sorted non- GC B cells cultured with or without LiCl for 30 min and probed for cyclin D3 protein. Suresh Chintalapati and Matthew Cato performed the western blots.

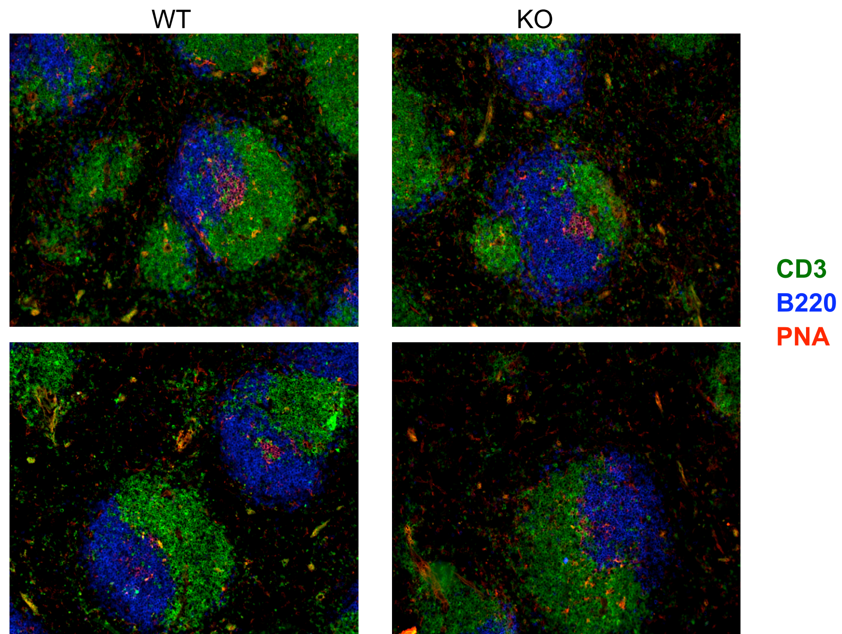


Figure 31. ICOSL on B cells is not required for GC reactions. Spleen sections of uMT mice that had either WT or ICOSL deficient (KO) B cells adoptively transferred intravenously prior to immunization.

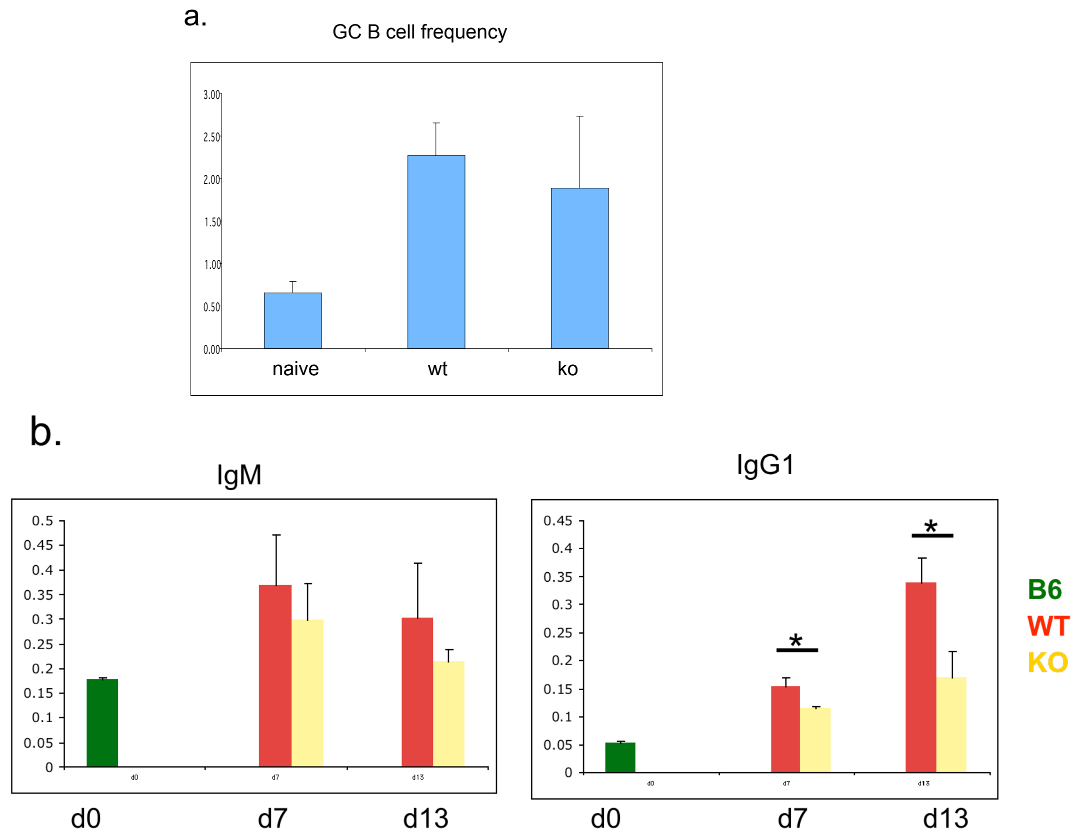


Figure 32. GC B cell frequency and antigen specific serum Ig levels. Either WT or ICOSL deficient (KO) B cells were adoptively transferred intravenously into uMT mice prior to immunization. **a,** GC B cells (GL7+Fas+) frequency were determined by flow cytometric analysis and plotted on the graph. **b,** NP-specific serum IgM and IgG levels were determined by ELISA.

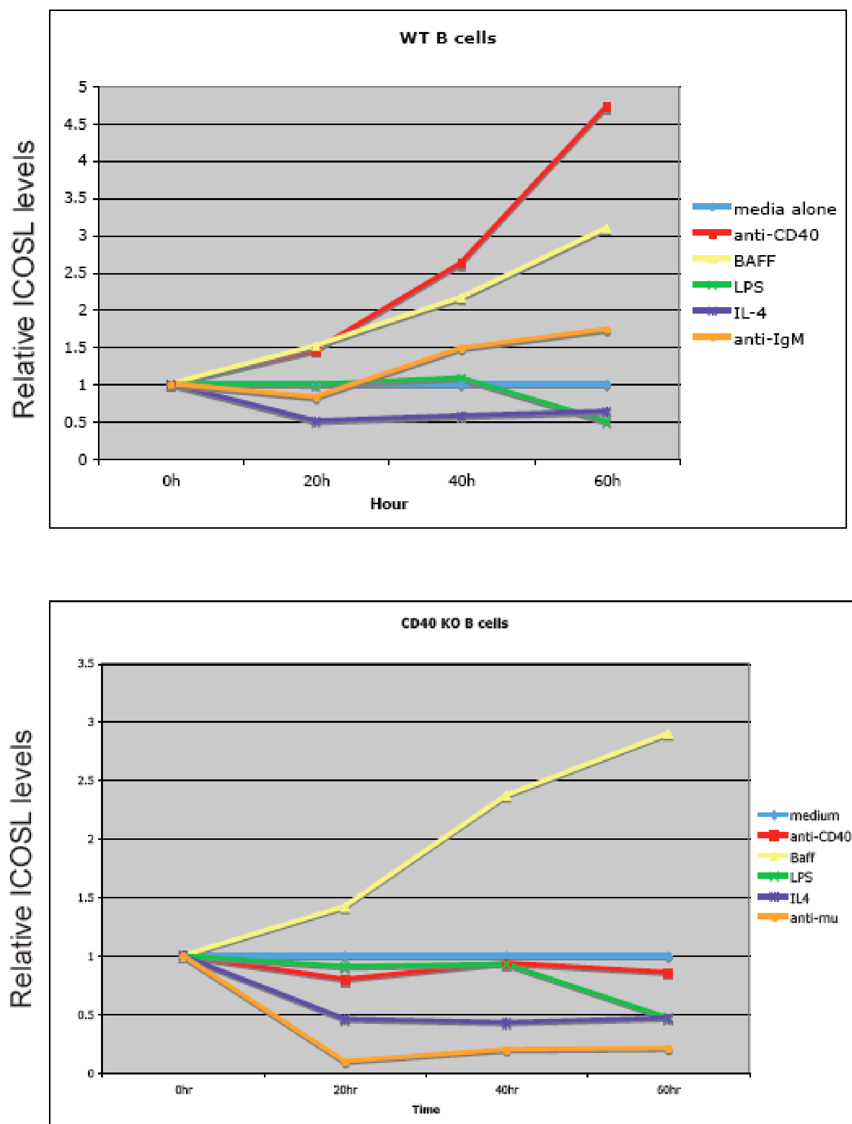


Figure 33. Surface ICOSL induction by BAFF. WT (top) or CD40 deficient (bottom) B cells were sorted and cultured with the various reagents and examined for ICOSL expression over a 60-hour period. Relative ICOSL levels were measured and graphed by taking the ICOSL MFI ratio of B cells cultured in reagents versus medium alone.

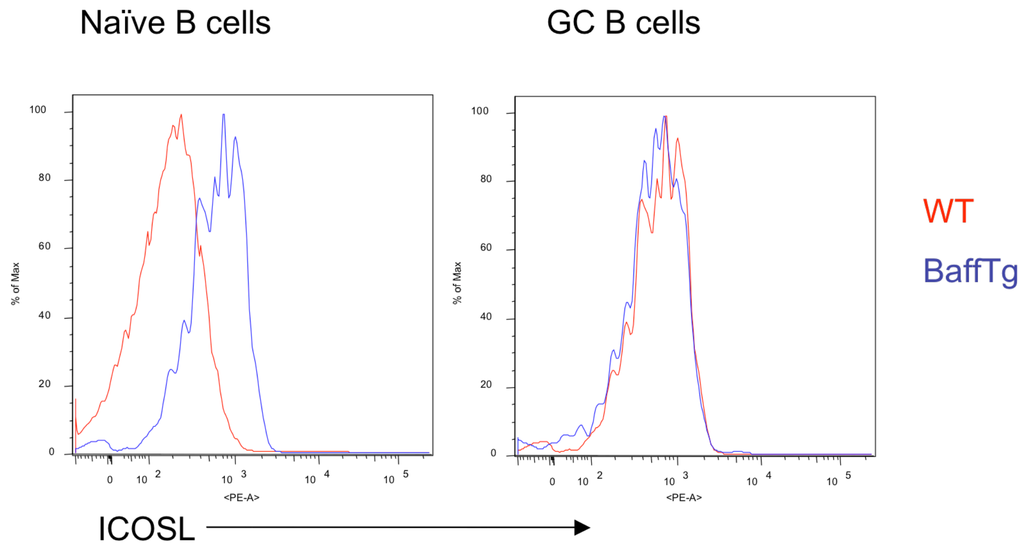


Figure 34. ICOSL expression on naïve and GC B cells. Overlay histograms of ICOSL expression on naïve or GC B cells from WT and BAFF Tg mice.

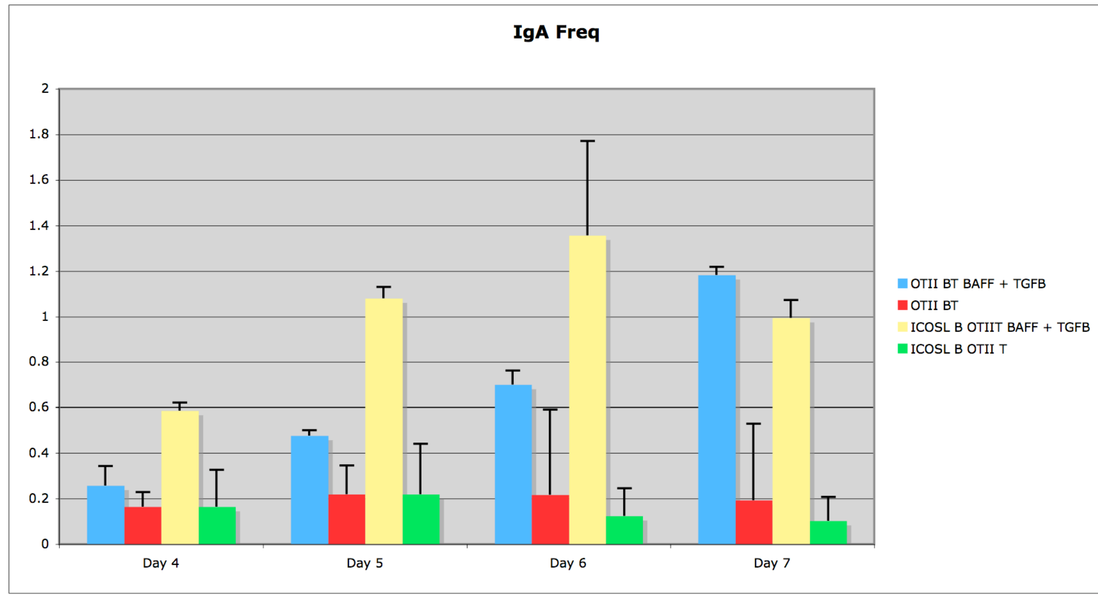


Figure 35. T cell-B cell collaboration. B cells and T cells were isolated from OTII and ICOSL KO mice and cultured with or without soluble BAFF and TGFbeta. IgA-switched B cells were assessed by flow cytometric analysis on Day 4-7 and the frequency was plotted on the graph.

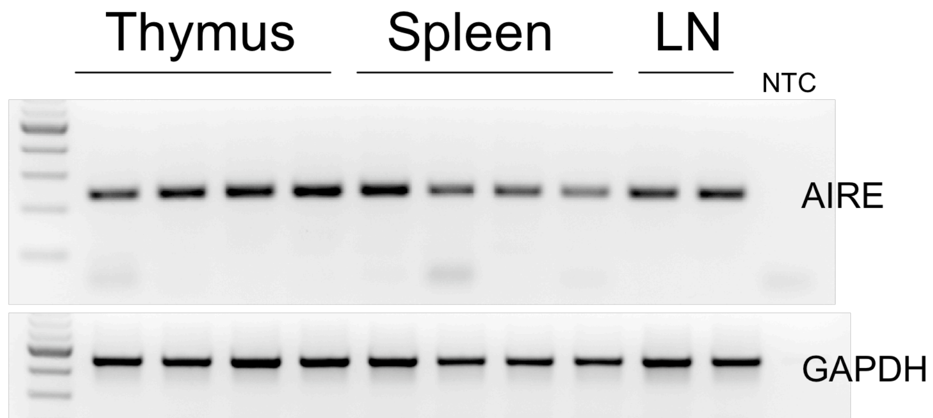
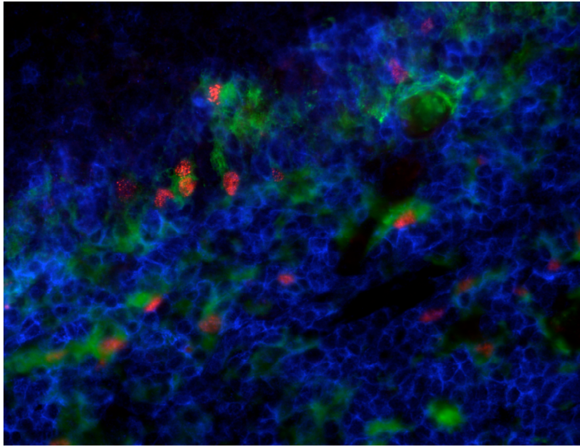


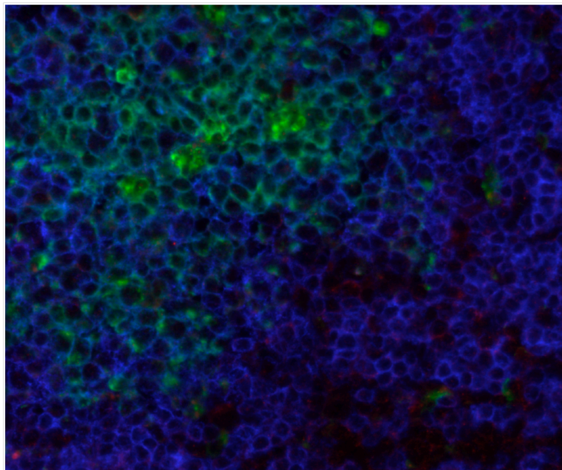
Figure 36. RT-PCR of AIRE transcripts using cDNA prepared from thymocytes and stromal cells from the spleen and lymph node from WT mice.

Thymus



CD3e
MHCII
AIRE

Spleen



B220
PNA
AIRE

Figure 37. AIRE expression. Immunohistochemistry of thymus (top) and spleen (bottom) sections from an immunized WT mouse. AIRE detection coincides with MHCII-expressing mTECs in the thymus, but not in PNA^+ GC in the spleen.

Chapter 4 Conclusion

Here, I show that self-antigen retained on FDCs mediates the elimination of self-reactive B cells at the transitional stage. While the T1 B cell subset remains intact, there is a sharp reduction of T2 self-reactive B cells indicating a breach in development from the T1 to T2 stage. My model is the first to show that late-stage self-reactive transitional B cells can be eliminated in the spleen where B cells naturally complete their maturation and collaborate with other cell types to mount an immune response. Furthermore, it is the first model to show that self-retained antigen specifically on FDCs can mediate effective elimination of self-reactive B cells. It would be of interest to examine how frequently self-antigen binds to FDCs in mouse models and healthy individuals.

Previous B cell tolerance models have also shown a block in transitional B cell development although their encounter of self-antigen is at various locations that may or may not be physiologically relevant. The SW_{HEL}xML5 mice most likely encounter soluble HEL during development in the BM (40). The 2-12Htg mice generate B cells that can bind ssDNA and smith antigen, similar to what is found in SLE patients and may encounter DNA and cellular components during development in the BM or during maturation in the spleen (96). In IgD superantigen mice, self-reactive B cells can encounter ubiquitously-expressed self-antigen as soon as they start expressing surface IgD in the periphery, which includes development in the spleen but may be elsewhere (97). It is curious that although antigen encounter takes place in different locations and at various developmental stages, these B cells are all blocked at the

transitional stage, when B cells are at the cusp of becoming fully immunocompetent, but are still sensitive to BCR-mediated apoptosis.

Future experiments of interest would be to determine whether T cell help could rescue the elimination of self-reactive B cells in *Cd21*^{cre}mDEL^{loxp} mice. Past work addressing rescue of tolerant B cells by T cells have yielded opposing results. Using the soluble HEL transgenic system and HEL-binding B and CD4^{+} T cells, Rathmall et al. determined that anergic HEL-binding B cells were eliminated by FAS ligand-expressing CD4+ T cells (98). Furthermore, FAS-mediated apoptosis could be overcome with constitutive expression of CD86 costimulatory molecule, which is normally upregulated on activated B cells but not on anergic B cells (65, 99).

On the other hand, tolerant HEL-binding B cells from soluble HEL transgenic mice differentiated into plasma cells in the presence of membrane HEL and T cell help, which indicates a break in tolerance, indicating that strength of signaling is an important component in whether interaction with T cells leads to apoptosis or survival (100). Although CD86 levels in those experiments were not specifically examined, signaling with membrane HEL restored BCR signal transduction and presumably would restore upregulation of the T cell costimulatory molecule.

Another important factor in determining the outcome of T cell interaction with tolerant B cells is timing. Cooke et al. hypothesize that the avidity or quality of antigen binding determines the length of the "window of opportunity" for productive T cell interaction (101). The group showed that a portion of immature HEL-binding B cells, but not mature anergic HEL-binding B cell from a soluble HEL (sHEL) transgenic mouse that were transferred into an mHEL mouse along with provision of T cell help

could survive and proliferate rather than undergo elimination. The mature HEL-binding B cells had presumably longer and more frequent interaction with self-antigen and therefore could no longer productively interact with helper T cells.

In my mouse model, membrane DEL engagement would transduce a weaker signaling than with membrane HEL engagement. If FDCs in our model were to express membrane HEL, than stronger signal through the BCR upon engagement is expected. This stronger BCR signal would result in a more rapid elimination of HEL-binding B cells, but a stronger signal could also result in upregulation of CD86, which would rescue them with T cell help if reached in time. In support of this hypothesis is that CD86 levels were upregulated in the presence of a strong BCR stimulant (data not shown), but only weakly upregulated in the presence of soluble HEL or DEL (Fig. 21a). HEL-binding B cells directly ex-vivo from our system express slightly higher levels of CD86 similar to what is reported in anergic HEL-binding cells from MD4XML5 mice (99). From the results of previous groups, it would be hypothesized that the presence of CD4 T cells would lead to FAS-mediated apoptosis of HEL-binding B cells in *Cd21*$^{\mathrm{cre}}$mDEL^{loxp} mice because these self-reactive cells are unable to upregulate the proper T cell costimulatory molecules needed for survival. The transitional self-reactive B cells would be especially vulnerable to FAS-mediated apoptosis since it is reported that transitional cells are unable to regulate CD86 (3, 9). However, if T cell-help were available to the more mature HEL-binding B cells shortly after they encounter mDEL binding, than it would be hypothesized that these B cells may be able to survive. I do observe a fraction of antigen-experienced HEL-binding B cells in *Cd21*$^{\mathrm{cre}}$mDEL^{loxp} mice that are more mature (CD24low or

AA4.1low). Although a majority of antigen-experienced low-HEL-binding B cells have higher Bim levels, a fraction is still Bim low. It would be hypothesized that these cells have not had as frequent or long encounters with mDEL and can still interact with T helper cells.

Work is ongoing to determine whether BAFF overexpression can rescue self-reactive B cells from elimination in our peripheral tolerance mouse model. Elevated BAFF levels are seen in patients with various autoimmune diseases such as SLE and Sjogren's syndrome. Previous work using various tolerance mouse models have shown that elevated BAFF can break tolerance induction of self-reactive B cells depending on the B cell stage of arrest (41). SW_{HEL} B cells in the presence of increased BAFF levels that were exposed to membrane HEL during development in the BM were not rescued, which is in agreement with the fact that the receptor for BAFF, BAFFR, is not expressed until the T2 stage of B cells development (15, 102). On the other hand, HEL-binding B cells exposed to soluble HEL during development could mature into FO and MZ B cells in the presence of BAFF overexpression. In the IgD-superantigen tolerance model, BAFF overexpression could not rescue self-reactive B cells, although an increase in MZ and plasma cells were seen (97).

Preliminary results in our model indicate that self-reactive B cells progress through the T2 stage; nevertheless, they are eliminated at a more mature stage. BAFF signaling do not seem to overcome the apoptotic signals from repeated antigen encounter. Increased plasma cells were also seen in our model in the presence of elevated BAFF levels, indicating that BAFF overexpression allows the survival of self-reactive B cells to differentiate into plasma cells or allows the survival of plasma

cells, or a combination of both. It is worth exploring the mechanism behind BAFF-mediated plasma cell increase seen our mouse model.

Other spatial and temporal aspects of peripheral tolerance can be addressed in the future using mDEL^{loxp} mice as this mouse model conditionally expresses mDEL. For example, mDEL^{loxp} mice could be crossed to *Mx1*-cre mice in which DEL would only be expressed upon interferon induction. Immunizing these mice and inducing interferon production can be employed to study tolerance mechanisms in the GC. Breeding mDEL^{loxp} mice with villin-cre mice can be used to study B cell tolerance in the gut, an area of interest that has not been well studied. Additionally, osteoblast cre-expressing mice (OC-*Cre*) could be used to study developing, memory, or plasma cell encounter with self-antigen in the bone marrow. *mDEL*^{loxp} mice are currently bred with col2a-cre mice, which secrete cre in chondrocytes, to use as a model for arthritis.

Self-reactive B cells can originate from somatically hyper mutated GC B cells during a GC reaction. Although our attempts at identifying a novel mechanism (AIRE) for GC B cell tolerance were unsuccessful, general "safety" mechanisms are in place throughout the course of a GC reaction to prevent autoimmunity from arising out of a normal immune response. For example, GC B cells must receive survival signals from cognate T cells or undergo apoptosis attributed to high Bim levels and concurrent low Bcl2 levels (103). FAS also contributes to the selection of autoreactive B cells. Mice deficient in either FAS or FASL develop SLE-like symptoms (103). Tfh cells seem to also play a role in GC B cell selection as higher numbers of Tfh cells are detected in several mouse models of SLE (76). ICOS is an important molecule needed on Tfh

cells in GC reactions and levels of ICOS is reported to be higher on T cells from SLE and RA patients (104, 105).

Most isotype-switched high affinity antibodies are formed as a result of T-cell dependent immune responses. Recent work has shown that B cells can become activated in a TLR-dependent fashion as well, which can lead to the activation of autoreactive B cells in the absence of T cell help. Indeed, SLE-like symptoms, which occurs in BAFF transgenic mice, develops independent of T cells, but does require the TLR-associated adaptor protein MyD88 (92). TLR9-deficient and TLR7-deficient lupus prone MRL. Fas lpr mice both lacked anti-DNA and RNA antibodies, respectively (106). Although the TLR studies show that self-reactive B cells can be activated in the absence of T cells, this phenomenon does not lead to autoimmunity in humans with normal immunity. Exposure of TLR ligands to B cells would either require a true pathogen (i.e. LPS) or a deregulation in apoptotic cell clearance (i.e. mfge8), which would expose DNA and RNA to self-reactive B cells.

It is important to understand the contribution of B cells in autoimmune disease so that effective therapies can be established. Rituximab is a powerful monoclonal antibody therapy that depletes peripheral transitional and mature B cells, but not plasma cells and therefore, serum antibodies do not considerably decrease. It was originally used to treat patients with lymphomas (107), but more recently, Rituximab has been evaluated for treating patients with autoimmunity. An increase in serum BAFF levels is seen in patients as a result of Rituximab treatment and therefore, could have dire effects for the elimination of autoreactive B cells as it has been shown in mouse models that BAFF can lead to the persistence of self-reactive B cells (20, 41).

Given our preliminary results that increased plasma cells are found in mice expressing self-antigen and increased BAFF, it would not be surprising to find higher plasma cell frequencies in some patients treated with Rituximab alone. It would be interesting to explore this concept further as a cause for the failure of Rituximab treatment in SLE patients in a phase III clinical trial (108). It is suggested that Rituximab should be combined with other treatments such as with Belimumab, which targets BAFF (109).

B cells are involved in autoimmune pathogenesis through autoantibody production and by aiding autoreactive T cell activation. Much of the insights gained in autoimmune diseases have relied on animal models where disease initiation and progression in a site-specific manner can be studied. The study of autoimmune pathogenesis as well as the principles governing B cell tolerance in healthy individuals should continue as pathogenesis is different with each disease and therefore, treatment should be altered accordingly. FDCs have been implicated in the retention of HIV particles and prion replication involved in Creutzfeldt-Jakob disease (110, 111). Our findings suggest that simply targeting FDCs for depletion could have detrimental effects in eliminating self-reactive B cells. Through the generation and study of self-antigen retaining FDC mouse model, I find that tolerance mechanisms in the form of elimination can be conferred on late-stage transitional B cells, adding another dimension to the importance of FDCs in the follicle.

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