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Abstract

The biochemical pathways critical to B cell development remain poorly defined. Here I characterize a critical role for two separate families of proteins, Akt and Presenilin in the development and activation of B cells. The absence of Akt1 and Akt2 leads to a block in marginal zone (MZ) and B1B cell development, as well as decreased cellularity of splenic follicular B cells. In addition, I find the combined loss of Akt1 and Akt2 causes altered B cell receptor repertoire and poor competitive ability when matched against wild-type B cells. Similar to deficiencies in the Akt pathway the combined loss of Presenilin1 and Presenilin2 results in defective MZ, B1B cell development, and altered BCR repertoire selection. Furthermore, I find that these defects are independent of the Notch pathway and that Presenilins are required for optimal responses to cross-linking of the BCR. Collectively, these findings identify and phenotypically characterize two novel pathways important to B cell development and function.

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ABSENCE OF AKT OR PRESENILIN

Marco Calamito
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IN
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Supervisor of Dissertation:

David M. Allman, Ph.D.

Graduate Group Chairperson:

Steven L. Reiner, M.D.

Dissertation Committee

Jonathan S. Maltzman, MD, Ph.D

Michael P. Cancro, Ph.D

Jan S. Erikson, Ph.D

Christopher A. Hunter, Ph.D

Dedication

This work is dedicated to my family. To my parents, Joseph and Frances Calamito, who encouraged my curiosity from an early age, providing the opportunities ultimately leading to this degree, and without whose continued support I would never have made it through this process. To my wife, Thanh Calamito, for her support and understanding during the demands of graduate school.

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ABSTRACT

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Marco Calamito

David M. Allman, Ph.D.

The biochemical pathways critical to B cell development remain poorly defined. Here I characterize a critical role for two separate families of proteins, Akt and Presenilin in the development and activation of B cells. The absence of Akt1 and Akt2 leads to a block in marginal zone (MZ) and B1B cell development, as well as decreased cellularity of splenic follicular B cells. In addition, I find the combined loss of Akt1 and Akt2 causes altered B cell receptor repertoire and poor competitive ability when matched against wild-type B cells. Similar to deficiencies in the Akt pathway the combined loss of Presenilin1 and Presenilin2 results in defective MZ, B1B cell development, and altered BCR repertoire selection. Furthermore, I find that these defects are independent of the Notch pathway and that Presenilins are required for optimal responses to cross-linking of the BCR. Collectively, these findings identify and phenotypically characterize two novel pathways important to B cell development and function.

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Chapter 1: INTRODUCTION

1.1 Overview

A diverse array of microorganisms present a constant threat to the survival of higher order animals. The short reproductive cycle of microorganisms endows these potential pathogens with the ability to rapidly mutate and evolve mechanisms to evade host clearance. Mammalian evolution's answer to the threat posed by bacteria, virus, fungi, and parasites is a complex and multi-faceted immune system. Both innate and adaptive mechanisms combine to ensure host survival and reproduction.

As part of adaptive immunity, the humoral immune response is mediated by antibody producing B cells, capable of recognizing an infinite number of foreign antigens and providing both short and long term protection to pathogenic challenge. A growing body of literature suggests that there is a division of labor within the B cell lineage such that specific subpopulations distinguished by surface marker expression, activation state and physiological function are required to combat particular subsets of pathogens at different time points during the course of infection (Martin, Oliver et al. 2001; Lopes-Carvalho, Foote et al. 2005; Baumgarth, Choi et al. 2008). These B cell populations can be divided in two distinct groups B1 and B2, with the B2 subset further divided into follicular (FOL) and marginal zone (MZ) B cells. FOL B cells make up the overwhelming majority of B-lymphocytes in the mouse, and cooperate with T-cells to form germinal centers eliciting highly specific responses to protein antigens. While

this function may be critical to the survival of the host, primary FOL B cell responses take on the order of five to seven days to become firmly established (Jacob, Kassir et al. 1991). It is the responsibility of the innate immune system to protect the host during this lag phase of the humoral response. MZ and B1 B cells generate their B cell receptors (BCR) through recombination of immunoglobulin (Ig) genes and are firmly established in the adaptive arm of the immune system, yet examination of their function and role in the immune response demonstrates an “innate-like” quality. MZ and B1 B cells generate short lived plasma cells with much faster kinetics than do FOL B cells (Martin, Oliver et al. 2001). Furthermore, the nature of the antigen that MZ and B1 B cells respond to is often a non-protein, such as conserved glycolipids or carbohydrate motifs found on the majority of pathogenic organisms (Kearney 2005). It is thought, that the ability of these innate-like B cells to recognize and secrete large quantities of antibody towards these common microorganism components “bridges the gap” between the early innate immune response and late adaptive immune response (Martin and Kearney 2002).

Although MZ and B1 B cells exhibit innate like functions and make up only a small percentage of the total B cell pool, their development requires a complex array of molecules and signaling pathways activated by the BCR. A central feature of B cell development is the requirement to purge the BCR repertoire of auto-reactive clones (negative selection). In addition to negative selection, developing B cells must generate clones with sufficient BCR signaling capacity to ensure survival i.e. positive selection/tonic signaling. Of particular importance to

this complex and poorly defined process, is the lipid kinase phosphoinositide 3-kinase (PI-3K). Indeed, mice deficient in the catalytic subunit of PI-3K, p110 δ , fail to develop MZ or B1 B cells while maintaining a FOL B cell pool albeit with decreased numbers (Clayton, Bardi et al. 2002). PI-3K signaling is a downstream target of multiple receptors including antigen, cytokine, and chemokine receptors, including B cell activating factor (BR3), and Notch receptors. (Okkenhaug, Ali et al. 2007; Cardoso, Girio et al. 2008; Woodland, Fox et al. 2008). Recent work has shown that active PI-3K is sufficient to rescue B cells development and maintenance in the absence of a surface BCR (Srinivasan, Sasaki et al. 2009). A critical downstream effector of PI-3K is the serine/threonine kinase Akt. While recent studies have elucidated a function for Akt in T cell development (Juntilla, Wofford et al. 2007; Mao, Tili et al. 2007) the role of Akt signaling in the development and activation of B cells remains undefined.

The data and analysis in Chapter 3 characterizes B cell development in the combined absence of Akt1 and Akt2, revealing defective peripheral B cell development, including dramatic decreases in MZ and B1 B cell compartments, reminiscent of the phenotype observed in PI-3K deficient models (Fruman 2004). These observations suggest that Akt is a major and required target of PI-3K signaling in B cell biology. In addition, I demonstrate that Akt1/2 deficient FOL B cells are poor competitors when mixed with wild-type cells *in-vivo* and exhibit defective proliferation to various stimuli *in-vitro*. Furthermore, the defective MZ B cell compartment observed in the absence of Akt1/2 is not rescued by enforced

expression of the pro-survival molecule B-cell lymphoma-extra large (Bcl-x_L). Finally, I examine the potential connection between Notch and Akt in MZ B cell development.

In Chapter 4, I expand upon earlier work performed in this lab demonstrating crosstalk between the BCR and Notch and ultimately uncover a Notch independent function for the γ -secretase complex and its catalytic subunits Presenilin 1 (PS1) and Presenilin 2 (PS2) in B cell development and activation. In sum, my work contributes to our understanding of the factors controlling B cell biology by further defining the molecules critical to B cell development, activation, and BCR repertoire formation.

1.2 Mature peripheral B cell subsets: phenotype and function

Naive mature B lymphocytes are classically divided based upon surface marker expression and function into one of three distinct populations; follicular (FOL), marginal zone (MZ) and B1 B cells (**Fig 1**). Together these subsets form the cellular foundation of the humoral immune response, allowing protection to a wide array of antigens.

1.2.1 Follicular B cells

The adult mouse spleen contains roughly 30 million B cells, the majority of which are small long lived (~4.5months) re-circulating FOL B cells (Lopes-Carvalho and Kearney 2004). FOL B cells reside in the follicle of the lymph node and spleen, adjacent to T cell zones enabling these two populations to interact at the

interface between the two areas during a thymus dependent (TD) response (Pillai and Cariappa 2009). Antigen receptor stimulation of FOL B cells in conjunction with T cell help leads to proliferation, plasmablast formation and the eventual formation of a germinal center where somatic hypermutation and isotype switching occur. (Benson, Erickson et al. 2007). These processes result in the generation of affinity matured long lived plasma and memory cells conferring immunity to future exposure with that antigen.

1.2.2 Marginal Zone B cells

In contrast to FOL cells MZ B cells inhabit a distinct anatomical niche located between the red and white pulp of the spleen. It is here that arterial blood empties into the marginal sinus and is filtered by resident cells including macrophages, dendritic and MZ B-cells. Thus, MZ B cells are uniquely positioned to contact blood borne pathogens (Lopes-Carvalho and Kearney 2004). Indeed, mice lacking MZ B-cells succumb to streptococcus pneumoniae within days of infection (Tanigaki, Han et al. 2002). Interestingly, MZ B-cells express higher basal levels of the activation markers CD80, CD86, and MHC class II. In addition, MZ B-cells are more effective antigen presenting cells than FOL B-cells, inducing robust T-cell responses (Attanavanich and Kearney 2004). MZ-B cells were long thought to be sessile, and while it remains true that these cells do not exit the spleen, recent evidence shows that MZ B-cells are highly motile, migrating every few hours from the marginal sinus area to the B-cell follicle (Cinamon, Matloubian et al. 2004; Cinamon, Zachariah et al. 2008).

Despite representing only five percent of the splenic B cell pool, MZ cells along with B1 cells dominate early humoral immune responses to T-independent antigens (Martin, Oliver et al. 2001). In addition, MZ B-cells are hyper-responsive to toll-like receptor (TLR) stimuli, and upon activation quickly differentiate into IgM secreting plasma cells (Oliver, Martin et al. 1997). (Functional differences summarized in **Table 1**). These observations have led to a model in which MZ and B1 B cells function to bridge the gap from the early humoral response to the late affinity matured FOL B-cell dominated response. Finally, FOL and MZ B-cells differentially express surface IgM, the complement receptor CD21/35, and low affinity IgE receptor CD23, enabling identification through flow cytometric based strategies summarized in **Table 2**.

1.2.3 B1 B cells

B1 B cells are highly enriched in peritoneal and pleural cavities, contribute to T-independent responses, and can be subdivided based on expression of the surface marker CD5 into CD5⁺ B1a and CD5⁻ B1b B cells. B1a B cells spontaneously secrete IgM providing a first line defense against encapsulated bacteria, and are thought to provide a more innate like function (Haas, Poe et al. 2005). B1b cells can be rapidly induced to secrete antibody to T-independent antigens helping to clear pathogen and aid in long term immunity (Alugupalli, Leong et al. 2004). As mentioned above, B1 and MZ B cells tend to express BCRs that recognize conserved components found on microorganisms. Interestingly, many B1 B cells lack N-additions, and thus possess germ-line

encoded antigen receptors that afford protection against a wide range of pathogens (Feeney 1990; Gu, Forster et al. 1990). For example B1 B cells are enriched for a V_HV_L pair with specificity for phosphocholine (T15 idiotype), a common component of bacterial cell walls (Gu, Forster et al. 1990; Feeney 1991). Adoptive transfer of T15 antibody affords passive immunity to streptococcus pneumoniae (Briles, Nahm et al. 1981). These observations imply that germ-line encoded BCRs have an innate-like ability to recognize microbial products and as such could be selected for during evolution.

In contrast to the accepted role for B1 cells in the early humoral response, the development of these cells is anything but clear. Two models have been advanced to explain the ontogeny of the B1 compartment, the lineage model and the selection model (Dorshkind and Montecino-Rodriguez 2007). The lineage model is based upon the observation that fetal liver progenitors are best able to reconstitute the B1 compartment when compared to adult marrow progenitors, and that B1 cells possess the capacity for self-renew (Kantor, Stall et al. 1992; Kantor, Stall et al. 1995; Montecino-Rodriguez, Leathers et al. 2006). The enhanced ability of fetal progenitors to form B1 B cells is perhaps the result of ligand/cytokine variations in fetal vs. adult B cell development that influence expression of genes important to the B1 fate such as terminal deoxynucleotidyl transferase (TdT).

In contrast, the selection model proposes that both the B1 and B2 lineage share a common precursor cell, and signals derived from the BCR will direct cell fate (Pennell, Mercolino et al. 1989; Arnold, Pennell et al. 1994; Lam and

Rajewsky 1999). This notion is supported by data demonstrating that B2 cells can be induced to up-regulate surface expression of the B1 marker CD5 upon BCR cross-linking (Wortis, Teutsch et al. 1995).

Table 1: Characteristics of naïve mature B cell populations

Functional Characteristics	FOL	MZ	B1
Localization	Spleen Lymph Node Bone Marrow	Spleen	Peritoneal Cavity Spleen
T Independent Response	Weak	Strong	Strong
T Dependent Response	Strong	Weak	
BCR Response	Proliferation	Death	Death
TLR-Ligand Response	Mild Proliferation	Strong Proliferation	Strong Proliferation

Table 2: Surface marker expression of murine B cell subsets

Tissue	Cell Surface Marker				
Bone Marrow	CD19	B220	CD43	AA4	IgM
Pro-B	+	+	+	+	-
Pre-B	+	+	+/-	+	-
Immature B	+	+	-	+	+
Mature B	+	+	-	-	+

Spleen	CD19	CD23	CD21/35	AA4	IgM
Transitional 1	+	-	+/-	+	+++
Transitional 2	+	+	+	+	+++
Transitional 3	+	+	++	+	++
MZP	+	+	+++	+/-	+++
FOL	+	+	++	-	++
MZ	+	-	+++	-	+++

Peritoneal Cavity	CD19	B220	CD43	CD5	IgM
B1a	+	+/-	+	+	+++
B1b	+	+/-	+	-	+++

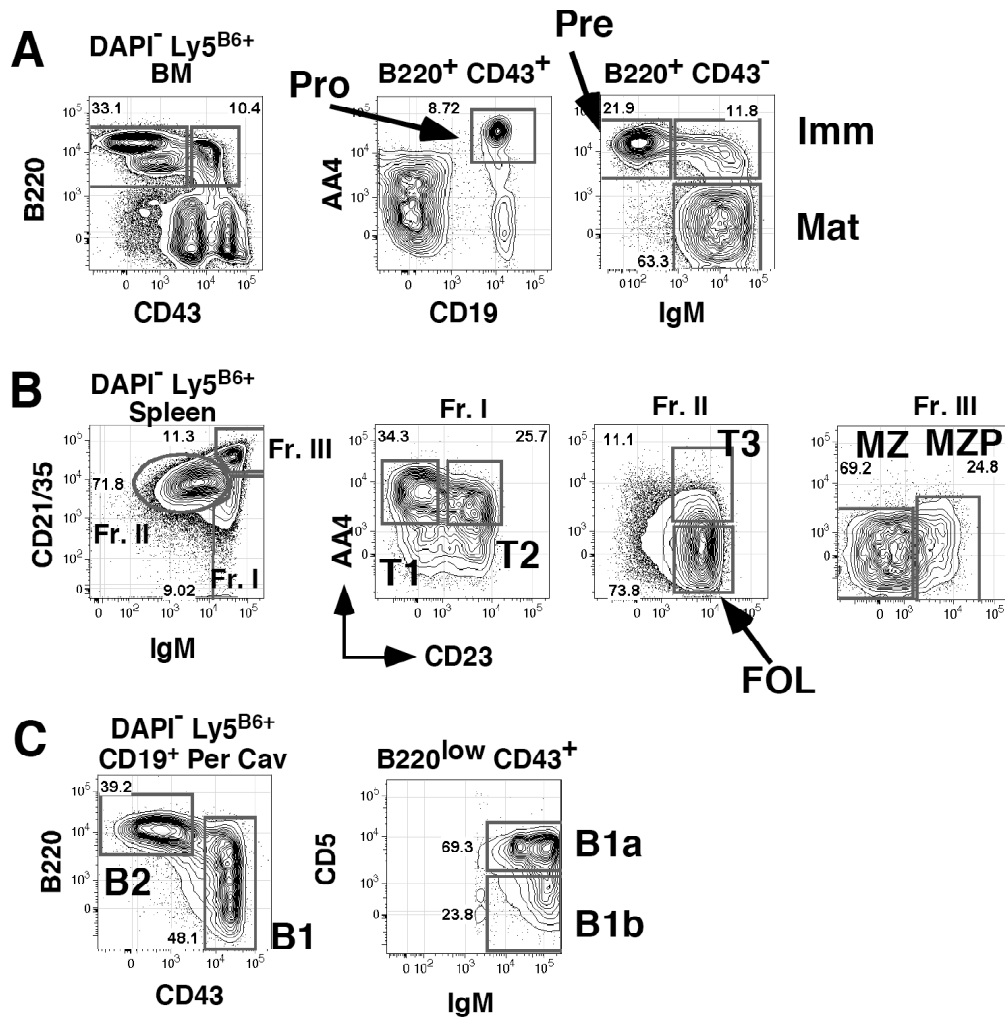


Figure 1: Gating scheme for flow cytometric analysis of murine B cell subsets. (A) Flow cytometric analysis of BM cells from c57/B6 mice aged 12 weeks. The left-most plots are gated on viable donor-derived (DAPI⁻ Ly5^{B6+}) cells. Numbers in plots indicate the frequency of events in the indicated gate as a function of the indicated parent population. Pro-B, CD43⁺ B220⁺ CD19⁺ AA4⁺; pre-B, CD43^{low} B220⁺ IgM⁻ AA4⁺; immature B, CD43⁻ B220⁺ IgM⁺ AA4⁺; mature B, CD43⁻ B220⁺ IgM⁺ AA4⁻. **(B)** Fr.I, IgM⁺⁺⁺ CD21/35⁺, Fr.II IgM⁺⁺⁺ CD21/35⁺⁺⁺, Fr.III IgM⁺⁺⁺ CD21/35⁺⁺⁺, (T1 CD23⁻ AA4⁺) (T2 CD23⁺ AA4⁺) (T3 CD23⁺ AA4⁺) (FOL CD23⁺ AA4⁻) (MZP CD23⁺ AA4^{+/+}) (MZ CD23⁻ AA4⁻). **(C)** B2 CD43⁻ B220⁺; B1 CD43⁺ B220^{Int-Low}; within in B1 gate B1a (CD5⁺) B1b(CD5⁻).

1.3 B cell development

1.3.1 B cell development in the bone marrow

Peripheral B cell compartments derive from B-lineage committed precursors resident in the liver during fetal life, and then in the bone marrow of adult mice. Precursors expressing high levels of the early B cell factor (EBF) restrict lineage potential and in conjunction with Pax-5 commit progenitors to the B cell fate (Cobaleda, Schebesta et al. 2007; Pongubala, Northrup et al. 2008).

Early B cell populations in the bone marrow sequentially transit a series of phenotypically distinct stages consisting of pro, pre, and immature B cell pools. Each of these populations can be defined by differential expression of surface markers consisting of CD19, B220, CD43, AA4 and IgM (Hardy, Li et al. 2000), [see **Table 2** and **Fig 1** for surface marker classification scheme]. These early precursors initiate expression of recombinase-activating genes (RAG), undergo rearrangement of germline encoded diversity (D) and joining (J) Ig heavy chain genes. Active rearrangement of variable (V) heavy chain genes marks pro-B cells (Nemazee 2006). In frame recombination of V_H with existing D_HJ_H gene segments will lead to expression of an intact Ig heavy chain, which pairs with a surrogate light chain (SLC) to form a pre-BCR. The pre-BCR signals through Ig- α and Ig- β resulting in a rapid decrease in RAG protein levels and cessation of heavy chain rearrangement (Jung, Giallourakis et al. 2006). Cells at this stage also grow in size and undergo an intensive proliferative burst. B cells deficient in the ability to successfully generate or transmit signals through the pre-BCR, as is the case in RAG, Ig α , or Ig β deficient mice are blocked at this early stage of B

cell development (Herzog, Reth et al. 2009). These observations underscore the fundamental importance the Ig heavy chain plays in the survival of the earliest B cells.

As cells exit cell cycle and conclude the massive wave of proliferation sparked by pre-BCR signaling, CD43 expression is decreased and the RAG proteins are re-expressed. At this stage Ig light chains commence rearrangement. Two different clusters of Ig genes are capable of generating light chains, the kappa (κ) and lambda (λ) loci. Light chain rearrangement proceeds in an ordered fashion with the κ -locus preceding the λ -locus. Productive rearrangement and pairing of heavy and light chain leads to surface expression of a mature BCR in the form of IgM. Surface IgM expression defines entry into the immature stage of bone marrow B cell development. It is at this stage that the BCR specificity of each respective B cell clone is first screened for auto-reactivity. Clones with BCRs recognizing multivalent self-antigens undergo clonal deletion or receptor editing (Nemazee and Burki 1989; Hartley, Crosbie et al. 1991). Recent work has demonstrated that, for membrane bound self antigens, receptor editing is the dominant mechanism by which auto-reactive specificities are purged from the immunoglobulin repertoire (Halverson, Torres et al. 2004). Clones possessing weak reactivity to self or recognizing soluble antigens are capable of reaching peripheral B cell compartments, but these cells are nonfunctional (anergic) with high turnover rates if matched against wild-type competitors (Cyster, Hartley et al. 1994). Consequently, though the adult murine bone marrow produces roughly 15 million immature B cells per day, only 10% of

these cells leave the bone marrow (Allman, Ferguson et al. 1993; Rolink, Andersson et al. 1998). The short half-life of immature B cells and extended life span of mature B cell compartments implies tremendous selective pressure is placed on immature B cells, and show active negative selection in B cell development. Those rare clones that successfully transit negative selection in the bone marrow are able to home via the blood to peripheral lymphoid organs such as the spleen.

1.3.2 Peripheral B cell development in the spleen

Immature B cells in the spleen are termed transitional cells; phenotypically these cells are subdivided into three distinct subsets based on termed transitional 1 (T1), transitional 2 (T2), and transitional 3 (T3). These cell types are differentiated based on differential surface expression of AA4, CD23, IgM and CD21/35 (see **table 2, Fig1/2**). The majority of transitional B cell in the spleen of an adult mouse will give rise to mature FOL B cells, and a minor fraction of transitional cells will transit through a marginal zone precursor (MZP) stage of development en route to a mature MZ B cell (**Fig 2**). Functionally, these cells are defined by their rapid turnover and apoptotic response to BCR cross-linking. Transitional B cells respond to TLR agonists and CD40 receptor cross-linking, demonstrating these cells are capable of proliferating to certain stimuli (Allman, Ferguson et al. 1992). The BCR mediated cell death that transitional B cells experience is in part due to defective generation of nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) targets A1 and Bcl-x_L (Castro, Wright

et al. 2009). The rapid cell turnover and apoptotic BCR response suggest that transitional B cell populations are also actively undergoing negative selection, purging auto-reactive specificities from the developing B cell pool and ensuring a self tolerant mature B cell compartment. However, there is a growing body of evidence demonstrating the importance of BCR specificity in positive selection to the B1, MZ or FOL B cell compartments.

The notion that positive selection exists for B cells, is supported by a landmark study demonstrating that the BCR is required for the survival and persistence of mature B cell populations (Lam, Kuhn et al. 1997). This work was later extended to show a requirement for BCR signaling in of itself for proper B cell maintenance (Kraus, Alimzhanov et al. 2004). In addition, light chain V gene segments while diverse in the pre-B and transitional pools, are skewed towards certain V genes in the mature compartment (Gu, Tarlinton et al. 1991; Levine, Haberman et al. 2000), implying both positive and negative selective pressure on certain V_H V_L pairs. Furthermore, evidence for B cell positive selection was substantiated by the observation that B cells specific for the Thy-1 glycoprotein, a self antigen, can be selected into the B1 compartment in normal mice and into the MZ B cell pool in mice expressing low levels of Thy-1 (Hayakawa, Asano et al. 1999; Wen, Brill-Dashoff et al. 2005). These studies imply that BCR specificity, and perhaps signal strength play a role in the B1, MZ or FOL cell fate decision of those cells gaining access to long lived B cell pools.

Casola et al demonstrated that stronger BCR signals lead to the generation of B1, while weaker BCR signaling was conducive to formation of MZ

and FOL B cell populations (Casola, Otipoby et al. 2004). In addition, while the FOL B cell compartment is intact, albeit compromised, in mice deficient in molecules critical to BCR signaling such as Bruton's tyrosine kinase (Btk) or CD19, these cells fail to compete with wild-type B cells in mixed marrow chimeras (Sprent and Bruce 1984; Otero, Anzelon et al. 2003). These observations suggest that the "fitness" of a BCR is sensed through transient interaction with self ligands and/or tonic BCR signaling, and that this in turn governs the make up of the B cell pool.

A key receptor that works in concert with the BCR to maintain the peripheral B cell repertoire is the B cell-activating factor receptor (BR3), expressed on FOL, MZ, and late transitional cells and its ligand B lymphocyte stimulator (Blys) (Moore, Belvedere et al. 1999; Harless, Lentz et al. 2001). Mice deficient in BR3 or in Blys have decreased MZ and FOL B cell numbers that can be partially rescued by enforced expression of the pro-survival molecule Bcl-x_L (Lentz, Cancro et al. 1996; Lentz, Hayes et al. 1998; Batten, Groom et al. 2000; Harless, Lentz et al. 2001; Amanna, Dingwall et al. 2003). Interestingly, BR3 signaling is not required for the generation or survival of B1 B cells (Lentz, Hayes et al. 1998). Recent studies by Stadanlick et al. have established a molecular mechanism of BCR-BR3 cross-talk (Stadanlick, Kaileh et al. 2008). This work demonstrates that tonic signaling through the BCR generates the classical NF κ B target p100, p100 then serves as a substrate for processing by BR3 leading to increased expression of pro-survival molecules Bcl-x_L and Pim2; thus regulating peripheral B cell numbers. These observations support the notion that BCRs are

not only screened for auto reactivity, but also for “fitness” as assayed by the ability to generate p100.

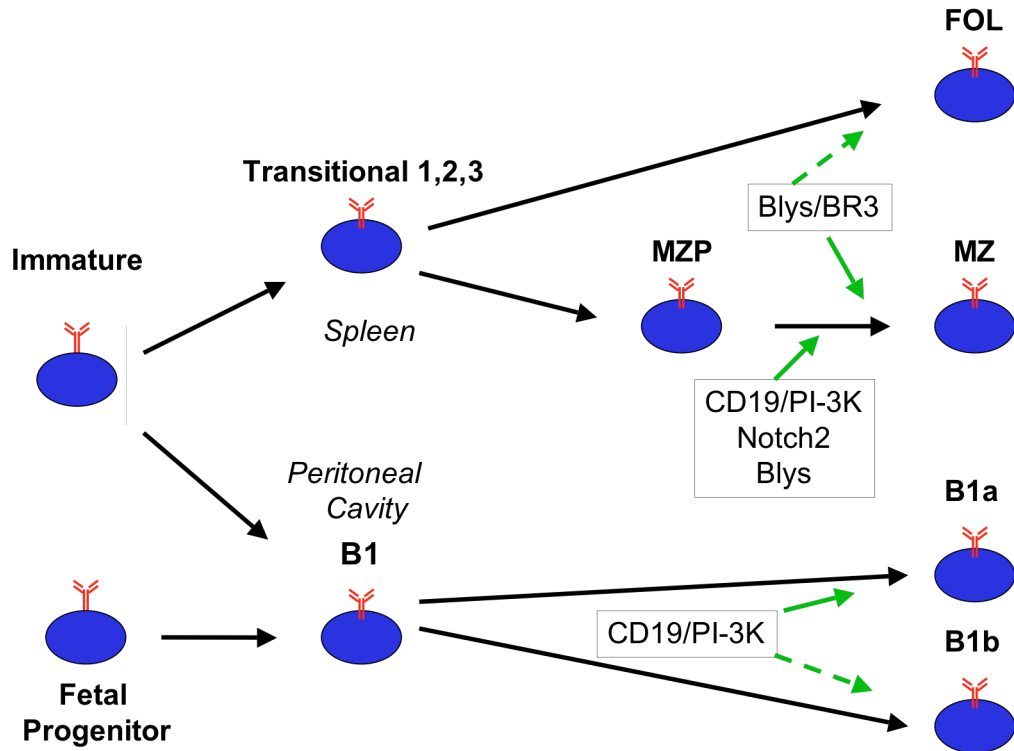


Figure 2: Peripheral B cell subsets and development. Immature B cells from the bone marrow enter the spleen (top) via the blood and are termed Transitional (T1 and T2) T2 B cells mature into either the follicular (FOL) or marginal zone (MZ) fates. This decision is dictated in part by BCR signal strength. MZ development requires CD19/PI-3K, Notch2, and Blys whereas normal FOL development requires Blys. B1 B cells home to the peritoneal cavity (bottom) and are most efficiently generated during fetal life. Some immature B-cells harbor BCRs of sufficient BCR signal strength to drive the B1 B cell fate. B1s can be further subdivided into the B1a or B1b lineage.

1.4 BCR signaling pathways and the importance of PI-3K.

Though a wealth of knowledge regarding the signaling pathways activated downstream of the BCR exists, questions remain regarding the how these effectors are utilized in the context of B cell development and peripheral B cell fate decisions. The BCR signaling complex is composed of membrane bound IgM associated with the signal transducing Ig α -Ig β heterodimer (Tolar, Sohn et al. 2008). BCR aggregation leads to the recruitment of numerous kinases, adaptors, and lipid modifying enzymes that serve to propagate activation signals to downstream secondary effectors. A key molecule in this signaling cascade is the lipid modifying enzyme phosphoinositide 3-kinase (PI-3K). PI-3K phosphorylates the inositol group of phosphatidylinositol (4,5)-bisphosphate (PIP₂) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). BCR oligomerization leads to rapid PI-3K recruitment via the B cell cytoplasmic adaptor protein (BCAP). PI-3K proceeds to generate PIP₃ which can serve as a docking site at the plasma membrane for pleckstrin homology (PH) containing proteins (Fruman 2004). Molecules such as Btk and phospholipase C gamma 2 (PLC γ 2) are recruited through their PH domains to the plasma membrane where they are phosphorylated by spleen tyrosine kinase (Syk) or other receptor associated tyrosine kinases. Activated Btk phosphorylates PLC γ 2 leading to increased generation of the secondary messengers inositol triphosphate (IP₃) and diacylglycerol (DAG) (Watanabe, Hashimoto et al. 2001). These molecules then activate calcium mobilization and protein kinase C (PKC) respectively,

leading to the activation of NF κ B and nuclear factor of activated T cells (NFAT) transcription factor families (**Fig 3**) (Scharenberg, Humphries et al. 2007).

There exist three different classes of PI-3K molecules. In cells of the immune system class I family members are the best characterized. Class I PI-3K molecules consist of a catalytic subunit that carries out generation of PIP₃ from PIP₂, referred to in general terms as p110. Of the three p110 isoforms (p110 α , p110 β , p110 δ) p110 δ is highly expressed and is the best characterized catalytic subunit in lymphocytes. Catalytic subunits pair with an adaptor termed p85, capable of binding to BCAP and localizing PI-3K at the plasma membrane (Okkenhaug and Vanhaesebroeck 2003). PI-3K activity is opposed by phosphatase and tensin homolog deleted on chromosome 10 (PTEN), an enzyme that converts PIP₃ into PIP₂ (Suzuki, Kaisho et al. 2003).

Deletion of the p110 δ subunit from the B-lineage results in a block in the generation of MZ and B1 B cells, FOL B cell numbers in the spleen are decreased, in addition, *in-vitro* proliferative responses to BCR cross-linking are diminished (Clayton, Bardi et al. 2002; Okkenhaug, Bilancio et al. 2002). Loss of p85 results in decreases in cellularity for transitional and mature populations, but an increase in pro-B cells suggesting a bottleneck in development (Fruman, Snapper et al. 1999; Suzuki, Terauchi et al. 1999). Interestingly, while Btk is a target of PI-3K signaling, the phenotype of p85 α -Btk double deficient B cells is more severe than deletion of either p85 α or Btk alone, suggesting Btk independent phenotypes associated with the loss of PI-3K activity. (Donahue, Hess et al. 2004).

Co-ligation of the BCR and its co-receptor CD19 enhances recruitment of PI-3K to the plasma membrane and decreases the threshold for BCR activation by 100 fold (Carter and Fearon 1992). Deletion of CD19 from mice results in an overlapping phenotype with PI-3K deficient B cells, mainly loss of MZ and B1 B cells, and an inability to compete with wild-type progenitors in mixed marrow chimeras (Rickert, Rajewsky et al. 1995; Otero, Anzelon et al. 2003). Interestingly, mice deficient in PTEN exhibit increased B1 and MZ B cell numbers, and crossing PTEN deficient mice onto a CD19 deficient background partially rescues MZ development (Anzelon, Wu et al. 2003). Furthermore, immature B cells deficient in PTEN do not undergo apoptosis in response to BCR cross-linking, and in contrast to wild-type cells proliferate (Browne, Del Nagro et al. 2009). Recent work by Srivinson et al. demonstrated that expression of an activated form of PI-3K was sufficient to rescue survival of mature B cells in which the BCR was conditionally deleted (Srinivasan, Sasaki et al. 2009). These observations highlight the importance of the PI-3K signaling pathway and its downstream effectors in BCR signaling and B cell biology. One major target of PI-3K activation is the Akt pathway, to date the direct role of Akt in B cell development and activation remains undefined.

1.5 The PI-3K target Akt

Oligimerization of the BCR *in-vitro* leads to transient activation of the Akt pathway. Coordinate co-ligation of the BCR and CD19 results in enhanced and prolonged activation of the Akt pathway (Otero, Omori et al. 2001). Akt, also

known as protein kinase B (PKB) is expressed in three distinctly coded isoforms, Akt1, Akt2, Akt3. All three Akt isoforms display similar structure and are recruited/activated via their PH domain by PI-3K activity (Plas and Thompson 2005; Franke 2008). Akt1 is ubiquitously expressed, Akt2 expression is largely restricted to the pancreas and liver, and Akt3 expression is limited to the neural tissue (Juntilla and Koretzky 2008). Deletion of Akt1 causes a dwarf phenotype, in which mice on average are 20% smaller than wild-type aged matched controls (Cho, Thorvaldsen et al. 2001). Deficiency in Akt2 leads to Insulin resistance and a diabetes mellitus-like syndrome (Cho, Mu et al. 2001). Mice deficient in Akt3 are grossly normal, though brain size is reduced 20% (Easton, Cho et al. 2005). The combined loss of Akt1 and Akt2 results in neonatal lethality (Peng, Xu et al. 2003). Akt signaling regulates a diverse array of cellular outcomes including metabolism, proliferation, and survival (**Figure 3**) (Manning and Cantley 2007). In addition to BCR mediated activation, Akt is downstream of BR3 leading to enhanced metabolic fitness. (Patke, Mecklenbrauker et al. 2006; Otipoby, Sasaki et al. 2008). The relative importance of Akt in the metabolic fitness and survival of B cells downstream of signaling mediated by BR3, remains poorly defined.

Two recent papers characterized thymocyte development in the absence of Akt signaling. Collectively this work demonstrated a profound defect at the β -selection checkpoint, a stage in which the T cell receptor (TCR) beta chain undergoes VDJ recombination to generate a pre-TCR and is required for subsequent proliferation and differentiation of early thymocytes. Progenitors

deficient in Akt1 and Akt2 showed an increased propensity for apoptosis after TCR activation, leading to a paucity of mature peripheral T cells (Juntilla, Wofford et al. 2007; Mao, Tili et al. 2007).

One potential explanation for the decreased survival of Akt1/2 deficient T cells is an inability to properly regulate the pro-survival molecules Bcl-x_L and A1, common targets regulated by Akt (Veis, Sentman et al. 1993; Mandal, Borowski et al. 2005). Another possibility is that the decreased glucose uptake and cell size observed in Akt1/2 deficient thymocytes results in sub-optimal metabolism. Given that cells at the β -selection checkpoint are about to undergo intense proliferation that may require optimal energy levels for cell cycle progression and maturation of the cell. The role of Akt1/2 in an analogous stage of early B cell development, the pre-BCR mediated proliferative burst, is unknown.

Another receptor critical to T-cell development and capable of activating Akt is Notch. Ciofani et al showed enhanced Akt activation and glucose metabolism in thymocytes cultured with Notch ligands and a constitutively active form of Akt could overcome the requirement for Notch during T cell development (Ciofani and Zuniga-Pflucker 2005). These data suggest that pre-TCR and Notch signaling converge on the PI-3K/Akt pathway. In peripheral B cell development, signaling from the Notch2 receptor is required for the generation of MZ B cells (Saito, Chiba et al. 2003). Furthermore, studies have recently demonstrated that Notch signaling can synergize with the BCR to enhance Akt activation (Thomas, Calamito et al. 2007). A potential interplay between the BCR, Notch and Akt remains unexplored in MZ B cell development.

1.6 The gamma-secretase substrate Notch

MZ B-cell development possesses a unique requirement for Notch signals (Saito, Chiba et al. 2003). Notch is expressed in four isoforms (Notch 1-4) with Notch2 preferentially expressed in mature B cells. Notch signaling is initiated by binding to one of its 5 ligands, Jagged 1/2 or Delta-like 1/3/4, leading to shedding of the extracellular domain via the activity of ADAM (A Disintegrin and Metalloproteinase) metalloproteases. Shedding enables the remaining transmembrane stalk to be opened up to intramembrane cleavage by the gamma secretase (γ -secretase) complex. The cytoplasmic cleavage product, intracellular Notch (ICN), translocates to the nucleus and binds the transcription factor CSL (for human, CBF1; *Drosophila*, Suppressor of Hairless; *C. elegans*, Lag-1) and the co-activator mastermind-like protein (MAML). This complex initiates transcription of Notch target genes, including members of the hairy and enhancer of split (HES) family (Maillard, Fang et al. 2005). Conditional deletion of Notch2, CSL, Notch ligand delta-like1 (DL1), or expression of a dominant negative MAML (DNMAML), which blocks canonical Notch signaling through all four Notch receptors, causes the specific loss of MZ B-cells (Tanigaki, Han et al. 2002; Saito, Chiba et al. 2003; Hozumi, Negishi et al. 2004; Maillard, Weng et al. 2004). Notch signaling may down modulate PTEN expression, and thus enhance PI-3K activity, via HES1, in T-lymphomas (Palomero, Sulis et al. 2007; Calzavara, Chiaramonte et al. 2008). Furthermore, in-vitro studies from our lab have shown that FOL-B cells stimulated through the BCR and co-cultured with OP9 stromal cells expressing-DL1 (OP9-DL1) have increased levels of activated Akt

compared to FO-B cells cultures with control OP9-GFP cells (Thomas, Calamito et al. 2007). These observations coupled with the requirement for PI-3K activity in MZ B cell generation raise the prospect of an important role for Akt downstream of Notch in B cell biology. Furthermore, splenic B cells express mRNA for DL1 (Hozumi, Negishi et al. 2004). The role B cell intrinsic Notch ligands play in B cell activation remains undefined.

1.7 The γ -secretase complex and Presenilin

Within the immune system γ -secretase is mainly associated with Notch signaling, as Notch activation requires the γ -secretase-dependent cleavage of Notch receptors within the plasma membrane (Maillard, He et al. 2003). The γ -secretase complex is a membrane-embedded aspartyl protease composed of four different proteins: Presenilin (PS), Nicastrin, and Anterior Pharynx-Defective-1 (Aph-1), and Presenilin Enhancer 2 (PEN2) (Parks and Curtis 2007). The catalytic activity of γ -secretase is contained in the PS proteins, consisting of two separately encoded isoforms Presenilin 1 (PS1) and Presenilin 2 (PS2). PS proteins undergo endoproteolysis and the amino and carboxy terminal fragments function together as a heterodimer in the mature γ -secretase complex. Nicastrin is responsible for the recognition and binding of substrates and along with PEN2 and APH1 play a role in the stability of the complex (Li, Wolfe et al. 2009). The complex is thought to contain a proteolytic pore that is protected from the lipid bilayer and provides the hydrophobic environment required for catalytic activity. Interestingly, the complex drives cleavage of a large number of

single-pass transmembrane proteins, over 80 to date, with rather promiscuous cleavage site specificities and is expressed at the plasma membrane in lipid rafts, endoplasmic reticulum (ER) and the golgi. (Parks and Curtis 2007; Li, Wolfe et al. 2009). Furthermore, a large body of work has demonstrated that Presenilin proteins have both catalytic dependent and independent functions affecting cell adhesion, protein localization/trafficking, apoptosis, development and calcium regulation (Selkoe and Wolfe 2007). Mutations in PS1 and PS2 have been linked to familial Alzheimer's disease (FAD) (Hass, Sato et al. 2009). Recent work has demonstrated that deletion of both PS1 and PS2 in developing thymocytes compromised TCR-mediated positive selection (Laky and Fowlkes 2007), suggesting a role for Presenilin and by extension γ -secretase activity in antigen receptor activation and lymphocyte selection during development. A recent paper by Yagi et al further demonstrated the importance of Presenilin proteins in B cell development, as PS1 PS2 double deficient cells failed to generate the MZ B lineage, presumably due to failed Notch2 activation (Yagi, Giallourakis et al. 2008). These authors also showed that Syk activation downstream of the BCR was compromised in the absence of PS1 and PS2. Given that the catalytic subunit of the γ -secretase complex, Presenilin, can be isolated from various compartments of the cell, and that the complex can cleave numerous substrates, the potential exists for both Notch dependent and Notch independent functions for Presenilin and by extension γ -secretase in B cell development and activation that to date are ill defined.

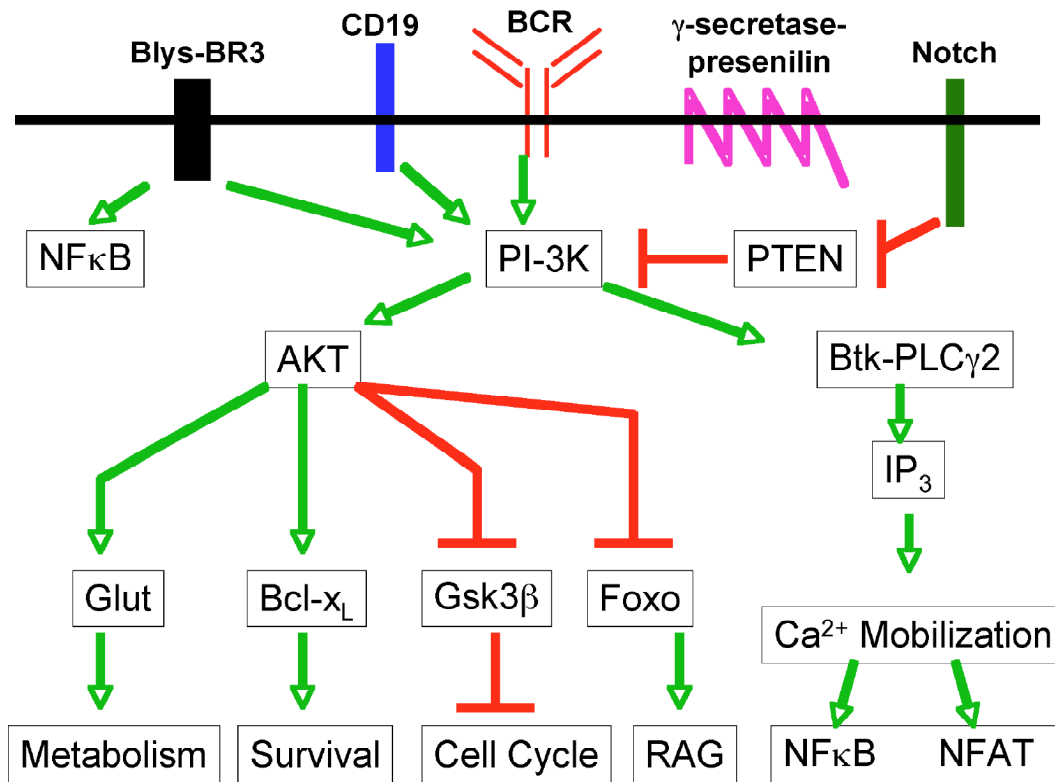


Figure 3: PI-3K mediated signaling cascades. PI-3K is recruited to the plasma membrane by the activity of the BCR, CD19, Notch and BR3. PI-3K mediated generation of PIP₃ is modulated by the activity of PTEN. PIP₃ concentrations influence the activation of Akt dependent and Akt-independent pathways. Akt dependent effects include increased metabolism, enhanced survival, positive regulation of cell cycle progression, and down-modulation of Foxo family transcription factors allowing decreased expression of RAG. Akt independent pathways include activation of Btk and PLC γ 2 enabling generation of IP₃ leading to mobilization of endoplasmic reticulum Ca²⁺ stores and transcription factor activation.

1.8 Summary Chapter 1

The biochemical pathways critical to B cell development remain poorly defined. Central to B lineage generation and cell fate decisions is the BCR. The fact that active PI-3K signaling can substitute for the BCR in the survival of mature B cells demonstrates its essential role in B cell biology. PI-3K's ability to activate numerous downstream effectors, including Akt, raises questions about the Akt dependent functions of PI-3K. I aimed to explore the role of Akt1 and Akt2 in B cell development and activation. In Chapter 3 I describe an essential and non-redundant function for signals derived from Akt1 and Akt2 in the development of MZ and B1 B cells. I also show that Akt signaling is critical to BCR repertoire formation, as measured by λ -light chain frequencies, and the competitive fitness of peripheral B cell populations. Furthermore, Akt is required for normal proliferation to BCR cross-linking. I demonstrate, through use of a Bcl-x_L expressing retrovirus, that poor survival is not the sole reason for the block in MZ B cell development (Calamito, Juntilla et al. 2009). Finally, constitutively active Akt signaling, while sufficient to drive developing B cells to the MZ fate, is insufficient to rescue a Notch loss of function approach.

Data published from our lab demonstrated a synergy between γ -secretase substrate Notch and the BCR, enabling increased Akt signaling and proliferation (Thomas, Calamito et al. 2007). In Chapter 4 I further explore this connection as it relates to BCR activation and find that while Notch enhances BCR mediated proliferation it is not required. Strikingly, in experiments initially performed with γ -secretase inhibitors (GSI) and then with a genetic loss of function approach I

uncover an unanticipated requirement for the γ -secretase complex (GS) in optimal BCR activation independent of the Notch pathway. In addition, I show that conditional deletion of the γ -secretase complex subunit Presenilin from the B cell lineage results in a profound block in the development of B1 and MZ B cells. Finally, I observe altered BCR repertoire formation and abnormal calcium mobilization downstream of the BCR in B cells deficient in Presenilin proteins (Calamito et al. submitted 2010).

Years of intense study by numerous labs have uncovered a great deal of information about the factors critical to B cell development and thus the humoral immune response. My work, contributes to our understanding of these requirements, defining for the first time the importance of Akt in B cell development and uncovering an unexpected role for the Presenilin family of proteins in B cell biology independent of the Notch pathway.

CHAPTER 2: Materials and Methods

2.1 Animals

C57BL/6 and CD19-Cre mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6.Ly5^{SJL} females were obtained from the National Cancer Institute (Frederick, MD). Mice deficient in Akt1 or Akt2 were provided by Dr. Morris Birnbaum (University of Pennsylvania), Rosa26-DNMAML and floxed Presenilin 1 Presenilin 2 null mice were provided by Dr. Warren Pear (University of Pennsylvania) and Dr. BJ Fowlkes (NIH), respectively. Myristoylated-Akt mice provided by Dr. Hooman Noorchashm (University of Pennsylvania). All animal procedures were approved by University of Pennsylvania Institutional Animal Care and Use Committee.

2.2 Chimera Construction

Fetal liver cells from B6.Ly5^{SJL}, or B6-backcrossed (Ly5^{B6+}) embryos lacking one or two alleles of Akt1 and/or Akt2 were harvested at day 14.5-16.5 postcoitum, and cultured overnight in α MEM with 20% FBS, 2.2g/liter sodium bicarbonate, 2mM glutamine, penstrep, 10ng/mL IL-6, 20ng/mL IL-3 and 100 μ g/mL stem cell factor. Host B6.Ly5^{SJL} female mice were irradiated with two doses of 550 rads at least four hours apart. Six hours after the second irradiation 2.5×10^5 cells were injected intravenously. For double chimeras B6.Ly5^{SJL} progenitors were mixed 50:50 with Akt deficient cells before injection into irradiated B6.Ly5^{SJL} females. Hosts were maintained on water containing a Bactrim suspension (400 mg

sulfamethoxazole and 80 mg trimethoprim per 500 ml water) for two days prior to, and at least two weeks following irradiation. Water was changed and a new dose of Bactrim added every two to three days.

2.3 Flow Cytometry

For flow cytometric analyses RBC-depleted cell suspensions from BM, spleen or peritoneal cavity were prepared and stained with optimal dilutions of directly conjugated antibodies in FACS buffer (1xPBS, 5% BSA, 1mM EDTA) before analysis a 14-color LSR II flow cytometer (BectonDickinson, San Jose, CA) equipped with four lasers for excitation of UV, violet, blue, and red-excited dyes. $1-2 \times 10^6$ cells were stained with optimal dilutions of antibodies in a 96-well round bottom plates in a final volume of 50 μ L. Antibodies used included fluorescein (FL), phycoerythrin (PE), PE-Cy5.5, PE-Cy7, allophycocyanin (APC), APC-Cy5.5, APC-Alexa750 or biotin (BI)-conjugated versions of monoclonal antibodies to the following cell surface antigens: Gr-1 (8C5), F4/80 (BM8) Ter-119, CD3 (2C11), CD127/IL-7Ra (A7R34), CD135/Flt3 (A2F10), CD117/c-kit (2B8), Sca-1/Ly6 A/E (E13-161.7) B220 (RA3-6B2), CD43 (S7), CD23 (B3B4), IgM (331), κ (187.1), λ 1-3 (R26-46), λ 1-2 (JC5-1), CD21/35 (7G6), Ly5^{B6} (104), Ly5^{SJL} (A20), CD11b (M1/70), C1qR/AA4 (AA4.1), CD5 (53-7.3) and CD19 (6D5). Polyclonal FL-conjugated Fab goat-anti-IgM antibodies (Jackson ImmunoResearch) were also employed. BI-conjugated antibodies were revealed with streptavidin (SA) coupled to PE-TexasRed (Caltag, Burlingame, CA). All directly conjugated antibodies were purchased from eBiosciences (San Diego,

CA) except for APC-Cy5.5 CD19 (Caltag), PE κ (BD Bioscience) Fitc λ 1-3 (R26-46) (BD Bioscience), PE λ 1-2 (JC5-1) (Southern Biotech), and PE-Cy5.5 CD21/35 which was purified and conjugated by standard methods in our laboratory. Non-viable cells were excluded from all analyses by staining with the UV-excited DNA dye DAPI. All flow cytometric data were analyzed by uploading files into FlowJo 8.8 (Tree Star, Inc., San Carlos, CA). Cell sorting was performed on a FACS Aria three lasers for excitation of violet, blue, and red-excited dyes.

2.4 Magnetic activated cell sorting enrichment of splenocytes

Follicular splenic B cells were isolated by first mechanical dissociation between two frosted glass slides. The resulting mixture was then passed through 80 μ M filter (Fisher Scientific). Red blood cell lysis with ACK (NH_4Cl , KHCO_3 , $\text{Na}_2\text{-EDTA}$) for 5 minutes on ice and washed in FACS buffer (1xPBS, 5% BSA, 1mM EDTA). Cells were spun down and the supernatant decanted. Cells re-suspended in 2mL of FACS buffer per spleen and 5ul of anti-CD23 biotin (lab made) added for 15 minutes at 4°C. Cells then washed in 13mL of FACS buffer, spun down and the supernatant decanted and re-suspended in 1mL per spleen of FACS buffer. 80 μ L/mL of streptavidin microbeads was added and enrichment of FOL B cells a positive selection using BI-CD23 antibodies and SA microbeads with (MACS) LS columns (Miltenyi Biotec Auburn, Ca) according to the manufacturer's instructions resulting in purity greater than 95%.

2.5 In-vitro B cell cultures

To assess cell division, splenic B cells isolated as above, were labeled with 5 μ M CFSE (Invitrogen, Carlsbad, CA) for 2 minutes in PBS. Subsequently 2 x10⁴ CD23⁺ cells per well were cultured in a 96 Ill plate in RPMI 1640 with 10% FBS, 2mM L-glutamine, 0.1mM nonessential amino acids, 1xOPI, 55 μ M b-ME, and 10mM HEPES. Cells were stimulated with F(ab')₂ goat anti-mouse IgM (Jackson ImmunoResearch), LPS (Sigma), CpG (IDT). Cultures in which γ -secretase inhibitors (GSI, compound E, Calbiochem) were used, GSI was at 100nM. For [³H]-thymidine experiments, 2x10⁴ sorted B cells per well were cultured in triplicate per condition for 3 days before being pulsed with 1 μ Ci (0.037MBq) before harvesting. Cells were collected onto Filtermat (PerkinElmer Life Sciences) and analyzed on a MicroBeta Trulux scintillation counter (PerkinElmer Life science). For mixing experiments, 2x10⁴ total cells per Ill (3:1 ratio PS1/2 DKO: WT^{SJL}) were cultured for three days and harvested. For staining cells were first incubated on ice for 15min with FcR Block (2.4G2), and then stained with Ly5^{B6} (104) and Ly5^{SJL} (A20) and CFSE dilution assessed on a FACS Canto.

2.6 Quantitative real-time PCR

Cells from at least three C57BL/6 mice were pooled and 1x10⁵ cells from each respective population sorted on a FACS Aria. Tissue samples from liver and brain were not sorted but isolated in bulk. Qiagen (Valencia, CA) RNeasy Mini Kits were used to isolate RNA as per instructions provided with the kit, and RNA

was reverse transcribed using first strand cDNA synthesis kit, protocol provided in kit (GE Healthcare). Quantitative RT-PCR (qRT-PCR) was performed using pre-validated Taqman primer/probes set for Akt1, Akt2, and Akt3 with 18s used as an endogenous control (Applied Biosystems, Foster City, CA). Data were normalized to respective Akt isoform expression in follicular B cells. In addition, delta-CT values were calculated by subtracting the average Ct for the 18s primer/probe for each population from the respective Akt isoform. For Akt1^{-/-} Akt2^{-/-} B cell populations were sorted as above and Akt3 transcript levels compared to the corresponding population from wild type mice.

2.7 Retroviral supernatant

293T cells were co-transfected with either MigR1-GFP, MigR1-Bcl-x_L-GFP, or MigR1-DNMAML-GFP and helper plasmid as per protocols outlined in (Pearlman et al., 2001). Fugene (Roche) mediated transfection was performed with 8-12µg MigR1 constructs and 4-6µg helper plasmid. Cell culture supernatants harvested 24 and 48 hours post transfection and stored at -80°C.

2.8 Retroviral transduction

Bcl-x_L

MigR1-GFP or MigR Bcl-x_L GFP constructs were transduced by retro-virus into AKT DKO day 15 fetal liver cells. Fetal liver cells were harvested as above and placed into aMEM media supplemented with 10% FCS, 2mM glutamine, 1% penstrep, 10ng/mL IL-6, 20ng/ml IL-3, and 100µg/mL stem cell factor. 1 day after harvest cells were subject to spinfection with respective virus + 10µg/mL

polybrene for 90 min (2500PRM) at 25°C, then placed at 37°C overnight. Spinfection was repeated the next day, and cells rested for an additional 6 hrs. Cells were washed 3x in 1xPBS in a total volume of 50mL each wash, and 2.5×10^5 cells injected into irradiated B6^{SJL} hosts as above.

DNMAML

MigR1-GFP or MigR-DNMAML GFP constructs were transduced into Class II controlled Myr-Akt bone marrow similar to above, B220, CD3, Gr-1 depleted adult bone marrow used in place of fetal liver.

2.9 Tat-Cre Incubation

For induction of Rosa26^{+DNMAML-GFP} cells, splenic CD23⁺ B cells were isolated as above and washed in HyClone Serum Free media three times and re-suspended at a final concentration of 5×10^6 /mL with 50µg/mL Tat-Cre for 45min at 37°C. Pulse was stopped by addition 10x volume of Hyclone media with 10% FBS. Cells washed twice and rested for 16hrs before sorting live B cells (B220⁺ Dapi⁻) on FACS Aria.

2.10 Calcium Mobilization

10×10^6 /mL splenic B cells isolated by positive selection as described above in RPMI 1640 with 10% FBS and loaded with Indo-1AM (invitrogen) 7µg/mL for 45min at 37°C, mixing every 15min. Cells were washed twice in media and run on an LSR II flow cytometer (Beckton Dickinson). Indo-1 fluorescence was analyzed using a 450-DC-LP dichroic beam splitter and emission filters at 530/30

and 405/20 for free and bound probes respectively. Each sample was run for 60s to collect baseline, then 15 μ g/mL F(ab')₂ goat anti-mouse IgM (Jackson ImmunoResearch) was added and collected for an additional 150s at which time the tube was removed and 1 μ g/mL ionomycin (Sigma) was added and the sample collected for an additional 60s. Thapsigargin 1 μ M (Fisher Scientific) treated samples were collected similar to above. Measurement of anti-IgM or thapsigargin mediated intracellular Ca²⁺ store release was performed by adding 12.5 mM EGTA (Fisher Scientific). All samples collected in linear mode on FACSDiva (Beckton Dickinson) software and analyzed in FlowJo 8.8 (Tree Star, Inc., San Carlos, CA) using kinetic analysis.

2.11 Statistical Analysis

All cell percentages and cell numbers were evaluated with unpaired two-tailed t-test using Excel software. Absolute cell numbers were calculated by multiplying the frequency of cells within each B cell subset by the total cells harvested from each respective organ.

CHAPTER 3 Akt signaling promotes peripheral B cell development and competitive fitness

3.1 Introduction: Importance of Akt signaling in B cell development

The serine/threonine kinase Akt consists of three individually encoded family members termed Akt1, Akt2, or Akt3 (Easton, Cho et al. 2005; Plas and Thompson 2005). All three isoforms are similar in structure but vary in expression pattern across different tissues (Manning and Cantley 2007). Regardless of cell type or tissue context, Akt signaling is an important mediator of survival, metabolism, and cell cycle progression (Yang, Tschopp et al. 2004). Akt lies downstream of phosphatidylinositol-3 kinase (PI-3K), a critical target of antigen receptor signaling in both B cells and T cells; in addition to Akt, PI-3K also activates other downstream effectors pathways including Tec family kinases and PLC γ (Okkenhaug and Vanhaesebroeck 2003; Fruman 2004).

In B cells, PI-3K is recruited to the plasma membrane upon cross-linking of surface BCR molecules, and recruitment of PI-3K is enhanced by the B cell specific co-receptor CD19 (Del Nagro, Otero et al. 2005). Antigen receptor mediated stimulation of PI-3K leads to a transient activation of Akt in B cells, co-ligation of the BCR and CD19 results in the sustained activation of Akt (Otero, Omori et al. 2001). In B cells, both PI-3K and CD19 are dispensable for early B cell development in the bone marrow but are required for the normal generation of peripheral B cells, such as the B1 and MZ B cell compartments (Otero, Anzelon et al. 2003; Otero and Rickert 2003). The role of Akt, downstream of PI-3K in B cell development remains unclear.

In this chapter I present results that demonstrate a critical and non-redundant role for Akt1 and Akt2 in the development of the peripheral B cell pool. To elucidate these findings I generated chimeras established with Akt1 and Akt2 deficient fetal liver and found that B1a and MZ B cell development was profoundly blocked in Akt1/2 deficient chimeras. In contrast to peripheral B cell development, immature B cells in the bone marrow were only mildly perturbed. In addition, Akt1/2 deficient progenitors are poor competitors starting at the late transitional stage of peripheral B cell development. The BCR repertoire is altered and proliferative responses to BCR cross-linking are dampened in Akt1/2 deficient FOL B cells. Examination of Akt mRNA expression across multiple subsets of B cells by qRT-PCR revealed that Akt3 expression is reduced in MZ and B1 subsets relative to FOL B cells. Interestingly, though Akt3 expression decreases in the B cell subsets most affected by the loss of Akt1 and Akt2 I observed that the overall level of Akt3 mRNA was significantly increased compared to Akt2 (Calamito, Juntilla et al. 2009).

Given the role of Akt in cell survival, I examined the effect of ectopic expression of the pro-survival gene *Bcl-x_L*, through retroviral transduction of Akt1/2 deficient progenitors and found it insufficient to rescue MZ B cell development. Furthermore, the Akt pathway has previously been implicated as downstream of the Notch family of receptors in thymocyte development (Ciofani and Zuniga-Pflucker 2005) and enhanced Akt activation has been reported in B cells exposed to cross-linking anti-IgM antibody in the presence of Notch ligands (Thomas, Calamito et al. 2007). In addition, Notch signaling is required for the

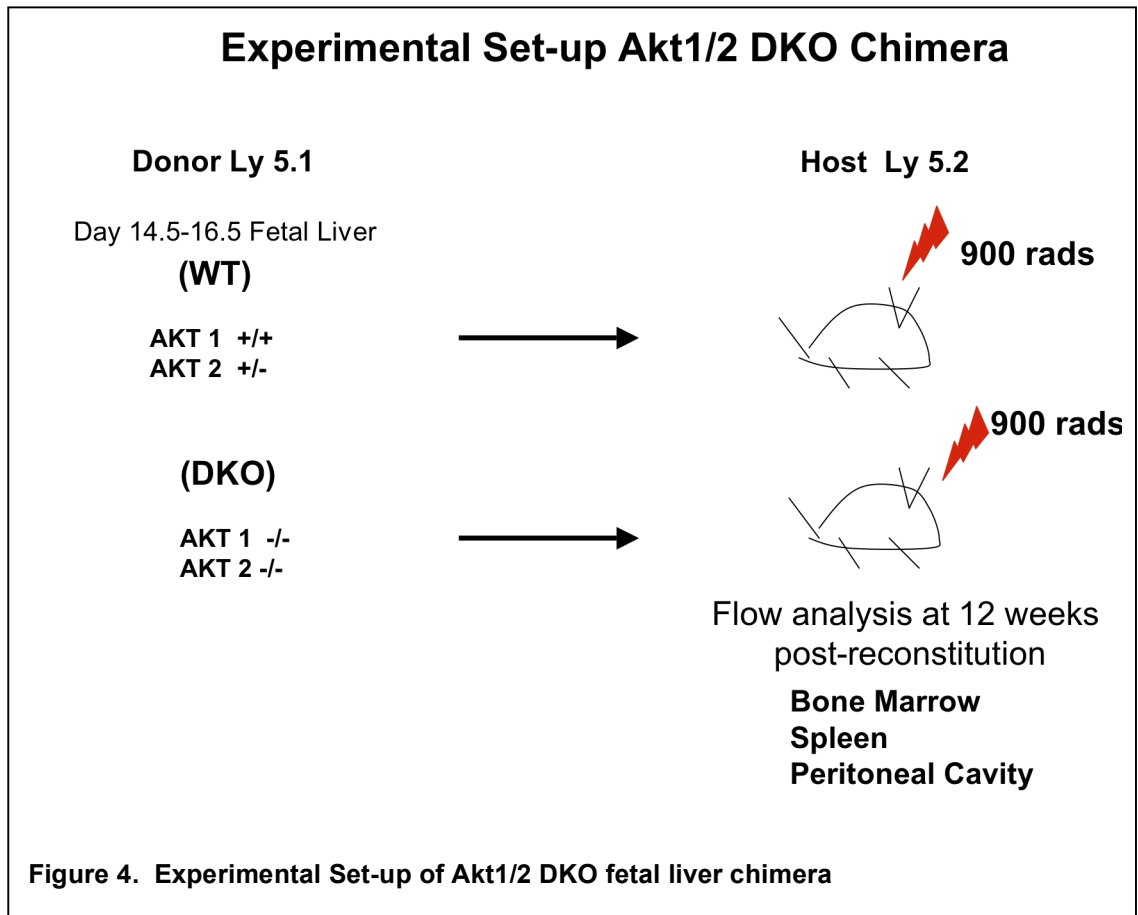
generation of MZ B cells, but dispensable for other B cell lineages (Tanigaki, Han et al. 2002; Saito, Chiba et al. 2003; Hozumi, Negishi et al. 2004; Maillard, Weng et al. 2004; Wu, Maillard et al. 2007). To examine the role of Akt downstream of Notch I introduced a dominant negative mastermind (DNMAML), capable of blocking all Notch receptor signaling, into bone marrow progenitors from mice harboring a class II promoter driven transgene harboring a constitutively activate form of Akt (Kouskoff, Fehling et al. 1993; Tuttle, Gill et al. 2001; Maillard, Weng et al. 2004) This system demonstrated that while an active Akt drives B cells toward the MZ B cell fate, the presence of this transgene is not sufficient to rescue a Notch loss of function.

3.2 Chapter 3 Results

3.2.1 Early B cell development without Akt1 and Akt2.

Developing B cells undergo intense proliferation at the pro to pre B transition (Nemazee 2006), given Akt's documented role in cell cycle progression and survival I sought to examine the role of Akt in early B cell development. Bone marrow B cell populations were not affected in the absence of a single isoform of Akt1 or Akt2 (not shown). To assess the role of Akt1 and Akt2 in B cell development, I transferred fetal liver cells carrying null mutations for Akt1 and/or Akt2 into lethally irradiated Ly5 congenic hosts, and allowed 12-14 weeks for full reconstitution (**Fig 4**). Use of the fetal liver chimeras was required due to the neonatal lethal phenotype of Akt1^{-/-} Akt2^{-/-} (Akt1/2 DKO) mice (Peng, Xu et al. 2003; Juntilla, Wofford et al. 2007). Although combined expression of Akt1 and Akt2 is required for thymopoiesis beyond the pre-TCR checkpoint, Akt1/2 DKO progenitors readily generated pre-BCR-dependent pre-B cells in the bone marrow as well as downstream immature B cells (**Fig. 5A**). Indeed, if anything frequencies of Akt1/2 DKO donor-derived pro- (B220^{low} CD43⁺ AA4⁺ CD19⁺), pre- (B220^{low} CD43⁻ AA4⁺ CD19⁺ sIgM⁻) and immature (B220^{low} CD43⁻ AA4⁺ CD19⁺ sIgM⁺) BM B cells were elevated. Numbers of pro-, pre-, and immature B cells were increased ~3, ~4, and ~2 fold, respectively, compared to chimeras reconstituted with Akt1^{+/+} Akt2^{+/-} progenitors (**Fig. 5B**). In contrast, frequencies and cell numbers of mature recirculating BM B cells derived from Akt1/2 DKO progenitors were decreased significantly compared to Akt1^{+/+} Akt2^{+/-} progenitor

controls (**Fig. 5A/B**). I conclude that the combined expression of Akt1 and Akt2 are not required to generate pre-B and immature B cells.



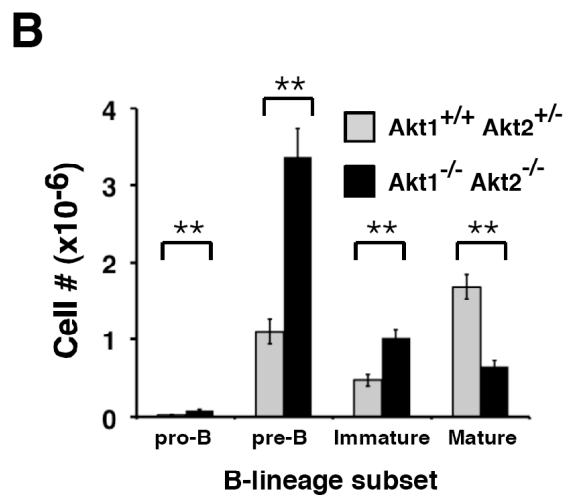
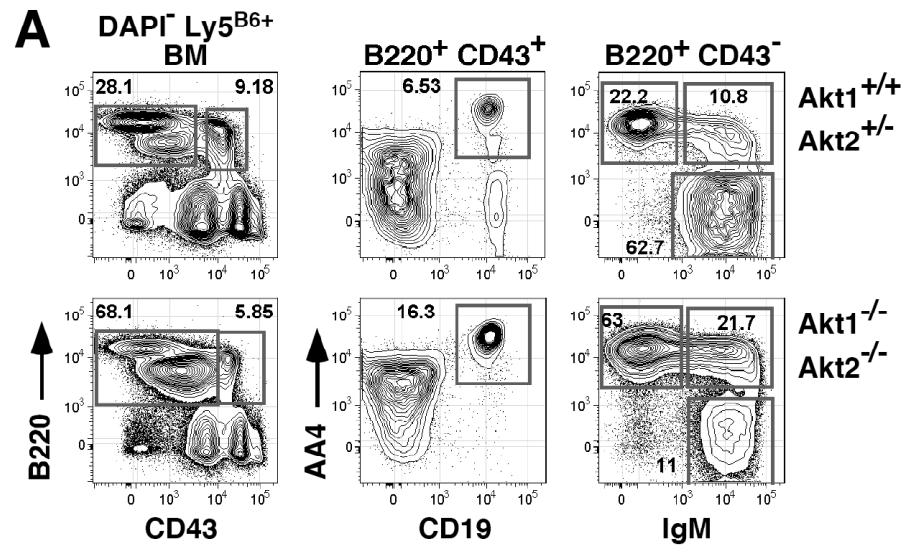


Figure 5. Akt-deficient progenitors generate marrow B-lineage precursors. (A) Representative flow cytometric analysis of BM cells from chimeras established with Akt1^{+/+} Akt2^{+/-} (top) or Akt1^{-/-} Akt2^{-/-} (bottom) progenitors 12-14 weeks previously. The left-most plots are gated on viable donor-derived (DAPI⁻ Ly5^{B6+}) cells. Numbers in plots indicate the frequency of events in the indicated gate as a function of the indicated parent population. Pro-B, CD43⁺ B220⁺ CD19⁺ AA4⁺; pre-B, CD43^{low} B220⁺ IgM⁻ AA4⁺; immature B, CD43⁺ B220⁺ IgM⁺ AA4⁺; mature B, CD43⁺ B220⁺ IgM⁺ AA4⁻. **(B)** Absolute cell numbers for respective BM populations were calculated using the gates shown in (A) (see methods). Error bars indicate the SEM for each group, n=5. Data representative of two separate experiments. **= p<.01

3.2.2 Akt1-2 deficient precursors fail to generate MZ B cells.

The decreased numbers of Akt1/2 DKO mature B cells in the BM raised the possibility of a role for Akt in peripheral B cell development. To examine this issue I assessed splenic B cell compartments in Akt1/2 DKO reconstituted animals. In chimeras established with Akt1/2 DKO progenitors, the donor-derived follicular B cell pool was decreased only 2-fold compared to chimeras generated with Akt1^{+/+} Akt2^{+/-} progenitors. Furthermore frequencies of AA4⁺ transitional B cell subpopulations, defined by differential expression levels for CD23 and IgM (termed T1, T2, and T3), were largely unaffected. In contrast, the development of MZ B cells was highly dependent on signaling derived from Akt1 and Akt2, as I was unable to detect donor-derived CD21^{high} IgM^{high} CD23^{low} MZ B cells in chimeras established with Akt1/2 DKO progenitors (**Fig. 6**). Numbers of splenic MZ B cell precursors (MZPs), considered immediate precursors for MZ B cells, were decreased 3-fold compared to control chimeras (**Figure 6B**). Analyses of mice deficient in either Akt1 or Akt2 exhibited a normal MZ B cell population (not shown), suggesting that Akt1 and Akt2 perform overlapping functions required for MZ B cell development. Altogether, these data show that MZ B cell development is critically dependent on the combined expression of Akt1 and Akt2.

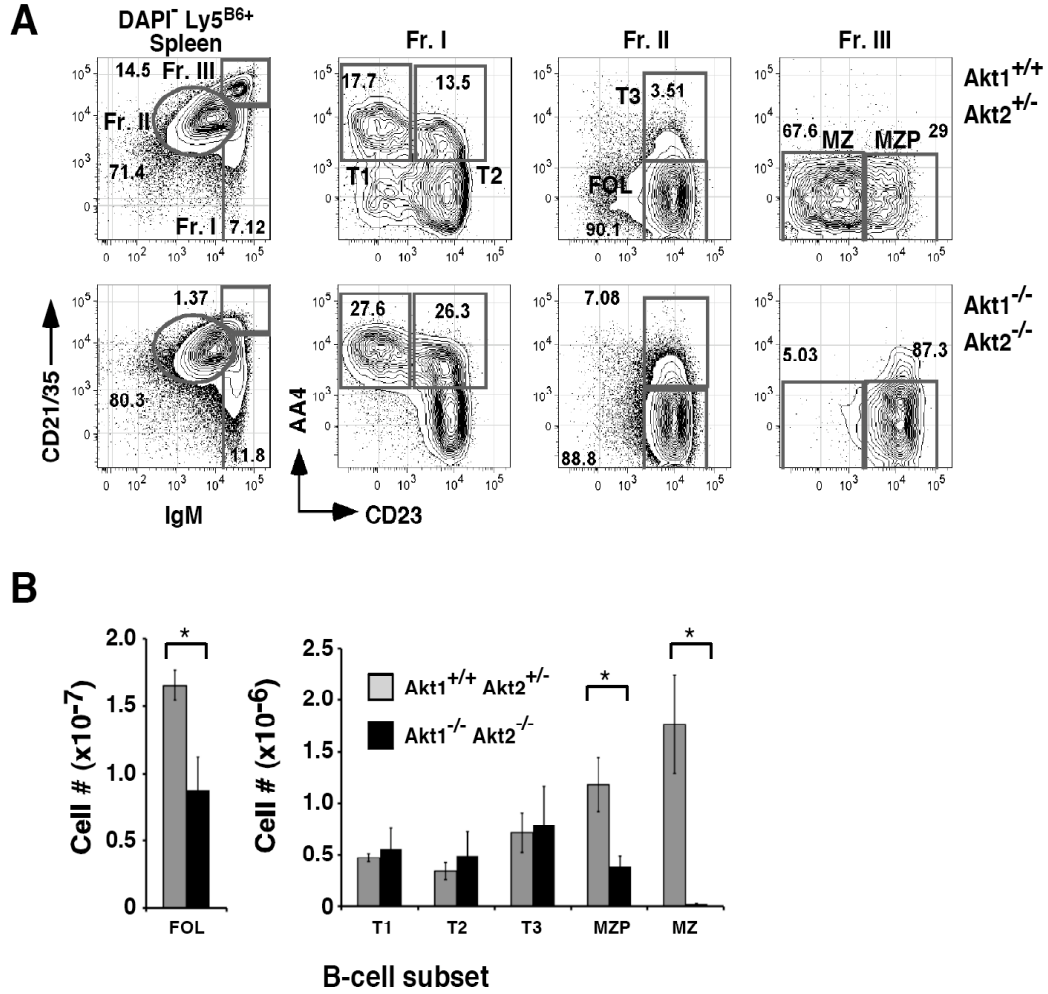
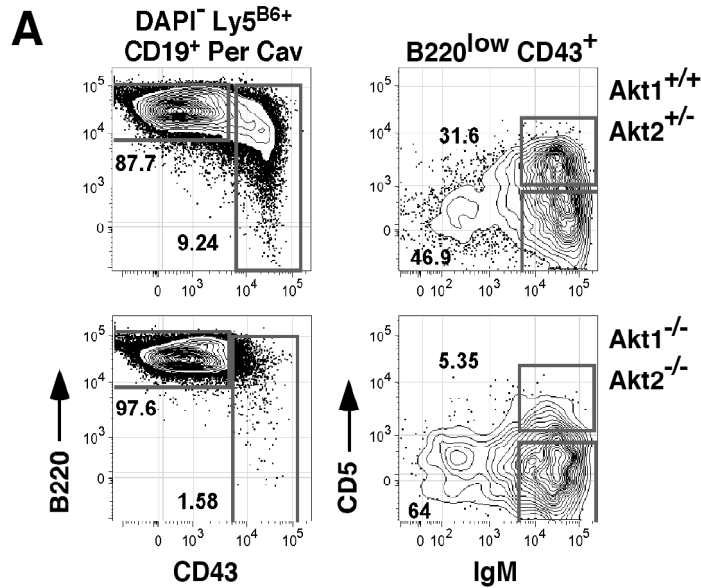


Figure 6. MZ B cell development is highly dependent on Akt1/2. (A) Representative analysis of splenocytes from chimeras established with Akt1^{+/+} Akt2^{+/-} (top) or Akt1^{-/-} Akt2^{-/-} (bottom) progenitors 12-14 weeks previously. Viable donor-derived cells were gated as in Figure 1. Numbers in plots show the frequency of events as a function of the indicated parent gate. (B) Absolute cell numbers for respective splenic B cell subsets were calculated using the gates shown in (A) (see methods). Error bars indicate the SEM for each group, n=5. Data representative of two separate experiments. *=p<.05

3.2.3 Defective B1 B cell development in Akt1/2-deficient chimeras.

Mice lacking CD19 or the catalytic subunit of PI-3K, p110 δ , fail to generate MZ and B1 B cells (Clayton, Bardi et al. 2002; Jou, Carpino et al. 2002; Okkenhaug, Bilancio et al. 2002), given that Akt is a major down stream target of the PI-3K pathways I hypothesized that B1 B cell development might be compromised in the absence of optimal Akt expression. Therefore I analyzed frequencies of donor-derived B1 B cells in the peritoneal cavity. As shown (**Fig. 7**), whereas animals reconstituted with Akt1^{+/+} Akt2^{+/-} progenitors generated abundant numbers of CD19⁺ CD43⁺ B1 peritoneal cavity B cells, the donor-derived B1 B cell pool was detectable but significantly diminished in chimeras established with Akt1/2 DKO progenitors. Further, frequencies of CD5⁺ B1a cells were severely decreased, whereas CD5⁻ CD43⁺ B1b cells were reduced only 2-fold. I conclude that the generation of B1a B cells is also highly dependent on the Akt pathway and this interpretation is in agreement with previous observations demonstrating the importance of the PI-3K signaling pathway in the generation of both MZ and B1 B cells.



B

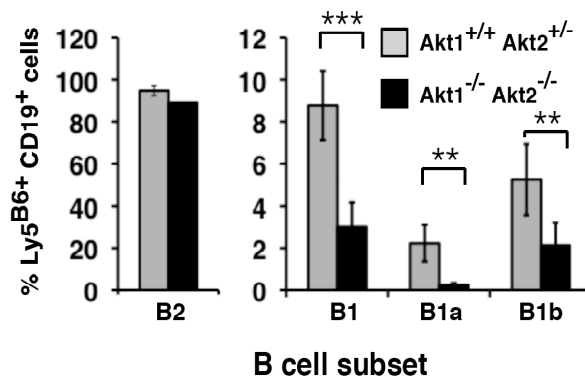


Figure 7. Requirement for Akt1/2 for B1 B cell development. (A) Representative analysis of peritoneal cavity lymphocytes from chimeras established with Akt1^{+/+} Akt2^{+/-} (top) or Akt1^{-/-} Akt2^{-/-} (bottom) progenitors 12-14 weeks previously. The left-most plots are gated on viable donor-derived B cells (DAPI⁻ Ly5^{B6+} CD19⁺). Numbers in plots show the frequency of events as a function of the indicated parent gate. (B) Average frequencies for the indicated subsets are calculated using the gates shown in (A). B2, CD19⁺ B220⁺ CD43⁻; B1, CD19⁺ B220⁺ CD43⁺; B1a, CD19⁺ B220⁺ CD43⁺ CD5⁺; B1b, CD19⁺ B220⁺ CD43⁺ CD5⁻. Error bars indicate the SEM for each group, n=5. Data representative of two separate experiments. **=p<.01, ***=p<.005

3.2.4 Diminished proliferation to BCR aggregation without Akt1/2.

B1a and MZ B cell development both require optimal selection via the BCR. In addition, past studies suggest that BCR aggregation leads to activation of Akt (Craxton, Jiang et al. 1999). I therefore assessed BCR-mediated activation and proliferation in Akt1/2 deficient B cells. I isolated CFSE-labeled CD23⁺ splenic B cells from Akt1/2 DKO or control reconstituted chimeras and stimulated them with increasing concentrations of anti-IgM antibodies. After 3 days, Akt1/2 deficient B cells exhibited decreases in the number of cell divisions induced by IgM aggregation and markedly decreased survival as assessed by uptake of the DNA dye 7-AAD (**Fig. 8A**), resulting in significant decreases in numbers of viable B cells (**Fig. 8B**). Furthermore, while the number of cell divisions occurring in response to the TLR-4 agonist lipopolysaccharide (LPS) was largely unchanged between Akt1/2 DKO and Akt1^{+/+} Akt2^{+/-} B cells, overall cell recoveries for Akt1/2 DKO follicular B cells were significantly reduced, likely due to increased cell death (**Fig. 8**). These data suggest that, optimal BCR-mediated B cell proliferation requires Akt1 and Akt2.

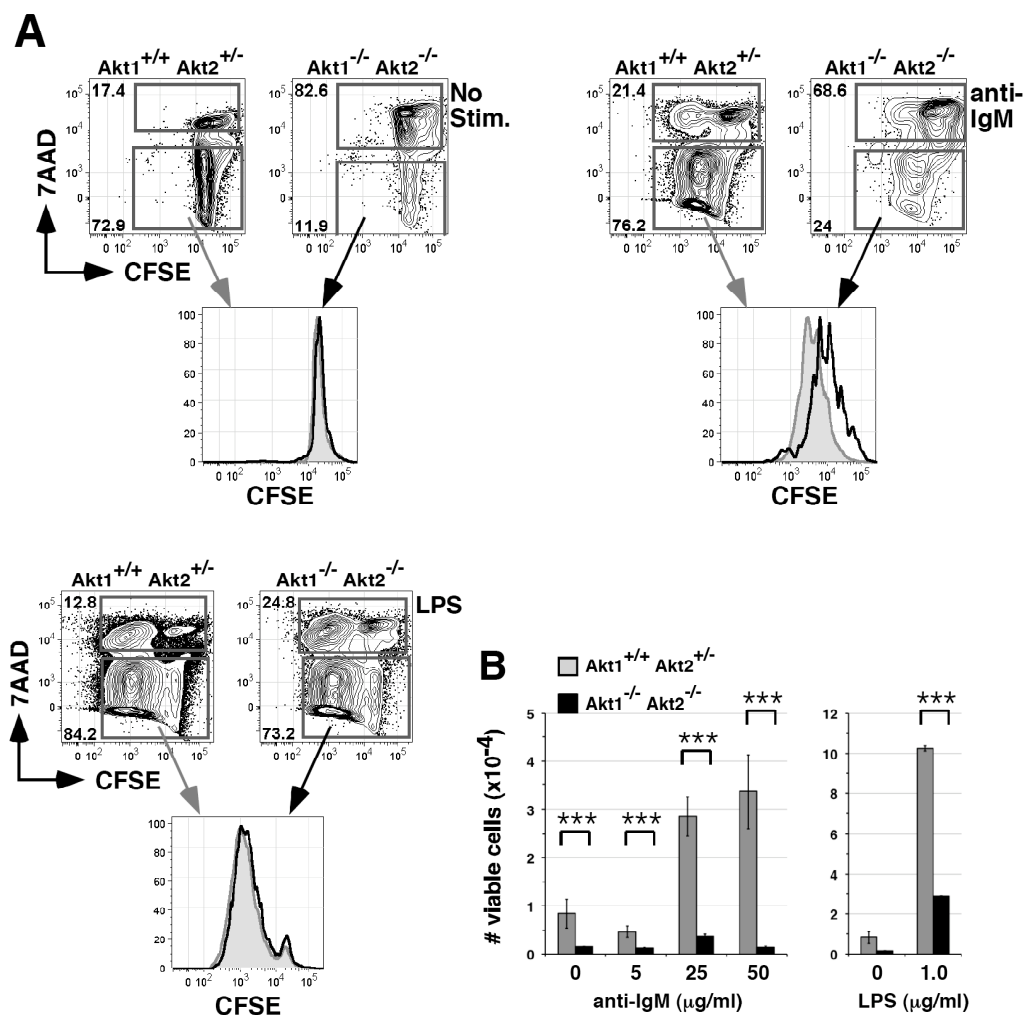


Figure 8. Akt1/2-deficient B cells exhibit a defective BCR-mediated proliferative response. (A) CFSE-labeled CD23⁺ follicular B cells were left unstimulated (top plots) or stimulated with 50mg/ml anti-IgM antibodies (middle) or 1ug/mL of LPS (bottom) for 3 days, stained with the viability dye 7AAD, and analyzed by flow cytometry. The right-most overlay histograms were gated on viable (7AAD⁻) cells using the gates indicated in the corresponding plots. Black line, Akt1^{-/-} Akt2^{-/-}; gray filled curves, Akt1^{+/+} Akt2^{+/-}. (B) Mean numbers of viable Akt1^{-/-} Akt2^{-/-} or Akt1^{+/+} Akt2^{+/-} B cells recovered from triplicate cultures stimulated with the indicated concentrations of anti-IgM antibodies or LPS were calculated by flow cytometry using the 7AAD⁻ gates shown in (A). Error bars indicate SEMs from 4 animals per group. Representative of two separate experiments. ***=p<.005

3.2.5 The Akt pathway promotes competitive fitness of mature B cells *in vivo*.

Past work illustrates that mature B cells lacking CD19 or the tec tyrosine kinase Btk are unable to compete against wild-type (WT) B cells *in vivo* (Sprent and Bruce 1984; Otero, Anzelon et al. 2003; Lindsley, Thomas et al. 2007). This feature correlates with the inability of mature B cells from these mutants to undergo cell division upon BCR aggregation *in vitro*. Given the suboptimal survival of Akt1/2 DKO B cells in response to BCR cross-linking, I reasoned that Akt1/2 DKO B cells might also survive poorly when forced to compete against WT B cells. To test this possibility, I generated double chimeras in which equal numbers of C57BL/6 (Ly5^{B6}) backcrossed Akt1/2 DKO or control Akt1^{+/+} Akt2^{+/-} fetal liver progenitors were mixed with wild type progenitors from congenic B6.Ly5^{SJL} mice before transplantation into irradiated B6.Ly5^{SJL} adults. Four months later recipients were assessed for the representation of Akt1^{+/+} Akt2^{+/-} or Akt1/2 DKO-derived (both Ly5^{B6+}) versus B6.Ly5^{SJL}-derived (Ly5^{SJL+}) B-lineage cells including BM pro- and pre-B cells, immature (AA4⁺ IgM⁺) B cells in the BM and spleen, and mature splenic follicular, MZP, and MZ B cells. To control for engraftment of progenitors from each donor, I also assessed the relative contribution of C57BL/6 versus B6.Ly5^{SJL} progenitors to LSK (Lineage⁻ c-Kit⁺ Sca1⁺) BM progenitor pool, which contains hematopoietic stem cells and early multipotent progenitors.

In the BM, Akt1/2 deficient cells were highly competitive with WT cells within both the LSK pool and within all B-lineage precursor populations including

immature IgM⁺ B cells, as the degree of chimerism for each of these populations derived from Akt1/2 DKO progenitors paralleled that observed for Akt1^{+/+} Akt2^{+/-} progenitors (**Fig. 9B**). In sharp contrast, all mature B cell populations including follicular and MZP B cells in the spleen and mature BM B cells were dramatically under represented in Akt1/2 DKO + WT compared to Akt1^{+/+} Akt2^{+/-} + WT double chimeras (**Fig. 9A and 9B**). In addition, whereas Akt1/2 DKO and Akt1^{+/+} Akt2^{+/-} cells were equally competitive within the least mature (T1) splenic transitional subset, I noted a partial loss of Akt1/2 DKO B cells within the more mature T2 transitional subset. Furthermore, in additional double chimeras, Akt1^{-/-} or Akt2^{-/-} mature B cells displayed a substantial albeit incomplete inability to compete against WT cells (not shown). Altogether these data suggest that the Akt pathway initiates cellular processes required for competitive survival as immature B cells transit from the T2 population into mature B cell pools.

Blys and BR3 are major determinants of peripheral B cell numbers and recent publications demonstrate that Akt is downstream of BR3 (Patke, Mecklenbrauker et al. 2006; Otipoby, Sasaki et al. 2008; Woodland, Fox et al. 2008). This raised the possibility that BR3 signaling is defective in the absence of Akt1 and Akt2 thus providing a mechanistic explanation for the inability of Akt1/2 deficient cells to compete with WT cells. To address this question, I isolated FOL splenic B cells from control or Akt1/2 DKO chimeras and cultured them in the presence or absence of Blys for 72 hrs assaying survival and metabolic fitness. I found that survival as examined by exclusion of the viability dye 7-AAD, was enhanced after culture with Blys (**Fig 10A**). Furthermore, Akt1/2

deficient cells increased their cell size after culture with Blys, suggesting enhanced metabolic activity (**Fig 10B**). Therefore Akt1/2 deficient FOL B cells are responsive to Blys in terms of survival and metabolism suggesting that BR3-Akt1/2 signaling does not regulate FOL B cell survival in-vivo. It is possible that the concentration of Blys utilized in these experiments (100ng/mL) is in such excess as to mask any defect downstream of BR3. Alternatively, the absence of Akt1/2 downstream of BCR signaling could lead to poor generation of the Blys-BR3 substrate p100 compromising the ability of Akt1/2 deficient B cells to respond to Blys.

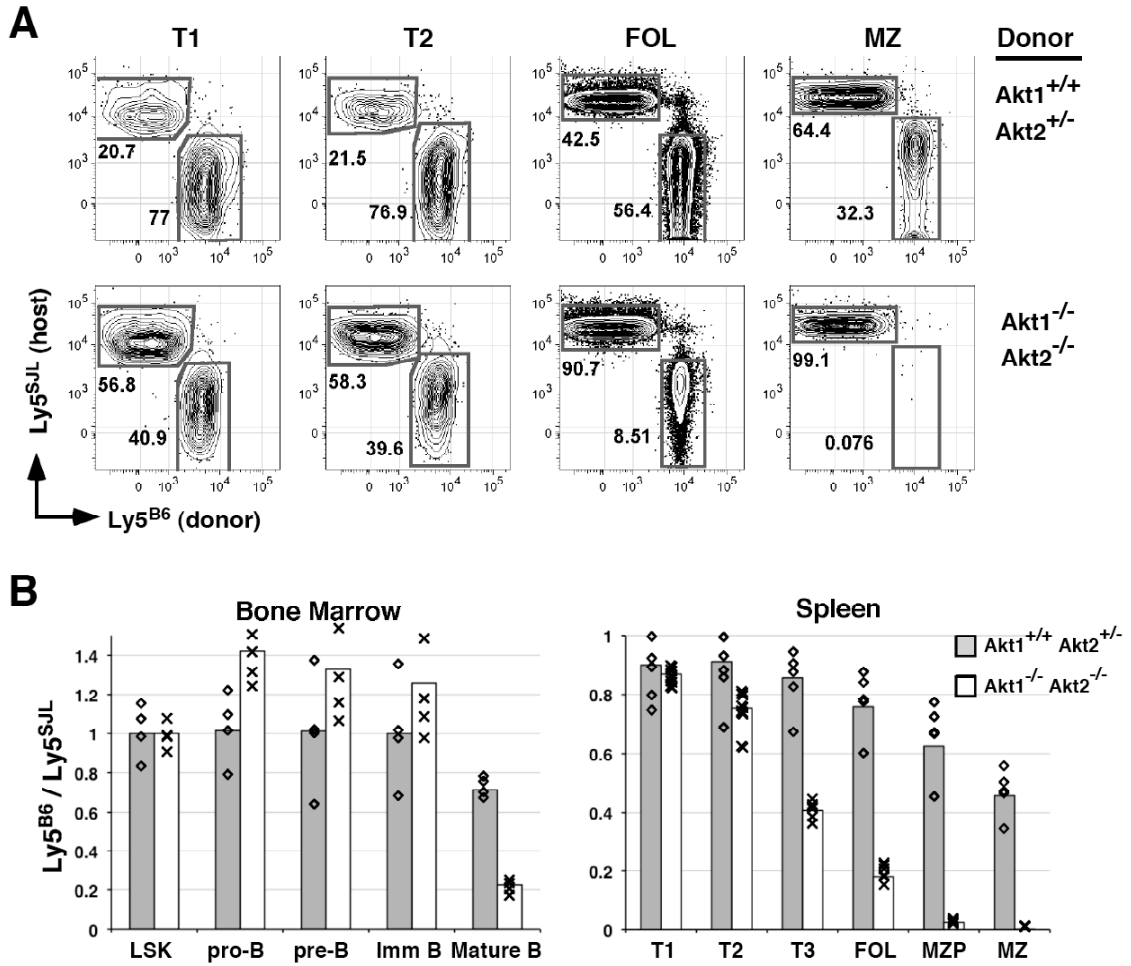


Figure 9. Akt1/2-deficient B cells compete poorly with wild-type B cells. (A) Double chimeras were analyzed at 16 weeks post transplantation. **(A)** Representation of the indicated B cell subset derived from WT (Ly5^{SjL}⁺) or Akt1^{+/+} Akt2^{+/-} (top) or Akt1^{-/-} Akt2^{-/-} (bottom) progenitors. Each subset was gated as shown in Figure 2. **(B)** The average ratio of cells in the indicated subpopulation derived from Ly5^{B6}⁺ and Ly5^{SjL}⁺ progenitors in double chimeras established with Akt1^{+/+} Akt2^{+/-} (gray columns) or Akt1^{-/-} Akt2^{-/-} (white columns) progenitors was calculated using gates shown in Figures 1 and 2 with 4 or 5 animals per group. Diamond and “X” symbols indicate individual mice. LSK, Lineage⁻ c-Kit⁺ Sca-1⁺. Representative of two separate experiments.

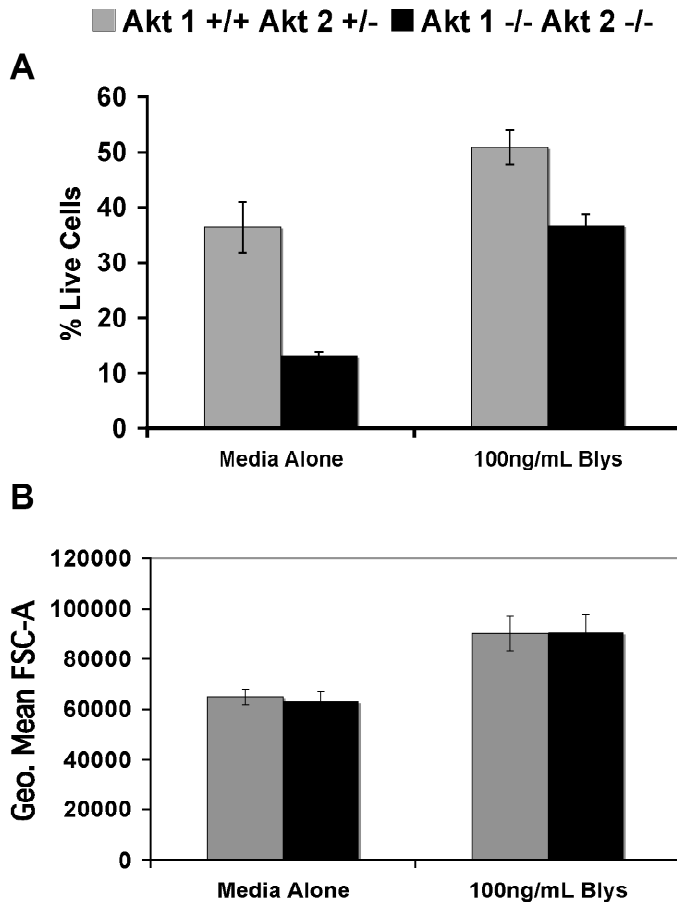


Figure 10: Survival mediated by Blys is Akt1 Akt2 independent. (A) Survival of CD23⁺ splenocytes from control (Grey) or Akt1/2 DKO (black). Viability assayed by exclusion of 7-AAD after 72hrs *in-vitro* culture. **(B)** Cells assayed as above, cell size of live (7-AAD⁻) cells.

3.2.6 Altered BCR repertoire in Akt1/2 deficient B cells

B cells deficient in CD19 or the downstream PI3K have altered λ^+ light chain repertoires (Diamant, Keren et al. 2005; Llorian, Stamataki et al. 2007), given that our studies to this stage had demonstrated overlapping phenotypes between CD19, PI-3K and Akt. I sought to determine if Akt1/2 deficient B cells demonstrated a similar BCR repertoire phenotype. Consistent with the notion of Akt being a major downstream target of PI3K, splenic FOL B cells ($CD19^+ AA4^- IgM^{int} CD21/35^{int}$) from Akt1/2 deficient chimeras revealed a dramatic increase in the percentage of λ^+ B cells (**Fig. 11A** and **Table 3**). The increase in λ^+ B cells was also detected in both the T1 ($CD19^+ AA4^+ CD23^-$) and T2 ($CD19^+ AA4^+ CD23^+$) splenic transitional B cell populations. Furthermore, immature B cells in the bone marrow ($B220^+ CD43^- AA4^+ IgM^+$) also displayed increased frequencies of λ^+ cells, suggesting that Akt1 and Akt2 are required at these early stages of B cell development to generate and shape the BCR repertoire.

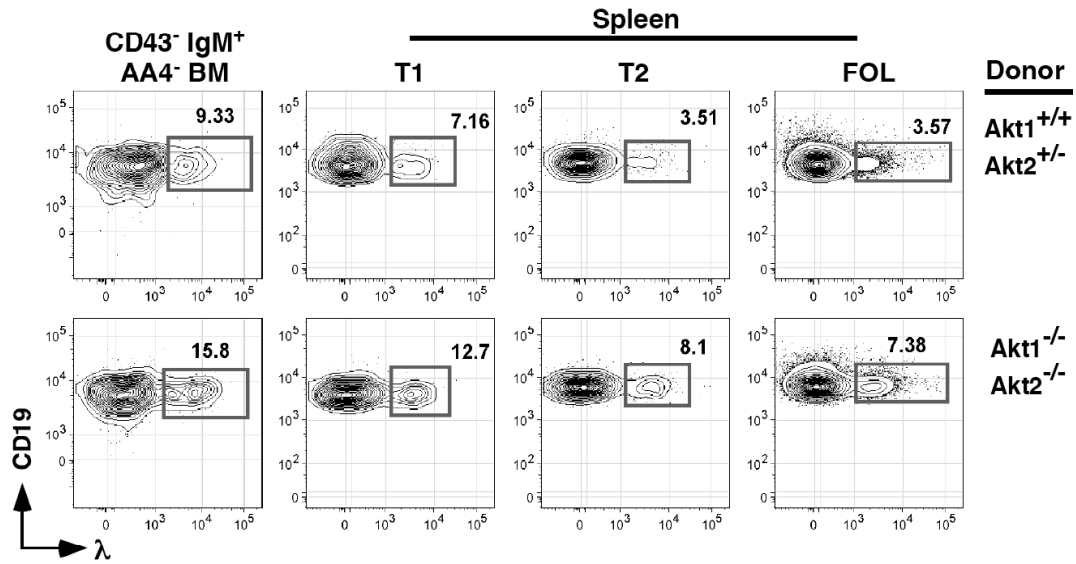


Figure 11: Altered BCR repertoire in Akt 1/2 deficient B cells Representative analysis of bone marrow or splenocytes from chimeras established with Akt1^{+/+} Akt2^{+/-} (top) or Akt1^{-/-} Akt2^{-/-} (bottom) progenitors 12-14 weeks previously. Viable donor-derived cells were gated as in Figure 4 and 5. Numbers in plots show the frequency of events as a function of the indicated parent gate. Number of animals equals two (Akt1^{+/+} Akt2^{+/-}) or four (Akt1^{-/-} Akt2^{-/-}).

Table 3 Frequency of λ^+ B cells in Akt1/2 deficient B cells

Tissue	Akt1 ^{+/+} Akt2 ^{+/-}	Akt1 ^{-/-} Akt2 ^{-/-}
Bone Marrow	% λ Positive	
Immature B	8.28 (.91)	13.5 (1.2)
Spleen		
T1	8.0 (1.2)	12.2 (1.1)
T2	6.3 (2.4)	12.6 (2.1)
FO	4.6 (.59)	8.4 (.41)

B cell subsets were defined as illustrated in figure 4 and figure 5. Mean percent of viable gated populations of two (Akt1^{+/+} Akt2^{+/-}) or four (Akt1^{-/-} Akt2^{-/-}). Numbers in parentheses represent SEM.

3.2.7 Akt isoform expression across B cell subsets.

Since pre-B cell frequencies were increased and MZ, MZP, and B1 B cell frequencies decreased upon deletion of Akt1 and Akt2, I next sought to better define the expression of each Akt isoform throughout the B cell lineage. My initial analyses sought to address expression of each isoform in each immature and mature B cell subpopulation. As shown in **Fig 12A**, Akt1 and Akt3 transcripts were present at 3-6 fold higher levels for wild type pro- and immature B cells in the BM relative to follicular B cells in the spleen. Though Akt3 transcripts were expressed at higher levels in immature relative to follicular B cells, the expression of Akt3 was still lower than brain, a tissue shown previously to express high levels of Akt3 (Easton, Cho et al. 2005). Interestingly, Akt1 and Akt3 expression appeared to be down regulated both as pro-B cells give rise to pre-B cells, up-regulated in immature B cells, and again down regulated as immature B cells give rise to mature B cells. In addition, relative to follicular B cells, transcript abundance for Akt1 and Akt3 was decreased further in MZ B cells and their immediate precursors (MZPs) in the spleen, and in both peritoneal cavity B1 subpopulations. These observations contrast with the expression pattern for Akt2, which did not vary substantially among any of the B-lineage subsets examined (**Fig. 12A**) and was expressed at lower overall levels compared to Akt1 and Akt3.

Given that past studies have shown that Akt3 is expressed in unfractionated splenic B cells (Juntilla, Wofford et al. 2007), and pre-B cell development in wild type and Akt1/2 DKO precursors may be highly dependent

on Akt3, I sought to further quantify overall expression of each Akt isoform in each B-lineage population relative to 18s RNA. To this end, for each B-lineage population I divided the mean signal (Ct) for each Akt isoform by the mean Ct for 18s RNA, thus yielding a delta-Ct for each Akt isoform within each population. As shown in **Figure 12B**, whereas Akt1 and Akt3 are expressed at similar levels among BM and peripheral B cell subsets, Akt2 is expressed at substantially lower levels (resulting in a significantly higher delta-Ct) in every population. In contrast, samples from mouse liver, a source of abundant Akt2 transcripts (Altomare, Lyons et al. 1998) demonstrated significantly lower delta-Ct values.

Finally, to address whether Akt3 expression increases in B-lineage cells such as pre-B cells lacking Akt1 and Akt2, I compared Akt3 transcript abundance in wild type and Akt1/2 DKO B-lineage cells. As shown in **Figure 12C**, Akt3 expression was only modestly increased (1.6-fold) in Akt1/2 DKO pre-B cells compared to their wild type counterparts. Interestingly, whereas Akt3 transcript expression was unchanged in most other B-lineage cells, Akt3 transcripts were increased 3-fold in Akt1/2 DKO MZPs compared to wild type MZPs (**Fig. 12C**). Thus, while the absence of Akt1 and Akt2 in MZPs appears to result in increased mRNA levels for Akt3, this increase is insufficient to rescue MZ B cell development. Altogether, these data show that while Akt3 mRNA is expressed at substantial levels in developing and mature B cells, Akt3 expression alone is unable to drive MZ and B1 B cell development and follicular B cell competitive fitness.

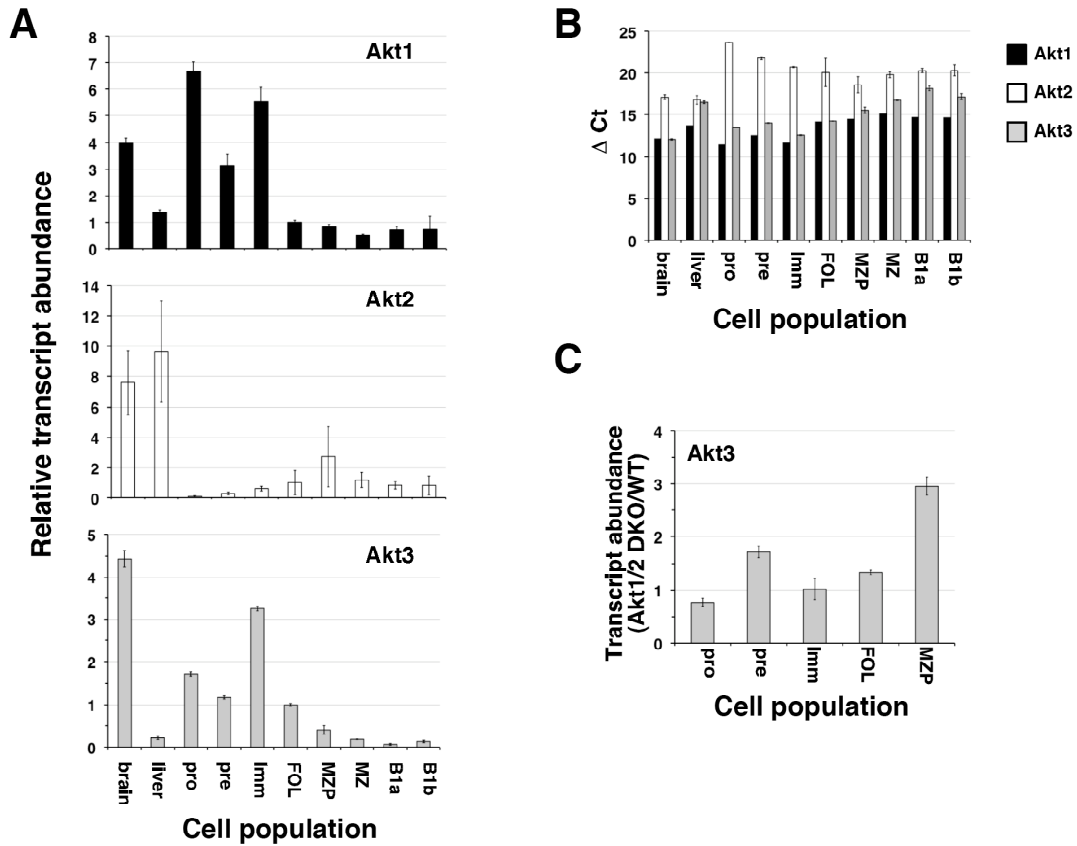


Figure 12. Expression of Akt isoforms in B-lineage Cells. (A) Relative Akt isoform expression in sorted BM, splenic and peritoneal B cell populations measured by qRT-PCR. Data are expressed relative to wild type follicular B cells. Brain and liver cDNA serve as positive controls for Akt3 and Akt2 mRNA, respectively. Error bars indicate the relative quantity minimum and relative quantity maximum for each sample. (B) Delta Ct values for Akt1, Akt2, and Akt3 relative to endogenous control 18s, within each of the indicated B cell populations as in (A). (C) Akt3 transcript abundance in sorted B cell populations from Akt1/2 DKO chimeras relative to the corresponding wild-type population.

3.2.8 Ectopic Bcl-x_L expression is insufficient to rescue marginal zone B cell development in the absence of Akt1 and Akt2.

Given that Akt plays a critical role in the regulation of pro-survival molecules down stream of PI-3K (Franke 2008) and transgenic expression of Bcl-x_L was able to mediate rescue of splenic B cell numbers in PI-3K deficient mice (Suzuki, Matsuda et al. 2003), I sought to examine the impact of retroviral mediated over-expression of Bcl-x_L in Akt1/2 DKO progenitors. Ectopic expression of Bcl-x_L was able to rescue a substantial portion of mature recirculating B cells in the bone marrow demonstrating the potency of the Bcl-x_L retrovirus. I noted a decrease in the percentage of GFP⁺ pre and immature B cells to levels more representative of a wild-type bone marrow compartment (**Fig 13A,C**). While Bcl-x_L did provide limited rescue to bone marrow B cell populations it was not sufficient to rescue MZ B cells in the spleen (**Fig 13B,C**). These data demonstrate that the pro-survival function of Bcl-x_L is insufficient to rescue MZ B cell development the absence of Akt1 and Akt2. Yet in recirculating B cell populations enhanced survival does provide benefit, highlighting the context dependent requirements for Akt in B cell development. While it is possible that Akt still plays a critical role in the survival of MZ B cells these data suggest that Akt interacts with other biochemical pathways critical to the generation of MZ B cells.

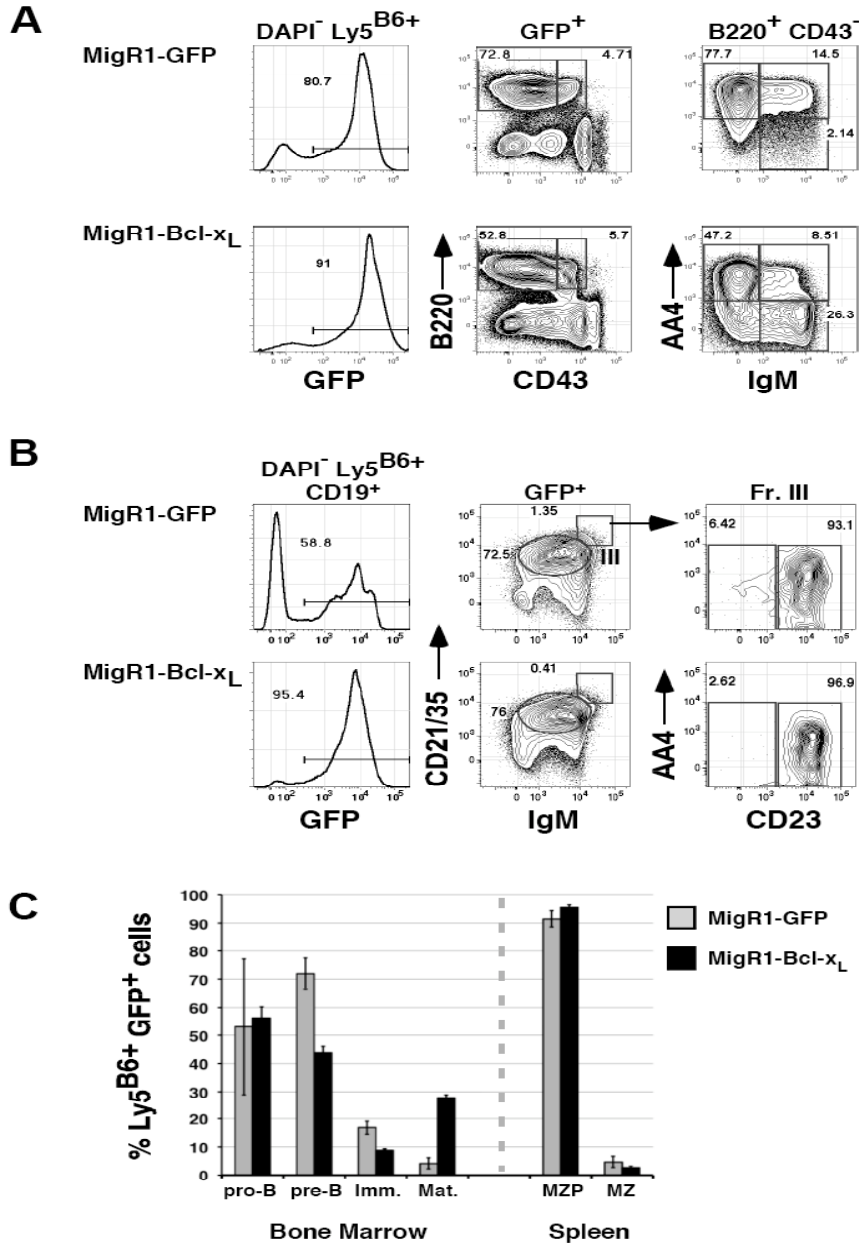


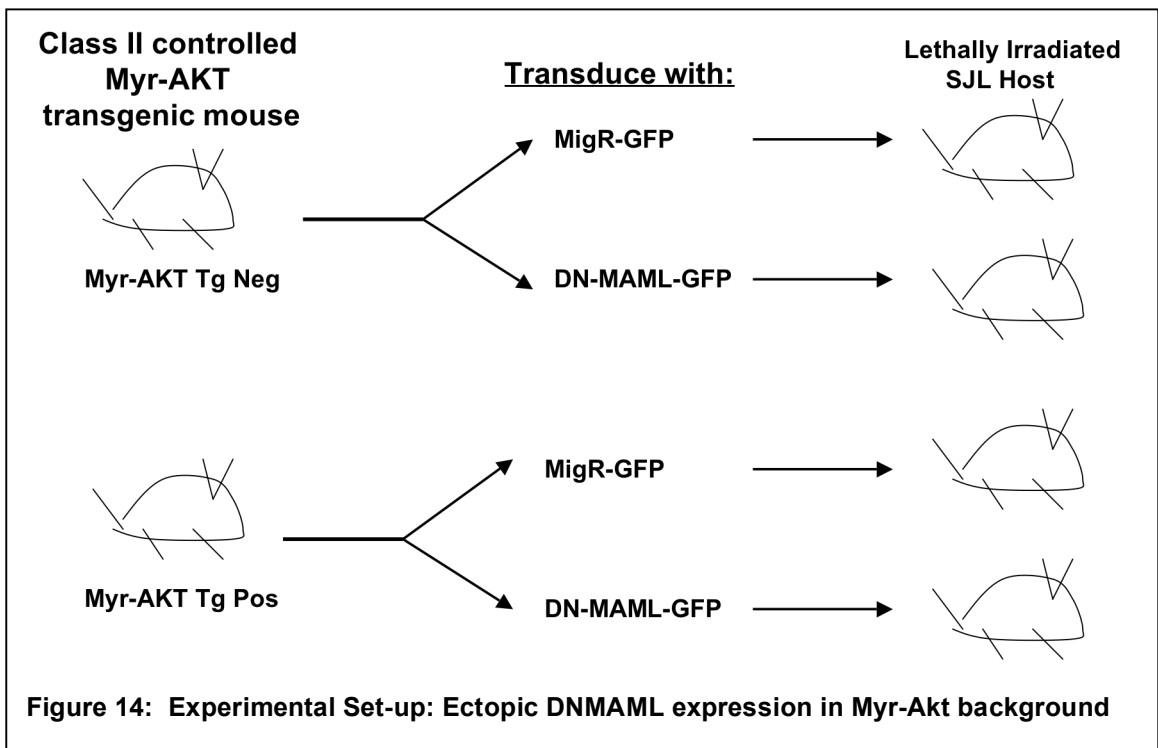
Figure 13: Impact of enforced Bcl-x_L expression fails on BM and peripheral B cell development from Akt1/2-deficient precursors. (A) Flow cytometric analysis of BM cells from chimeras established with Akt1^{-/-} Akt2^{-/-} progenitors transduced with retrovirus expressing MigR1-GFP control (Grey) or MigR1-Bcl-x_L (Black) 8 weeks previously. The left-most plots are gated on viable donor-derived (DAPI⁻ Ly5^{B6+}) cells. Numbers in plots indicate the frequency of events in the indicated gate as a function of the indicated parent population. Pro-B, CD43⁺ B220⁺ CD19⁺ AA4⁺; pre-B, CD43^{low} B220⁺ IgM⁻ AA4⁺; immature B, CD43⁻ B220⁺ IgM⁺ AA4⁺; mature B, CD43⁻ B220⁺ IgM⁺ AA4⁻. (B) Flow cytometric analysis of spleen cells from chimeras in (A); MZ B cells are gated as in Figure 2. For (A) and (B) numbers in plots indicate the frequency of events as a function of the indicated parent population. (C) Percent of donor-derived GFP⁺ cells within the pro, pre, immature, mature B cells pool of the BM and within the MZ precursor (MZP) and MZ B cell gates. Error Bars represent SEM for 2 mice in MigR1-GFP and 3 mice in MigR1-Bcl-x_L.

3.2.9 The requirement for Notch in MZ B cell development is Akt independent.

A factor critical to MZ B cell development is the γ -secretase substrate Notch (Tanigaki, Han et al. 2002; Saito, Chiba et al. 2003; Hozumi, Negishi et al. 2004; Maillard, Weng et al. 2004; Yagi, Giallourakis et al. 2008). Recent experiments in thymocytes and follicular B cells support a role for the activation of Akt downstream of the Notch pathway (Ciofani and Zuniga-Pflucker 2005; Thomas, Calamito et al. 2007). Given my observation that Akt1 and Akt2 are required for the generation of MZ B cells, I next sought to examine a potential Notch-Akt connection in MZ B cell development. To test this hypothesis I utilized mice harboring a constitutively active N-terminal myristoylated Akt1 transgene (herein termed Mry-Akt) controlled by the MHC class II promoter (Kouskoff, Fehling et al. 1993; Tuttle, Gill et al. 2001). This construct is expressed in a manner consistent with endogenous MHC class II genes and therefore will be expressed in mature B cells which maintain MHC class expression basally (Glimcher and Kara 1992). Notch signaling was inhibited by retroviral expression of a dominant negative mastermind version of the Notch/CSL co-activator MAML1 (DNMAML) transduced into Myr-Akt positive or littermate control Myr-Akt negative bone marrow (**Fig 14**). The DNMAML construct has previously been shown to block MZ B cells development and recapitulate Notch loss of function studies (Maillard, Weng et al. 2004).

Splenic B cells from chimeras established with control MigR1-GFP in Myr-Akt positive progenitors demonstrated consistently increased frequencies of MZ

B cells demonstrating the activity of the Myr-Akt transgene and its positive effect on MZ B cell development (**Fig 15A**). Despite the ability of Myr-Akt to positively impact MZ B cell development/ maintenance, expression of DN MAML in Myr-Akt positive B cells continued to block MZ B cell development. (**Fig 15B**) This data suggests, that while Notch can activate Akt in B cells, Akt activation alone is insufficient to replace Notch signaling.



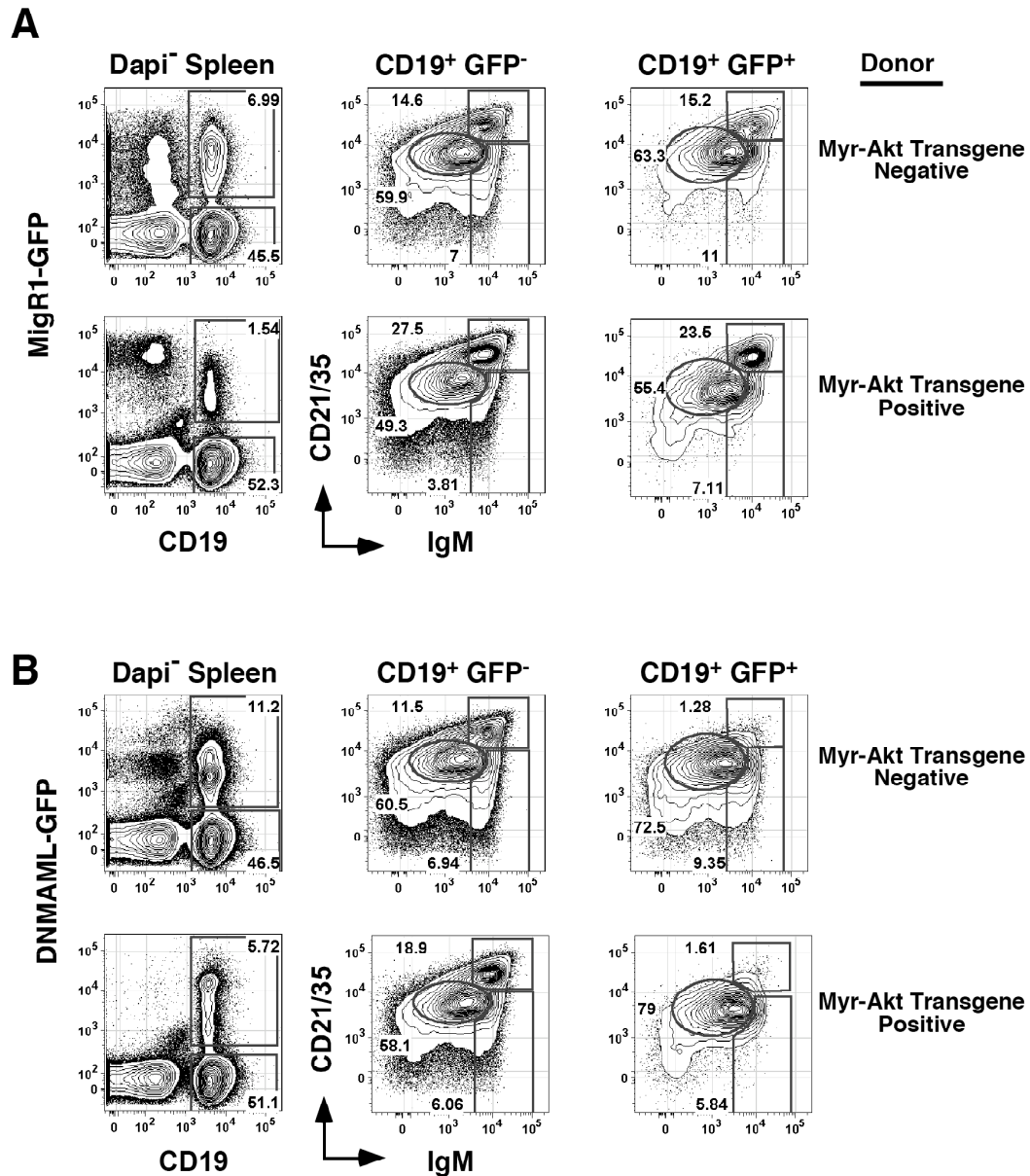


Figure 15: Myristoylated Akt increases MZ B cell frequency but is insufficient to rescue Notch inhibition. (A) Flow cytometric analysis of splenic cells from chimeras established with MigR1-GFP transduced bone marrow from Myristoylated-Akt (Myr-Akt) negative littermate control (top) or Myr-Akt positive (bottom) twelve weeks post reconstitution. The left most plots are gated on live (Dapi⁻) splenocytes. Middle plots gated on CD19⁺ GFP⁻ cells, right most plots gated on CD19⁺ GFP⁺. Fraction I, II, and III gated as in figure 4. Numbers represent frequencies of parent gate. **(B)** Flow cytometric analysis of splenic cells from chimeras established with dominant-negative mastermind (DNMAML) transduced bone marrow from Myr-Akt negative littermate control (top) or Myr-Akt positive (bottom) twelve weeks post reconstitution. Gating as in (A). Representative of two (MigR1-GFP control) or three (DNMAML) mice.

3.3 Chapter 3 Summary-Brief Discussion

I have characterized B cell development in the absence of Akt1 and Akt2. I find that signals derived from Akt1 and Akt2 are not required for the pro to pre B transition, indeed the total cell counts for each of these respective pools is increased relative to controls. In the periphery, MZ and B1 cell development is dramatically impaired, and the competitive fitness of FOL B cells is compromised in the absence of Akt1 and Akt2 (**Fig 16**). This phenotypic characterization is reminiscent of the B cell phenotypes observed in PI-3K and CD19 loss of function animals (Rickert, Rajewsky et al. 1995; Donahue, Hess et al. 2004; Okkenhaug, Ali et al. 2007) and supports the hypothesis that Akt is a critical target of the PI-3K pathway in peripheral B cell development. The increased frequency and number of pro, pre and immature B cells in the bone marrow of Akt1/2 deficient chimeras could suggest a bottleneck in development by which the remaining Akt3 is unable to efficiently and effectively signal maturation in the absence of Akt1 and Akt2. Alternatively, Akt3 may activate distinct downstream signaling pathways that exert positive effects on early B cell survival and/or proliferation. Clearly signals from Akt1 and Akt2 at these developmental stages are not required for development or survival of these cells. This is in contrast to observations made in thymocyte development, in which a severe arrest occurs at the DN3 stage in Akt1/2 deficient thymocytes (Juntilla, Wofford et al. 2007). These differences may reflect Akt3 expression pattern variations as Akt3 is expressed at detectable levels in early B cells, but down regulated dramatically in developing thymocytes. In this model, the aggregate Akt signaling required to

generate T cells is not reached in the absence of Akt1 and Akt2, but is achieved for early B cells. Alternatively, the phenotype could reflect the outcome of specific downstream targets of Akt3 vs Akt1 and/or Akt2 and their ability to support early B cell generation. Examination of Akt 1-3 triple knockout B cells will be required to elucidate Akt3's role in early B cell development. Interestingly, I find that the BCR repertoire of Akt1/2 deficient B cells is altered with increased frequencies of λ^+ cells, in both mature and immature B cell subsets, these observations are in agreement with CD19 deficient B cells, which also display increased frequencies of λ^+ cells across immature and mature B cells subsets (Diamant, Keren et al. 2005). The increased λ^+ frequency could represent changes in BCR selection as a result of altered BCR complex signaling outcomes during development in the absence of Akt. A notion supported by our in-vitro data illustrating defective proliferative responses to BCR cross-linking. Alternatively, Akt signaling could be important in shutting down Rag transcription as observed by Amin and colleagues (Amin and Schlissel 2008) as such it is possible that cells in the pro and immature B cell stages can not properly regulate Rag, resulting in continued expression and exhaustion of the κ locus and eventual progression to the λ locus.

In the periphery, B1 and MZ B cells are thought to be a selected pool, requiring stronger signals through the BCR (Lopes-Carvalho and Kearney 2004), given the ability of CD19 to increase the sensitivity of the BCR signaling (Fujimoto, Poe et al. 2002) it is possible that the loss of MZ and B1 B cells in Akt 1/2 DKO chimeras reflects an inability to generate the "proper" strength of signal

through the BCR-CD19 complex. I can conclude that the block in MZ development is not the result of poor survival, as ectopic expression of Bcl-x_L was unable to rescue development of these cells. Interestingly, it was previously reported that a Bcl-2 transgene could rescue MZ development in CD19 deficient animals (Otero, Anzelon et al. 2003). These data suggest that Akt may be required in both a CD19 dependent and independent manner during the generation of MZ B cells. Furthermore, Suzuki et al. demonstrated that a Bcl-x_L transgene could rescue B220⁺ cells in PI-3K deficient splenocytes, however these workers did not subset B cell populations and thus the MZ B cell compartment was not examined (Suzuki, Matsuda et al. 2003). It should be noted that numerous receptors possess the capacity to activate PI-3K and hence Akt, including but not limited to chemokine, cytokine, toll-like and co-stimulatory receptors (Fruman, Snapper et al. 1999; Jou, Carpino et al. 2002; Okkenhaug and Vanhaesebroeck 2003; Donahue, Hess et al. 2004; Fruman 2004). One such pathway I explored was the Notch pathway, previously shown to activate Akt in developing thymocytes (Ciofani and Zuniga-Pflucker 2005; Palomero, Sulis et al. 2007). My results indicate that the presence of a constitutively active form of Akt selectively expressed in mature B cells failed to rescue MZ development in the presence of DNMA1L, suggesting that any potential Notch-Akt signaling axis does not require activation of Akt to generate MZ B cells.

This similarity of developmental phenotypes and defective in-vitro proliferative responses of Akt1/2 deficient and CD19/PI-3K deficient animals supports the hypothesis that Akt is a critical downstream target of the PI-3K in

the context of B cell development and activation. Clearly, additional experiments addressing the potential of myr-Akt to rescue the CD19 deficient phenotype or assessment of positive/negative selection of BCRs with heavy chain transgenic systems will be required to more definitively demonstrate the role of Akt1/2 downstream of the BCR/CD19 in the context of B cell development.

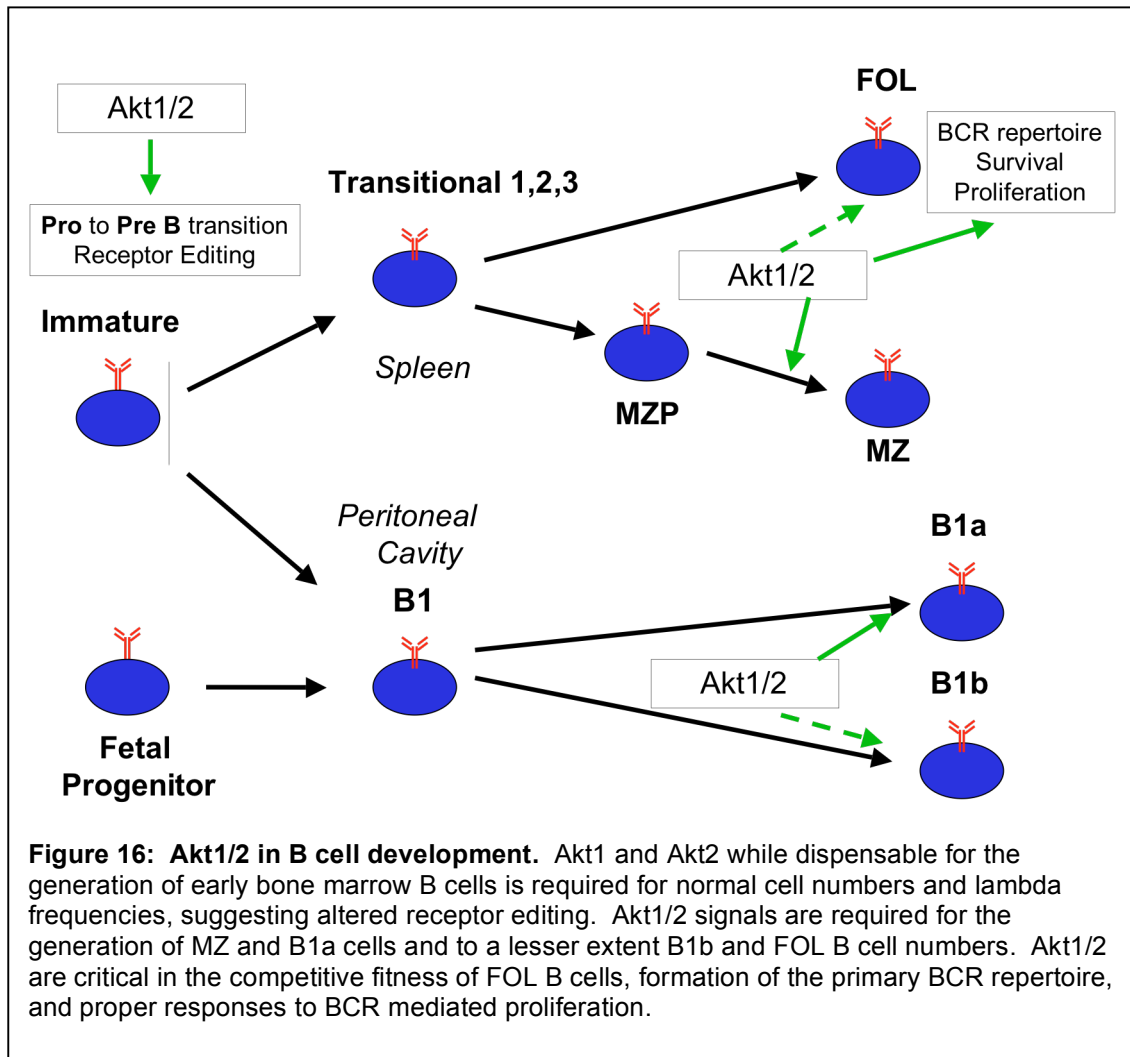


Figure 16: Akt1/2 in B cell development. Akt1 and Akt2 while dispensable for the generation of early bone marrow B cells is required for normal cell numbers and lambda frequencies, suggesting altered receptor editing. Akt1/2 signals are required for the generation of MZ and B1a cells and to a lesser extent B1b and FOL B cell numbers. Akt1/2 are critical in the competitive fitness of FOL B cells, formation of the primary BCR repertoire, and proper responses to BCR mediated proliferation.

Chapter 4. Notch independent γ -secretase functions in B cell development and activation

4.1 Introduction: Role γ -secretase complex in lymphocytes.

The Notch pathway has a well documented role in the development of MZ B cells and though dispensable for the development of FOL B cells it does enhance their activation (Tanigaki, Han et al. 2002; Saito, Chiba et al. 2003; Hozumi, Negishi et al. 2004; Thomas, Calamito et al. 2007). Notch signaling is initiated by receptor-ligand binding. This binding enables metalloproteases to cleave the extracellular domain of the Notch receptor and leads to ectodomain shedding. Shedding opens the remaining intramembrane region of the Notch receptor to be cleaved by the γ -secretase complex, freeing the intracellular domain of Notch to translocate to the nucleus and activate Notch target genes (Maillard, Fang et al. 2005). While Notch is a critical target of γ -secretase activity in MZ B cell development, numerous reports demonstrate the importance γ -secretase activity on signaling pathways independent of Notch (Citron, Vigo-Pelfrey et al. 1994; Kopan and Ilagan 2004; Parks and Curtis 2007; Hass, Sato et al. 2009; Li, Wolfe et al. 2009). Perhaps the best example is the amyloid precursor protein (APP), which when mutated, promotes the formation of a 42 amino acid γ -secretase cleavage product at the expense of the normal 40 amino acid product. This larger cleavage product more readily forms plaques and is associated with early onset Alzheimer's disease (Citron, Vigo-Pelfrey et al. 1994). The γ -secretase complex is a multi-protein membrane-imbedded aspartyl protease, consisting of four proteins: Presenilin, Presenilin enhancer (PEN2),

Nicastrin, and Anterior Pharynx-Defective-1 (Aph-1) (Li, Wolfe et al. 2009). The catalytic activity of γ -secretase, which is contained in the PS1 and PS2 proteins, drives the cleavage of a large number of single-pass transmembrane proteins of which over 80 substrates have been identified (Parks and Curtis 2007; Li, Wolfe et al. 2009). The large number of γ -secretase targets has caused it to be loosely referred to as the “proteasome of the membrane” (Kopan and Ilagan 2004). To add an additional layer of complexity, γ -secretase can be isolated from the plasma membrane, the endoplasmic reticulum and the golgi, potentially implicating its function in a diverse array of cellular processes. These observations raise the possibility that the γ -secretase complex could play both Notch dependent and Notch independent roles in B cell development and activation.

Two recent publications have elucidated a role for the catalytic components (Presenilin 1 and Presenilin 2) of the γ -secretase complex, in the development of thymocytes and MZ B cells (Laky and Fowlkes 2007; Yagi, Giallourakis et al. 2008). However, these studies did not differentiate between Notch dependent and Notch independent functions of Presenilin proteins. Indeed, their respective observations are in agreement with Notch loss of function approaches suggesting that the loss of Presenilins and thus γ -secretase activity alters T and B cell development at the expected stages, yet the decreased calcium mobilization in splenocytes activated through the BCR in Presenilin deficient B cells (Yagi, Giallourakis et al. 2008) is not observed in B cells with a Notch loss of function (Tanigaki, Han et al. 2002). To clarify these

issues, I examined B cell subset development and BCR and TLR-mediated B cell activation upon deletion of PS1 and/or PS2 or inhibition of Notch activity in developing and mature B cells. Our studies suggest that Presenilins promote B cell activation, B1 B cell development, and establishment of the primary BCR repertoire without engagement of the Notch pathway.

4.2 Chapter 4 Results.

4.2.1 Optimal BCR mediated proliferation requires γ -secretase in a Notch independent manner.

Recent studies from this lab demonstrated a role for γ -secretase activity in B cell activation through a synergistic effect of the γ -secretase substrate Notch and BCR (Thomas, Calamito et al. 2007). These experiments utilized a co-culture system in which B cells were added to OP9 stromal cells engineered to express the Notch ligand Delta-like 1 (OP9-DL1). Given that splenic B cells express mRNA for DL1 (Hozumi, Negishi et al. 2004), one possibility is that BCR-induced B cell proliferation is amplified via interactions between Notch2 and DL1 on neighboring B cells. To address this possibility I first tested addition of γ -secretase inhibitor (GSI, compound E) to purified CFSE-labeled B cells stimulated with anti-IgM antibody or TLR ligand and measured proliferation *in vitro*. After 3 days in culture, GSI treated cells demonstrated significantly reduced proliferation to various concentrations of anti-IgM antibody (**Fig. 17A**). Notably, this effect was specific to stimulation through the BCR, as proliferation in response to LPS and CpG was not significantly altered (**Fig. 17B**). Importantly, exposure of CD23⁺ B cells to GSI was not toxic to B cells when compared to vehicle controls (**Fig. 17C**).

Because multiple γ -secretase substrates outside the Notch pathway have been identified, I next sought to test whether specific inhibition of Notch activity also diminished BCR-induced proliferation. To this end, I harvested follicular B cells from mice carrying an inducible (Cre-dependent) allele encoding a dominant

negative version of the Notch/CSL coactivator MAML1 in the Rosa26 locus. This dominant negative form of MAML1, termed DNMA11, recapitulates all known loss-of-function mutations associated with the Notch pathway in the hematopoietic system, and is expressed as a GFP fusion protein upon Cre-mediated deletion of a transcriptional stop cassette in the Rosa26 promoter (Tu, Fang et al. 2005). Nearly half of follicular B cells from Rosa26^{+ /DNMA11-GFP} mice pulsed with purified Tat-Cre fusion proteins and rested for 16 hours became GFP⁺ (**Fig. 18A**). This approach allowed me to examine the impact of DNMA11 expression in isolated B cells without potential complications arising from inhibiting Notch activity during B-lineage development and selection in the BM. Thus, GFP⁺ and GFP⁻ CD19⁺ cells from Tat-Cre cultured Rosa26^{+ /DNMA11-GFP} B cells were stimulated with increasing doses of anti-IgM or the TLR4 agonist LPS with or without addition of GSI. Three days later cells were pulsed with ³H-thymidine. As shown (**Fig. 18B**), inhibition of CSL-dependent Notch signals had no affect on BCR-induced proliferation across increasing doses of anti-IgM antibody. Significantly however, exposure of both GFP⁺ and GFP⁻ cells to GSI resulted in a dramatic drop in ³H-thymidine incorporation (**Fig. 18B, D**). Furthermore, I also observed normal BCR-induced proliferation in follicular B cells harvested from CD19^{+ /Cre} Rosa26^{+ /DNMA11-GFP} mice (not shown). Altogether, these data further demonstrate that γ -secretase activity is required for optimal B cell proliferation in response to BCR cross-linking, and indicate that γ -secretase promotes optimal BCR signaling independently of CSL mediated Notch activity

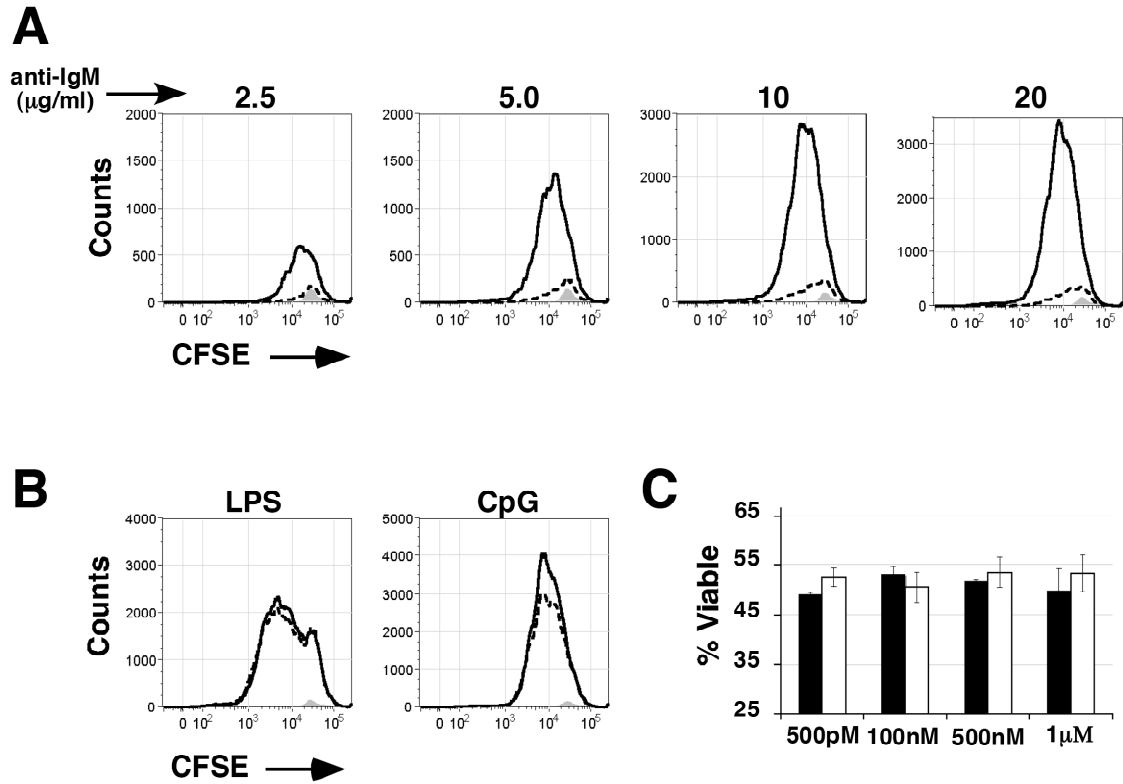


Figure 17. γ -Secretase inhibitors diminish BCR-mediated proliferative responses (A) CFSE-labeled $\text{CD}23^+$ follicular B cells are stimulated with increasing concentrations (as indicated) of anti-IgM antibody (DMSO/black line) or γ -secretase inhibitor (100nM GSI, dotted line) **(B)** Cells isolated as in (A) stimulated with LPS (1 $\mu\text{g/ml}$) or CpG (0.1 μM) (DMSO/black line) or (GSI, dotted line). **(C)** $\text{CD}23^+$ splenic B cells cultured *in-vitro* for 24hrs in the indicated doses of GSI and viability assessed.

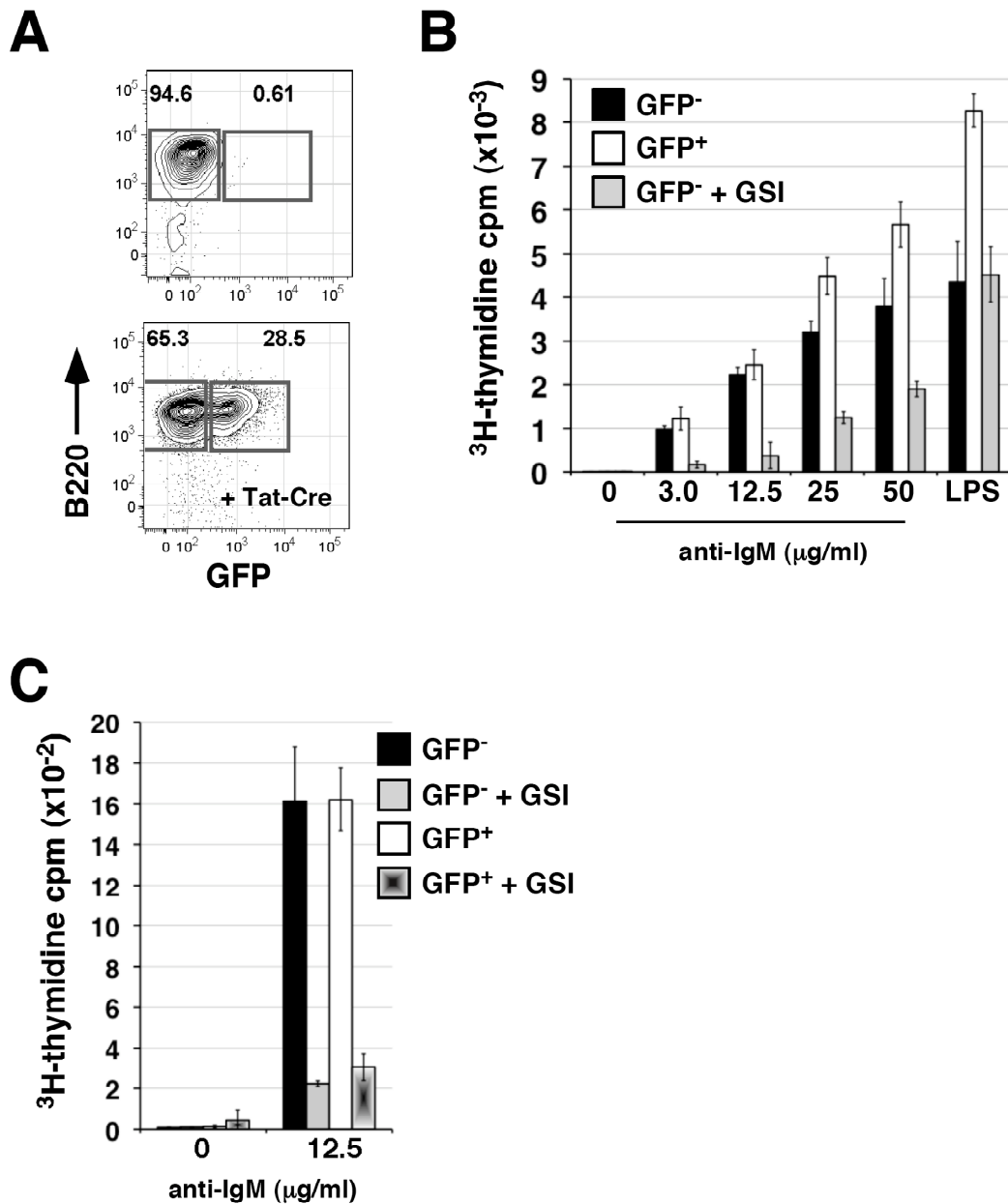


Figure 18: GSI diminishes BCR mediated proliferation is Notch independent. (A) CD23⁺ splenic B cells from Rosa26^{+/DNMAML-GFP} mice were pulsed with Tat-Cre for 16 hours before sorting DAPI⁻ B220⁺ GFP⁺ and DAPI⁻ B220⁺ GFP⁻ cells. (B) ³[H]-thymidine uptake by sorted GFP⁺ or GFP⁻ cells from (B) after three days stimulation with the indicated doses of anti-IgM antibodies or LPS (1μg/ml). Solid black bar, GFP⁻; solid white bar, DNAMAML-GFP⁺; solid grey bar, DNAMAML-GFP⁻ plus 100nM GSI. (C) ³[H]-thymidine uptake by sorted GFP⁺ or GFP⁻ from (B) treated for three days in the presence of 12.5μg/ml anti-IgM. Bars as in (C) except the grey bar with black shading indicates DNAMAML-GFP⁺ cells cultured with GSI. Results are representative of 2-3 separate experiments.

4.2.2 Presenilins promote optimal BCR but not TLR-induced proliferation.

Given that GSI negatively affects anti-IgM but not LPS or CpG-induced B cell proliferation when added to wild type or Notch-inhibited (DNMAML1-GFP⁺) B cells (**Fig. 17 and 18**), I tested whether B cells lacking the catalytic components of γ -secretase exhibit suboptimal proliferative responses to BCR cross linking and whether this effect is unique to the BCR. In this regard, one recent study from Yagi et al. showed that B cells lacking Presenilin 1 (PS1) and Presenilin 2 (PS2) fail to enter the cell cycle upon either anti-IgM or LPS stimulation (Yagi, Giallourakis et al. 2008). However I find that GSI fails to impact B cell proliferation in response to LPS or CpG stimulation (**Fig. 17**). To address these issues I first generated C57BL/6-backcrossed CD19^{+Cre} mice harboring two floxed PS1 alleles and two null alleles for PS2. For simplicity I will refer to the resulting CD19^{+Cre} PS1^{ff} PS2^{-/-} mice as PS1/2 DKO mice throughout this dissertation.

To clarify the cell-intrinsic role of Presenilins in BCR and TLR-mediated B cell proliferation, I mixed CFSE-labeled CD23⁺ splenic B cells from PS1/2 DKO (Ly5^{B6}) and wild type B6.Ly5^{SJL} mice at a 3:1 ratio of PS1/2 DKO and wild type cells, and stimulated these cells with increasing doses of anti-IgM antibodies or LPS or CpG. After three days of stimulation with multiple doses of anti-IgM antibodies, wild type (Ly5^{SJL+}) B cells grew to dominate these cultures (**Fig. 19A**). Further, examination of CFSE dilution curves for each population revealed that a substantially smaller number of PS1/2 DKO B cells completed one or more rounds of cell division compared to wild type cells cultured under identical

conditions. Indeed, after three days cell recoveries for PS1/2 DKO B cells were 10-fold lower than wild type cells, whether such cells were mixed before stimulation or cultured separately (**Fig. 20A,B**). Furthermore, addition of GSI to PS1/2 DKO B cells did not result in additional decreases in anti-IgM induced proliferation, indicating that GSI specifically inhibits Presenilins activity in this context (**Fig. 20C**). However, proliferative responses of PS1/2 DKO B cells upon LPS or CpG stimulation were unchanged relative to wild type controls (**Fig. 19B**). These data show that γ -secretase activity is required for optimal BCR-mediated but not TLR-mediated B cell activation, and contrast with a recent study suggesting that Presenilins regulate responsiveness to TLR ligands such as LPS.

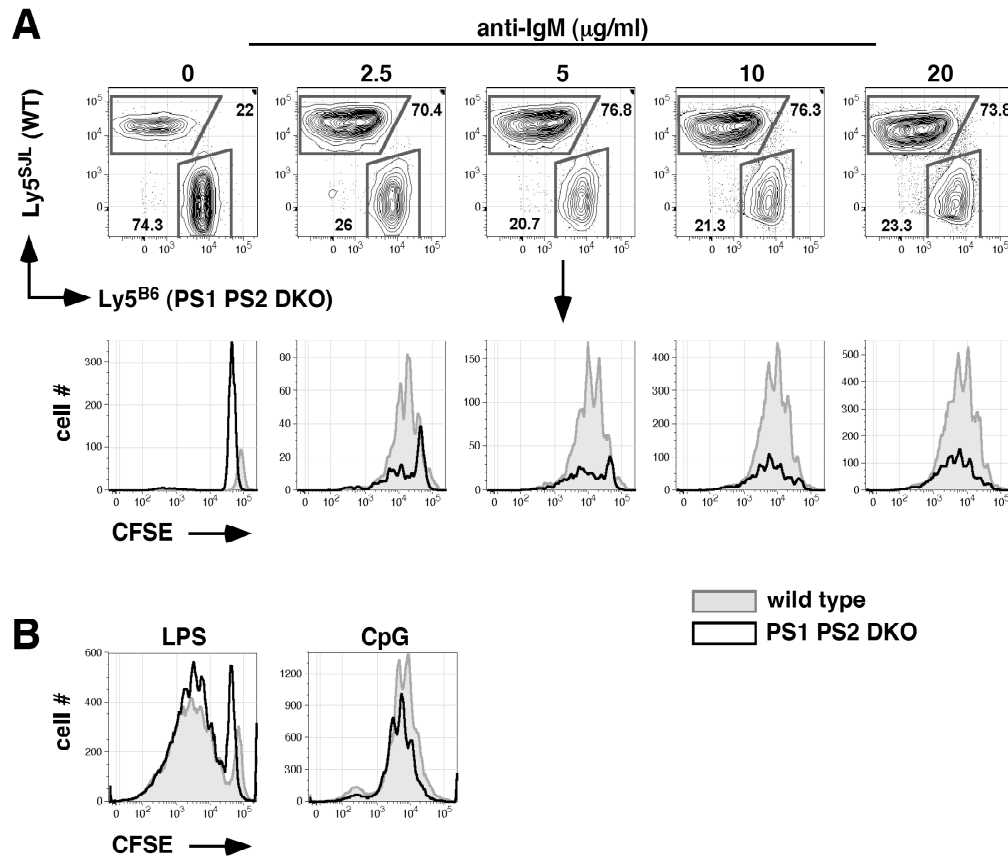


Figure 19: Presenilins promote BCR but not TLR induced proliferation in follicular B cells. (A) Ly5^{B6+} (PS1/PS2 DKO) and Ly5^{SJL+} (control) CD23⁺ splenic B cells are CFSE labeled, mixed at a 3:1 ratio, and stimulated with the indicated doses of anti-IgM antibody for three days (top panel). Plots gated on live (DAPI⁺) cells. Histograms illustrate CFSE dilution curves for PS1/PS2 DKO (black) versus wild type (grey) cells for the corresponding dose of anti-IgM antibodies. (B) CFSE-labeled CD23⁺ splenic B cells cultured with LPS (1 $\mu\text{g/ml}$) or CpG (0.1 μM) for three days. Plots gated on live (DAPI⁺) cells, PS1/PS2 DKO (black) versus wild type (grey). Representative of three experiments.

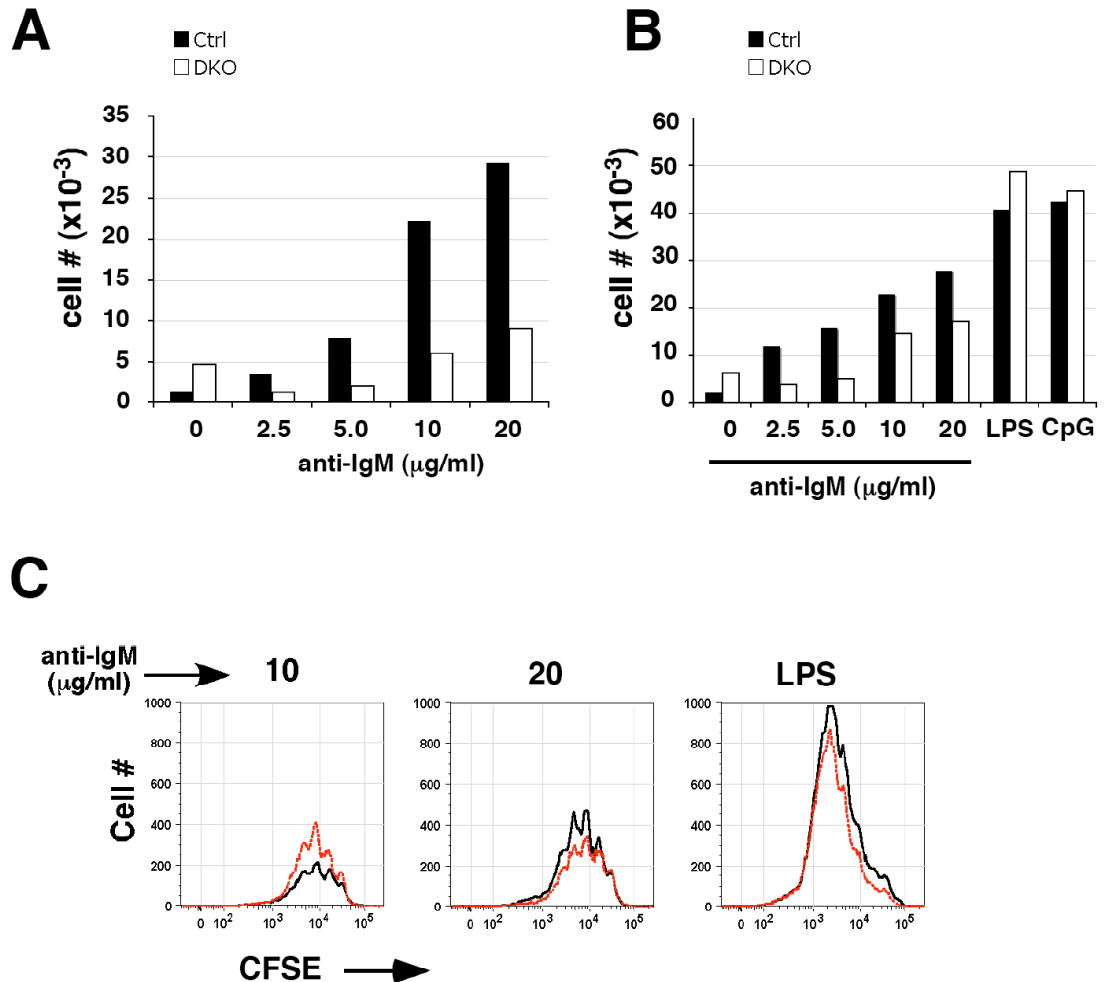


Figure 20. Presenilin proteins are required for optimal BCR mediated proliferation. (A) Viable cell recoveries of Ly5^{SJL+} (black) or Ly5^{B6+} CD19^{+Cre} PS1^{f/f} PS2^{-/-} CD23⁺ cells in a mixed *in-vitro* culture after three days. (B) Viable cell recoveries of CD19^{+Cre} PS1^{+/+} PS2^{+/+} (black) or CD19^{+Cre} PS1^{f/f} PS2^{-/-} (white) after three day *in-vitro* culture, representative of four experiments. (C) CFSE-labeled CD23⁺ splenic B cells from CD19^{+Cre} PS1^{f/f} PS2^{-/-} mice were cultured *in-vitro* for three days in the presence of vehicle control (solid black line) or 100nM GSI (red line)

4.2.3 Compromised calcium mobilization in Presenilin deficient B cells.

Increases in intracellular calcium levels enact a diverse range of functions and occur due to the induced release of calcium from the endoplasmic reticulum (ER), and the subsequent intake of extracellular calcium through channels in the plasma membrane in response to depletion of ER calcium stores (Scharenberg, Humphries et al. 2007; King and Freedman 2009). A wealth of data indicate that Presenilins regulate the release and utilization of calcium from the endoplasmic reticulum (ER) in neurons (Cai, Lin et al. 2006; Tu, Nelson et al. 2006; Cheung, Shineman et al. 2008) and recent data indicate that Presenilin-deficient thymocytes exhibit compromised calcium mobilization upon aggregation of the T cell receptor (Laky and Fowlkes 2007). To test whether diminished BCR-induced B cell activation correlates with suboptimal calcium responsiveness, I isolated CD23⁺ splenic B cells from PS1/PS2 DKO mice as well as CD19^{+Cre} and CD19^{+Cre} Rosa26^{+DNMAML1-GFP} controls, labeled these cells with Indo-1AM, and added anti-IgM cross linking antibodies.

In media containing free calcium I observed delayed and marginally decreased calcium mobilization in PS1/PS2 DKO B cells relative to all controls including GFP⁺ cells from CD19^{+Cre} Rosa26^{+DNMAML1-GFP} mice (**Fig. 21A**). As expected, calcium mobilization was not depressed in Notch-inhibited B cells. Indeed, in many experiments DNMAML1-GFP⁺ B cells exhibited a slightly increased calcium response (**Fig 21A**). To test directly whether Presenilins regulate calcium release from the ER, I chelated extracellular calcium by adding EGTA before aggregation of the BCR. As shown (**Fig. 21B**), PS1/PS2 DKO B

cells displayed a modest but consistent decrease in ER calcium store release upon BCR stimulation

With separate samples I also tested whether Presenilins influence the intake of extracellular calcium by adding thapsigargin, which depletes ER calcium stores by blocking SERCA pumps in the ER. As shown (**Fig. 21C**), CD19^{+Cre} control and PS1/PS2 DKO B cells exhibited similar levels of calcium uptake after adding thapsigargin, suggesting that Presenilins do not play a major role in calcium homeostasis downstream of the ER. Furthermore, initial calcium levels within the ER of unstimulated cells appeared normal, as baseline calcium levels were comparable for CD19^{+Cre} and PS1/PS2 DKO B cells (**Fig. 21A, B**), and in mutant and control samples increases in intracellular calcium levels were not significantly different upon adding thapsigargin in the presence of EGTA (**Fig. 21D**). These data are consistent with the notion that Presenilins promote the BCR-mediated release of intracellular calcium stores from the ER.

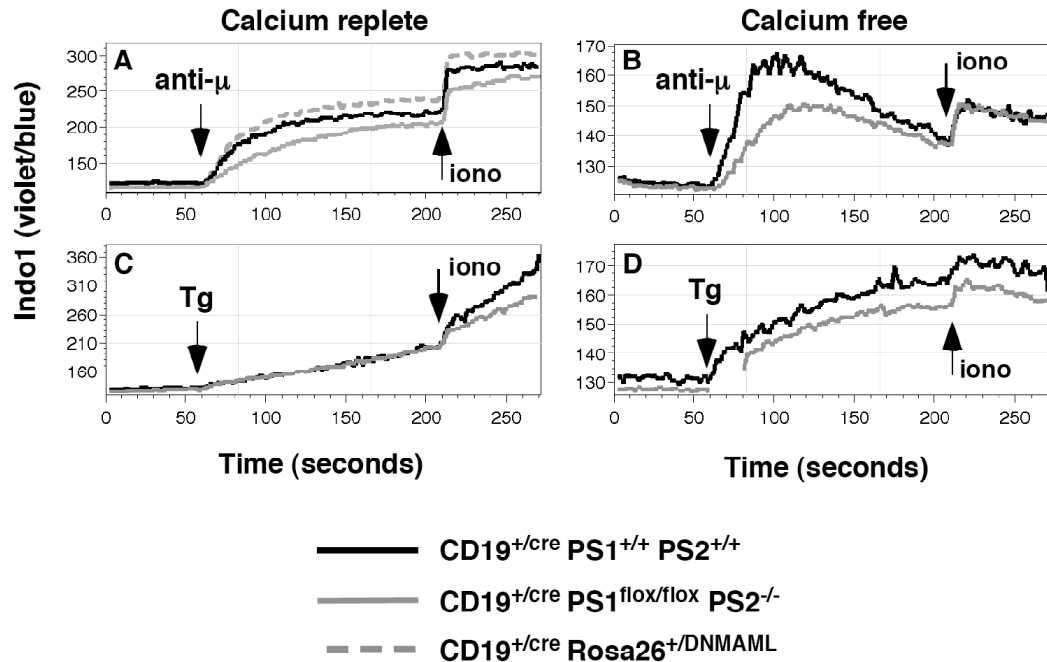


FIGURE 21. Presenilins promote optimal BCR-induced ER Ca²⁺ release. (A) Mean of calcium mobilization measurements in Indo-1AM loaded CD23⁺ splenic B cells isolated from control (solid black line), PS1/PS2 DKO (solid grey line), and CD19^{+/Cre} Rosa26^{+/DNMAML-GFP} (dashed grey line) after stimulation with 15 μ g/ml anti-IgM antibody in calcium-containing media. (B) Calcium mobilization cells isolated and stimulated as in (A) in the presence of 12.5 mM EGTA. (C) Calcium mobilization analysis of cells isolated as in (A) treated with 1 μ M thapsigargin in calcium-containing media. (D) Analysis of calcium mobilization in cells isolated as in (A) and stimulated with 1 μ M thapsigargin in the presence of 12.5 mM EGTA.

4.2.4 Presenilins are critically required for the development of B1 B cells.

Previous reports suggest that enhanced BCR signaling drives the formation of B1 B cells (Lam and Rajewsky 1999; Casola, Otipoby et al. 2004). Given that PS DKO B cells exhibited suboptimal responsiveness to BCR aggregation, I assessed frequencies of all peripheral B cell subsets including B1 B cells in the peritoneal cavity of mice lacking PS1 and PS2 or either PS1 or PS2 alone. Consistent with the expected loss of Notch2 function, the splenic MZ B cell pool was markedly decreased in PS1/PS2 DKO mice (**Fig 22A**). In addition, frequencies of immature transitional and mature follicular B cells in the spleen and B-lineage progenitors in the BM were unaffected, although I consistently noted a decline in surface CD21 levels in PS1/PS2 DKO B cells as reported to occur in Notch-deficient B cells (Saito, Chiba et al. 2003), and I also noted an unexplained decline in surface CD23 levels as recently noted upon enforced survival of mature B cells lacking a BCR (**Figure 22B**) (Srinivasan, Sasaki et al. 2009).

In the peritoneal cavity PS1 and PS2-deficient mice as well as PS1/PS2 DKO mice exhibited a significant decline in frequencies of all CD19⁺ B220^{low} CD43⁺ B1 B cells (**Fig. 23A**). Furthermore, whereas both B1a (CD5⁺) and B1b (CD5⁻) cells, the two chief subsets of B1 B cells (Dorshkind and Montecino-Rodriguez 2007), were equally compromised in PS1- and PS2-deficient mice, frequencies of B1a cells were especially depressed in PS1/PS2 DKO mice (**Fig. 23A**). Notably and consistent with past studies, Notch inhibition did not affect the

size of the B1 pool (**Fig. 23B**). I conclude that Presenilins promote B1 B cell development independently of the Notch pathway.

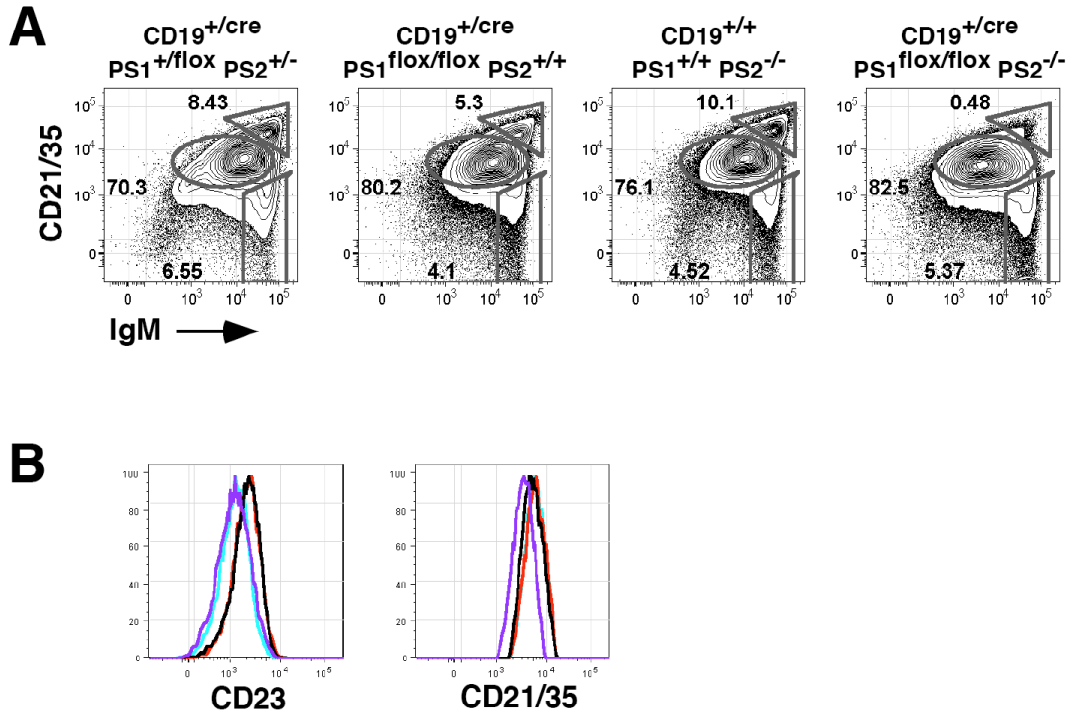


Figure 22. Impact of loss of Presenilins on splenic B cell subset composition. (A) Splenic B cells from CD19^{+Cre} PS1^{+/+} PS2^{+/+} (far left), CD19^{+Cre} PS1^{f/f} PS2^{+/+} (second from left), CD19^{+/+} PS1^{+/+} PS2^{-/-} (third from left), or CD19^{+Cre} PS1^{f/f} PS2^{-/-} (far right) were stained with the indicated antibodies before analysis of 250,000 events on an LSR2 flow cytometer. **(B)** Overlay histograms for the indicated surface proteins. Cells were gated on IgM^{int} CD21⁺ follicular B cells as shown in (A). Black CD19^{+Cre} PS1^{+/flox} PS2^{+/+}, Red CD19^{+Cre} PS1^{flox/flox} PS2^{+/+}, Blue CD19^{+/+} PS1^{+/+} PS2^{-/-}, Purple CD19^{+Cre} PS1^{flox/flox} PS2^{-/-}. Data are representative of at least three animals per group and two separate experiments.

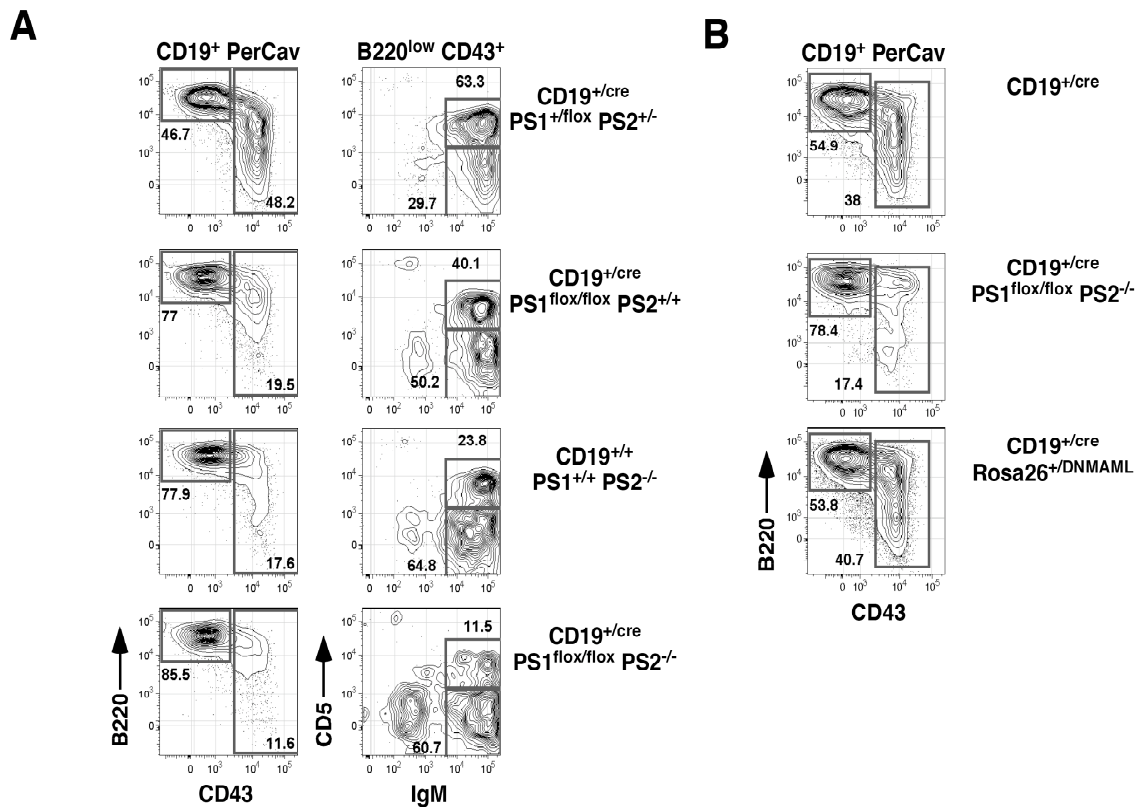


Figure 23. Defective B1 B cell development without Presenilins. (A) Peritoneal cavity lymphocytes from the indicated mice were stained with the indicated antibodies before collection of 100,000 events on an LSR2 flow cytometer. The left-most plots are gated on viable B cells (DAPI⁻ CD19⁺). Numbers in plots show the frequency of events as a function of the indicated parent gate. Representative of at least three animals per group and two separate experiments. (B) Peritoneal cavity lymphocytes from the indicated additional 12-week old mice were stained and analyzed as in (A). All panels gated on DAPI⁻ CD19⁺ cells, CD19^{+/Cre} Rosa26^{+/DNMAML}-GFP mice were also gated on GFP⁺ cells. Representative of three separate experiments.

4.2.5 The absence of Presenilin proteins leads to decreased λ^+ B cells.

Defects in B1 development, compromised anti-IgM proliferative responses, and altered Ca^+ collectively suggest that Presenilins potentiate optimal BCR signaling. Hence, given that tonic BCR signaling plays a fundamental role in formation and maintenance of the primary BCR repertoire, I reasoned that Presenilins might also play a role in BCR repertoire selection. Consistent with this possibility, mature splenic B cells ($\text{CD19}^+ \text{AA4}^-$) revealed a dramatic decrease in the percentage of λ^+ B cells (**Fig. 24A**). The ratio of mature B cells bearing κ versus λ light chains was raised from 19.61 \pm (.35) in controls to 50.32 \pm (4.9) in PS DKO B cells (**Table 4**). The decrease in λ^+ B cells was also detected in the splenic transitional B cell pool ($\text{CD19}^+ \text{AA4}^+$) from PS1/PS2 DKO mice, suggesting that the generation of λ^+ B cells is defective in the absence of Presenilin proteins. Similar observations were evident with the loss of a single isoform of Presenilin 1 or Presenilin 2 (**Fig. 24A & Table 4**). Further, examination of the BM, spleen and peritoneal cavity B cell pools demonstrated decreased frequencies of λ^+ B cells across all tissues in the absence of Presenilins (**Fig. 25**). In contrast, frequencies of λ^+ B cells in $\text{CD19}^{+/Cre}$ $\text{Rosa26}^{+/DNMAML-GFP}$ mice did not differ from the $\text{CD19}^{+/Cre}$ controls across all B cell subsets (**Fig. 25**). These data indicate that Presenilins play a fundamental role in shaping BCR repertoire without engaging the Notch pathway.

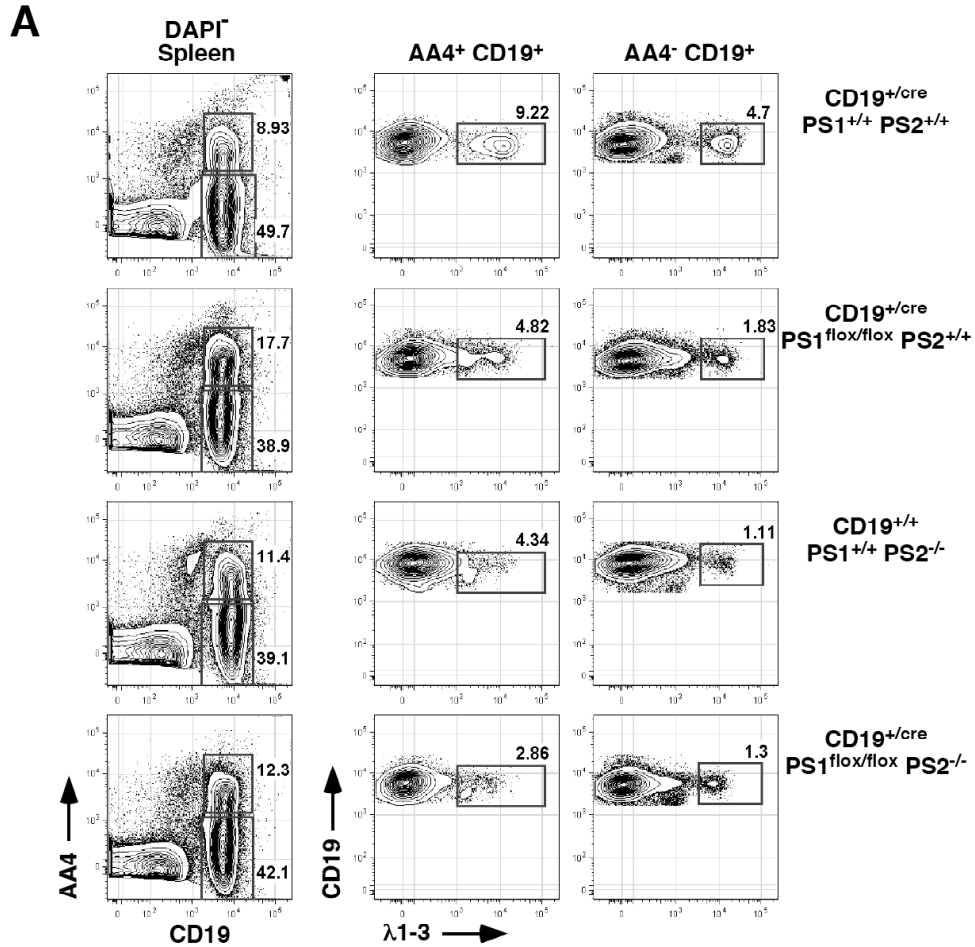


Figure 24. Generation of λ -chain positive B cells with and without Presenilins. (A) Splenocytes from 12-week old mice of the indicated genotypes were stained with the indicated antibodies before collection of 300,000 events on an LSR2 flow cytometer. The left-most panels are gated on DAPI⁻ cells, and the indicated parent gates were used to examine frequencies of I⁺ cells in transitional and mature B cells. Flow plots representative of at least three animals per group. All data representative of three separate experiments.

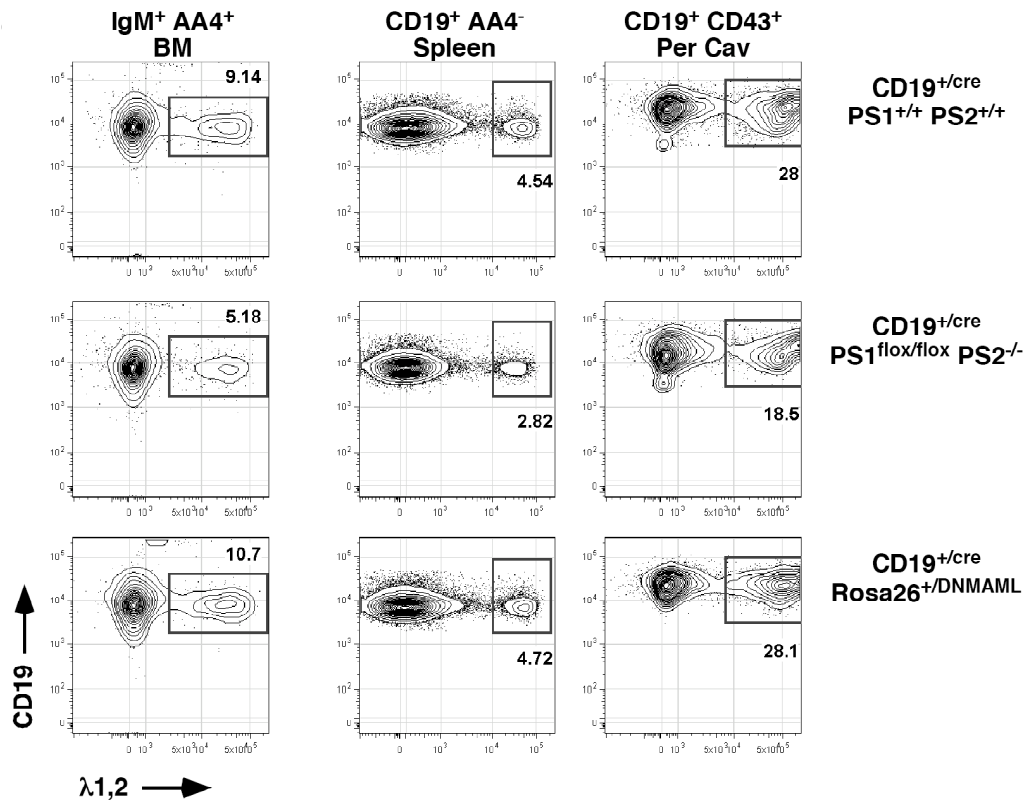


Figure 25. Decrease in λ -chain positive cells is Notch independent and occurs within the immature bone marrow B cell compartment. Representative flow cytometric data of BM immature (CD19⁺ IgM^{high} AA4⁺, left column), mature splenic (CD19⁺ AA4⁻, center column), and peritoneal cavity B1 (CD19⁺ CD43⁺, right column) B cells from the indicated mice. All plots also gated on viable (DAPI⁻) cells. For (A) and (B) numbers within plots represent the frequency of events as a function of the indicated parent gate

Table 4. Altered kappa/lambda ratios in Presenilin-deficient mice

B Cell Subset	Genotype	% λ	% κ	Ratio κ/λ
Immature	CD19 ^{+Cre} PS1 ^{+/+} PS2 ^{+/+}	8.12 (.37)	85.80 (.85)	10.62 (.60)
	CD19 ^{+Cre} PS1 ^{f/f} PS2 ^{+/+}	4.40 (.96)	80.13 (7.68)	20.66 (5.64)
	CD19 ^{+/+} PS1 ^{+/+} PS2 ^{-/-}	6.28 (2.48)	70.76 (7.02)	15.59 (5.75)
	CD19 ^{+Cre} PS1 ^{f/f} PS2 ^{-/-}	2.79 (.26)	78.58 (4.76)	28.97 (2.65)
Mature	CD19 ^{+Cre} PS1 ^{+/+} PS2 ^{+/+}	4.75 (.07)	93.13 (.20)	19.61 (.35)
	CD19 ^{+Cre} PS1 ^{f/f} PS2 ^{+/+}	1.39 (.35)	88.43 (6.17)	70.37 (13.58)
	CD19 ^{+/+} PS1 ^{+/+} PS2 ^{-/-}	.95 (.13)	79.73 (6.21)	85.47 (7.27)
	CD19 ^{+Cre} PS1 ^{f/f} PS2 ^{-/-}	1.71 (.14)	84.08 (5.10)	50.32 (4.9)

Immature B cells (CD19⁺ AA4⁺) and mature B cells (CD19⁺ AA4⁻) were gated as shown in Figure 20A. Percentages are means for 3-5 animals per group, SEMs are indicated within parentheses. Ratio calculated by dividing the frequency of κ ⁺ cells by the frequency of λ ⁺ cells for each B cell subset.

4.3 Chapter 4 Summary-Brief Discussion

In this chapter I demonstrate that FOL B cells co-cultured with GSI exhibit decreased proliferative responses to BCR cross-linking, furthermore I show that GSI is not overtly toxic to B cells. Previous studies documented a similar phenotype in T-cells stimulated through the T cell receptor (TCR) in the presence of GSI, leading to the interpretation that the Notch pathway was critical to TCR mediated proliferation (Palaga, Miele et al. 2003). Given that the γ -secretase complex can catalyze the cleavage of multiple single pass transmembrane proteins I sought to examine the involvement of the Notch pathway in GSI treated B cell cultures. To this end I employed an inducible DNMM1 construct, and demonstrate that canonical Notch signaling is dispensable for normal BCR driven proliferation. This observation does not contradict previous work from our lab highlighting a role for the Notch pathway in enhancing BCR driven responses, as the data presented herein only rule out a requirement for the Notch pathway in B cell activation.

To extend our observations beyond inhibitor based studies I choose to utilize a loss of function approach in which the catalytic components of the γ -secretase complex (Presenilins) were conditionally deleted from the B-lineage. Using FOL B cells from PS1 PS2 deficient mice I demonstrated a cell intrinsic defect in BCR responsiveness, especially at suboptimal doses of anti-IgM cross-linking antibody. These data demonstrate a role for Presenilin proteins in modulating BCR activation and agree with and extend the recent findings of Yagi et al. In contrast to observations made by Yagi et al. I do not find that B cells

lacking Presenilin proteins exhibit defective proliferative responses to LPS stimulation (Yagi, Giallourakis et al. 2008). A potential explanation for this inconsistency is that their data did not control for differences in splenic B cell subset composition in Presenilin-deficient and wild type control animals. Indeed, it is well established that MZ B cells are highly enriched for LPS-responsive cells (Oliver, Martin et al. 1997), and this population is absent from PS1/PS2 DKO B cell pools, presumably due to failed Notch2 activation. In contrast all experiments assessing B cell activation herein employed positively selected CD23⁺ B cells, which are highly enriched for FOL B cells and lack MZ B cells. Therefore I posit that the previous data suggesting that LPS responsiveness in B cells requires Presenilins instead reflect differences in B cell subset composition among Presenilin-deficient and control splenocytes.

Given the suboptimal BCR responsiveness of Presenilin deficient B cells I next examined calcium mobilization downstream of the antigen receptor. My data suggests that the magnitude of the initial release of ER calcium stores is dampened in the absence of Presenilin proteins. This observation is consistent with the notion that Presenilins modulate BCR signaling. My observation that the B1 B cell compartment is dramatically reduced in Presenilin deficient mice also supports this hypothesis, as B1's are thought to require stronger signaling through the BCR for their generation (Casola, Otipoby et al. 2004). An alternative explanation for the dearth of B1 B cells in these mice is that the maintenance and not the generation per se of the B1 B cell pool is perturbed, such that the self-renewing capacity of the B1 pool is compromised.

Finally, I observed that while FOL B cells develop in the absence of Presenilins, the BCR repertoire of this pool as indicated by the decreased frequency of λ^+ cells, is altered. Furthermore, this decrease in λ^+ cells is observed within the immature B cell populations of the spleen and bone marrow suggesting that the generation and not the maintenance of λ^+ bearing cells is defective in PS1/2 deficient cells.

In conclusion, this chapter provides the first evidence of a role for Presenilin proteins in the development, activation, calcium signaling and BCR repertoire formation of B-lymphocytes independent of the canonical Notch signaling pathway. Clearly, future studies are required to dissect how the described phenotypes are dependent or independent of the enzymatic activity of Presenilin proteins. Given that GSI is currently in clinical trials to treat Notch driven T-cell leukemia, my work emphasizes the need to further understand Presenilin and γ -secretase complex function in B cell biology.

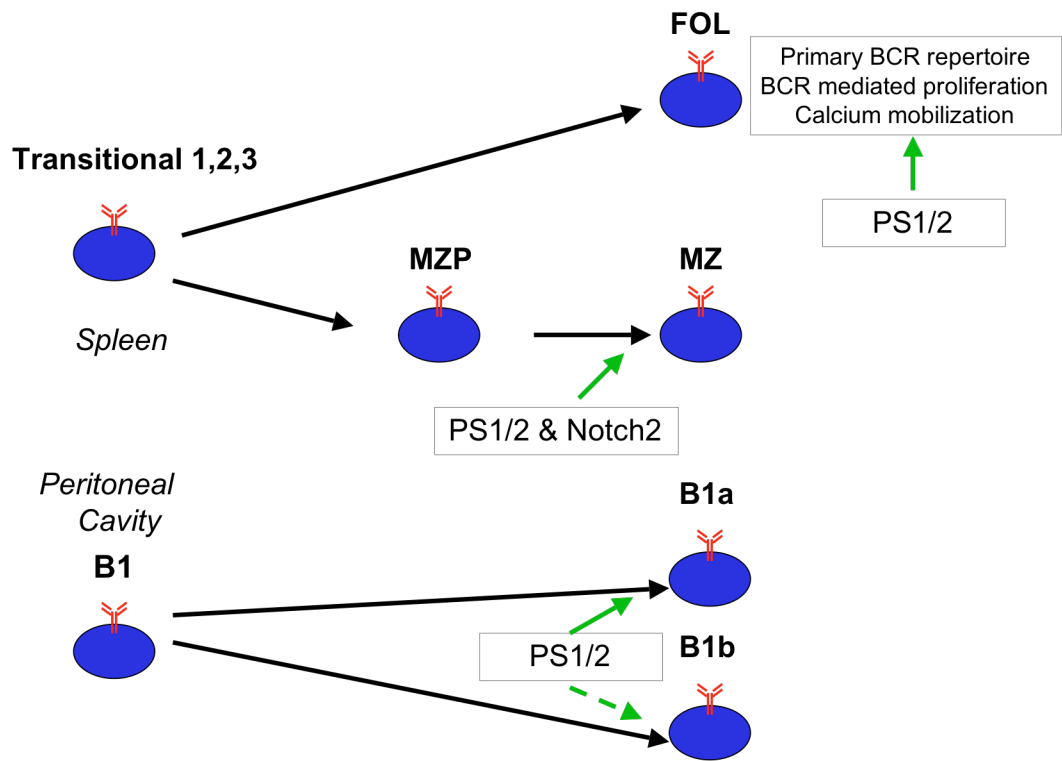


Figure 26. Role of Presenilins in B cell biology. Presenilins are required for normal B1a, B1b and BCR repertoire development independent of the Notch pathway. In addition, Presenilins promote BCR mediated proliferation and normal calcium mobilization in response to antigen receptor cross-linking. Presenilin mediated cleavage of Notch drives the MZ B cell fate.

Chapter 5: Overall Discussion

5.1 Akt1 and Akt2 in bone marrow B cell development

In this thesis, I have shown that Akt1 and Akt2 are dispensable for the development of pro, pre, and immature B cells (**Fig 5**). This result was in some ways surprising given the requirement for Akt1/2 dependent signals in analogous stages of thymocyte development (Juntilla, Wofford et al. 2007; Mao, Tili et al. 2007). The simplest explanation for my finding is that the remaining Akt3, is sufficient to promote the early stages of B cell development. Indeed, I find Akt3 mRNA in pro, pre and immature B cell populations (**Fig 12**), this is in contrast to thymocytes that express negligible levels of Akt3 mRNA and protein (Juntilla, Wofford et al. 2007). A drawback of my study is that I did not examine Akt3 protein expression of in these early B cell progenitors. Therefore it remains to be seen if the Akt3 mRNA is actually translated into protein in these cells. These data would further support the hypothesis that Akt3 is sufficient to mediate early B cell development in the absence of Akt1 and Akt2. An alternative explanation is that early B cell populations develop independently of Akt signaling in general. This notion is supported by the observation that deletion of molecules upstream of Akt activation, for example p110 δ or CD19, contain intact pro, pre and immature B cell compartments in steady state animals (Rickert, Rajewsky et al. 1995; Clayton, Bardi et al. 2002; Otero and Rickert 2003). Clearly, an Akt 3 single knockout as well as an Akt1-3 triple knockout will be required to more clearly define the role of Akt3 in bone marrow B cell generation. Interestingly, a

B cell specific Akt1-3 deficient mouse has been generated and is currently being examined (**Rickert,R personal communication**).

The increased frequency and cell numbers of these early B cell pools is a peculiar phenotype given Akt's positive regulation of cellular metabolism, survival and cell cycle. If anything, I would expect these pools to be diminished with the loss of two Akt isoforms. A potential explanation for this observation is that while Akt is dispensable for the survival of early B cells it is important for their developmental differentiation. In this model, the absence of Akt1 and Akt2 leads to an inefficient transition from the immature to mature B cell pool by an unknown mechanism, perhaps activation of transcription factors downstream of Akt signaling. For example, recent work demonstrates a role for Akt mediated degradation of the Forkhead (Foxo) family of transcription factors at the pro and immature stage of B cell development. Foxo expression at these stages increases Rag transcription. First pre-BCR and then mature BCR signaling at these respective stages activates Akt, causing Foxo degradation and cessation of Rag transcription (Amin and Schlissel 2008). This predicts that in the absence of Akt1 and Akt2 Rag down-regulation is inefficient, and by extension the regulation of other genes critical to B cell development may be abnormal. In turn this could lead to a slower developmental kinetic for B cells in the immature stage. This notion is supported by three independent but related observations. First, BCR signaling at the immature stage of development activates PI-3K and is important for promoting positive selection (Verkoczy, Duong et al. 2007). Furthermore, immature B cells treated with PI-3K inhibitors display the capacity

for “back-differentiation,” i.e. expression of surface markers and genes typically associated with the pro and pre B cell stages of development (Tze, Schram et al. 2005). Finally, mice deficient in p110 δ or CD19 possess immature B cells with increased levels of Rag mRNA and contain an increased percentage of λ + cells, thus PI-3K-Akt signaling may be critical to prohibiting receptor editing in positively selected B clones (Diamant, Keren et al. 2005; Llorian, Stamataki et al. 2007). The immature B cell populations in chimeras established with Akt1/2 deficient progenitors have increased frequencies of λ + cells, similar to CD19 deficient animals, suggesting enhanced receptor editing; possibly the result of crippled BCR positive selection in the absence of Akt1 and Akt2. If Akt1/2 deficient immature B cells are not signaling differentiation efficiently then they might be susceptible to the “back-differentiation” observed by Tze et al. and this may explain the increased percentage and numbers of cells found in the pro and pre B cell gates with flow cytometric analysis. This model may also address the increased representation of Akt1/2 deficient B cells in competitive mixed fetal liver chimeras (**Fig 9B**). Assessment of turnover rates in the pro, pre and immature B cell compartments by Brd-U pulse-chase, will further understanding of the kinetics at which cells exit these respective pools. My hypothesis is that if Akt signals promote efficient differentiation, then turnover rates in each respective compartment will be decreased. In addition, if Akt regulates Rag expression, then sorted immature Akt1/2 deficient B cells should express increased Rag transcripts. If these predictions hold true, one could speculate

that ectopic expression of an siRNA capable of Foxo knockdown might restore the bone marrow B cell compartment to normal proportions.

Recent data from Derudder et al. indicate that NF- κ B deficiency leads to increased frequencies of pre-B cells (Kane, Shapiro et al. 1999; Derudder, Cadera et al. 2009). Given that Akt signaling promotes NF- κ B activity in other contexts (Kane, Shapiro et al. 1999), the increased numbers of pre-B cells produced by Akt1/2 DKO progenitors may reflect decreased NF- κ B activity in pro- and/or pre-B cells. Thus experiments testing whether enforced NF- κ B activity decreases numbers of pre-B cells and increases numbers of MZ and B1 B cells in the context of Akt deficiency might provide important information concerning the mechanism of Akt-dependent development and selection within B-lineage precursors in the marrow.

Finally, an alternative explanation for the increased frequency of λ -bearing cells within the immature B cell compartment, is that Akt signaling influences the accessibility of the RAG complex to the light chain loci. In this model the altered κ/λ ratio is a product independent of selection mediated by the BCR, but instead reflects defective cytokine or receptor-ligand interactions critical for opening the κ -locus or repressing the λ -locus.

5.2 Normal B1 and MZ B cell development requires Akt1 and Akt2.

I find that B1a and MZ B cell development is blocked, and B1b development is diminished in the absence of Akt1 and Akt2 (**Fig 6 & 7**). These findings are consistent with previous reports demonstrating a lack of B1 and MZ

B cells in mice deficient for CD19 or components of the PI-3K pathway, suggesting that Akt is a critical downstream target of these molecules. In addition, these data demonstrate that Akt3 alone is insufficient to mediate development of these subsets. This may reflect the decreased expression of Akt3 in MZ and B1s relative to the FOL pool (**Fig 12**) such that the required aggregate Akt signaling does not reach a required threshold. Alternatively, the results may indicate that Akt1 or Akt2 can regulate downstream pathways that Akt3 does not in the context of B1 and MZ development. Furthermore, I observed an increased frequency of MZ B cells among naïve CD19⁺ B cells, in mice engineered to conditionally express a constitutively active form of Akt (Myr-Akt) in the mature B-lineage (**Fig 15A**). Collectively, these data demonstrate the importance of the Akt pathway in B1a and MZ B cell development, and indicate a non-redundant role for Akt1 and Akt2 in the peripheral B cell compartment.

Given Akt's capacity to regulate a diverse number of biochemical pathways, I questioned what the mechanistic function of Akt is for MZ B cell development. Studies by Otero et al demonstrated that MZ B cell development in CD19 deficient animals could be rescued by transgenic expression of the pro-survival molecule Bcl-2 (Otero, Anzelon et al. 2003). The similarities between CD19 deficient and Akt1/2 deficient B cells to this point were striking, so I decided to test the hypothesis that survival was the critical downstream effect of CD19-Akt signaling. To this end, I transduced Akt1/2 deficient fetal liver progenitors with Bcl-x_L. While ectopic expression of Bcl-x_L enhanced frequencies of recirculating B cells in the bone marrow, it was not sufficient to rescue MZ B

cell development in the absence of Akt1 and Akt2 (**Fig 13**). It is possible that my retro-viral approach did not produce sufficient levels of Bcl-x_L to mediate rescue. However, I can offer indirect evidence of the constructs efficacy; first it was able to mediate increases in mature bone marrow B cells (**Fig 13A**). Second, *in-vitro* assays demonstrated that cells expressing Bcl-x_L survived radiation induced apoptosis again suggesting the construct afforded a survival advantage (not shown). If I accept that the Bcl-x_L construct had efficacy, then the discrepancy between our findings and Otero et al could reflect inherent differences in the functions of Bcl-2 vs. Bcl-x_L. This possibility will need to be addressed in future experiments. Should enforced expression of Bcl-2 fail to rescue the MZ lineage in an Akt1/2 deficient background it raises the prospect of CD19 independent functions for Akt. Given that the BCR can transiently activate Akt this is a likely possibility (Li, Davis et al. 1999). Crossing B lineage restricted Myr-Akt mice onto the CD19 deficient background, followed by an assessment of MZ and B1 development should help to elucidate the Akt dependent functions of CD19.

Notch receptor signaling has the potential to activate Akt in T cells and B cells, and is absolutely required for MZ B cell generation (Tanigaki, Han et al. 2002; Saito, Chiba et al. 2003; Maillard, Weng et al. 2004; Ciofani and Zuniga-Pflucker 2005; Thomas, Calamito et al. 2007). To determine if Myr-Akt could by pass the Notch requirement for MZ B cell development I introduced a dominant negative MAML construct, capable of inhibiting all canonical Notch signaling, into B lineage restricted Myr-Akt expressing cells. These data show that Myr-Akt, while sufficient to increase the frequency of MZ B cells in a wild-type background,

is insufficient to rescue MZ B cell development in the absence of Notch signaling (**Fig 15B**). Therefore, Notch in all likelihood activates pathways in addition to Akt and perhaps these either independently or combined can regulate Notch's effect on MZ B cell development. It is worth noting that Moran et al found a genetic link between the NF κ B and the Notch pathway (Moran, Cariappa et al. 2007), and perhaps future studies could place an NF κ B gain of function approach on a Notch loss of function and examine the effect on MZ B cell biology.

I also examined cell size as a crude measure of metabolic fitness in Akt1/2 deficient FOL and transitional B cell subsets, and found no difference in comparison to wild-type cells, suggesting that poor metabolism was not responsible for the block in MZ development.

B1 and MZ B cells pools are positively selected on self-ligand or strong tonic signals, and Akt may play an important role in this process. Indeed, BCR-CD19 activation mediates positive selection events in the periphery (Martin and Kearney 2000; Diamant, Keren et al. 2005). Furthermore, mice deficient in PTEN have increased frequencies of B1 and MZ, and possess FOL B cells that fail negative selection in tolerance models (Anzelon, Wu et al. 2003; Browne, Del Nagro et al. 2009). These observations, coupled with the fact that Akt is downstream of the BCR (Otero, Omori et al. 2001), suggest that Akt derived signals are critical to negative and positive selection in the periphery. Indeed, I observe a change in the peripheral BCR repertoire as indicated by an increased frequency of λ + naïve FOL B cells (**Fig 11**). Furthermore, I find that proliferative responses to BCR cross-linking on mature B cell in Akt1/2 deficient splenocytes

are defective (**Fig 8**), further supporting that the B cell abnormalities observed in Chapter 3 of this thesis reflect crippled BCR signaling in the absence of Akt1/2.

In mice deficient in the tyrosine phosphatase CD45 (a positive regulator of BCR signaling), B cells expressing an Ig transgene with specificity for hen egg lysozyme (HEL) are positively selected in the presence of soluble antigen, in contrast to wild-type Ig HEL+ B cell which are rendered anergic in this system (Cyster, Healy et al. 1996). It would be interesting to breed the Ig HEL+ transgenic BCR onto Akt1/2 deficient soluble and membrane bound HEL expressing mice and examine the fate of peripheral Ig HEL recognizing clones.

5.3 Akt 1 and Akt2 are critical for the competitive fitness of peripheral B cell populations.

Decreased FOL B cell numbers, poor survival *in-vitro*, and a block in B cell populations that require stronger BCR signals suggested that Akt1/2 deficient progenitors may be weak competitors in mixed marrow chimeras. Indeed, the absence of Akt1 and Akt2 resulted in an absolute defect in the ability to generate MZ or marginal zone precursor (MZP), furthermore a paucity of donor derived cells were represented within the FOL B cell pool when matched with wild-type competitors (**Fig 9**). This data is reminiscent of competitive chimeras in which mice deficient in Btk or CD19 were matched with wild-type cells (Sprent and Bruce 1984; Otero, Anzelon et al. 2003). These data show that Akt is critical to the competitive fitness of peripheral B cell populations, and demonstrates an inability to efficiently gain access to or utilize resources crucial to survival. I

noticed that Akt1/2 deficient B cells started losing representation as early as the T2 stage of peripheral B cell development. Interestingly, it is at this stage that BR3 is up-regulated and begins to govern the size of the peripheral B cell pool (Stadanlick, Kaileh et al. 2008). Given that Blys-BR3 signaling can activate Akt (Patke, Mecklenbrauker et al. 2006; Otipoby, Sasaki et al. 2008; Woodland, Fox et al. 2008) I decided to explore the ability of Akt1/2 deficient FOL B cells to respond to Blys, finding that these cells do in fact respond to Blys (**Fig 10A**). This observation suggests that Akt3 may be sufficient for Blys mediated survival, alternatively, the Akt pathway while activated by Blys does not mediate survival functions downstream of this pathway. It is interesting to note that BR3 deficiency does not lead to defects in the B1 pool (Lentz, Hayes et al. 1998), but Akt1/2 deficient chimeras do show B1 abnormalities. Furthermore, enforced expression of Bcl-x_L was previously shown to enhance the MZ compartment in BR3 deficient mice (Amanna, Dingwall et al. 2003). If a BR3-Akt axis was critical to the maintenance of MZ B cells my over-expression of Bcl-x_L should have rescued this population. These observations, coupled with *in-vitro* data, allows me to make the general conclusion that Akt1 and Akt2 are not the principle mediators of survival downstream of Blys signaling. Perhaps an assessment of Akt1/2 deficient B cell's capacity to generate the BR3 substrate p100, a classical NF κ B target downstream of the BCR, would more thoroughly examine the role of Akt in BCR-BR3 cross-talk. Given the inability of Akt1/2 deficient progenitors to compete with wild-type cells, one might expect p100 generation, while not absent, could be decreased in response to tonic BCR signals. This experiment

will help elucidate the mechanism by which Akt1/2 deficient B cells fail to compete and survive when matched with wild-type cells. Collectively, our results demonstrate that Akt is critical for competitive fitness, but this requirement is most likely independent of direct BR3 receptor pathway activity.

A final thought on p100 generation in peripheral B cell populations. The capacity of the MZ B cell population to generate p100 in tonic or BCR cross-linking studies remains unexplored. A central question to the FOL vs MZ B cell fate decision is the role of BCR signal strength. It is known that MZ B cells are more dependent on Blys than FOL B cells (Thien, Phan et al. 2004). Whether this enhanced requirement reflects too much or too little p100 generation is unknown, but it is interesting to note that p100 is a target of the classical NF κ B pathway. As such p100 levels are in part dictated by p50. MZ B cell development is blocked in the absence of p50 (Moran, Cariappa et al. 2007). Furthermore, Notch signaling is required for MZ B cell generation and Notch was recently shown to stabilize p50 levels (Shin, Minter et al. 2006; Moran, Cariappa et al. 2007). Does this imply that Notch and the BCR communicate to generate a specified level of p100 such that BR3 can mediate processing and eventual transcription of Bcl-x_L and Pim2? This could be tested by first examining the capacity of MZ B cells to generate p100 downstream of BCR cross-linking, this experiment may require use of a Bcl-x_L transgenic mouse as MZ B cells undergo apoptosis in response to BCR oligomerization. If p100 is generated, the extent of p100 generation should be examined in MZ B cells co-cultured with OP9 stromal cells expressing the Notch ligand DL1 by both western and qRT-PCR.

5.4 Notch independent functions of the γ -secretase complex in BCR mediated proliferation.

Previous observations demonstrated a synergy between Notch-DL1 and BCR signaling that resulted in enhanced proliferative and isotype switching responses (Thomas, Calamito et al. 2007). These experiments forced Notch signaling upon B cells by co-culturing them with OP9- stromal cells engineered to express the Notch ligand DL1. Interestingly, DL1 mRNA is expressed intrinsically on splenic B cells (Hozumi, Negishi et al. 2004). This observation raised questions regarding the role for B-cell restricted Notch ligand expression in BCR mediated activation. To inhibit Notch signaling, I cultured FOL B cells in the presence of GSI or vehicle control. As described in the introduction, GSIs block the enzymatic activity of γ -secretase, which is required for Notch pathway activation. These experiments showed that GSI greatly diminished the ability of FOL B cells to proliferate in response to antigen receptor stimulation (**Fig 17**). This effect was specific to the BCR, as stimulation through TLR4 via LPS did not perturb CFSE dilution. In addition, I also showed that GSI was not inherently toxic to B cells as various doses of GSI resulted in the same cell viability when compared to the vehicle control DMSO. Furthermore, both a Bcl-x_L transgene and retrovirus could not rescue proliferation in GSI treated B cells (data not shown). Collectively these results suggested that the Notch pathway could not only enhance BCR mediated proliferation as per earlier published studies, but that Notch was essential to proliferation via the antigen receptor. A second and distinct hypothesis was that the effect I observed was Notch independent. This

was not unreasonable, as over 80 different substrates have been identified as targets of the γ -secretase complex. To better understand the mechanism causing decreased proliferation in GSI treated cultures I decided to target the Notch pathway specifically. To this end I employed a system in which a DNMAML construct could be induced upon pulsing with a cell permeate Tat-Cre, and its expression tracked/sorted via GFP. An advantage of this system was that it allowed B cell development to occur in the presence of normal Notch signaling, negating any selective bias that could be introduced as a result of Notch signaling blockade. As demonstrated in **Figure 18** inhibition of canonical Notch signaling did not have an effect on BCR mediated proliferation across multiple doses of anti-IgM cross-linking antibody, suggesting that Notch signaling is not required for normal BCR mediated activation. Furthermore, addition of GSI to DNMAML expressing or non-expressing cells could repress BCR mediated proliferation (**Fig 18**).

These data were compelling, but a concern was that even though I could track DNMAML expression by GFP and this level of expression was sufficient to block Notch signaling as required for MZ B cell development, it was possible that the level of DNMAML reached during the 16hr incubation period before sorting and subsequent three days in culture was insufficient to block Notch signaling. To address this concern I harvested FOL B cells from $CD19^{Cre} ROSA26^{+/DNMAML}$ mice, these FOL B cells develop in the presence of DNMAML and are at steady state. Cells in this pool have a life span in excess of three months, thus I'm confident that FOL B cells from these mice express levels of DNMAML sufficient

to block canonical Notch signaling. Experiments examining proliferation in these cells resulted in the same conclusion as those experiments utilizing Tat-Cre; Notch signaling is not required for BCR driven proliferation.

Previous work performed and published from our lab showed that Notch could enhance BCR signals. My current results do not refute this finding, as those studies described a function for Notch in the enhancement of proliferation, i.e. what Notch “can do”. In contrast, the experiments in this thesis examine the requirement for Notch in BCR driven proliferation and concludes that Notch is dispensable for BCR mediated activation. Furthermore, my work, for the first time in B cells, suggests that the γ -secretase complex has Notch independent functions in B cell activation.

A limitation of my experimental approach was that it hinged upon chemical inhibitor based studies that could have off target effects. To address the γ -secretase specific phenotype I generated mice with conditional deletion of both isoforms of the catalytic subunit of γ -secretase, Presenilin 1 and Presenilin 2. Conditional deletion by a B cell specific Cre was required for the PS1 floxed gene as conventional PS1 or PS1/2 DKO mice are embryonic lethal (Feng, Rampon et al. 2001). To examine the B cell intrinsic effects of Presenilin deletion on BCR mediated activation I performed a mixing experiment in which PS1/2 DKO FOL B cells were co-cultured with congenic wild-type FOL B cells. These experiments recapitulated the GSI studies demonstrating defective proliferation in response to BCR cross-linking in the absence of Presenilin proteins and demonstrate that the defect is indeed B cell intrinsic (**Fig 19A/B**). During the course of my thesis work

Yagi et al published that proliferative responses to BCR stimulation were abnormal in the absence of both PS1 and PS2, but these authors did not show that the defect was cell intrinsic. Furthermore, they concluded that Presenilin proteins were required for proliferation in response to LPS stimulation (Yagi, Giallourakis et al. 2008). While my observations agree with the BCR mediated proliferation defects, I do not find that Presenilins are required for LPS responsiveness. I'd like to point out that these workers did not control for differences in splenic B cell subset composition in Presenilin-deficient and wild type control animals. Indeed, it is well established that MZ B cells are highly enriched for LPS-responsive cells (Oliver, Martin et al. 1997), and this population is absent from PS1/PS2 DKO B cell pools, presumable due to failed Notch2 activation. In contrast all experiments assessing B cell activation herein employed positively selected CD23⁺ B cells, which are highly enriched for FOL B cells and lack MZ B cells. Therefore, previous data suggesting that LPS responsiveness in B cells requires Presenilins instead reflect differences in B cell subset composition among Presenilin-deficient and control splenocytes.

The BCR specific defects suggested that downstream signaling could be altered. A large body of evidence in neurons implicated Presenilin proteins in calcium regulation (Cai, Lin et al. 2006; Tu, Nelson et al. 2006; Cheung, Shineman et al. 2008). To gain a qualitative measure of signaling downstream of the BCR in the absence of Presenilins I examined calcium mobilization (**Fig 21**). These experiments collectively demonstrated that BCR signaling responses are decreased both in terms of magnitude and kinetics in the absence of Presenilin

proteins. Experiments performed in calcium free media suggest that the defect localizes to the initial BCR signaling cascade, implying that BCR activation is suboptimal. These defects are in agreement with observations made by Yagi et al showing that Syk phosphorylation is decreased immediately after BCR cross-linking (Yagi, Giallourakis et al. 2008). Presenilin proteins do have functions independent of their catalytic activity, therefore future experiments utilizing a catalytically dead Presenilin mutant must be performed to ascertain the requirement for enzymatic activity in normal calcium mobilization.

Previous studies, some using GSI, concluded that Notch was a critical factor in the Th1 vs Th2 decision (Minter, Turley et al. 2005; Tu, Fang et al. 2005; Fang, Yashiro-Ohtani et al. 2007). My results justify reexamination of this conclusion, as Minter et al may have been observing a Notch independent function of the inhibition of the γ -secretase complex. My results demonstrate that further understanding of the γ -secretase complex is warranted as off target effects may be more severe than anticipated.

5.5 Requirement for Presenilin in B1a B cell development and BCR repertoire formation

Mutations or deletion of proteins that strengthen BCR signaling, such as Btk, often coincide with defective calcium mobilization and a block B1 B cell development (Khan, Alt et al. 1995; Takata and Kurosaki 1996). Therefore, I evaluated the peripheral B compartment of PS1/2 deficient mice and discovered a severe block in the generation of B1a B cells and overall decrease in the B1

compartment (**Fig 23A**). Furthermore, loss of a single isoform of PS1 or PS2 resulted in a similar albeit less dramatic decrease in the B1 pool. I also noted that CD19^{+Cre} Rosa26^{+DNMAML} mice do not show a defect in B1 B cell development (**Fig 23B**), again demonstrating that the effect is Notch independent. These data, combined with the proliferation and calcium defects characterized above, imply that Presenilins function to optimize BCR signaling and potentially promote the selection of B1a and to a lesser extent B1b B cells.

An alternative explanation is that B1 B cell generation is normal in the absence of Presenilin proteins, but that the maintenance of this subset is defective, perhaps through defective self-renewal. Future experiments will examine the B1 compartment in young mice to determine if B1 B cells are present at this early stage. This is a critical experiment in that my current data only supports the hypothesis that abnormal BCR signaling is responsible for generating B1a cells, the possibility remains that altered self-renewal is the defect.

Additional support for a Presenilin dependent function in BCR signaling and B cell development came from examination of the BCR repertoire as measured by λ frequencies. As shown in **Figure 24** of PS1 and PS2 resulted in decreased frequencies of λ bearing cells. Furthermore, this effect was also observed in single knockouts. As λ frequencies are thought to be representative of the extent of receptor editing (Nemazee 2006), these data suggest that receptor editing is diminished in the absence of Presenilin. This may represent altered negative/positive selection as a result of abnormal BCR signaling.

Strikingly, studies from Laky et al showed that positive selection of developing T cells is compromised in PS1/PS2-deficient thymocytes, and led to models proposing that Notch activity regulates TCR signal strength during thymocyte development (Izon, Punt et al. 2001; Laky and Fowlkes 2007). However, in combining these observations with my data, it suggests that many of these effects may instead reflect Notch-independent functions for Presenilins. Therefore, whereas my results do not minimize the importance of Notch signaling in T and B cell development and function, they emphasize the need to understand the additional roles played by Presenilins in the immune system.

5.6 Concluding remarks

My work shows that Akt1, Akt2 and Presenilins are critical factors in the generation of positively selected subsets, the peripheral BCR repertoire and BCR driven proliferation (**Fig 27**). My data combined with observations from other labs suggests that these molecules are critical targets of BCR signaling, and may serve to strengthen antigen receptor signals. An unanswered but important question is how CD19 and the BCR converge on Akt to drive the B1 and MZ fate. The data presented in this thesis suggests that survival and metabolism are not solely responsible for the block in the generation of these subsets. For Presenilin a clear question that needs to be addressed is whether the defects observed in B cell development depend upon the catalytic activity of the γ -secretase complex, and if so what is the substrate (**Fig 27**)? It would also be advantageous to generate mice deficient in Akt or Presenilins harboring Ig heavy chains such as M167 or 81x with idiotypes that drive defined cell fates within the B-lineage,

studies such as these will more precisely determine the role of these molecules in negative and positive selection.

Some twenty years ago Herzenberg and colleagues proposed the idea of a “layered immune system” in which the evolutionary most primitive cellular subsets would arise first. From this foundation additional layers could be built increasing in complexity and providing survival and reproductive advantage to the organism (Herzenberg 1989). In relation to B cell biology, B1 and MZ B cells with their “innate-like” T- cell independent functions may serve as the foundation of the humoral immune response. From this foundation, the more complex T- dependent, germinal center reactions dominated by FOL B cells evolved. The biochemical pathways utilized by this evolutionary process are poorly understood. Examination of previously published data and the work presented within this thesis demonstrates that though B1 and MZ cells may function in a more primitive way, the developmental requirements of these cells may be more complex than that of FOL B cells. This implies that evolution favored “relaxing” positive selection standards to increase the diversity of antigens recognized and enlarge the overall B cell repertoire.

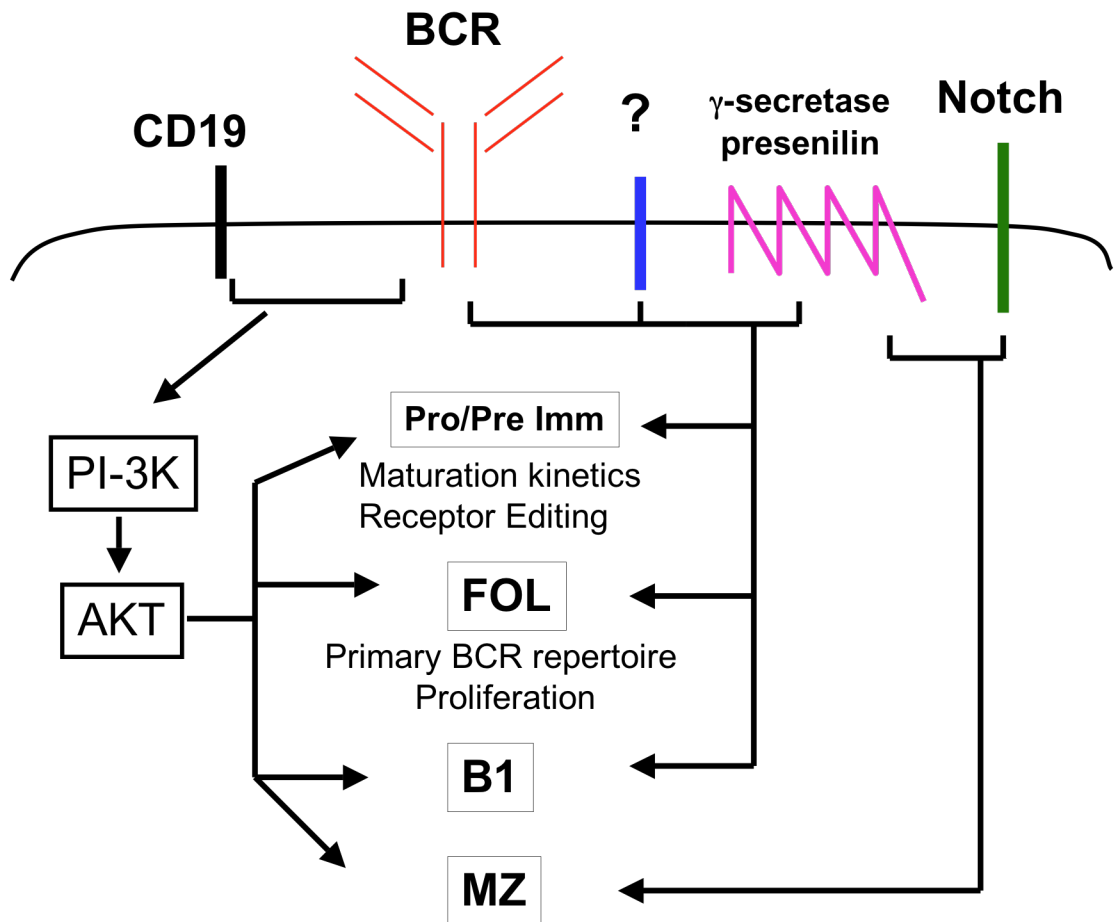
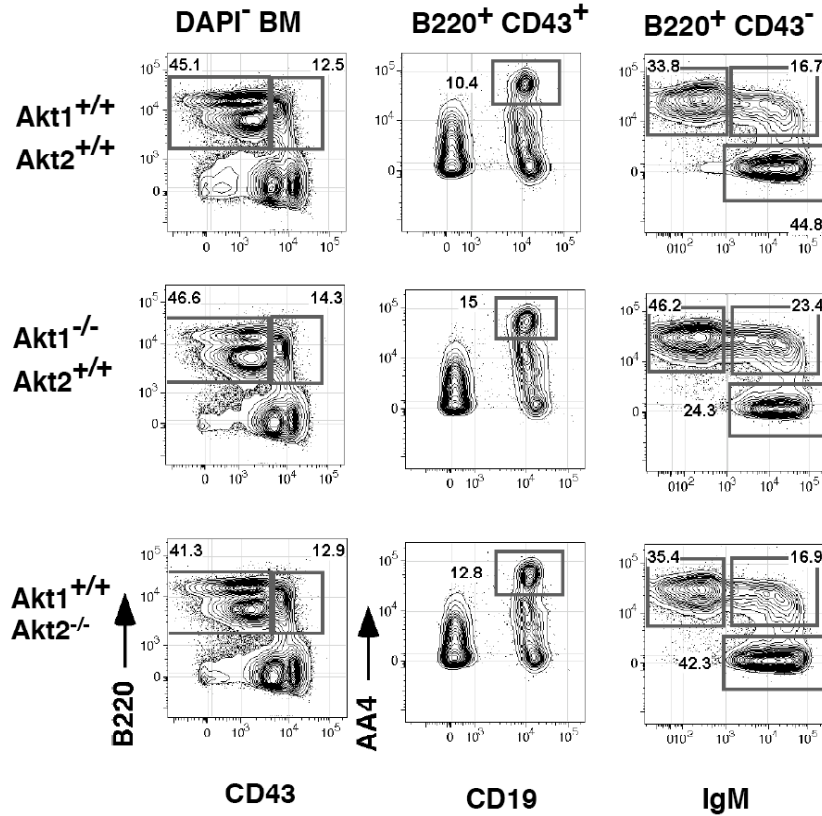
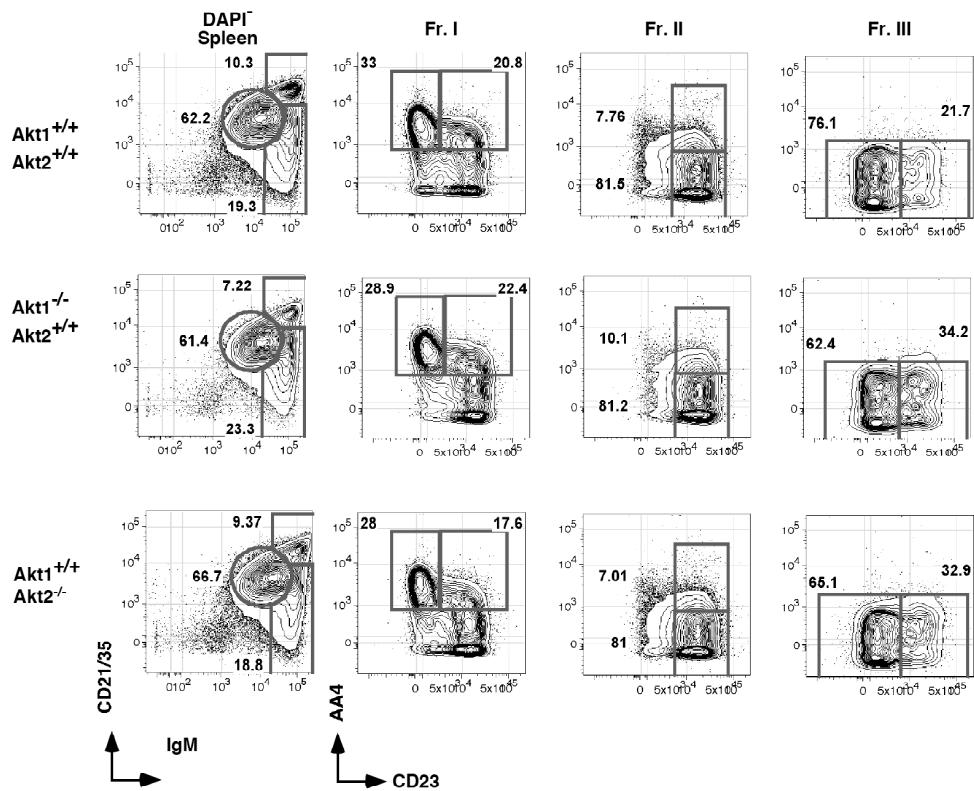


Figure 27. Akt and presenilin promote BCR signaling and support generation of innate-like B cell populations. B cells deficient in Akt1/2 are phenotypically very similar to CD19 or PI-3K loss of function cells. These observations suggest that Akt is a required downstream target of these molecules in the generation of the normal peripheral B cell pool. Presenilins while required to generate MZ in a Notch dependent manner, also play critical Notch independent functions downstream of the BCR in the development of B1 B cells and BCR repertoire formation. Collectively, Akt1/2 and Presenilins are important molecules in the generation and propagation of signals derived from the BCR and thus play a critical role in the formation of the humoral arm of the immune system.

Appendix A.



Appendix Figure 1: Early B cell development is normal in the absence of Akt1 or Akt2. (A) Representative flow cytometric analysis of BM cells from wild-type (top) Akt1^{+/+} (middle) Akt2^{-/-} (bottom). The left-most plots are gated on viable donor-derived (DAPI⁻) cells. Numbers in plots indicate the frequency of events in the indicated gate as a function of the indicated parent population. Pro-B, CD43⁺ B220⁺ CD19⁺ AA4⁺; pre-B, CD43^{low} B220⁺ IgM⁻ AA4⁺; immature B, CD43⁻ B220⁺ IgM⁺ AA4⁺; mature B, CD43⁻ B220⁺ IgM⁺ AA4⁻.



Appendix Figure 2: MZ B cell development is intact with loss of a single isoform of Akt1 or Akt2. Representative analysis of splenocytes from wild-type (top), Akt1^{-/-} (middle), or Akt2^{-/-} (bottom) mice. Viable donor-derived cells were gated as in Figure 1. Numbers in plots show the frequency of events as a function of the indicated parent gate.

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