THE ROLE OF FOXO TRANSCRIPTION FACTORS IN B CELL DEVELOPMENT AND ACTIVATION

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To my grandfather, Harvey Hinman, and his “nine lives” of inspiration.

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THE ROLE OF FOXO TRANSCRIPTION FACTORS IN B CELL DEVELOPMENT AND ACTIVATION

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A functional immune system depends on a diverse, self tolerant B cell repertoire. Mature B cells distributed throughout secondary lymphoid organs respond to antigenic stimuli by dividing and differentiating into plasma cells and other effector cell types. Signaling from the B cell receptor (BCR) plays a critical role at several points during this developmental process. Cell survival, proliferation, differentiation, death, anergy, and receptor editing may occur in response to BCR stimulation. A variety of factors, including signal strength and duration, cytokine presence, and co-stimulation determine the ultimate B cell fate.

In this thesis, the roles Foxo transcription factors play in maintaining B cell homeostasis will be explored. Foxo1, Foxo3, and Foxo4 have both anti-mitogenic and pro-apoptotic properties. The transcription factors are posttranslationally controlled via Akt. When a mature B lymphocyte is stimulated through the BCR, Akt-mediated phosphorylation of Foxos results in their exclusion from the nucleus. In the absence of Foxo nuclear activity, the B cell progresses into the cell cycle.

We have discovered a second PI3K-dependent means of control for Foxos, at the level of mRNA expression. Downstream of the BCR, this means of control is unique and functionally relevant. Mature B cells proliferating in response to anti-IgM downregulate Foxo mRNA expression. This is via activation of the PI3K/Btk/BLNK/PLCγ2 pathway. Conversely, Foxo mRNA expression is upregulated in immature B cells, both when the tonic/basal signal through the BCR is disrupted and when the BCR is engaged with anti-IgM. Overexpression of Foxo3 mRNA in an immature B cell line promotes anti-IgM induced apoptosis. Primary immature B cells from Foxo3-/- mice have decreased apoptotic response to BCR crosslinking. Thus, at the immature stage of development our work has revealed a potential role for Foxo3 in promoting clonal deletion. Foxo3-/- mice also have reduced frequencies of pre-B and mature recirculating B cells in the blood and bone marrow. The mice demonstrate increased basal levels of IgG2a, IgG3, and IgA. Thus, Foxo3 deficiency affects numerous aspects of B cell development.

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LIST OF ABBREVIATIONS

α – anti

Ab – antibody

Ag – Antigen

BAFF – B cell activating factor belonging to the tumor necrosis factor family

BCR – B cell receptor

BLNK – B cell linker protein

BSA – bovine serum albumin

Btg – B cell translocation gene

Btk – Bruton's tyrosine kinase

cDNA – complementary DNA

C. elegans – *Caenorhabditis elegans*

CLP – common lymphoid progenitor

DAG – 1,2-diacylglycerol

ds – double stranded

DBD – DNA binding domain

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

FBS – fetal bovine serum

FITC – Fluorescein isothiocyanate

FKH – forkhead binding domain

FO – follicular

Foxo – Forkhead box class O

GFP – green fluorescent protein

Grb – growth factor receptor binding protein

h – hour

IFN - interferon

Ig – Immunoglobulin

IL – interleukin

IP3 – inositol 1,4,5-triphosphate

ITAM – immunoreceptor tyrosine-based activation motif

LPS – lipopolysaccharide

mRNA – messenger RNA

MZ – marginal zone

NES – Nuclear export signal

NFκB – nuclear factor κB

NLS – Nuclear localization signal

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PE -phycoerythrin

PH domains – pleckstrin homology domains

$PI(3,4)P_2$ - phosphatidylinositol 3,4-biphosphate

PIP_3 - phosphatidylinositol 3,4,5-triphosphate

PIP4 - phosphatidylinositol 4-phosphate

PIP5k - phosphatidylinositol 4-phosphate 5-kinase

$PI(4,5)P_2$ - phosphadylinositol 4,5-bisphosphate

PI3K – phosphoinositide 3-kinase

PKB – protein kinase B

PLC – phospholipase C

PMA – phorbol 12-myristate 13-acetate

Pten – phosphatase and tensin homolog

Q-PCR – quantitative real-time polymerase chain reaction

RNA –ribonucleic acid

S1P – Sphingosine-1 phosphate

SD – standard deviation

SGK – serum- and glucocorticoid- induced kinase

SHIP - phosphatase SH2 domain-containing inositol phosphatase

SLE – systemic lupus erythematosus

SOS – Son of Sevenless

SRF – serum response factor

ss – single stranded

TCR – T cell receptor

TLR – Toll-like receptor

TNF – tumor necrosis factor

XLA – X-linked agammaglobulinemia

CHAPTER I

INTRODUCTION

I.a. Overview

Within the immune system, the maintenance of homeostasis relies on the interplay between survival, proliferation, and death. The number of T and B cells that enter the periphery is only a fraction of that initially generated. Programmed cell death eliminates lymphocytes with nonfunctional or autoreactive antigen receptors. T and B cells circulating in the periphery are kept quiescent, dividing and differentiating only upon encounters with their cognate antigen or another external stimulus such as LPS. A proportion of these activated cells later undergo apoptosis at the termination of the immune response.

The Forkhead box class O (Foxo) subfamily of transcription factors has been the subject of much interest with regard to cellular homeostasis. In numerous cell types, including those of the immune system, these pro-apoptotic and anti-mitogenic molecules are known to be posttranslationally controlled via the kinase Akt. When a mature B lymphocyte is stimulated through the B cell receptor (BCR), Akt-mediated phosphorylation of Foxos results in their exclusion from the nucleus. The Foxo proteins are subsequently degraded. In the absence of Foxo activity, the B cell enters the cell

cycle. Conversely, the overexpression of a constitutively active/nuclear Foxo protein promotes cell cycle arrest and apoptosis (Yusuf et al. 2004).

In our work, we identify a second PI3K-dependent mechanism for control of Foxo transcription factors at the level of mRNA expression. Downstream of the BCR, this means of control is via a unique pathway from that established for the protein phosphorylation. It is functionally relevant. And while Foxo1, Foxo3, and Foxo4 have been shown to interact preferentially with the same DNA binding motif (Furuyama et al. 2000), our analysis of Foxo3-/- mice demonstrates that this Foxo family member has unique functions in B cell development and activation.

I.b. Forkhead box class O (Foxo) subfamily of transcription factors

More than 100 different members of the forkhead box (Fox) family of transcription factors have been identified in species ranging from yeast to human (Kaufmann et al. 1996). This diverse group of proteins is characterized by the presence of a highly conserved, monomeric DNA-binding domain. This domain, or forkhead box, is often referred to as a winged helix motif. The 110 amino acid domain forms a butterfly-shaped structure made up of three tightly packed N-terminal α-helices, three β-sheets, and loop regions located at the C terminal end that shape the 'wings' of the structure (Clark et al. 1993). The orientation of this domain, and in particular the third α-helix, is not identical in all family members. The core DNA binding sequence of 7 nucleotides is variable: T(G/A)TT(G/T)(G/A)(C/T) (Kaufmann et al. 1994, Kaufmann et al. 1995,

Overdier et al. 1994, Pierrou et al. 1994). The consequences of Fox binding to DNA can be either the activation or inhibition of gene transcription. Fox family members have been divided into subfamilies ranging from Foxa to Foxq. They are required for a range of phenotypes from craniopharyngeal development (FOXE1), to speech and language development (FOXP2) and hearing (Foxi1) (Lehmann et al. 2003). Several of these transcription factors, including Foxp3, Foxn1, Foxj1, and the entire Foxo subfamily are known to play roles in the immune system (Table 1.1).

Work presented in this thesis focuses on the forkhead box class O (Foxo) subfamily of transcription factors and their function in the immune system. This subfamily, including the murine genes Foxo1, Foxo3 and Foxo4, are grouped based on their shared regulation via the PI3K and the Akt signaling pathways (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999, Rena et al. 1999, Takaishi et al. 1999, Tang et al. 1999). They have been shown to regulate cell cycle progression, metabolism, oxidative stress resistance, and/or promote apoptosis (Birkenkamp and Coffer 2003, Burgering and Kops 2002, Tran et al. 2003). A role for Foxo transcription factors in the maintenance of cellular quiescence was first suggested through studies of the *Caenorhabditis elegans* homologue DAF-16. When the worm is in a nutrient-rich environment, DAF-16 is phosphorylated by Akt and sequestered to the cytoplasm. However, under conditions that are unfavorable to growth, such as food deprivation, adverse temperatures, or high population densities, DAF-16 is dephosphorylated and enters the nucleus. The transcription factor promotes a Daur or non-aging larval stage in the worm, in which the

Table 1.1: Fox gene knockout mice and their immune-related phenotypes.

Gene	KO Viable?	Phenotype	Role in Immune System	References
Foxp3	Yes	Scurfy-mouse phenotype; aggressive lymphoproliferative autoimmune syndrome.	Development and function of CD4+ CD25+ T-reg cells	Brukow et al. 2001, Fontenot et al. 2003
Foxn1	Yes	Nude phenotype; congenital absence of hair and severe immunodeficiency.	Differentiation of thymic epithelial cells	Balciunaite et al. 2002, Nehls et al. 1994
Foxj1	No	Embryonic lethal; fetal-liver chimerization results in systemic autoimmune inflammation.	Suppression of T cell activation and prevention of autoimmunity	Chen et al. 1998, Brody et al. 2000, Lin et al. 2004(b)
Foxo1	No	Embryonic lethal due to defects in angiogenesis; regulates B cell development, as well as B and T cell migration.	Regulates IL-7R and RAG expression in B cells. L-selectin expression in B and T cells.	Furuyama et al. 2004, Dengler et al. 2008, Fabre et al. 2008
Foxo3	Yes	Defects in ovarian follicle development leading to age-dependent infertility. Background dependent spontaneous lymphoproliferation.	Plays a role in B cell development and migration. Regulates DC cytokine secretion. Probable role in mucosal immunity.	Castrillon et al. 2003, Hinman et al. 2009, Dejean et al. 2009, Snoeks et al. 2009
Foxo4	Yes	Reduced smooth muscle cell migration in response to injury.	Probable role in regulating mucosal immunity.	Hao et al. 2007, Zhou et al. 2009

C. elegans displays both reduced metabolic activity and increased resistance to oxidative stress (Lin et al. 1997, Ogg et al. 1997).

Four consensus sequences for phosphorylation by Akt have been identified in the DAF-16 protein. Three of the four are conserved in Foxo1, Foxo3, and Foxo4 (Furukawa-Hibi et al. 2005, Fig. 1.1). These sites may also be phosphorylated by SGK (serum- and glucocorticoid- induced kinase), another target of PI3K (Brunet et al. 2001). In mammals, cytokines and other growth factors such as insulin and insulin-like growth factor-1 signal for Foxo protein phosphorylation via either Akt or SGK in a PI3K dependent manner (Biggs et al. 1999, Brunet et. al 1999, Brunet et al. 2001, Buse et al. 1999, Kobayashi et al. 1999, Kops et al. 1999, Park et al. 1999, Rena et al. 1999, Takaishi et al. 1999, Tang et al. 1999). The Foxo3 protein may also be phosphorylated by IκB at a site separate from those described above (Hu et al. 2004). The phosphorylated Foxo1, Foxo3, or Foxo4 protein in turn interacts with the adaptor 14-3-3. 14-3-3 promotes Foxo relocalization to the cytoplasm. It is proposed to do this through a conformational change that exposes the nuclear-export signal and masks the nuclear-localization signal within the protein (Brunet et al. 2002). The Foxo protein is subsequently degraded. Absence of Foxo activity within the nucleus promotes cell cycle entry (Figure 1.2). Conversely, expression of a constitutively active/nuclear Foxo mutant which lacks the ability to be phosphorylated at these site results in either cell cycle arrest or apoptosis depending on cell type (Kops et al. 2002, Medema et al. 2000, Nakamura et al. 2000). Other posttranslational modifications of the Foxo proteins have been proposed to stabilize their nuclear localization. For example, protein arginine methyltransferases (PRMTs) catalyze

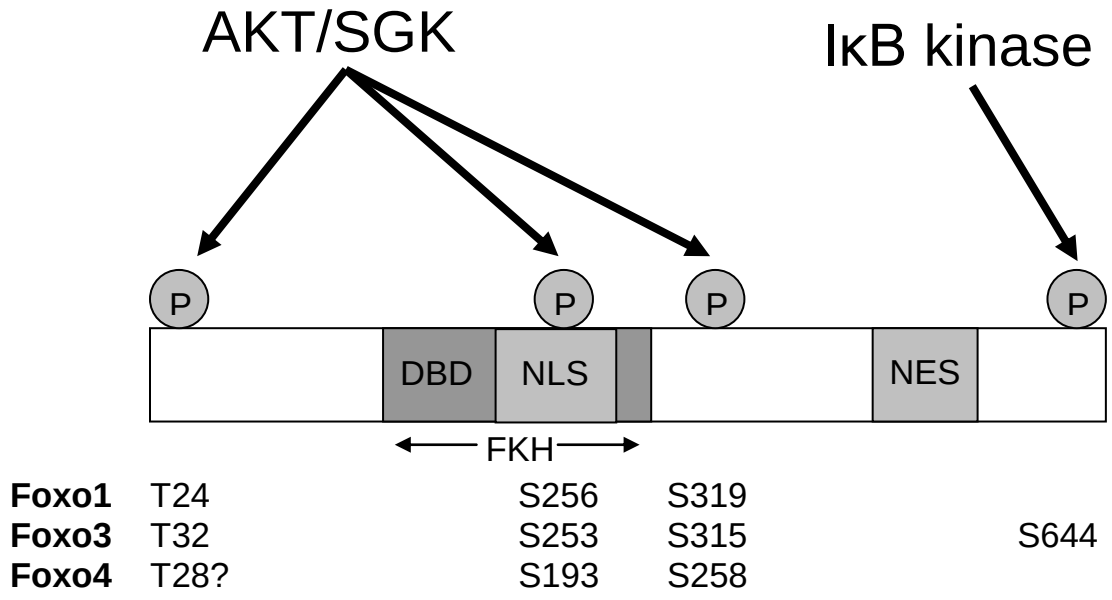


Figure 1.1: Regulation of Foxo protein phosphorylation.
Schematic representation of the phosphorylation sites in the Foxo1, Foxo3, and Foxo4 proteins. The kinases that mediate this phosphorylation are shown. The identity of the phosphorylated amino acids and their locations are indicated. FKH, forkhead binding domain; DBD, DNA binding domain; NLS, nuclear localization signal; NES, nuclear export signal.

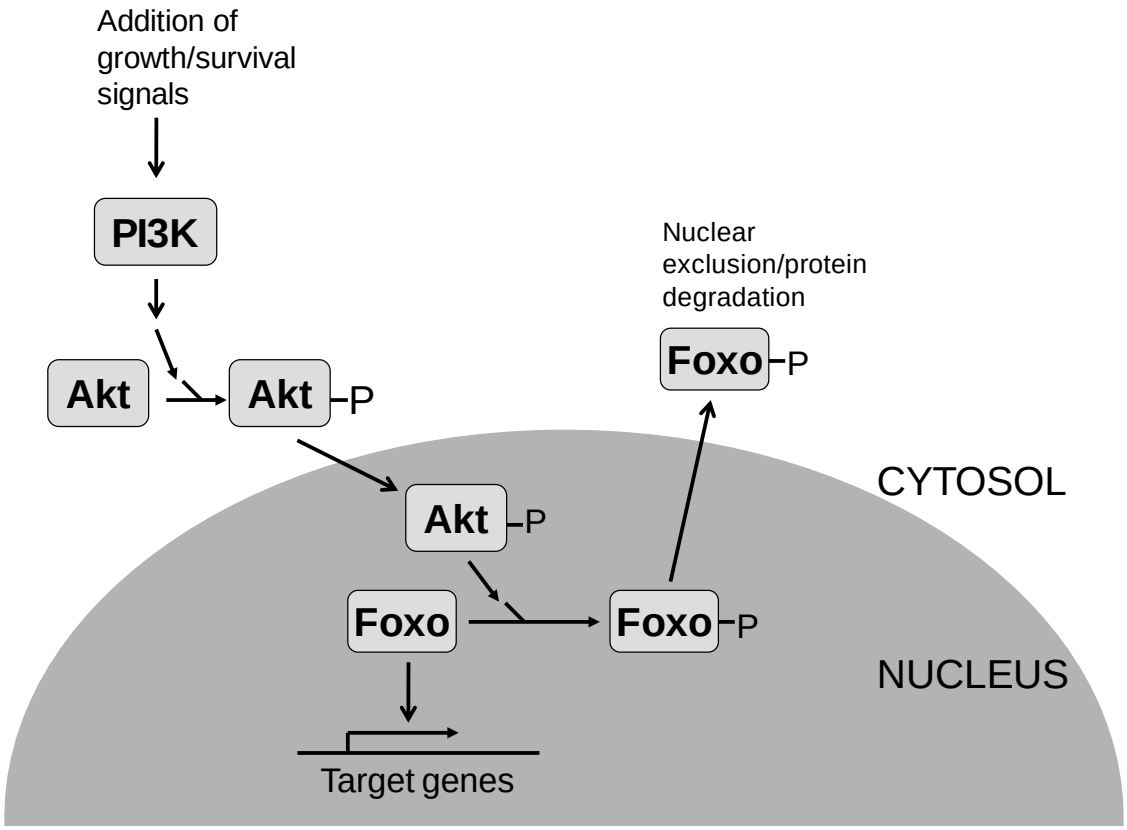


Figure 1.2: Simplified diagram of Foxo protein phosphorylation and movement within the cell.
In mammals, cytokines and other growth factors such as insulin and insulin-like growth factor-1 signal for Foxo protein phosphorylation via Akt in a PI3K dependent manner. The phosphorylated Foxo1, Foxo3, or Foxo4 protein in turn interacts with molecules such as the adaptor 14-3-3 (not shown in this diagram). The phosphorylated Foxo protein is subsequently shuttled out of the nucleus and into the cytoplasm, where it may be degraded. Absence of Foxo activity within the nucleus promotes cell cycle entry.

methylation of the terminal nitrogens on the guanidinium side chains of arginine. Oxidative stress has been shown to cause induction of PRMT1, resulting in the methylation of two arginine residues present in Foxo1. This directly inhibits Akt-mediated phosphorylation and thus enhances nuclear localization of Foxo1 and apoptosis (Yamagata et al. 2008). Overall, these means of posttranslational control for the Foxo subfamily of transcription factors are well defined. The impact of other levels of regulation, such as mRNA expression, remains largely unexplored.

Foxo1, Foxo3, and Foxo4 share overlapping patterns of tissue expression and interact preferentially with the same DNA binding motif 5'-TTGTTTAC-3' (Furuyama et al. 2000). They are capable of regulating gene expression in both positive and negative manners. Like most transcription factors, Foxo1, Foxo3, and Foxo4 mediate their activity as part of protein complexes. To date, at least 24 transcription factors have been shown to bind to the Foxo proteins. These include a number of nuclear hormone receptors, β-catenin, C/EBP, PPAR and the PPARγ coactivator PGC-1α, as well as Smad3 and Smad4 (van der Vos and Coffer 2008). Foxo1 has been shown to be able to carry out chromatin opening as a 'gateway' transcription factor (Hatta and Cirillo 2007). However, not all Foxo activity is dependent on direct DNA binding. Ramaswamy et al. demonstrated that a Foxo3 mutant, in which DNA-binding was abolished, could still effectively regulate a specific subset of genes when introduced into the 786-O cell line. Repression of the cell cycle progression proteins Cyclin D1 and Cyclin D2 was not altered. The cells still retained limited ability to upregulate Cyclin G2 and Btg-1 (Ramaswamy et al. 2002).

Foxo1, Foxo3, and Foxo4 have been linked to many of the same downstream targets, including those described above (Table 1.2). For example, in the halt of cell cycle progression all three family members downregulate Cyclin D2 levels (Schmidt et al. 2002, Park et al. 2005) and promote the expression of Cyclin G2 (Martinez-Gac et al. 2004, Chen et al. 2006), and $p27^{Kip1}$ (Banerji et al. 2001, Yang et al. 2005, Lees et al. 2008). In apoptotic cells, Bim (Mandal et al. 2008) and Puma (You et al. 2006) expression rises in a Foxo dependent manner. GADD45 is also a direct Foxo target associated oxidative stress resistance (Tran et al. 2002).

The overlap in target genes raises the question of functional redundancy. Yet, individual disruption of each gene results in a distinct phenotype (Hosaka et al. 2004). Murine knockouts of Foxo1 are embryonic lethal due to defects in angiogenesis (Furuyama et al. 2004). Knockouts of both Foxo3 and Foxo4 are viable, however Foxo3 deficient mice exhibit age-dependent infertility in females (Castrillon et al. 2003, John et al. 2007) as well as enhanced dendritic cell production of key inflammatory cytokines, including IL-6 (Dejean et al. 2009). An independently generated line of Foxo3 mutant mice on a mixed background, including 129, BALB/c and C57BL/6, showed spontaneous T-cell activation and lymphoproliferation over time (Lin et al. 2004). However, this phenotype has not been observed in the two other mouse models of Foxo3 deficiency on the C57BL/6 (Hosaka et al. 2004) and FVB (Castrillon et al. 2003) backgrounds respectively. It has suggested, therefore, that this phenotype is likely to be background specific. A genetic polymorphism found in 129 mice may act together with the loss of Foxo3 to dysregulate T cell homeostasis and cause autoimmune disease (Hedrick 2009).

Table 1.2: Foxo target genes

Target Gene	Up/Down Regulation of Gene Expression	Foxo Family Member	direct/ indirect binding effect	Cell Type	Reference(s)
CELL CYCLE					
Btg-1	↑	Foxo3	Direct	erythroid progenitors	Bakker et al. 2007
Cyclin G2	↑	Foxo3	Direct	NIH 3T3 cell line	Martinez-Gac et al. 2004
	↑	Foxo1 & Foxo3	Direct	B lymphocyte	Chen et al. 2006
Cyclin D2	↓	Foxo3 & Foxo4	Indirect	embryonic fibroblasts, NIH 3T3 cell line	Schmidt et al. 2002
	↓	Foxo1	Direct	granulosa	Park et al. 2005
p27Kip1	↑	Foxo1 & Foxo3	Direct	muscle precursor	Lees et al. 2008
	↑	Foxo4	Indirect	NIH 3T3 cell line	Yang et al. 2005
APOPTOSIS					
Bim	↑	Foxo3	?	T lymphocyte	Mandal et al. 2008
	↑	Foxo4	?	endothelial progenitor	Urbich et al. 2005
	↑	Foxo1	Direct	skeletal myoblasts	McLoughlin et al. 2009
Puma	↑	Foxo3	?	embryonic fibroblasts, T lymphocytes	You et al. 2006
OXIDATIVE STRESS AND DNA REPAIR					
GADD45	↑	Foxo3	Direct	embryonic fibroblasts	Tran et al. 2002
	↑	Foxo1 & Foxo4	Direct	C2C12 cell line	Furukawa-Hibi et al. 2002
ATM	↑	Foxo3	?	hematopoietic stem cells	Yalcin et al. 2008
METABOLISM					
glucose-6-phosphatase	↑	Foxo1	?	hepatic cells	Barthel et al. 2005
PEPCK	↑	Foxo1	?	hepatic cells	Barthel et al. 2005
IMMUNE SYSTEM RELATED					
IL-7R	↑	Foxo1	?	B and T lymphocytes	Dengler et al. 2008, Kerdiles et al. 2009
KLF4	↑	Foxo1 &Foxo3	Direct	B lymphocyte	Yusuf et al. 2008
KLF2	↑	Foxo1	?	T lymphocyte	Fabre et al. 2008
S1P1 (EDG1)	↑	Foxo3	?	B and T lymphocytes	Fabre et al. 2008, Hinman et al. 2009
RAG	↑	Foxo1	Direct	B lymphocyte	Amin and Schlissel et al. 2008
Bcl-6	↑	Foxo4	Direct	macrophages	Tang et al. 2002
	↑	Foxo1	?	EBV infected B lymphocytes	Shore et al. 2006

However, the presence or absence of the phenotype could also be an artifact of how each mutant line was generated. Foxo3 mutant mice in the study by Lin et al. had the T-cell phenotype and were created using retroviral gene trap techniques. Gene trap vectors are designed to insert into genomic sequence and interrupt transcription of the trapped gene. The retrovirus inserted between exon 2 and exon 3 (Lin et al.2004). Hosaka et al. also used gene trap techniques to generate their Foxo3 mutant mouse line. However in this case, the retrovirus inserted between exon 1 and exon 2 and no spontaneous T cell activation was observed (Hosaka et al. 2004). With targeted recombination, Castrillon et al. deleted exon 2 to create a Foxo3-/- mouse line. This line was likewise normal in regard to T cell activation (Castrillon et al. 2003). Mice deficient in Foxo4 display decreased migration of vascular smooth muscle cells (Hao et al. 2007). They also show exacerbated inflammatory responses to colonic injury (Zhou et al. 2009). All these phenotypic differences suggest that there are unique functions for Foxo1, Foxo3, and Foxo4 which remain to be teased out at the molecular level.

Further investigation of both Foxo control and function is particularly important in the immune system, where the maintenance of homeostasis relies on the interplay between survival, proliferation, and death inducing factors. Many of these factors in the B lymphocyte are linked to signals received through the BCR. The stage of B cell development, the strength and duration of the BCR signal, and the presence or absence of additional microenvironmental signals, cytokines and/or constimulation influence the ultimate B cell fate. Proliferation, differentiation, cell survival, cell death, anergy, or

receptor editing may occur. All will be discussed in greater detail below and in the chapters that follow.

I.c. The BCR in B cell development

The BCR is a multiprotein complex. The antigen binding subunit of this complex is composed of the heavy and light immunoglobulin chains. This subunit has the same antigen specificity as the secreted antibodies which the B cell may eventually produce. Indeed, it is identical to a secreted monomeric immunoglobulin, except that it is attached to the membrane through the carboxy termini of the paired heavy chains. Given that the portion of these heavy chains which extends into the interior of the cell is only a few amino acids in length; the subunit is incapable of signaling on its own. Instead it relies on the invariant accessory proteins, Igα and Igβ, to complete this task. Igα and Igβ each contain a single immunoreceptor tyrosine-based activation motif (ITAM) within their cytoplasmic tail. It is the phosphorylation of these ITAMs which is primarily responsible for BCR signal transduction (Clark et al. 1992, Dal Porto et al. 2004, Hombach et al. 1990).

The assembly of a functional, non self-reactive BCR drives B cell development. In the pool of mature B cells, there is enormous BCR variability. This variability is achieved via V(D)J recombination (Schlissel et al. 2003, Tonegawa et al. 1983). Through this process the assembly of the heavy and light immunoglobulin chains of the BCR occurs in a sequential manner, with developing B cells tested at defined checkpoints for

successful recombination events. Here we discuss this process, highlighting how the biological outcomes of BCR crosslinking change as the B cell matures (Fig. 1.3).

1.c.1. The pro-B cell

B cells arise from a common lymphoid progenitor (CLP) in the bone marrow. CLPs exist primarily at the outer edge of the bone cross-section. As B cells develop they migrate towards the central sinus and eventually exit through the blood, migrating to the spleen (Fleming and Paige 2002, Jacobsen and Osmond 1990). The earliest cell committed to the B cell lineage is the pro-B cell. Pro-B cells are dependent upon IL-7 for survival and maturation (Hayashi et al. 1990, Lee et al. 1989, von Freeden-Jeffry et al. 1995). The growth factor is produced by the reticular stromal cells in the bone marrow (Namen et al. 1988). Within an area of high IL-7 concentration, pro-B cells begin the assembly of a competent BCR. Recombination of the immunoglobulin heavy chain locus is initiated by the RAG1-RAG2 protein complex, which generates double stranded DNA breaks between gene segments and specific recognition sites. The cleaved DNA ends are then joined by the non-homologous end joining proteins, which repair double-stranded DNA breaks irrespective of sequence homology (Lieber et al. 2003, Oettinger et al. 1990, Schatz et al. 1989). The accessory proteins, Igα and Igβ, are already expressed on the cell surface, although their function at this stage of development is unclear (Nagata et al. 1997). Some theories suggest that they act as a pro-B cell receptor of sorts, which may be required for continued development.

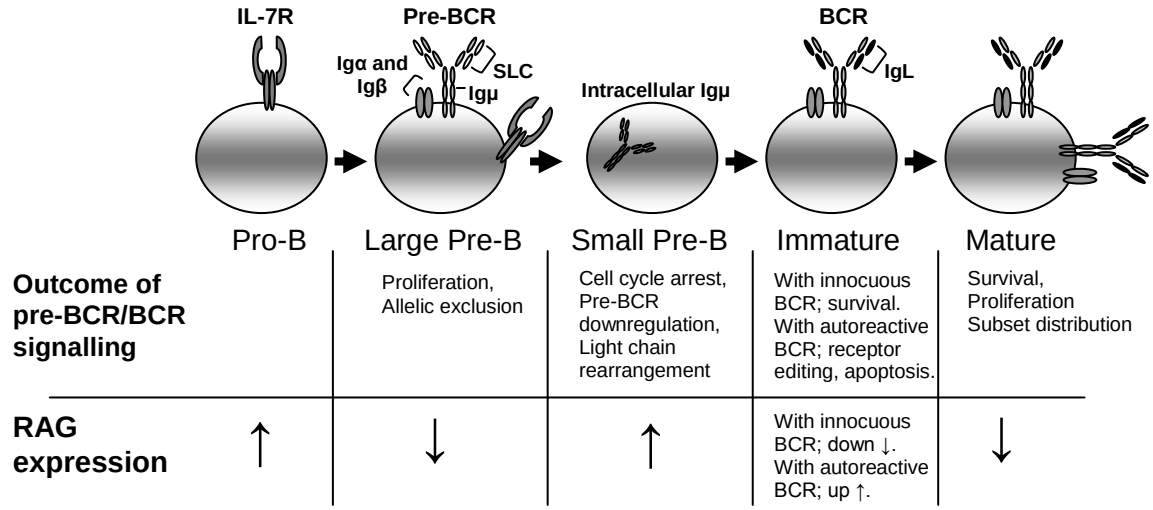


Figure 1.3: The pre-BCR/BCR in B cell development.
Rearrangement of the immunoglobulin heavy chain locus (IgH) is initiated at the pro-B cell stage in the bone marrow and, if successful, gives rise to the Igμ that is expressed on the cell surface in the form of the pre-BCR at the large pre-B cell stage. Signaling from the pre-BCR in turn provides feedback as to the functionality of the Igμ, inducing clonal proliferation, downregulation of the pre-BCR components and recombination of the immunoglobulin light chain genes (IgL). In-frame IgL gene rearrangements in small pre-B cells results in expression of a complete BCR on immature B cells. These immature cells are subjected to selection processes and eventually enter the pool of mature B cells in the periphery (Pictorial diagram adapted from Herzog et al. 2009).

Following recombination of the heavy chain locus, the pre-BCR complex is assembled. This complex includes the surrogate light chain proteins (VpreB and λ5), the μ-heavy chain, Igα, and Igβ. The complex is transiently expressed, and signals in a largely ligand-independent manner (Bankovich et al. 2007, Melchers 2005). Though several probable pre-BCR ligands have been identified, including galectin and heparan sulphate, none are required for *in vitro* pre-BCR induced proliferation or differentiation (Bradl et al. 2001, Bradl et al. 2003, Gauthier et al. 2002, Rolink et al. 2000). Intracellular signals generated by the pre-BCR provide rapid feedback as to the functionality of the μ-heavy chain. Only cells which express a signaling competent receptor can further mature. Successful pre-BCR signal transduction prompts upregulation of the IL-7 receptor. With greater receptor expression, the cell does not require high extracellular concentrations of IL-7 for continued survival. The pre-B cell becomes capable of migrating closer to the central sinus of the bone marrow, where the concentration of IL-7 is low (Fleming and Paige 2002, Marshall et al. 1998). With a functional pre-BCR it progresses to the large pre-B cell stage of development. Cells with non-functional pre-BCRs may attempt to migrate closer to the central sinus, but they cannot compete for the limited IL-7 in this local. Without sufficient IL-7, they cannot continue to survive and are eliminated.

1.c.2. The large and small pre-B cells

The recombination of the Ig heavy-chain locus, as described above, is most often non-productive. When a functional μ-heavy chain is produced, the resulting large pre-B cell undergoes allelic exclusion, followed by clonal expansion. The cytoplasmic tyrosine

kinase Syk is recruited to pre-BCR complex. In binding to the phosphorylated ITAMS of Igα and Igβ, the Syk protein itself is phosphorylated and in turn may phosphorylate neighboring pre-BCR complexes in a positive feedback loop (Bauer and Scheuermann 1993, Futterer et al. 1998, Kurosaki et al. 1995, Rolli et al. 2002, Rowley et al. 1995). Syk activation of the PI3K-Akt axis suppresses RAG activity (Herzog et al. 2008). Inhibition of further gene rearrangement, allelic exclusion, ensures that only one μ-heavy chain is expressed per B cell. The gene for the non-functional μ-heavy chain is silenced. A burst of cellular proliferation follows shortly thereafter; proliferation which does not occur in the absence of Syk (Cheng et al. 1995, Turner et al. 1995). This increases the number of pre-B cells with functional μ-heavy chains available for later rearrangement of the Ig light-chain locus.

At the small pre-B cell stage of development, the outcome of pre-BCR signaling shifts, first from cell proliferation to cell differentiation and then from downregulation to upregulation of the recombination machinery. Tight regulation of this shift is necessary to prevent the uncontrolled proliferation and genomic instability which are hallmarks of cancer. Subsequent rearrangement of the Ig light-chain locus is thought to be mediated via specific signals from the pre-BCR and/or the termination of surface pre-BCR expression due to downregulation of the surrogate light chain. Bruton's tyrosine kinase (Btk), BLNK, and PLCγ2 are all part of the signalosome proposed to play a role in this process (Herzog et al. 2009). In humans, X-linked agammaglobulinemia (XLA) results from a loss in Btk function. The loss can vary in severity. In its most extreme

presentation, XLA patients have a block in B cell development prior to rearrangement of the Ig light-chain locus (Satterthwaite and Witte 2000).

The transition from the large to small pre-B cell stage involves cessation of proliferation. Given that Foxo transcription factors are known to have anti-mitogenic properties, these molecules will likely have a critical role in this process, as will be discussed in later chapters.

1.c.3. The immature B cell

The BCR complex of immature B cells contains either a κ or λ light chain that replaces the surrogate light chain. IL-7 is no longer required for survival and IL-7 receptor is not expressed. This allows immature B cells to move closer to the central sinus of the bone marrow where IL-7 is not present (Fleming et al. 2002). It is at this stage of development where the BCR first interacts with its specific antigen. Whereas in large pre-B cells, it is only the capacity for signal transmission that is tested, here the receptor-ligand interaction itself is also of concern. The immature B cell stage is an important checkpoint in self-tolerance. If a functional, but non-self reactive receptor is expressed, it results in a tonic signal. This signal from the BCR, without antigen engagement, allows the cell to proceed in maturation (Meffre and Nussenzweig 2002, Shaffer, and Schlissel 1997, Tze et al. 2005, Schram et al 2008). However, either the absence of a tonic/basal BCR signal or BCR engagement with antigen, self or otherwise, leads to negative selection (Fig. 1.4, Cheng et al. 2009, Llorian et al 2007, Melamed et al, 1998, Schram et al 2008, Verkoczy et al 2007, King et al. 2000).

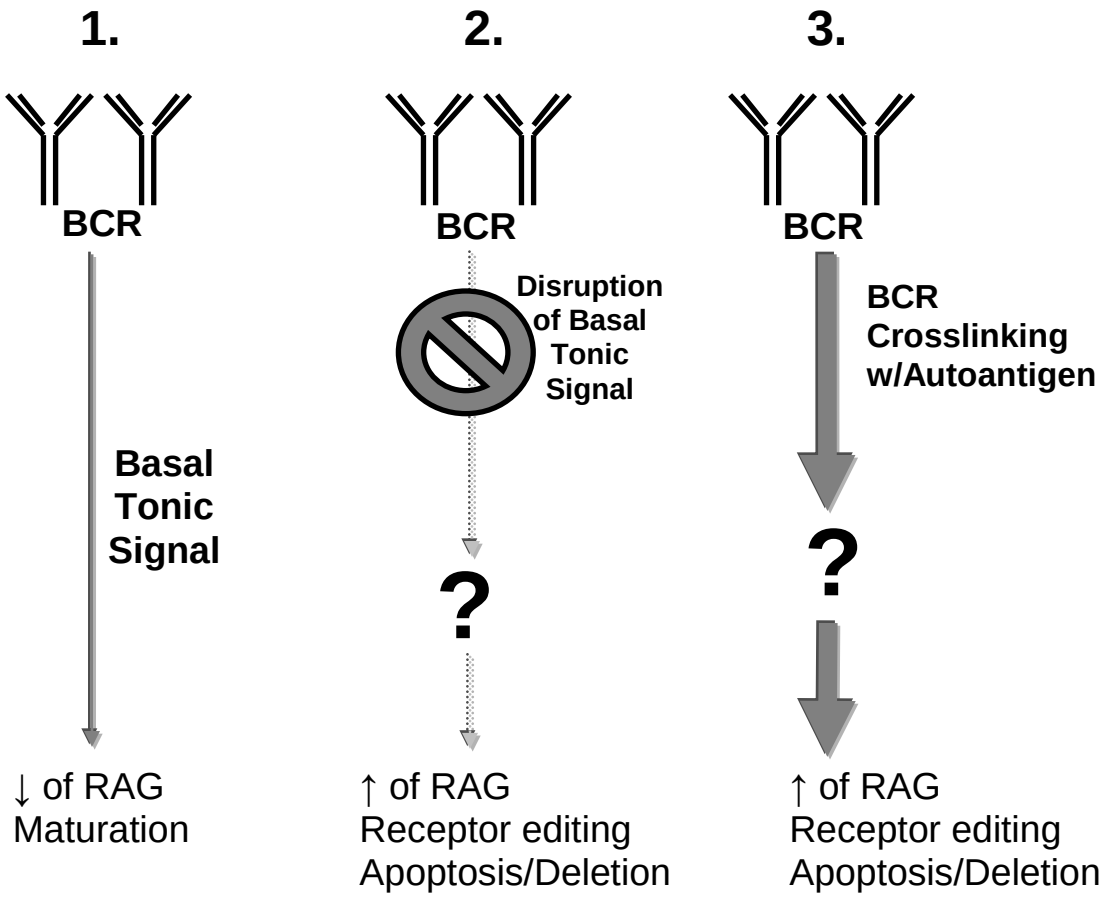


Figure 1.4: Model for immature B cell signaling through the BCR
In immature B cells, signals transmitted through the BCR have varying end results. **(1)** There is a tonic or basal signal necessary for downregulation of RAG and further B cell maturation. Either **(2)** disruption of the tonic signal through the unligated BCR or **(3)** strong engagement of the BCR with anti-IgM or antigen leads to a "negative" outcome of receptor editing, anergy, or clonal deletion. Whether this "negative" outcome occurs via the same or different mechanisms in **(2)** and **(3)** has yet to be determined.

Negative selection in immature B cells may refer to RAG-mediated receptor editing, clonal deletion, and/or anergy. Upon encounter with antigen or disruption of the tonic BCR signal, the immature B cell decreases surface BCR expression and there is continued rearrangement of the light chain locus. If the newly generated receptor is signaling competent and not autoreactive, the cell is "rescued" and development continues. Otherwise, the cell is driven towards apoptosis. A role for PI3K in regulating these outcomes will be discussed further below.

Immature B cells with a weakly self-reactive BCR may, in some cases, be rendered anergic. These cells mature, exit the bone marrow, and migrate to the secondary lymphoid organs, including the lymph nodes and spleen. Anergic cells cannot, however, upregulate activation markers, proliferate, or secret immunoglobulin upon BCR crosslinking (Goodnow 1988). The molecular mechanisms underlying this process are not fully understood, although they are thought to involve changes in signaling events both proximal and distal to the BCR. Vilen et al. have noted, for example, that the association of Igα and Igβ with the BCR complex is destabilized (Vilen et al. 1999). Only a small number of substrates are tyrosine phosphorylated after BCR aggregation, and NFkB and JNK are not activated (Dal Porto et al. 2004, Healy et al. 1997). The cells exhibit an elevated basal intracellular Ca^{2+} concentration, but no further increase in Ca^{2+} upon stimulation (Benschop et al. 2001, Healy et al. 1997). Anergic B cells also have a markedly short half-life relative to wild-type mature B cells. This is due, at least in part, to a greater requirement for B-cell-activating factor belonging to the TNF family (BAFF) (Thien et al. 2004). Several recent studies have proposed that the splenic T3 B cell

population is comprised of these anergic B cells (Merrell et al. 2006, Teague et al. 2007). T3 cells are thought to arise from the T2 population described below. The purpose of the T3 population in the immune response is still, as of yet, undetermined.

Immature B cells which escape negative selection in the bone marrow exit to the periphery. They home to the spleen, where they undergo additional differentiation through the T1 (CD93+, IgM^{high}, CD23-) and T2 (CD93+, IgM^{high}, CD23+) stages of development. The earliest emigrants to the spleen, the T1 cells, remain sensitive to tolerance induction and antigen induced cell death. T1 cells give rise to T2, which are susceptible to anti-IgM-mediated apoptosis as well (Allman et al. 2001, Chung et al. 2002). There are, however, conflicting studies which also indicate some proliferative potential for T2 cells (Loder et al. 1999, Petro et al. 2002, Su and Rawlings 2002). It is at the T2 stage of development that B cells acquire dependence on the cytokine BAFF for continued survival. T2 cells undergo rapid apoptosis in the absence of this molecule (Batten et al. 2000). BAFF-/- mice are thus deficient in mature B cells (Gross et al. 2001, Schiemann et al. 2001). Conversely, in transgenic mice overexpressing BAFF, B cell numbers are increased and the mice develop an autoimmunity resembling systemic lupus erythematosus (SLE). This due, in part, to the survival of self-reactive B cells which would normally be eliminated at the transitional stages of development (Gross et al. 2000, Mackay et al. 1999).

1.c.4. The mature B cell

A B cell which passes through the T1 and T2 stages of development has reached maturity. At maturation, there is a tonic/basal signal through the unligated BCR which is necessary for continued survival. Lam et al. demonstrated this requirement using transgenic mice in which BCR expression could be conditionally ablated by Cre-mediated gene disruption. 10-20 days after BCR expression was ablated, all pre-existing peripheral mature B cell subpopulations were eliminated. Only $CD43^{+}$ pro-B cells were present in the bone marrow (Lam et al. 1997).

B cells with a BCR capable of transmitting tonic signals may develop into one of two mature subsets, follicular (FO) or marginal zone (MZ) cells. FO cells are the larger of the two populations, at about 60% of the total B cells These cells recirculate throughout the periphery, remaining quiescent unless they encounter their cognate antigen. FO cells are capable of participating in both T-cell dependent and independent antibody responses and, in the former case can further differentiate in germinal centers to give rise to high-affinity, isotype switched plasma and memory cells (Lopes-Carvalho and Kearney 2004, Srivastava et al. 2005). In contrast, MZ cells permanently reside in the spleen, surrounding the splenic sinuses. Though these cells make up only approximately 5-10% of the total splenic B cell population, they are ideally located to respond rapidly to blood borne pathogens. The MZ BCR repertoire, unlike the FO, is biased towards T-cell independent, 'innate-like' antigens such as bacterial cell wall components. They are rapidly activated in response to these antigens to generate massive waves of short-lived IgM-secreting plasmablasts (Lopes-Carvalho et al. 2005). In vitro, MZ B cells are particularly sensitive to activation through the Toll-like receptors (TLRs). These

receptors designated TLR2, TLR4, TLR5, etc., each recognize a range of conserved molecules from a group of pathogens. TLR4, for example, binds to lipopolysaccharide (LPS) from Gram-negative bacteria (Pasare and Medzhitov 2005). MZ cells respond well to these ligands but, relative to FO B cells, proliferate poorly in response to BCR engagement alone (Lopes-Carvalho et al. 2005).

There appear to be two major factors that influence FO versus MZ differentiation; BCR specificity, as indicated above, and signal strength. In the latter case, the BCR as described above normally provides a baseline constitutive tonic survival signal. A relatively strong BCR signal commits the cell to a FO fate, whereas a weak signal is permissive for MZ development (Pillai et al. 2005). For example, in the absence of CD21/CR2, a known positive regulator of BCR signaling, MZ B cells are overrepresented (Cariappa et al. 2001). Mice lacking CD22, a negative regulator of BCR signaling, have the opposing phenotype. They have no MZ B cells (Samardzic et al. 2002). Non-BCR signals such as those mediated by Notch also promote the development of MZ B cells (Kuroda et al. 2003, Tanigaki et al. 2002).

A third population of mature B cells, the B-1cells, resides primarily in the pleural and peritoneal cavities. B-1 and MZ cells share many of the same functional and phenotypic characteristics. In comparison to FO cells, they have an ‘innate-like’ BCR repertoire and differential responses to TLR ligands vs. BCR engagement (Hardy and Hayakawa 2001, Martin and Kearney 2001, Rothstein 2002, Wortis and Berland 2001). It is the B-1 population which produces the majority of natural IgM, critical in the early phase of responses to bacterial, viral, and parasitic infections (Paciorkowski et al. 2000,

Martin et al. 2001, Baumgarth et al. 2000). These cells also give rise to mucosal IgA secreting plasma cells (Kroese et al. 1989), which are involved in neutralizing pathogens. The origin of B-1 cells is controversial. The lineage hypothesis states that B-1 and B-2 (FO and MZ) cells represent separate lineages, derived independently from hematopoietic stem cells (Hayakawa et al. 1985). Indeed, a progenitor which gives rise to B-1, but not B-2, cells was recently discovered in both fetal liver and adult bone marrow (Montecino-Rodriguez et al. 2006). However, there are also lines of evidence to suggest that with certain BCR specificities and signal strength, B1 cells can be induced to differentiate from the transitional B cell population of the spleen. Immunoglobulin (Ig) transgenes derived from B-1 cells, for example, force the majority of B cells expressing them to adopt a B-1 phenotype (Arnold et al. 1994, Chumley et al. 2000, Lam and Rajewsky 1999, Watanabe et al. 1999). Mice carrying mutations that reduce BCR signaling thresholds (Lyn−/−, CD22−/−, PD-1−/−, CD72−/−, CD19 transgene) also have increased numbers of B-1 cells (Hardy and Hayakawa 2001, Wortis and Berland 2001).

I.d. PI3K signaling in B cell development

As described above, the BCR is a central regulator of B cell fate. BCR signaling is required for the development, survival, and recirculation of naïve B cells, as well as for antigen-specific immune responses. Ligation of the BCR can have different outcomes in different contexts, including proliferation, differentiation, anergy or apoptosis. Given the multitude of signaling pathways which may be activated by the BCR, an important

question in the field has been to identify key components that determine B cell fate. Phosphatidylinositol 3-kinase (PI3K) is one such component (Fruman et al. 1999, Donahue et al. 2003).

The term PI3K refers to a family of enzymes that phosphorylate D-*myo*-phosphatidylinositol or its derivatives on the 3-hydroxl of the inositol head group. The 3-phosphorylated inositol lipids are membrane targeting signals that mediate membrane recruitment of selected proteins. PI3Ks are categorized in three classes, class I, II, and III, depending on the subunit structure, regulation, and substrate selectivity (Vanhaesebroeck et al. 2001, Fruman et al. 1998). In B cells, the most important type appears to be the class I PI3Ks, which are comprised of a p110 catalytic subunit and a tightly associated p85 regulatory subunit (Deane and Fruman 2004). Within this class there are three p110 isoforms (p110α, p110β, and p110δ) and two p85 isoforms (p85α and p85β). The p110δ and p85α isoforms are most dominant in B lymphocytes. Here we describe two major pathways downstream of these PI3Ks: the PI3K/Akt and PI3K/Btk/BLNK/PLCγ2 pathways (Fig. 1.5). How each controls the Foxo family of transcription factors in regulating B cell homeostasis will be detailed in later chapters.

1.d.1. Mature B cell activation

1.d.1.a. PI3K activation following BCR engagement

In the mature B cell, ligand-aggregated BCR partitions into the glycosphingolipid-rich microdomains of the plasma membrane. In these ‘lipid rafts’, there is an increased concentration of the *src*-family kinases Lyn, Fyn, Blk, and Lck

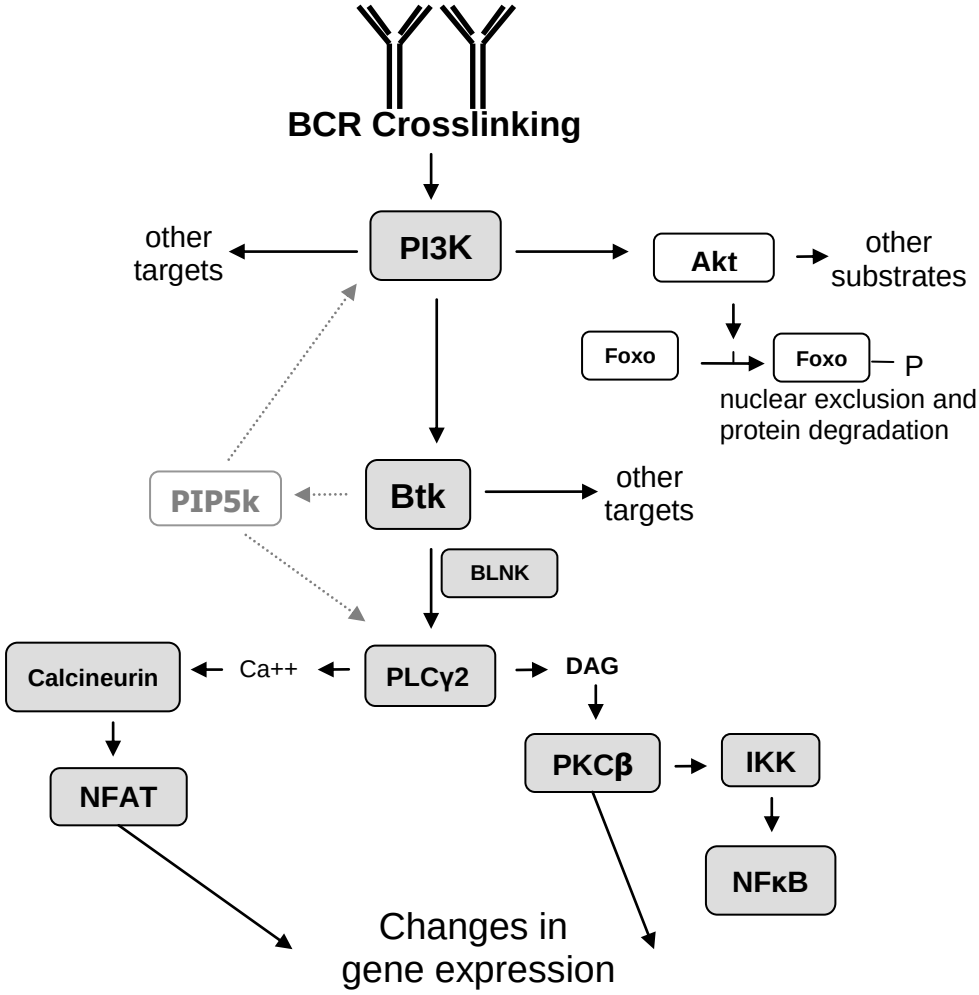


Figure 1.5: Simplified model for the regulation of signaling downstream of PI3K in BCR-stimulated mature B cells.
Following BCR stimulation, PI3K/Akt controls Foxo protein degradation as well as the activation of additional substrates. PI3K/Btk/BLNK/PLCγ2 promotes activation of NFAT and NFκB, leading to additional changes in gene expression that also promote B cell proliferation and survival.

(Cheng et al. 2001). These molecules act in a largely redundant manner to facilitate the phosphorylation of the Igα and Igβ ITAMs (Kurosaki 1999). Tyrosines within the YxxM motifs of the B cell coreceptor CD19 are also phosphorylated. It is this molecule which plays a principle role in recruitment of PI3K to the membrane. The two phosphorylated YxxM motifs on CD19 act as binding sites for the SH2 domains of p85 adaptor subunit of PI3K (Buhl and Cambier 1999, Fujimoto et al. 1998, Tuveson et al. 1993, Wang et al. 2002). The catalytic activity of the p110 subunit is subsequently increased. The p110 subunit interacts with p85 and is brought in close proximity to available substrate at the membrane (Gold et al. 2000, Wang et al. 2002). BCR ligation of CD19-deficient B cells does not, however, result in complete ablation of PI3K activation, suggesting some level of functional redundancy. Syk, which binds to the phosphorylated ITAMs of Igα and Igβ, is likely responsible for this residual PI3K activation. The expression of a dominant-negative Syk mutant in the A20 mature B cell line resulted in the partial inhibition of PI3K activation (Beitz et. al 1999). The phosphorylation of CD19 was not affected. Other proteins capable of bringing PI3K to the membrane include the adaptor molecule BCAP, the adaptor and ubiquitin ligase Cbl, and adaptor/scaffolding molecules Gab1 and Gab2 (Ingham et al. 1998, Kim et al. 1995, Okada et al. 2000).

At the B cell membrane, the principle substrate for PI3K is phosphadylinositol 4,5-bisphosphate ($PI(4,5)P_2$). PI3K mediated phosphorylation of $PI(4,5)P_2$ yields phosphatidylinositol 3,4,5-triphosphate (PIP_3). The SH2 domain-containing inositol phosphatase (SHIP) in turn removes the D5 phosphate from PIP_3 to generate phosphatidylinositol 3,4-biphosphate ($PI(3,4)P_2$). $PI(3,4)P_2$ can also result from the

phosphorylation of phosphatidylinositol 4-phosphate (PIP4) by PI3K (Gold et al. 2000, Fig. 1.6).

Both PIP_3 and $PI(3,4)P_2$ act as ligands for the pleckstrin homology (PH) domains that are found in a number of cytosolic signaling proteins, including Akt and Btk (Toker and Cantley 1997). These will be discussed in further detail below. PIP_3 and $PI(3,4)P_2$ bring these enzymes in close proximity to their membrane-associated substrates. PIP_3 and $PI(3,4)P_2$ also co-localize those PH-domain containing proteins which act together in the same signaling pathways. Either PIP_3 or $PI(3,4)P_2$ binding can induce conformational changes in the target molecule(s), affecting subsequent enzymatic activity and substrate interactions (Toker and Cantley 1997). It is intriguing to note that while Akt has a higher affinity for $PI(3,4)P_2$, both $PI(3,4)P_2$ and PIP_3 are required for optimal kinase activation. Btk, on the other hand, preferentially interacts with PIP_3. $PI(3,4)P_2$ is not required for its activity (Rameh et al. 1997). These observations suggest that SHIP, which generates $PI(3,4)P_2$ from PIP_3, could influence signal strength through either Akt or Btk by controlling substrate availability. Indeed, activation of SHIP upon cocrosslinking of the BCR with the inhibitory receptor FcγRIIb results in decreased membrane localization of, and signaling by, Btk (Bolland et al. 1998, Scharenberg et al. 1998).

The study of a B cell conditional knockout of the p85α subunit of PI3K emphasizes the importance of PI3K-derived lipids to B cell development and activation. B cells deficient in p85α cannot recruit PI3K to the plasma membrane. The development of the cells is seriously impaired. Cells which do make it to the periphery proliferate very poorly to BCR crosslinking (Fruman et al. 1999, Suzuki et al 1999). Mice with a null

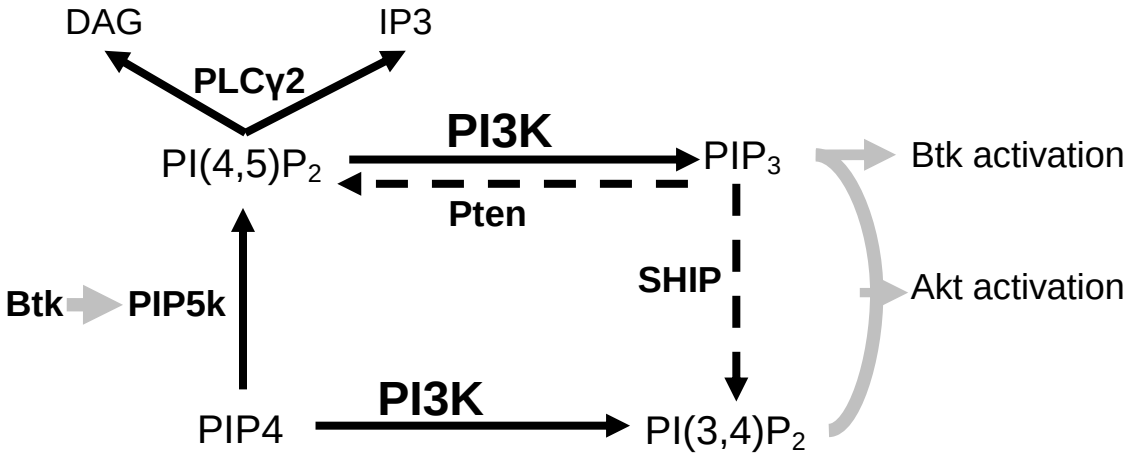


Figure 1.6: Phosphoinositide signaling pathways
This schematic illustrates some of the pathways leading to the various phosphorylated forms of PI. Phosphorylation reactions are indicated with solid black lines while dephosphorylation reactions are shown with dotted black lines.

mutation or a kinase-dead knock-in mutation of p110δ have been generated. They exhibit similar B cell defects to those in the p85α deficient mice (Okkenhaug et al. 2002, Clayton et al. 2002, Jou et al. 2002). This is in contrast to B cells lacking Pten, a key functional antagonist to PI3K which dephosphorylates PIP_3 to generate $PI(4,5)P_2$. These cells are hyperproliferative in response to mitogenic stimuli and exhibit a lower threshold for activation through the BCR (Anzelon et al. 2003).

1.d.1.b The PI3K/Akt pathway

The serine/threonine protein kinase Akt (also known as protein kinase B (PKB)) was originally identified as part of an oncogene responsible for spontaneously arising leukemias and lymphomas (Downward et al. 1998, Franke et al. 1995). Akt is a major target of PI3K signaling. Akt has a PH domain that binds PI3K derived lipids. PH domain binding to either PIP_3 or $PI(3,4)P_2$ recruits Akt to the plasma membrane and promotes Akt dimerization (Andjelković et al. 1997, Datta et al. 1995). The resulting conformational change enables protein phosphorylation at threonine 308 and serine 473 by PDK1 and PDK2, respectively. Akt then translocates to cytosol and to the nucleus (Astoul et al. 1999, Meier et al. 1997).

A number of Akt substrates have been identified. Many of these substrates are regulators of apoptosis and cell survival (Gold et al. 2000). They include Bad, a death-promoting member of the Bcl-2 family (Datta et al. 1997, del Peso et al. 1997). Bad promotes apoptosis by neutralizing bcl-xl, an anti-apoptotic member of the Bcl-2 family. Akt-mediated phosphorylation of Bad causes it to dissociate from bcl-xl and bind to 14-

3-3 proteins. 14-3-3 binding sequesters Bad. Akt also phosphorylates caspase-9 to prevent its cytochrome c-dependent proteolytic activation. Cytochrome c-dependent proteolytic activation of capase-9 is an important step that, if allowed to occur, commits a cell to apoptosis (Cardone et al. 2000). Other molecules directly regulated by Akt include E2F (Brennan et al. 1997) and glycogen synthase kinase-3 (Gold et al. 1999).

Akt has been shown to mediate Foxo protein phosphorylation in a number of cell types (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999, Rena et al. 1999, Takaishi et al. 1999, Tang et al. 1999). This phosphorylation promotes the exclusion of Foxos from the nucleus. They are subsequently degraded in the cytosol. As work presented in this thesis was commencing, a study by Yusuf et al. was published examining this phenomenon of Foxo protein phosphorylation in mature splenic B cells. BCR engagement triggered Akt-dependent phosphorylation and nuclear export of the Foxo1 protein, promoting cell survival and proliferation. Conversely, expression of a variant of Foxo1, which cannot be phosphorylated by Akt, induced partial arrest in G_1 phase of the cell cycle and increased apoptosis (Yusuf et al. 2004). This finding demonstrates that Foxo transcription factors play a critical role in regulating B cell homeostasis. The specifics of this role remain to be teased out and will be explored further in later chapters. The work of Yusuf et al. focuses on protein phosphorlyation in the control of Foxo activity. We hypothesize that other levels of Foxo regulation, such as mRNA expression, also have influence.

1.d.1.c The PI3K/Btk/BLNK/PLCγ2 pathway

BCR crosslinking activates the Tec kinase Btk via the concerted effort of PI3K and Src family kinases. The PI3K derived ligand PIP_3 is bound by the PH domain of Btk, and translocates Btk to the plasma membrane. The importance of this interaction is demonstrated by the observation that the naturally occuring xid mutation, a point mutation in the Btk PH domain which impairs PIP_3 binding, results in a phenotype almost identical to complete Btk deficiency (Rawlings et al. 1993). Btk-/- and xid mice have a block at the T2 to mature B transition in the periphery, and a lack of B-1 cells. These mice also have reduced levels of IgM and IgG3 in the serum. The mice fail to react to type II T-independent antigens. They also have poor mitogenic responses to the crosslinking of several cell surface receptors, including the BCR, CD38, RP-105, IL-5R, IL10-R and in some cases CD40 (Fruman et al. 2000, Khan et.al 1995, Satterthwaite et al. 2000). It is possible to uncouple the Btk-mediated processes of B cell development and function. Using transgenic mice expressing 0%, 25%, 50%, and 100% of endogenous Btk levels it has been shown that B cell functional responses are significantly more sensitive to changes in Btk dosage than is B cell development. 25% of endogenous Btk levels are sufficient to support the development of normal numbers of mature B cells when expressed from a transgene in Btk-/- mice. Yet, these cells remain functionally impaired. The study of the effects of Btk deficiency on downstream signaling events is therefore possible (Satterthwaite et al. 1997).

In wildtype mice, the Btk that is localized to the membrane via PI3K is transphosphorylated at Y551 by the Src family kinases (Bolland et al. 1998, Rawlings et al 1996, Scharenberg et al. 1998). This leads to Btk autophosphorylation at Y223, located

in the SH3 domain, resulting in full catalytic activation (Park et al. 1996). The most well characterized signaling function of Btk is to activate PLCγ2. Btk exerts this function both directly, by phosphorylating PLCγ2 (Humphries et al. 2004, Rodriguez et al. 2001, Watanabe et al. 2001), and indirectly, by increasing the local concentration of PLCγ2's substrate via Btk's interaction with PIP5K (Saito et al. 2003). Direct activation of PLCγ2 by Btk is facilitated by the adaptor protein BLNK (Hashimoto et al. 1999). Once activated, PLCγ2 cleaves $PI(4,5)P_2$ to produce DAG and IP3. DAG and IP3 activate PKCβ and initiate Ca^{2+} influx, respectively. PKCβ signaling leads to activation of NFkB via degradation of IkB, while the increase in intracellular Ca^{2+} activates the phosphatase calcinuerin, resulting in translocation of NFAT to the nucleus (Dal Porto et al. 2004, Fruman et al. 2000, Guo et al. 2004, Kurosaki et al. 2000). The changes in gene expression which follow serve to promote mature B cell proliferation and survival.

There is data to indicate that the individual components of the Btk/BLNK/PLCγ2 signalosome also have functions independent from one another following BCR stimulation. These functions are also important to the life of the B cell. Btk has, for example, been shown to interact with or phosphorylate a number of transcription factors, including transcription factor II-I (Novina et al. 1999), STAT-5a (Mahajan et al. 2001) and BRIGHT (Webb et al. 2000). None of these interactions have been shown to require BLNK. BLNK likewise can also physically associate with a number of other signaling molecules, including growth factor receptor-binding protein (Grb2), Vav and Nck (Fu et al. 1998) that are not necessarily involved in the regulation of the Btk/PLCγ2 pathway. Thus it is not surprising that mice lacking both Btk and BLNK have a more severe

phenotype than mice lacking either molecule alone. In BLNK-/-Btk-/- mice, there is a block at the pre-B cell stage of development associated with increased pre-BCR expression (Jumaa et al. 2001). We have also shown that Btk-/-PLCγ2-/- mice have a more severe phenotype than that of either individual knockout (Halcomb et al. 2007). It is PLCγ2, but not Btk, which contributes to the BCR induced activation of ERK in splenic B cells. In contrast, Btk, but not PLCγ2, suppresses pre-B cell tumors in BLNK-/- mice (Halcomb et al. 2007). These findings demonstrate that the components of the Btk/BLNK/PLCγ2 signalsome are capable of acting both in concert and independently throughout B cell development. And while the proximal signaling events are well defined, additional characterization of how these signals are ultimately transmitted into changes in gene expression and biological outcomes has only just begun. This is a focus of our work.

Upregulation of transcripts for both cyclin D2 (Glassford et al. 2003) and bcl-xl (Solvason et al. 1998) has been noted following signaling events downstream of the Btk/BLNK/PLCγ2 signalsome. These events are important for the proliferation and survival of mature B cells in response to BCR crosslinking. A bcl-xl transgene partially rescues some, but not all, aspects of the xid phenotype (Solvason et al. 1998). Cyclin D2 is a regulator of the G1/S transition (Ando et al. 1993). Splenic B cells from Cyclin D2-/- mice have impaired proliferative responses to BCR crosslinking. The mice also have reduced numbers of B-1 cells (Lam et al. 2000, Solvason et al. 2000). This phenotype is quite similar to that of mice expressing reduced Btk levels (Satterthwaite et al. 1997). Given the above, it is intriguing to note that in other cell types, Foxo transcription factors

inhibit cyclin D2 (Schmidt et al. 2002). Could it be the same for quiescent B cells? Then, following BCR stimulation, the PI3K/Btk/BLNK/PLCγ2 pathway would subsequently act to downregulate Foxo activity and increase Cyclin D2 expression. We will provide data demonstrating this to be the case in later chapters of this thesis.

1.d.1.d. Other pathways activated by PI3K

Akt and Btk are not the only molecules which act downstream of PI3K. Son of Sevenless (SOS) and Vav also contain PH domains capable of binding PI3K-derived lipids. This binding increases the ability of these enzymes to activate Rho family GTPases such as Rac1 (Nimnual et al. 1998, Han et al. 1998). Rho family GTPases have multiple roles in BCR signaling. Rac1 links the BCR to activation of the c-Jun N-terminal kinase (JNK) mitogen-activated protein (MAP) kinase (Hashimoto et al. 1998). The Rho family GTPases are also key players in a positive feedback loop. They regulate the PI4P 5-kinases that produce $PI(4,5)P_2$, the substrate for both PI3K and PLCγ2 (Chong et al. 1994, Tolias et al. 1995, Ren et al. 1996). This promotes sustained signaling through these molecules following BCR activation.

Inhibitor studies indicate that activation of ERK in response to BCR engagement is also PI3K-dependent. Intriguingly, pretreatment of B cells with stimuli such as IL-4 overcomes this requirement for PI3K in BCR-induced ERK activation (Guo and Rothstein 2005). This suggests that the functional outcome of PI3K signaling may vary depending on the prior experience of a B cell.

1.d.2 Immature B cell signaling through the BCR/PI3K

In mature B cells, engagement of the BCR by foreign antigen leads to cell survival and proliferation. This "positive" outcome enables the body to mount a protective immune response against pathogen. In immature B cells, signals transmitted through the BCR have a different end result. As described above, either disruption of the tonic signal through the unligated BCR or strong engagement of the BCR with anti-IgM leads to a "negative" outcome of receptor editing, anergy, or clonal deletion. Whether they reach this "negative" outcome through the same or separate signaling pathways remains to be determined (Fig. 1.4).

There are differences in the signaling cascades stemming from the BCR in mature and immature B cells (King and Monroe 2000). These variations can account for the opposing "positive" and "negative" outcomes. In immature B cells with an unligated BCR, there is a basal level of PI3K which is required for positive selection (Verkoczy et al. 2007, Llorian et al. 2007). This steady-state low level enzymatic activity promotes the downregulation of RAG expression, allowing for further cellular differentiation. Mice deficient in p85, the regulatory subunit of PI3K, have elevated RAG levels in immature B cells, excessive rearrangement of the light chain locus, and fail to adequately express cell surface differentiation markers *in vivo* and *in vitro* (Verkoczy et al. 2007). A similar phenotype was observed in p110δ-/- mice (Llorian et al. 2007).

When the BCR of immature B cells engages antigen, it does not move into membrane lipid rafts (Sproul et al. 2000). The BCR is thus theorized to have limited accessibility to the downstream signaling components which would normally promote

cell survival. Cheng et al. have also noted that there is a higher intracellular level of Pten, the functional antagonist of PI3K, in immature B cells. When the BCR is ligated by self-antigen, this increased Pten presence counteracts sufficient PI3K activation. The production of PIP_3 is subsequently inhibited and downstream anti-apoptotic signaling components, such as Akt, are not engaged as they would be in a mature B cell when PI3K activity is high (Cheng et al. 2009). Instead, the expression of Bim and other apoptotic genes rises promoting a "negative" outcome (Allman et al 2001, Norvell et al. 1995). In addition to the differences described above, immature B cells have been shown to have a higher basal expression of Lyn, PLCγ2, Btk, and BLNK, while Syk expression is decreased. The cells are also more sensitive to antigen stimulation as measured by changes in intracellular free Ca^{2+} (Benschop et al. 1999). This drives efficient clonal deletion.

1.d.3 Pre-B cell signaling through the BCR/PI3K

The components of the PI3K pathway also contribute to pre-BCR mediated events. As described above, pre-BCR signaling through PI3K/Akt downregulates Rag expression, allowing expansion of the large pre-B cell population. Btk, BLNK, and PLCγ2 on the other hand, promote pre-BCR downregulation, cessation of proliferation, and light chain rearrangement in small pre-B cells (Herzog et al. 2009). What regulates the switch between these two major outcomes downstream of PI3K is unclear but warrants further investigation. At later stages of B cell development, Foxo transcription factors have been shown to inhibit cell cycle progression (Yusuf et al. 2004). Therefore,

it would not be surprising to find that they play a similar role at the large to small pre-B cell transition.

1.e. Conclusion

Here we present evidence that BCR signaling through PI3K plays an important role at multiple stages of B cell development. Proliferation, quiescence, and apoptosis are all important processes for the generation of a diverse, self-tolerant mature B cell repertoire. We and others have shown that the pro-apoptotic/anti-mitogenic Foxo family of transcription factors plays an important role during this process. Work presented within this thesis demonstrates that PI3K regulates Foxo1, Foxo3, and Foxo4 through a previously undescribed means of control at the mRNA level, in addition to protein phosphorylation. It also demonstrates that there are unique roles for individual Foxo family members at multiple stages of B cell development.

CHAPTER II

METHODS

II.a. Mice

Animals were housed in the both the south and north campuses of UTSWMC. All animal experimentation was done at UTSWMC in accordance with protocols approved by the Institutional Animal Care and Use Committee. Foxo3-/- mice (Castrillon et al. 2003) and wild type controls to which they were compared were of the FVB genetic background. Wild-type mice of the C57BL/6 genetic background were used in the inhibitor studies and bone marrow isolation. *Btk*^{lo} mice (mice expressing 25% of endogenous Btk levels in B cells) are *Btk*-/- mice (Khan et al. 1995) carrying a wild-type *Btk* transgene driven by the IgH chain promoter and enhancer (Satterthwaite et al. 1997). *BLNK*-/- (Pappu et al. 1999) mice were mated to *Btk*^{lo} mice to generate progeny of the appropriate genotypes, as described in Whyburn et al. (Whyburn et al. 2003). Because *Btk*-/-, *Btk*^{lo}, *Btk*^{lo}*BLNK*+/-, *BLNK*-/- (Pappu et al. 1999), and *PLCγ2*-/- (Wang et al. 2000) mice were of mixed genetic background (C57BL/6 X 129), littermates were compared directly when possible and experiments were repeated with multiple litters. Mice were genotyped by PCR.

II.b. Cell lines

The WEHI 231 immature B cell line was a generous gift from C. Mohan (University of Texas Southwestern Medical Center). Phoenix 293T cells were provided by J. Schatzle (University of Texas Southwestern Medical Center).

II.c. Flow cytometry

Splenocytes, bone marrow, peritoneal exudate, and peripheral blood were depleted of red blood cells and stained with combinations of anti-CD21-FITC, anti-CD43-FITC, anti-IgM-PE, anti-CD23 PE, anti-CD5 PE, anti-B220-PerCP, anti-AA4.1-biotin, anti-CD23-biotin, anti-CD93-biotin, anti-CD138-biotin, anti-Igλ light chain-biotin, anti-CD22-biotin (eBioscience), and anti-IL-7Rα(CD127)-biotin (eBioscience). Antibodies were purchased from BD Pharmingen unless otherwise indicated. Biotinylated antibodies were detected with streptavidin-allophycocyanin (Caltag). Samples were run on a FACS Calibur (Becton Dickinson). Data were analyzed with CellQuest (Becton Dickinson) software. Live cells were gated based on forward and side scatter.

II.d. IL-7 cultures

Bone marrow was cultured at 10^6cells/mL in RPMI + 10% FBS + varying concentrations of IL-7 (R&D Systems) for 5 days. Proliferation was measured by [^{3}H]thymidine (Amersham) for an additional 24 hours. Additional analysis was

performed on other cell cultures expanded for 5 days in 10 ng/ml IL-7. B220+ cells were enumerated by flow cytometry, cDNA was prepared for Quantitative real-time PCR **(**Q-PCR) analysis of Rag2 expression, and cell cycle status was assessed by fixing cells in 70% ethanol for >24 hrs and then incubating with propidium iodide (Roche) and RNase A (Qiagen) for 20 minutes prior to analysis by flow cytometry.

II.e. Immature and mature recirculating B cell purification

Bone marrow was incubated for 5 minutes in 0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA to deplete red blood cells. B cells from the bone marrow were then purified by positive selection with anti-B220 magnetic beads using the IMag system (BD Pharmingen). Cells were washed twice with 1X IMag buffer (PBS, 0.5% BSA, 2mM EDTA) to remove all traces of media. They were then incubated in 50μl anti-B220 magnetic beads/10^7 cells for 30 minutes at 4°C. The volume was then brought up to 2-8x10^7 cells/ml and cells were transferred to tubes for magnetic separation. The tubes were placed on the provided magnet for 6 minutes. The unbound fraction was then removed and cells resuspended in 1ml IMag buffer per tube. The tubes were then placed on magnet for an addition 4 minutes and the negative fraction was subsequently removed. B cells were resuspended and stained with anti-IgM-PE, anti-B220-PerCP, and anti-AA4.1-biotin (BD Pharmingen). The biotinylated antibody was detected with streptavidin-allophycocyanin (Caltag). Immature ($B220^+$, IgM^+, $AA4.1^+$) and mature recirculating

($B220^+$, IgM^+, $AA4.1^-$) cells were then sorted on an Aria (Becton Dickinson) for later experimentation.

II.f. Immature and mature recirculating B cell stimulation and immature B cell survival.

Purified immature B cells were harvested immediately (time 0), or stimulated with 10 μg/ml goat anti-mouse IgM $F(ab')_2$ (Jackson ImmunoResearch Laboratories) for either 14 or 18 h at 10^6/mL in RPMI + 10% FBS. Apoptosis was measured for time zero and 18 h samples by staining with annexin V-FITC (BD Pharmingen). Purified mature recirculating B cells were harvested at time 0, or stimulated with 10 μg/ml goat anti-mouse IgM $F(ab')_2$ (Jackson ImmunoResearch Laboratories) for 6 h at 10^6/mL in RPMI + 10% FBS.

II.g. Retroviral constructs

We amplified Btg-1, Foxo3, and Foxo4 by RT-PCR and cloned them into the pMIG retroviral vector, a gift of J. Schatzle (University of Texas Southwestern Medical Center). The following primers were used; Btg-1 forward, 5'-ATC GCT CGT CTC TCT CTG TCT CT-3', Btg-1 reverse, 5'-ATC CTT TCC TTG GAC TCA CAG GCT-3', Foxo3 forward (1), 5'-AAG GGA GGA GGA GGA ATG TGG AA-3', Foxo3 reverse (1), 5'AAG GAA ATG CTC CTC GCG GCG G-3', Foxo3 forward (2), 5'-GAG CCA

GCA GCA TGG CCG AAT-3', Foxo3 reverse (2), 5'-CAT TGT GGC GCA CTA ACC TGC TTT-3', Foxo4 forward (1), 5'-GAG AAC GGA GTG AAA GGG ACA GTT-3', Foxo4 reverse (1), 5'-AGT GTG TTG CCA CCA ATC CTT AGC-3', Foxo4 forward (2), 5'-AGA GCT CTT GGT GGA TGC TGA A-3', Foxo4 reverse (2), 5'-CAG GAC AGA CGG CTT CTT CTT GGG-3'. In the case Foxo3 and Foxo4 we amplified each primer set, (1) and (2), separately. Overlapping restriction digest sites were then utilized to cut and ligate together the pieces to make a single transcript. All constructs were DNA sequenced to check for accuracy.

II.h. Calcium phosphate transfection of Phoenix 293T cells to generate retrovirus.

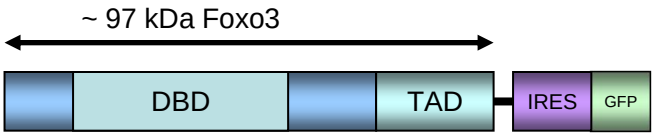
Phoenix 293T cells were plated out 24 hr prior to calcium phosphate transfection on 10cm plates. Cells were approximately 50-75% confluent at time of transfection. The media (DMEM + 10% FBS) was changed just prior to beginning the protocol. New media contained 25μM Choloroquine. 10μg of DNA per plate was resuspended in 450ul water. 50 μl 2.5M $CaCl_2$ was added to this solution. 500 μl 2x HeBs (0.28M NaCl, 0.05M HEPES, 1.5M Na_2HPO_4, pH 7.05) was placed in a separate tube and the DNA- $CaCl_2$ mixture was then added to this dropwise, while continually bubbling the liquid. Bubbling continued for 30 seconds. The solution was allowed to sit for an additional 5 minutes before being added, dropwise, to the Phoenix 293T cells. Cells were then incubated at 37 °C for 12 hrs. Media was changed and cells were allowed to make retrovirus for 48 hr

prior to harvesting the media and filtering it through a 0.22 mM low protein-binding filter (Millipore). Retroviral media not used immediately was kept frozen at -80 °C until use.

II.i. WEHI 231 retroviral infection and GFP+ sort.

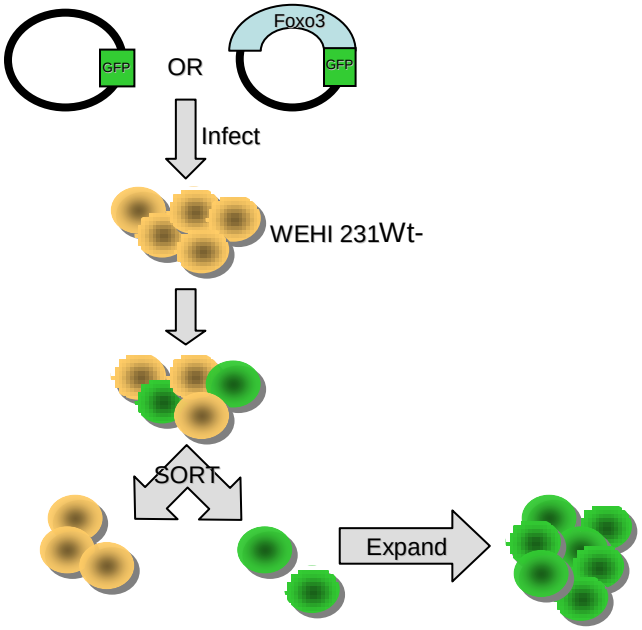
We followed a previously published protocol for WEHI 231 retroviral infection (Krebs et al. 1999). Cells were pelleted and resuspended at 10^6/mL in RPMI + 10% FBS. 0.5 ml of the WEHI 231 mixture was added to each well of a 6-well dish. Collected and/or thawed supernatant containing the desired retrovirus (Vector, Foxo3, Foxo4, or Btg-1 as described above) was passed through a 0.22 mM low protein-binding filter (Millipore) and 2.5 μl of 10 mg/ml polybrene (hexadimethrine bromide; dissolved in H_2O)(Sigma) was added to a 2 ml aliquot. The mixture was then put on the target cells and plate(s) were incubated at 37 °C. One hour later, a second 2 ml aliquot of retroviral supernatant plus polybrene was added to the cells. 16 h later, the cells were pelleted and resuspended in fresh RPMI + 10% FBS without polybrene. At 48 h post-infection, expression of the transferred gene could then be analyzed by flow cytometry. GFP+ cells (5-10% of the total culture) were subsequently sorted on an Aria (Becton Dickinson). The GFP+ cells were maintained in culture for a maximum of 3 weeks. The schematic for the sort is presented in Figure 2.1, along with a sample diagram of the Foxo3 construct and immunoblot showing upregulation of Foxo3 protein expression following retroviral infection.

A.



DBD = forkhead DNA Binding domain
TAD = transactivation domain
IRES = internal ribosome entry site
GFP = green fluorescent protein

B.



C.

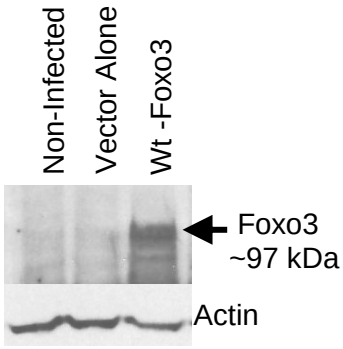


Figure 2.1: WEHI 231 retroviral infection and GFP+ sort with Foxo3 construct.
A) Diagram of the Foxo3 retroviral construct in the pMIG vector. **B)** The retroviral infection of WEHI 231 cells and subsequent GFP+ sort. **C**) Sorted GFP+ (Vector Alone and Wt-Foxo3) and GFP- (Non-Infected) WEHI 231 cells were harvested. Whole cell lysates were resolved by SDS-PAGE and immunoblotted for total Foxo3. The blot was also probed for actin to determine equal loading. The immunoblot shows higher levels of Foxo3 protein expression in WEHI 231 following retroviral infection with a Wt-Foxo3 construct.

II.j. WEHI 231 stimulation and survival

WEHI 231 cells were incubated at 0.5 x 10^6/mL in RPMI + 10% FBS alone or stimulated with 10 μg/ml goat anti-mouse IgM F(ab')$_2$ (Jackson ImmunoResearch Laboratories) for either 24 or 48 h. For inhibitor studies, cells were pre-treated with either 10 μM Ly294002 or 10 μM U0126 (Calbiochem) for 15 minutes prior to anti-IgM treatment**.** Apoptosis was measured for media alone, 24 and 48 h samples by staining with annexin V-PE (BD Pharmingen).

II.k. Splenic B cell purification

Splenocytes were incubated for 5 minutes in 0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA to deplete red blood cells. Splenic B cells were then purified by negative selection with anti-CD43 magnetic beads using the MidiMACS system (Miltenyi Biotec), according to the manufacturer's instructions. As measured by flow cytometry, purified B cells were >90% $B220^+$.

II.l. Splenic B cell stimulation for subsequent quantitative real-time PCR (Q-PCR) assays.

Purified B cells were harvested at time 0, incubated in medium alone, or stimulated with 10 μg/ml goat anti-mouse IgM F(ab')$_2$ (Jackson ImmunoResearch

Laboratories) for either 1, 6, 12, or 16 h at 10^6/mL in RPMI + 10% FBS. For inhibitor studies, cells were pre-treated with either 10 μM Ly294002, 50 μM Gö6850, 10 μM U0126 or 100 ng/ml Cyclosporin A for 15 minutes prior to anti-IgM treatment or 10 μM 11R-VIVIT for 2 hrs prior to anti-IgM treatment. All inhibitors were obtained from Calbiochem.

II.m. Splenic B cell stimulation for proliferation, survival, and differentiation assays

Splenic B cells were plated at 10^6/mL in RPMI + 10% FBS with media alone, 2 or 20 μg/mL anti-IgM $F(ab')_2$ fragments (Jackson ImmunoResearch Laboratories), 0.1, 1, or 10 ug/ml LPS (Sigma), 10 ng/mL PMA (Sigma) +1 μg/mL ionomycin (Calbiochem), or 10 ng/ml IL-4 (R&D Systems) + 1 μg/ml or 10 μg/ml anti-CD40 (BD Pharmingen) for 48 hours. Proliferation was measured by labeling cells with [^{3}H]thymidine (Amersham) for the final 8 hrs. Apoptosis was measured by staining with annexin V-PE and 7-AAD (BD Pharmingen). For differentiation studies, splenic B cells were plated at 5 x 10^5/ml in RPMI + 10% FBS with media alone, 20 μg/ml LPS (Sigma), or 1 μg/ml anti-CD40 (BD Pharmingen) + 10 ng/ml IL-4 (R & D Systems) for 72 hrs and subsequently stained with antibodies against B220 and CD138.

II.n. Quantitative real-time PCR (Q-PCR)

Total RNA was prepared using the RNeasy kit (Qiagen). cDNA was generated with a cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed in an Applied Biosystems 7300 Real Time PCR System using TaqMan reagents specific for mouse Btg-1, Cyclin D2, Cyclin G2, Foxo1, Foxo3, Foxo4, Edg1 ($S1P_1$), Rag2 and the internal control GAPDH (Applied Biosystems). Data were normalized to GAPDH using the delta comparative threshold cycle (Ct) method (Livak and Schmittgen 2001). Semiquantitative PCR was performed for 26–30 cycles (22 s at 95°C, 22 s at 59°C, 45 s at 72°C) using the following primers: Foxo1 forward, 5'-AAG AGC GTG CCC TAC TTC AAG GAT-3'; Foxo1 reverse, 5'-ATT TCA GAC AGA CTG GGC AGC GTA-3'; actin forward, 5'-GAG GCC CAG AGC AAG AGA G-3'; actin reverse, 5'-GTC ATC TTT TCA CGG TTG G-3'.

II.o. Immunoblots

Cells were lysed by boiling in SDS sample buffer for 10 min. Total cell extracts were electrophoresed by 8 or 10% SDS-PAGE gels and blotted to nitrocellose. Blots were blocked in 5% BSA in 10 mM Tris (pH 7.5) and 150 mM NaCl, and subsequently probed with anti-phospho-Foxo1 (Ser256), anti-Foxo1, anti-phospho-Akt (Ser473), anti-Akt (Cell Signaling Technology), anti-Foxo3 (Millipore), or anti-actin (Sigma-Aldrich) diluted in 10 mM Tris (pH 7.5), 250 mM NaCl, 0.05% Tween 20, and 0.2% sodium azide. A 1:1000 dilution was used for all antibodies with the exception of anti-actin. A 1:5000 dilution was used in this case. Blots were washed in 10 mM Tris (pH 7.5), 500

mM NaCl, and 0.05% Tween 20, and then incubated with HRP-conjugated goat anti-rabbit Ig (to detect anti-phospho-Foxo1 (Ser256), anti-Foxo1, anti-Foxo3, anti-phospho-Akt (Ser473), and anti-Akt) (Bio-Rad) or goat anti-mouse Ig (to detect actin) (Bio-Rad) diluted 1:5000 in 10 mM Tris (pH 7.5), 250 mM NaCl, and 0.05% Tween 20. Blots were washed in 10 mM Tris (pH 7.5), 500 mM NaCl, and 0.05% Tween 20, and HRP was subsequently visualized using an ECL kit (Amersham) according to the manufacturer's protocol. The program ImageJ was used to analyze relative band intensities.

II.p. Measurement of Ig levels by ELISA

Mice were bled prior to and one and two weeks after intraperitoneal immunization with either 10 μg TNP-Ficoll (Biosearch Technologies) in PBS or 100 μg NP-CGG (Biosearch Technologies) in Inject-Alum (Pierce). Serial dilutions of sera were assayed for total IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA by ELISA using the mouse clonotyping system (Southern Biotechnology Associates). Serum Ig concentrations were calculated based on purified Ig standards (Southern Biotechnology Associates). To assay anti-TNP and anti-NP antibodies, ELISAs were performed on serial dilutions of serum using plates coated with TNP- or NP-BSA (Biosearch Technologies) and secondary antibodies from the mouse clonotyping kit (Southern Biotechnology Associates). ELISAs were developed with an alkaline phosphatase substrate kit (Biorad) and OD405 read on a Bio-Tek Instruments microplate reader.

II.q. Measurement of anti-dsDNA and ssDNA IgM by ELISA

Mice were bled at 6-8 months and 9-11 months. To assay anti-dsDNA and anti-ssDNA antibodies, ELISAs were performed on serial dilutions of serum using plates coated with dsDNA or ssDNA (Sigma) and secondary IgM antibody from the mouse clonotyping kit (Southern Biotechnology Associates). ELISAs were developed with an alkaline phosphatase substrate kit (Biorad) and OD405 read on a Bio-Tek Instruments microplate reader.

CHAPTER III

IN MATURE B LYMPHOCYTES, BCR STIMULATION REGULATES FOXO PROTEIN PHOSPHORYLATION AND MRNA EXPRESSION VIA DISTINCT PATHWAYS.

Work presented in this chapter has been published in the *Journal of Immunology*, volume 178, pages 740–747.

Work presented in this chapter has also been published in *International Immunology*, volume 21(7), pages 831-842.

III.a. Introduction

When mature resting B cells encounter their cognate Ag, the BCR is engaged. As a result, the cells enter and progress through the cell cycle and receive a variety of survival signals. Each of these processes depends on PI3K (Fruman et al. 1999, Donahue et al. 2003). A major downstream target of PI3K signaling in BCR-stimulated B cells is Akt (Astoul et al. 1999, Deane et al. 2004, Gold et al. 1999). Yusuf et al. (Yusuf et al. 2004) have shown that BCR cross-linking leads to PI3K dependent phosphorylation of

Foxo1, a substrate of Akt (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999, Rena et al. 1999, Takaishi et al. 1999, Tang et al. 1999), and subsequent nuclear exclusion of the Foxo1 protein. In activated B cells, overexpression of either a constitutively active/nuclear Foxo1 or Foxo3 protein causes cell cycle arrest and apoptosis (Yusuf et al. 2004). Taken together, these results suggest that PI3K-mediated down-regulation of Foxo family members is important for the activation of resting B cells by Ag.

A second major pathway emanating from PI3K is mediated by Bruton's tyrosine kinase (Btk) (Scharenberg et al, 1998, Bolland et al. 1998, Deane et al. 2004, Fruman et al. 2000). Btk is a Tec family kinase that signals through the adaptor protein B cell linker protein (BLNK) and phospholipase C (PLC)γ2 to mediate BCR-induced $Ca2^{+}$ flux and activation of protein kinase C (PKC)β (Fruman et al. 2000, Hashimoto et al. 1999, Kurosaki et al. 2000). Btk is required for up-regulation of cell cycle regulators and anti-apoptotic genes in response to BCR cross-linking (Brorson et al. 1997, Khan et al. 1995, Solvason et al. 1998, Whyburn et al. 2003). This includes the cell cycle progression protein Cyclin D2 (Brorson et al. 1997, Glassford et al. 2003, Whyburn et al. 2003). The Foxo family of transcription factors negatively regulates Cyclin D2 (Schmidt et al. 2002). Work presented in this chapter demonstrates that BCR stimulation down-regulates Foxo mRNA via the PI3K/Btk/BLNK/PLCγ2 pathway in a manner distinct from that established for Foxo protein phosphorylation. Downstream of PI3K/Btk/BLNK/PLCγ2, the mRNA expression of Foxo1, Foxo3, and Foxo4 is, however, controlled by unique mechanisms. Do these Foxo family members, which share some target genes in common,

also have unique functions during B cell development and activation? This hypothesis will be further explored in later chapters.

III.b. Results

III.b.1. BCR stimulation regulates Foxo1 protein phosphorylation and mRNA expression via distinct pathways.

Stimulation of mature B lymphocytes through the BCR has previously been shown to trigger PI3K-dependent phosphorylation and nuclear exclusion of the Foxo1 protein (Yusuf et al. 2004). Our findings demonstrate that BCR signaling also regulates Foxo1 at the level of mRNA expression. Q-PCR showed a ~80% decrease in the levels Foxo1 mRNA transcript upon stimulation of purified wild-type splenic B cells with anti-IgM for 6 h (Fig. 3.1a). In contrast, incubation of cells with media alone had no effect on Foxo1 mRNA levels (Fig. 3.1a). Foxo1 mRNA downregulation was maintained for at least 12 h post-stimulation (Fig. 3.1b). This decrease was not observed in cells pretreated with the PI3K inhibitor LY294002 (Fig. 3.2a), indicating a previously unidentified role for PI3K in the contro1 of Foxo1 at the mRNA level in addition to its known posttranslational effects. These results were confirmed by microarray data generated through the Alliance for Cellular Signaling showing that stimulation of primary murine B cells with anti-IgM for 4 h induces down-regulation of Foxo1 mRNA (Zhu et al. 2004). Consistent with the change in mRNA levels, total Foxo1 protein was decreased at 16 h post-stimulation (Fig. 3.1c). Cells cultured in media alone also exhibitied decreased Foxo1 protein levels (data not shown). This is most likely due to the ability of growth factors present in the serum to induce the phosphorylation and degradation of Foxo1 protein (Puig and Tjian 2005). However, the downregulation of Foxo1 mRNA was

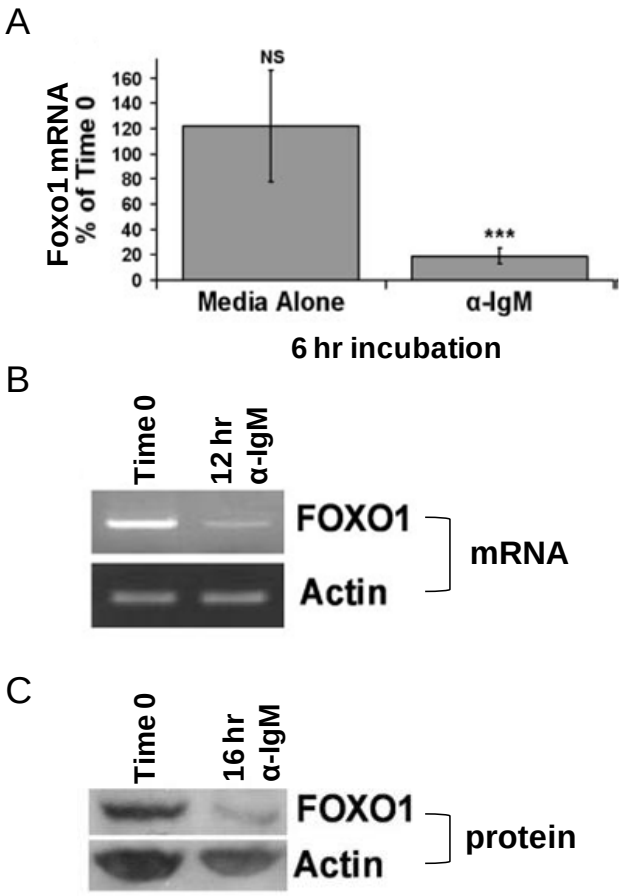


Figure 3.1: BCR signaling down-regulates Foxo1 mRNA expression.
A) Purified wild-type B cells were either harvested immediately (Time 0), incubated for 6 hours in media alone, or stimulated for 6 hours with 10 μg/ml anti-IgM $F(ab)'_2$ fragments. Independent RNA samples derived from at least 3 separate B cell preparations were an alyzed by Q-PCR for expression of Foxo1. Foxo1 expression levels were normalized to GAPDH using the delta Ct method and plotted as an average (+/- SD) percentage of the expression level at Time 0. To determine significance, P values in relation to Time 0 were calculated using a Student's t test and set to the following scale: not significant (NS) $p > 0.05$, *** $p < 0.0005$. **B)** RNA samples derived from B cells harvested immediately (Time 0) or B cells stimulated for 12 hours with 10 ug/ml anti-IgM $F(ab)'_2$ fragments were analyzed by semi-quantitative PCR for expression of Foxo1. Actin was used to determine equal loading. The gel is representative of three independent experiments, each performed at multiple cycles to ensure linearity. **C)** Whole cell lysates from B cells harvested immediately (Time 0) or B cells stimulated for 16 hours with 10 ug/ml anti-IgM $F(ab)'_2$ fragments were resolved by SDS-PAGE and immunoblotted for total Foxo1 protein. The blot was probed with actin to determine equal loading and is representative of three separate trials.

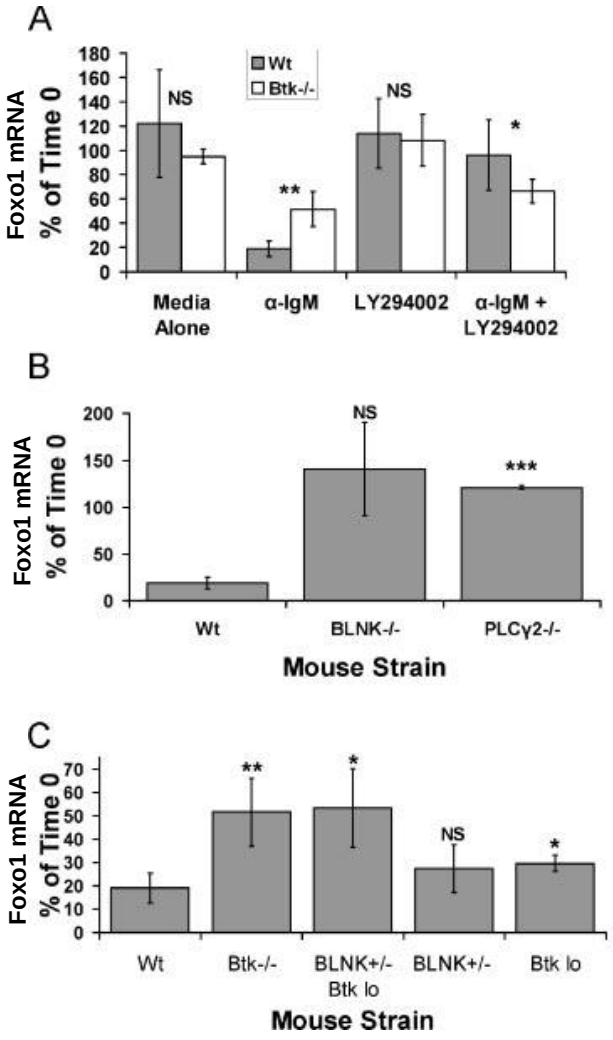


Figure 3.2: BCR induced downregulation of Foxo1 mRNA is mediated by the PI3K/Btk/BLNK/PLCγ2 pathway

A) Purified B cells of the indicated mouse strain were either harvested immediately (Time 0) or incubated for 6 hours in the presence and/or absence of 10 μg/ml anti-IgM F(ab)'$_2$ fragments and the PI3K inhibitor LY294002 (10 μM). **B, C)** Purified B cells of the indicated mouse strain were either harvested immediately (Time 0) or stimulated for 6 hours with 10 μg/ml anti-IgM F(ab)'$_2$ fragments. **A-C)** Independent RNA samples derived from at least 2-3 separate B cell preparations were analyzed by Q-PCR for expression of Foxo1. Foxo1 expression levels were normalized to GAPDH using the delta Ct method and plotted as an average (+/- SD) percentage of the expression level at Time 0 for the corresponding genotype. To determine significance, P values in relation to either **A)** the corresponding wild-type sample or **B-C)** anti-IgM stimulated wild-type cells were calculated using a Student's t test and set to the following scale: not significant (NS) $p > 0.05$, * $p < 0.05$, ** $p < 0.005$, *** < 0.0005.

specific for the BCR-induced signals and was not observed in the presence of media alone (Fig. 3.1a).

The Tec family kinase Btk acts downstream of PI3K (Scharenberg et al, 1998, Bolland et al. 1998, Deane et al. 2004, Fruman et al. 2000) and is required for proliferation and survival of B cells in response to BCR cross-linking (Brorson et al. 1997, Khan et al. 1995, Solvason et al. 1998, Whyburn et al. 2003). Previous research has suggested a connection between Btk and Foxo1. In quiescent cells, Cyclin D2 expression is blocked by Foxo1 (Schmidt et al. 2002), whereas Btk is known to promote Cyclin D2 expression in response to BCR cross-linking (Brorson et al. 1997, Glassford et al. 2003, Whyburn et al. 2003). We therefore asked whether BCR-mediated downregulation of Foxo1 could be mediated by Btk. While wildtype B cells exhibited a 5-fold average decrease in Foxo1 RNA levels upon BCR stimulation, a less than 2-fold reduction was found in *Btk-/-* B cells, as measured by Q-PCR (Fig. 3.2a and Fig. 3.2c).

Btk has been shown to activate PLCγ2 both directly, by phosphorylating it (Humphries et al. 2004, Rodriguez et al. 2001, Watanabe et al. 2001), and indirectly, by increasing the local concentration of its substrate via an interaction with phosphatidylinositol-4-phosphate 5-kinase (PIP5K) (Saito et al. 2003). Direct activation of PLCγ2 by Btk is facilitated by the adaptor protein BLNK (Hashimoto et al. 1999). We therefore asked whether BCR-induced down-regulation of Foxo1 was mediated by BLNK and/or PLCγ2 by examining B cells from *Blnk-/-* and *PLCγ2-/-* mice. Both failed to down-regulate Foxo1 mRNA in response to anti-IgM treatment (Fig. 3.2b).

Btk-/-, *BLNK-/-*, and *PLCγ2-/-* B cells are immature relative to wild-type B cells (Khan et al. 1995, Pappu et al. 1999, Wang et al. 2000), and immature B cells are known to respond differently than mature B cells to BCR cross-linking (Benschop et al. 1999, Yellen et al. 1991). As such, we expanded our studies to include a mouse model in which signaling through the Btk/BLNK pathway is prevented, but B cells remain phenotypically mature. *BLNK+/-Btk*^{lo} mice express low levels of both Btk and BLNK (Whyburn et al. 2003). It has been established that a transgene expressing 25% of endogenous Btk levels restores the development of normal numbers of mature B cells when crossed to *Btk-/-* mice. These cells, however, remain functionally impaired and have reduced, although measurable, responses to BCR cross-linking (Satterthwaite et al. 1997). Haploinsufficiency of *BLNK* further exacerbates this defect without affecting B cell development (Whyburn et al. 2003). BCR-stimulated down-regulation of Foxo1 mRNA was impaired to the same extent in *BLNK+/-Btk*^{lo} and *Btk-/-* B cells (Fig. 3.2c). B cells expressing either a reduced dosage of BLNK (*BLNK+/-*), or Btk (*Btk*^{lo}) were also examined. In these cells, the downregulation of Foxo1 mRNA upon BCR-stimulation was impaired, but not to the same degree as in *BLNK+/-Btk*^{lo} B cells. This suggests that BCR-mediated inhibition of Foxo1 mRNA expression could result from an additive effect of Btk and BLNK (Fig. 3.2c). Thus, the observed effect in *Btk-/-*, *BLNK-/-*, and *PLCγ2-/-* B cells is due to impaired signaling through the Btk/BLNK/PLCγ2 pathway rather than the relative immaturity of the cells.

Once activated via the Btk/BLNK complex, PLCγ2 cleaves phosphatidylinositol-4,5-bis-phosphate to produce 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate

(IP3). DAG and IP3 activate PKCβ and initiate Ca^{2+} influx, respectively (Fruman et al. 2000, Hashimoto et al. 1999, Kurosaki et al. 2000). To better define the mechanisms by which Btk signaling down-regulates Foxo1 mRNA expression, we stimulated wild-type cells through the BCR alone or in the presence of inhibitors of downstream components of the Btk/BLNK/PLCγ2 pathway. The PKCβ inhibitor Gö6850 had minimal effects on BCR-regulated Foxo1 down-regulation. These effects could not account for observations made using either the *Btk-/-* or the *BLNK+/-Btk^{lo}* model. However, the effects of CsA, an inhibitor of the Ca^{2+}-dependent phosphatase calcineurin, were roughly equivalent to that seen with the PI3K inhibitor, LY294002 (Fig. 3.3a), indicating that this branch of the BCR signaling cascade has a more prominent role in the BCR-induced down-regulation of Foxo1.

PI3K has also been shown to mediate BCR-induced activation of Erk (Jacob et al. 2002). To determine whether this pathway contributes to the down-regulation of Foxo1 mRNA expression, B cells were treated with the Erk inhibitor U0126 before incubation with anti-IgM. These cells showed normal levels of BCR-stimulated Foxo1 down-regulation (Fig. 3.3a). Q-PCR of the cyclin D2 gene, whose expression depends on Erk activity (Piatelli et al. 2002), confirmed that the inhibitor was effective (data not shown). Thus, the control of Foxo1 expression by BCR engagement is independent of Erk, but dependent on PI3K, Btk, BLNK, PLCγ2, and calcineurin. This observation is consistent with previous work from our lab and others demonstrating that BCR-induced Erk activation is unaffected in *Btk-/-*, *BLNK-/-*, and *BLNK+/-Btk^{lo}* B cells (Forssell et al. 2000, Tan et al. 2001, Whyburn et al. 2003).

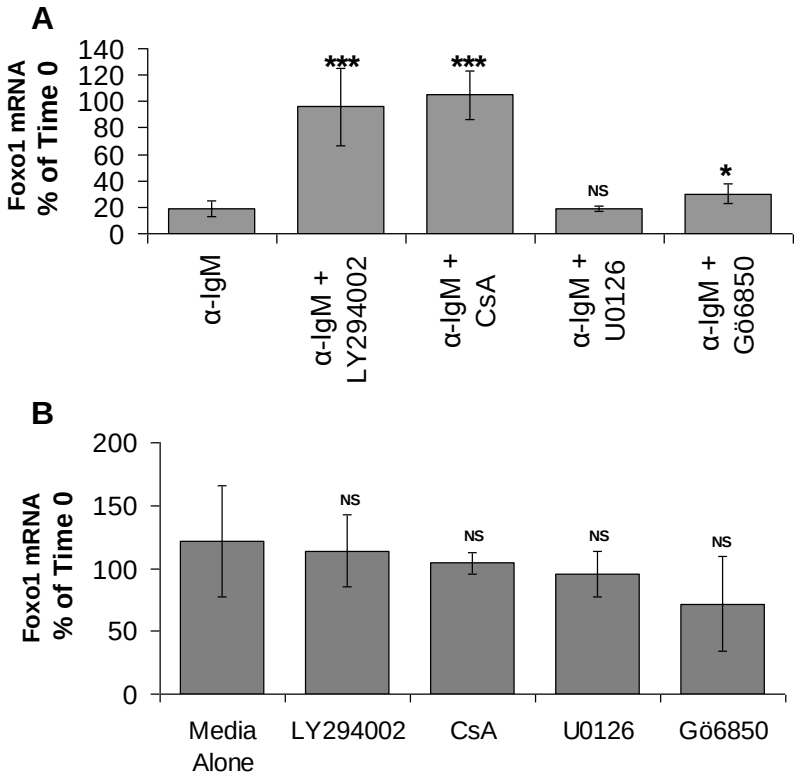


Figure 3.3: BCR induced downregulation of Foxo1 mRNA is mediated by PI3K and calcineurin.

A) Purified wild-type B cells were either harvested immediately (Time 0) or stimulated for 6 hours with 10 μg/ml anti-IgM F(ab)'$_2$ fragments in the presence or absence of LY294002 (10 μM), the calcineurin inhibitor CsA (100 ng/ml), the PKCβ inhibitor Gö6850 (50 μM), or the Erk inhibitor U0126 (10 μM). **B)** Purified wild type B cells were either harvested immediately (Time 0) or incubated for 6 hours with each inhibitor alone. **A-B)** Independent RNA samples derived from at least 2-3 separate B cell preparations were analyzed by Q-PCR for expression of Foxo1. Foxo1 expression levels were normalized to GAPDH using the delta Ct method and plotted as an average (+/- SD) percentage of Foxo1 expression at Time 0. To determine significance, P values in relation to either **A)** anti-IgM stimulated cells, or **B)** cells incubated in media alone were calculated using a Student's t test and set to the following scale: not significant (NS) $p > 0.05$, * $p < 0.05$, ** $p < 0.005$, *** < 0.0005.

It has been established that BCR stimulation induces PI3K-dependent phosphorylation and nuclear exclusion of the Foxo1 protein (Yusuf et al. 2004). To determine the contribution of Btk to this aspect of Foxo1 regulation, we first assessed Akt activation in *Btk-/-* B cells. Consistent with previous studies in *Btk-/-* (Forssell et al. 2000, Suzuki et al. 2003) and *BLNK-/-* (Tan et al. 2001) B cells, BCR-induced phosphorylation of Akt was normal in the absence of Btk (Fig. 3.4). Phosphorylation of Foxo1 at Ser256, an Akt consensus site, was also examined. Based on normal Akt activation, a similar 2- to 3-fold increase in Foxo1 phosphorylation was observed in wild-type and *Btk-/-* B cells (Fig. 3.5a-c). The PI3K inhibitor LY294002 blocked phosphorylation of both Akt and Foxo1 independent of Btk (Fig. 3.5a-c). Taken together, these observations indicate that Foxo1 protein phosphorylation and mRNA expression are controlled by distinct signaling pathways stemming from PI3K. This finding was further supported through the use of the calcineurin inhibitor, CsA. Although CsA was shown to block the down-regulation of Foxo1 mRNA (Fig. 3.3a), it had no appreciable effect on protein phosphorylation (Fig. 3.5a).

III.b.2. Differential regulation of the mRNA expression of Foxo1, Foxo3, and Foxo4 upon activation of mature B cells through the BCR

In the previous section, we demonstrated that downstream of PI3K, BCR-mediated down-regulation of Foxo1 mRNA is controlled by the Btk/BLNK/PLCγ2 pathway. Here, we extend this work to include Foxo3 and Foxo4. Wild-type, but not PLCγ2-deficient B cells displayed an average 3-5 fold decrease in mRNA levels of all

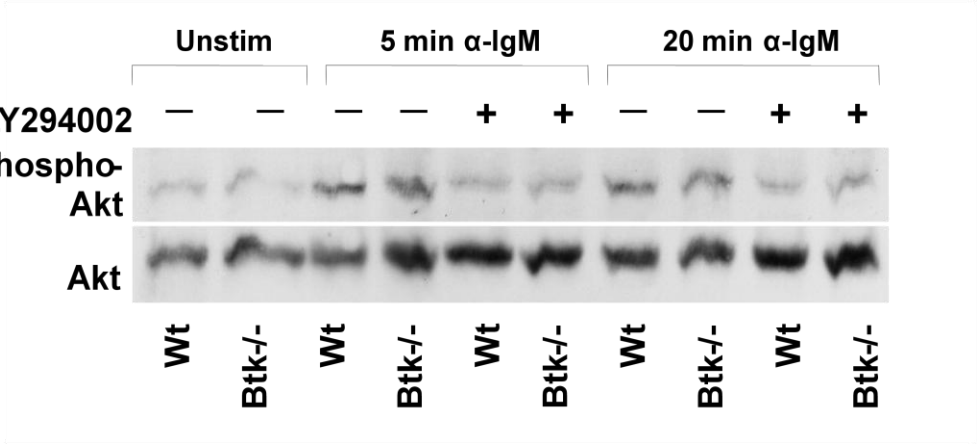


Figure 3.4: BCR stimulation induces PI3K-dependant phosphorylation of the Akt protein independent of Btk.
Purified B cells of the indicated mouse strains were either harvested immediately (Unstim), or stimulated with 10 μg/ml anti-IgM F(ab)'$_2$ fragments in the presence or absence of the PI3K inhibitor LY294002 (10 μM) for the indicated times. Whole cell lysates were resolved by SDS-PAGE and immunoblotted for phospho-Akt (Ser473) and total Akt. Blots were also probed for actin to determine equal loading.

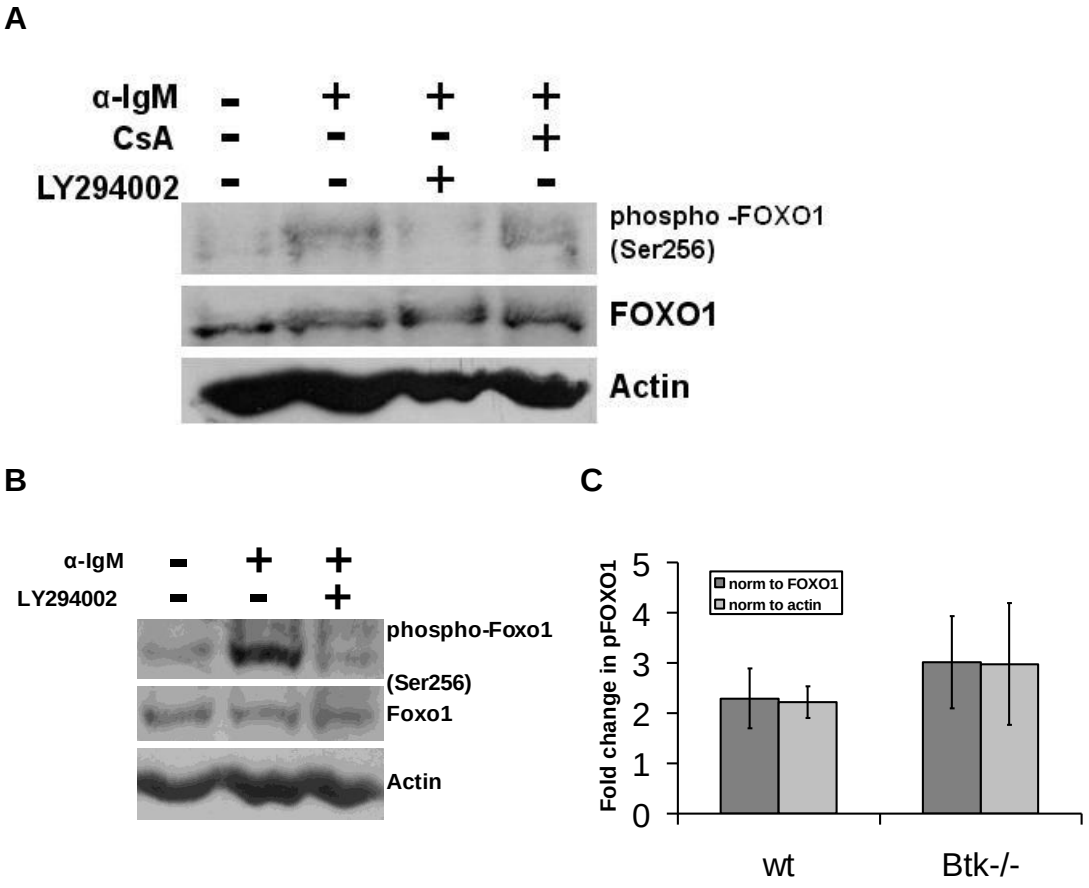


Figure 3.5: BCR stimulation induces PI3K-dependant phosphorylation of the Foxo1 protein independent of Btk.
A) Purified wild type B cells were either harvested immediately or stimulated for 1 hour with 10 μg/ml anti-IgM F(ab)'$_2$ fragments in the presence or absence of LY294002 (10 μM) or the calcineurin inhibitor CsA (100 ng/ml). **B)** Purified Btk-/- cells were either harvested immediately or stimulated for one hour with 10 μg/ml anti-IgM F(ab)'$_2$ fragments in the presence or absence of the PI3K inhibitor LY294002 (10 μM) Whole cell lysates from **A)** and **B)** were resolved by SDS-PAGE and immunoblotted for phospho-Foxo1 (Ser256) and total Foxo1. Blots were also probed for actin to confirm equal loading. Blots shown are representative of three separate experiments. In **C**) Image J was used to determine relative band intensities. The fold change in pFoxo1 upon anti-IgM stimulation was calculated for each mouse strain after normalizing to either total Foxo1 or actin as loading controls. Data are presented as mean +/- SD, n = 3 (wild type) or 4 (Btk-/-).

three family members following 6 hours of anti-IgM stimulation (Fig. 3.6a). However, downstream of PLCγ2, the pathways regulating expression of each Foxo family member differ. Foxo4 mRNA expression, like that of Foxo1 is largely unaffected by the presence of the PKCβ inhibitor Gö6850. Gö6850 does, however, prevent BCR-induced downregulation of Foxo3 mRNA (Fig. 3.6b). This indicates that PKCβ may have a selective role in the control of Foxo3. The calcineurin inhibitor CsA blocked the downregulation of Foxo1, Foxo3, and Foxo4 to a similar degree as the PI3K inhibitor LY294002. Yet, the effects of the NFAT inhibitor 11R-VIVIT were unique to each Foxo family member. 11R-VIVIT did not affect Foxo1 mRNA expression. It inhibited BCR induced Foxo4 mRNA downregulation and induced the upregulation of Foxo3 mRNA (Fig. 3.6b). This observation suggests that Foxo family members are uniquely regulated during B cell development or activation. Whether they have unique functions remains to be determined.

In support of this idea, we examined the mRNA expression patterns of cyclin G2 and Btg-1, two known direct targets of Foxo proteins (Bakker et al. 2004, Bakker et al. 2007, Bennin et al. 2002, Chen et al. 2006, Rouault et al. 1992). Cyclin G2 is an antimitogenic gene that inhibits cell cycle progression and contributes to the maintenance of the quiescent state of differentiated cells (Bennin et al. 2002). Btg-1 was initially isolated from a translocation break point in B-cell Chronic Lymphocytic Leukaemia. Like Cyclin G2, it is also known to have anti-proliferative properties. The box C region of Btg-1 binds to PRMT1, promoting growth arrest through arginine methylation (Rimokh et al. 1991). In splenic B cells treated with anti-IgM with or without the previously

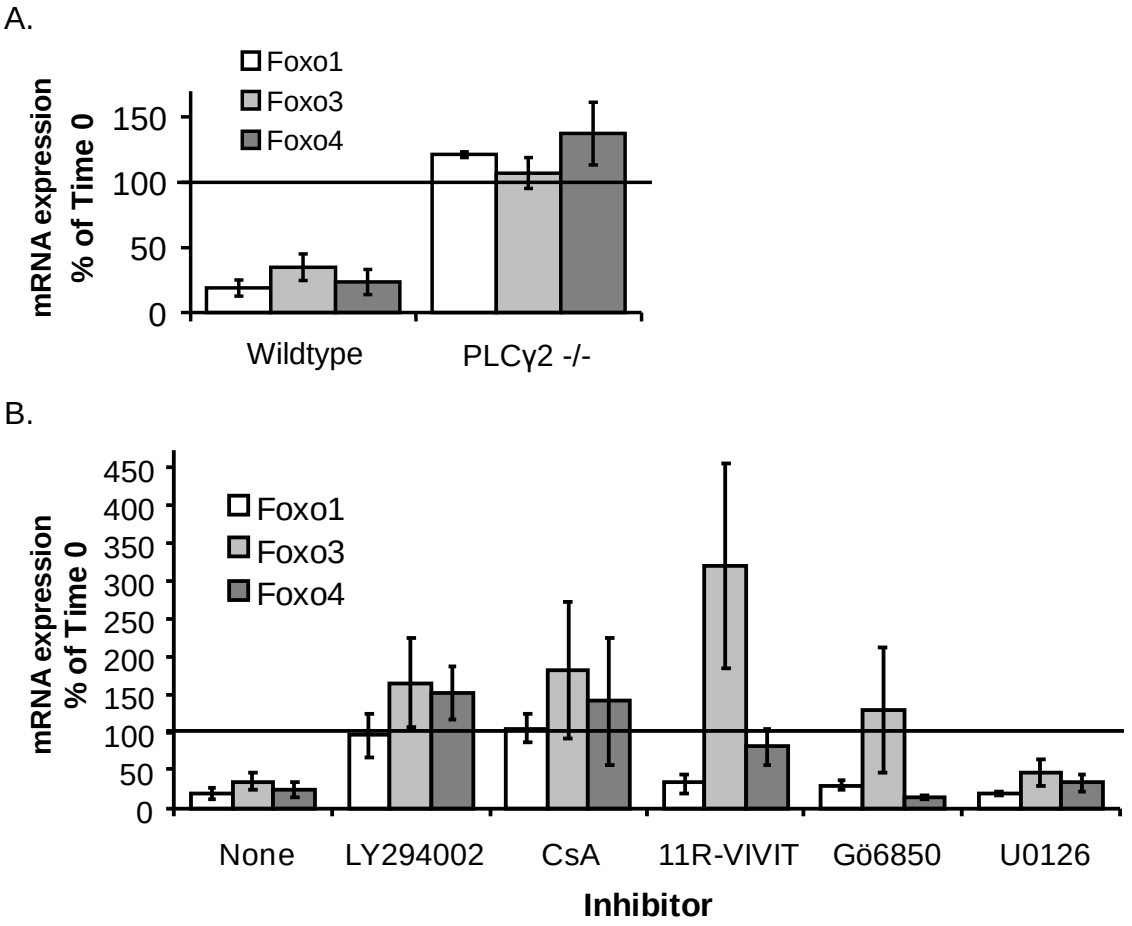


Figure 3.6: BCR-induced down-regulation of Foxo1, Foxo3, and Foxo4 mRNA is regulated by distinct signals downstream of PI3K and PLCγ2.
A) Purified B cells of the indicated mouse strain were either harvested immediately (Time 0) or incubated for 6 h in the presence 10 μg/ml anti-IgM $F(ab')_2$. **B)** Purified wild-type B cells were either harvested immediately (Time 0) or stimulated for 6 h with 10 μg/ml anti-IgM $F(ab')_2$ in the presence or absence of the PI3K inhibitor LY294002 (10 μM), the calcineurin inhibitor CsA (100 ng/ml), the NFAT inhibitor 11R-VIVIT (10 μM), the PKCβ inhibitor Gö6850 (50 μM), or the ERK inhibitor U0126 (10 μM). **A, B)** Independent RNA samples derived from at least two separate B cell preparations were analyzed by Q-PCR for expression of Foxo1, Foxo3, and Foxo4. Expression levels were normalized to GAPDH using the delta Ct method. Results from stimulated cells were then plotted as an average (±SD) percentage of the expression level at Time 0 in B cells of the corresponding genotype.

described panel of inhibitors, the expression patterns for both genes closely correlate with that of Foxo1, but not Foxo3 or Foxo4 (Fig. 3.7). This supports the idea that different Foxo family members may have unique targets in B cells. It is also interesting to note that CsA, which was shown in the previous section to have no appreciable effect on protein phosphorylation, does block the downregulation Cyclin G2 and Btg-1 following BCR stimulation. This demonstrates that Foxo regulation at the level of mRNA expression is functionally relevant.

III.b.3. Downregulation of Foxo mRNA expression in mature B lymphocytes is also observed in response to other stimuli.

The interaction of the BCR with its cognate Ag is not the only mitogenic signal capable of promoting B cell activation. Signals received via Toll-like receptors (TLRs) as well as the CD40/CD40L interaction between B cells and helper T cells (Richards et al. 2008) can also drive cell cycle entry. In order to determine whether downregulation of Foxo family mRNA is specific to stimulation through the BCR, we compared mature splenic B cells activated by LPS, CpG DNA and anti-CD40 for changes in Foxo mRNA expression. We focused on the Foxo1 and Foxo3 transcripts, as they are the most highly expressed in splenic B cells. Expression of downstream target gene Cyclin G2 was also analyzed. Downregulation of Foxo1, Foxo3, and Cyclin G2 mRNA was observed in all cases (Fig. 3.8), although whether this is via the same PI3K/Btk/BLNK/PLCγ2 pathway described in the previous sections remains to be determined.

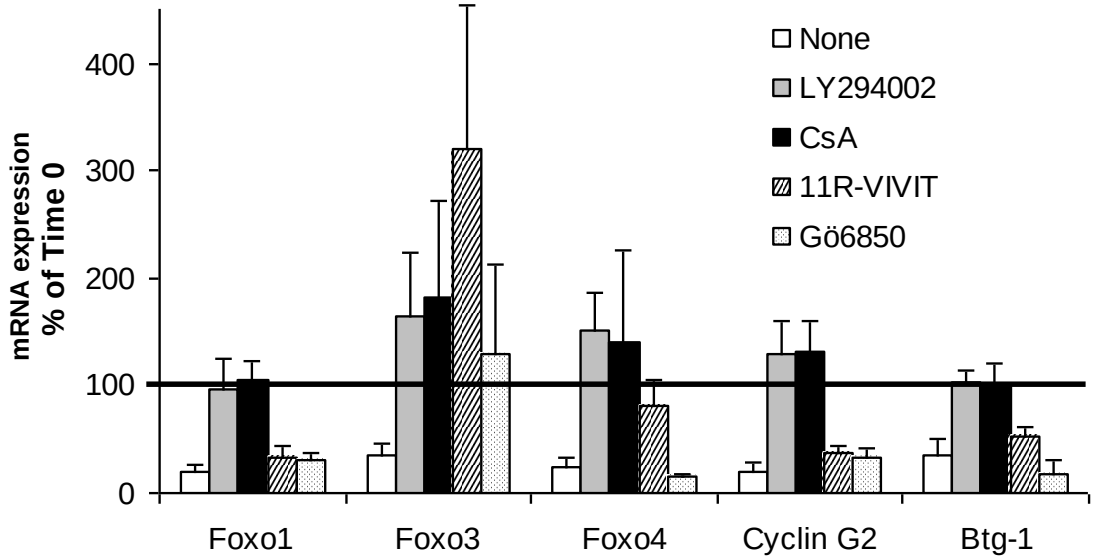


Figure 3.7: The mRNA expression patterns for the Foxo target genes Cyclin G2 and Btg-1 closely correlate with that of Foxo1, but not Foxo3 or Foxo4.
Purified wild-type B cells were either harvested immediately (Time 0) or stimulated for 6 h with 10 μg/ml anti-IgM $F(ab')_2$ in the presence or absence of the PI3K inhibitor LY294002 (10 μM), the calcineurin inhibitor CsA (100 ng/ml), the NFAT inhibitor 11R-VIVIT (10 μM), or the PKCβ inhibitor Gö6850 (50 μM). Independent RNA samples derived from at least two separate B cell preparations were analyzed by Q-PCR for expression of Foxo1, Foxo3, Foxo4 and the Foxo target genes Cyclin G2 and Btg-1. Expression levels were normalized to GAPDH using the delta Ct method. Results from stimulated cells were then plotted as an average (±SD) percentage of the expression level at Time 0.

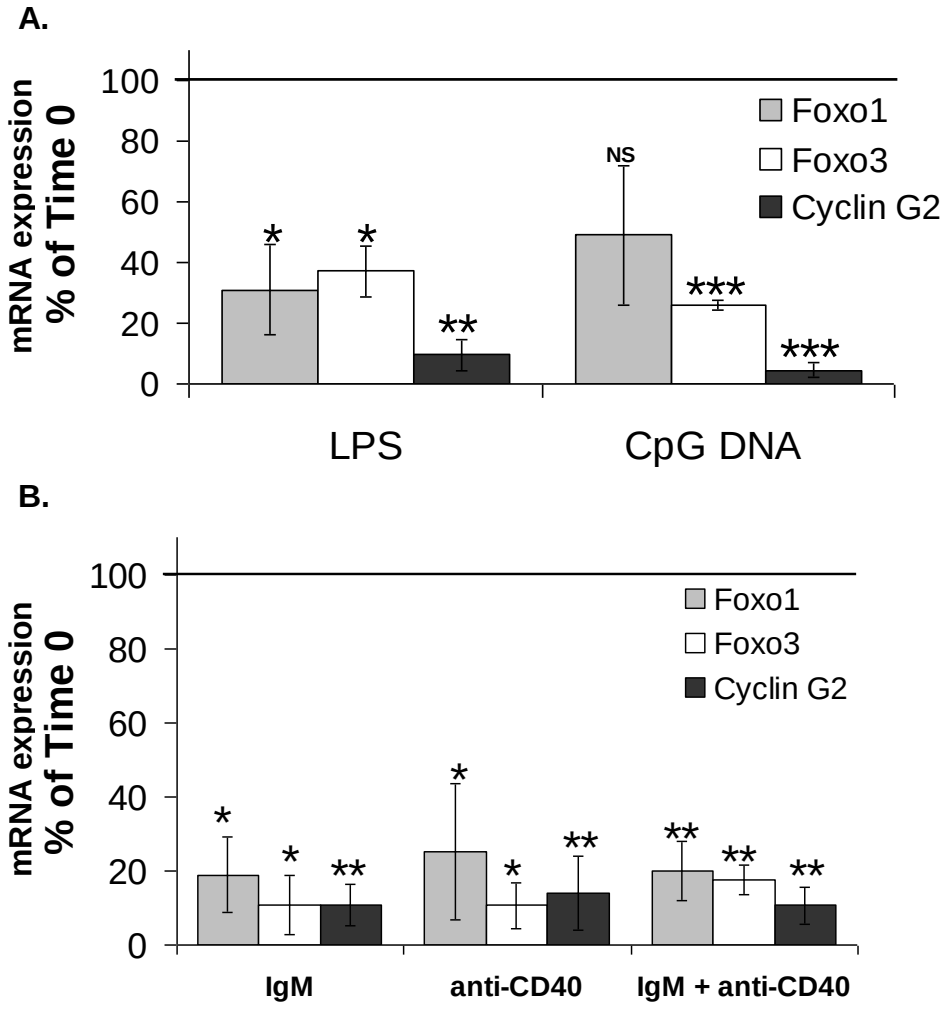


Figure 3.8: Other mitogenic signals, aside from BCR stimulation, are capable of inducing the downregulation of Foxo1, Foxo3, and Cyclin G2 mRNA expression. **A)** Purified wild-type B cells were either harvested immediately (Time 0) or incubated for 6 h in the presence either 10 μg/ml LPS or 0.1 μg/ml CpG DNA. **B)** Purified wild-type B cells were harvested immediately (Time 0) or stimulated for 6 h with either 10 μg/ml anti-IgM $F(ab')_2$, 10 μg/ml anti-CD40, or a combination of the two stimuli. **A, B)** Independent RNA samples derived from at least two separate B cell preparations were analyzed by Q-PCR for expression of Foxo1, Foxo3, and Cyclin G2. Expression levels were normalized to GAPDH using the delta Ct method. Results from stimulated cells were then plotted as an average (±SD) percentage of the expression level at Time 0. To determine significance, P values in relation to Time 0 were calculated using a Student's t test and set to the following scale: not significant (NS) $p > 0.05$, * $p < 0.05$, ** $p < 0.005$, *** < 0.0005.

III.c. Discussion

In numerous cell types, including B cells, activation of PI3K leads to phosphorylation and nuclear exclusion/degradation of the Foxo protein (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999, Rena et al. 1999, Takaishi et al. 1999, Tang et al. 1999, Yusuf et al. 2004). We have described another level of Foxo regulation. It is signaling through the BCR that downregulates Foxo mRNA expression via the PI3K/Btk/BLNK/PLCγ2 pathway. The discovery of this additional means of control emphasizes the importance of Foxo transcription factors in the maintenance of immune cell homeostasis.

Work presented in this chapter demonstrates that control of Foxo mRNA expression takes place via a separate signaling pathway from Foxo protein phosphorylation (Fig. 3.3, Fig. 3.4, Fig. 3.5a-c, Fig. 3.9). Both pathways involve PI3K, as demonstrated by Western and Q-PCR experiments using the PI3K inhibitor, LY294002. Akt appears largely responsible for the post-translational control of Foxos (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999, Rena et al. 1999, Takaishi et al. 1999, Tang et al. 1999, Yusuf et al. 2004), whereas the Btk/BLNK/PLCγ2 pathway down-regulates mRNA expression. These observations are consistent with previous studies of primary B cells showing BCR-stimulated activation of Akt to be independent of Btk and BLNK (Forssell et al. 2000, Suzuki et al. 2003, Tan et al. 2001). Western immunoblotting comparing *Btk*-/- and wildtype cells showed no significant difference in Foxo1 phosphorylation following BCR stimulation (Fig. 3.5a-c). Inhibition of calcineurin with

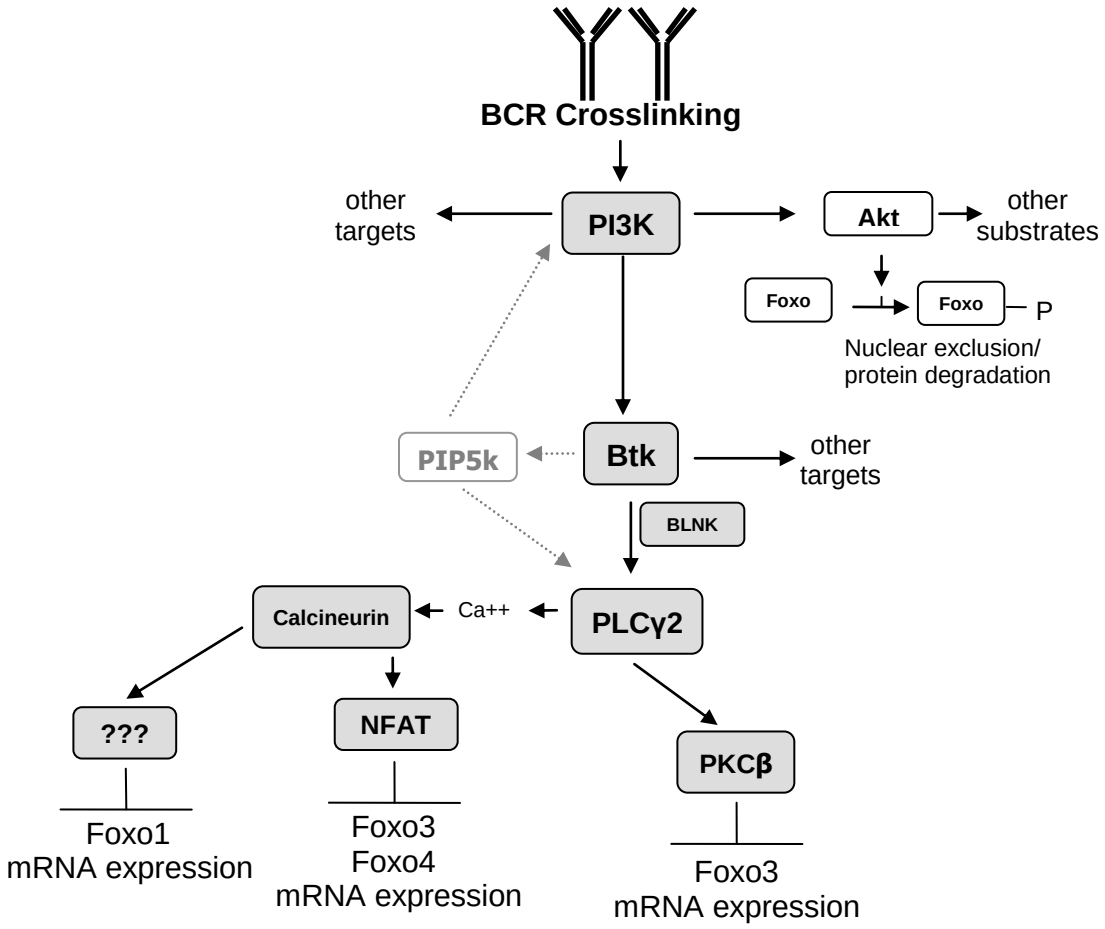


Figure 3.9: Model for the regulation of Foxo1, Foxo3, and Foxo4 by PI3K in BCR-stimulated B cells. Following BCR stimulation, PI3K/Akt controls initial Foxo protein phosphorylation and degradation. PI3K/Btk/BLNK/PLCγ2 down-regulates Foxo mRNA expression to prevent generation of additional Foxo. Each Foxo family member is influenced by a distinct set of mechanisms downstream of PLCγ2. BCR signaling induces downregulation of Foxo1 mRNA via calcineurin but not NFAT, Foxo3 mRNA via PKCβ and calcineurin/NFAT, and Foxo4 mRNA via calcineurin/NFAT.

CsA also blocked BCR-induced down-regulation of Foxo1 mRNA (Fig. 3.3a), but did not affect protein phosphorylation (Fig. 3.5a). Whether calcineurin exerts its effects on Foxo1 mRNA downstream of Btk via PLCγ2 and Ca^{2+}, downstream of PI3K independent of Btk (see below), or both, these results strongly support the idea that Foxo mRNA expression and protein phosphorylation are regulated differently.

It is important to note that our work does not rule out other roles for Btk in the posttranslational control of Foxo1, Foxo3, or Foxo4. Multiple sites for both phosphorylation and acetylation have been identified within the Foxo proteins (Furukawa-Hibi et al. 2005). The phospho-Foxo1 Ab used in this study is specific for Ser256, a site phosphorylated by Akt (Zhang et al. 2002). Btk may regulate phosphorylation at other sites that would not be detected with this reagent. For example, IκB kinase, which is downstream of Btk, is known to phosphorylate Foxo3 at Ser644 (Hu et al. 2004). Although this residue is not conserved in Foxo1, it is possible that there are additional IκB kinase-dependent phosphorylation sites in Foxo1 and/or Foxo4. Possible interactions with secondary molecules such as the 14-3-3 proteins that bind to phosphorylated Foxo protein and sequester it to the cytosol also remain to be investigated (Brunet et al. 2002).

LY294002 had a more profound effect on Foxo mRNA down-regulation than did Btk deficiency. Although much less effective than in wild-type cells, LY294002 did cause a slight increase in Foxo mRNA levels in *Btk*-/- cells stimulated with anti-IgM (Fig. 3.2a). This indicates that PI3K most likely has some Btk-independent contribution to the down-regulation of Foxo mRNA. This is not via the Erk pathway, as the Erk

inhibitor U0126 had no influence on BCR-induced Foxo1, Foxo3, or Foxo4 mRNA downregulation (Fig. 3.3a, Fig. 3.6b). The finding that inhibitors of PI3K and calcineurin have similar effects on Foxo mRNA levels (Fig. 3.3a, Fig. 3.6b) suggests that this alternative pathway lies between these two molecules. This Btk independent process could involve other Tec family kinases redundant with Btk for control of Ca^{2+} flux (Ca^{2+} mobilization is reduced, but not completely prevented, in B cells from *Btk*-/- or *BLNK*-/- *Btk*^{lo} mice (Forssell et al. 2000, Whyburn et al. 2003)) or a unique means of calcineurin activation via PI3K.

Another possible explanation for the apparently greater requirement for PI3K than Btk in mediating Foxo mRNA down-regulation is that Btk is acting upstream, rather than downstream, of PI3K. In addition to phosphorylating and activating PLCγ2, Btk can act in a kinase-independent manner to recruit PIP5K to the membrane, thus increasing the local production of phosphatidylinositol-4,5-bis-phosphate, the substrate for both PI3K and PLCγ2 (Saito et al. 2003) (Fig. 1.6, Fig. 3.9). In the absence of Btk, decreased substrate availability for PI3K would be predicted to reduce, but not completely inhibit, the ability of PI3K to signal. However, in primary B cell cultures, neither we (Fig. 3.4) nor others (Forssell et al. 2000, Suzuki et al. 2003) have observed a role for Btk in PI3K-dependant Akt phosphorylation or activation in response to BCR cross-linking. Our work also indicates that Btk does not regulate phosphorylation of Foxo1 protein at Akt consensus sites (Fig. 3.5a-c). Thus, if Btk is acting upstream of PI3K to regulate Foxo mRNA expression, it is most likely selectively affecting an Akt-independent function of

PI3K. The Btk/PIP5K interaction may also contribute to Foxo mRNA down-regulation by providing substrate for PLCγ2 (Saito et al. 2003) (Fig. 3.9).

Regardless of the relative order of PI3K and Btk in this pathway, the current findings suggest a model whereby PI3K-Akt controls the initial protein degradation, whereas PI3K, Btk, BLNK, and PLCγ2 later down-regulate mRNA expression to prevent generation of additional Foxo1, Foxo3, and Foxo4 (Fig. 3.9). Our work has shown that Foxo1 protein is phosphorylated 1 hour poststimulation, whereas Foxo1 mRNA levels are unchanged at this early time point (data not shown). The work of Donahue and Fruman (Donahue et al. 2003) supports this idea. This study demonstrated that sustained BCR signaling through PI3K is required for proliferation and survival of B cells. LY294002 and CsA were effective at blocking the activation of B cells at late time points in which Akt activation was weak and not PI3K dependent (Donahue et al. 2003). This suggests that newly generated Foxo protein may be inefficiently phosphorylated by Akt at late times post-BCR stimulation, necessitating the newly described PI3K/Btk/BLNK/PLCγ2-dependent mechanism for inhibiting further expression of Foxo mRNA.

It is intriguing to note that the mRNA expression of each Foxo family member is influenced by a distinct set of mechanisms downstream of PLCγ2. BCR signaling induces downregulation of Foxo1 mRNA via calcineurin but not NFAT, Foxo3 mRNA via PKCβ and calcineurin/NFAT, and Foxo4 mRNA via calcineurin/NFAT (Fig. 3.6b). Calcineurin is known to have functions independent of NFAT (Aramburu et al. 1999, Friday et al. 2000, Hao et al. 2003). For example, BCR-induced activation of serum response factor (SRF) is inhibited by CsA (Hao et al. 2003). However, SRF is unlikely to be the

downstream target of calcineurin responsible for the regulation of Foxo1 at the mRNA level. Whereas SRF activity is influenced by both PKCβ and Erk (Hao et al. 2003), we have shown that the mRNA expression of Foxo1 is unaffected by inhibitors of these molecules (Fig. 3.3a, Fig. 3.6b). Mef2c, another downstream target of calcineurin, seems a more probable candidate to be responsible for the downregulation of Foxo1 mRNA expression. In Mef2c-deficient B cells, Ca^{2+} flux is intact following BCR stimulation, however, the B cells do not proliferate and survive. The cells fail to upregulate Cyclin D2 (Wilker et al. 2008), a cell cycle progression protein for which Foxo transcription factors are known inhibitors (Schmidt et al. 2002).

The identity of this unknown calcineurin target has implications beyond just the control of Foxo1 mRNA expression. Our observations show the mRNA expression patterns for both Cyclin G2 and Btg-1, two known Foxo targets, closely correlate with Foxo1, but not Foxo3 or Foxo4 (Fig, 3.7) in splenic B cells. This implies that Foxo1, Foxo3a, and Foxo4 may each control a unique set of target genes, making a more complete understanding of their own regulation all the more relevant.

The question remains as to whether Foxo mRNA upregulation in response to BCR stimulation is controlled at the level of transcription or mRNA stability. In terms of transcription, promoter studies in a mature B cell line would permit the identification of the specific cis-acting elements and transcription factor(s) that control expression of each Foxo family member. However, basal levels of Foxo mRNA expression in the A20 cell line are low and remain unchanged in response to BCR stimulation (data not shown). Consistent with this observation, in studies of the promoter region of the Foxo target

Cyclin G2 in this cell line it was only after co-transfection of Foxos with the reporter construct that significant luciferase activity was observed (Chen et al. 2006). These findings are not surprising given the known anti-mitogenic properties of Foxo family members and the difference in cell cycle status between continuously proliferating cell lines and resting mature primary B cells. The murine promoter sequences for the Foxo1, Foxo3, and Foxo4 genes have not been clearly identified or studied in detail. Looking several kilobases upstream of the transcriptional start sites for Foxo1, Foxo3, and Foxo4, we could not find classical binding sites for NFAT, NFκB, or Mef2c. These transcription factors may still exert their effects through other means. They could regulate the expression of other genes which in turn affect Foxo mRNA levels.

Whether mature B lymphocytes activated by LPS, CpG DNA or anti-CD40 (Fig. 3.8) downregulate Foxo transcription factors by the same mechanism(s) described in response to BCR stimulation remains to be determined. These stimuli also affect PI3K signaling (Deane et al. 2004). Yet, a role for other effector or adaptor molecules aside from those in the PI3K/Btk/BLNK/PLCγ2 pathway is probable. For example, Btk deficient B cells fail to undergo anti-IgM induced proliferation, however their response to anti-CD40 is normal (Khan et al. 1995, Satterthwaite et al. 1997). This suggests that the method of Foxo mRNA regulation may be stimulus dependent.

Regardless of the stimuli, these findings are also potentially intriguing in relation to other cell types. In the literature, control of Foxos at the mRNA level has been largely unexplored. The model proposed here (Fig. 3.9) may be B cell specific, or this signaling pathway could control cell cycle progression in other cell types, particularly those of the

immune system. Fabre et al. (Fabre et al. 2005) have demonstrated that in T cells contacting APCs there is a sustained activation of PI3K, resulting in the sequestration of Foxo transcription factors outside the nucleus in a manner that permits cell growth. Is Foxo expression also controlled at the mRNA level in these cells, and, if so, what are the global consequences of the deregulation of this pathway?

CHAPTER IV

IN IMMATURE B LYMPHOCYTES, BCR STIMULATION INDUCES THE UPREGULATION OF FOXO MRNA EXPRESSION WHICH PROMOTES APOPTOSIS

IV.a.Introduction

The BCR has the ability to transmit signals for both cell survival and death, depending on context. In mature B cells, engagement of the BCR by foreign antigen leads to cell survival and proliferation. This "positive" outcome enables the body to mount a protective immune response against pathogens. In immature B cells, signals transmitted through the BCR have a different end result. It is at this stage of development that the BCR is first fully assembled and tested for functionality. A basal or tonic signal through the unligated BCR is necessary for continued cell survival and maturation (Meffre and Nussenzweig 2002, Shaffer, and Schlissel 1997, Tze et al. 2005, Schram et al 2008). This is mediated by PI3K (Verkoczy et al 2007, Llorian et al 2007). Thus, disruption of this signal (i.e. inhibition of PI3K or its downstream effectors) leads to a negative selection outcome of receptor editing, anergy, or clonal deletion (Verkoczy et al 2007, Llorian et al 2007, Schram et al 2008). Strong engagement of BCR by self-antigen also has a similar effect (Melamed et al, 1998, Verkoczy et al. 2007, Cheng et al. 2009), though whether it is achieved through the same or separate signaling pathways has yet to be determined.

The immature stage of B cell development is an important tolerance checkpoint in the maintenance of homeostasis. The observation that PI3K signaling regulates Foxo transcription factors both at the level of protein phosphorylation and mRNA expression (Hinman et al. 2007, Yusuf et al. 2004, Fig. 3.9) suggests that there may be a role for Foxos in mediating "negative" outcomes of BCR signaling.

In chapter three, we demonstrated a previously undescribed means of control for Foxo transcription factors in the mature B cell at the level of mRNA expression (Fig. 3.9). Foxo mRNA expression was downregulated following BCR crosslinking. Here, these findings are extended to the immature B cell, showing in this case the upregulation of Foxo mRNA expression. This occurs following either disruption of the tonic signal through the unligated BCR or strong engagement of the BCR with anti-IgM. A likely role for Foxos in apoptosis and the maintenance of tolerance at this stage of B cell development is also explored. We show that overexpression of Foxo3 in an immature B cell line promotes anti-IgM induced apoptosis, while primary immature B cells from Foxo3-/- mice have a decreased apoptotic response to BCR crosslinking. This indicates that Foxo3 may play a specific role in promoting clonal deletion. This is in contrast to Foxo1, which has been implicated in other studies to play a role in receptor editing (Amin and Schlissel 2008, Dengler et al. 2008). Here, we provide evidence that the editing process not impaired in Foxo3-/- mice. It could potentially be enhanced.

IV.b. Results

IV.b.1. BCR stimulation induces upregulation of Foxo mRNA expression in immature B lymphocytes

Unlike mature B cells, immature B cells may undergo apoptosis upon antigen stimulation. Deletion of autoreactive B cells via this mechanism is a critical checkpoint that contributes to B cell tolerance (Nemazee and Burki 1989, Hartley et al, 1991, Hartley et al, 1993, Chen et al 1994, Fang et al. 1998, Norvell et al 1995, Melamed et al 1998). Since Foxo family members can promote apoptosis (Burgering and Kops 2002, Jonsson et al. 2005, Modur et al. 2002, Tang et al 2002), we next examined Foxo mRNA expression at this stage in B cell development. Primary immature B cells were sorted from freshly isolated C57BL/6 bone marrow and stimulated with anti-IgM for 14 hours. In these cells, Foxo1, Foxo3, and Foxo4 mRNA expression was upregulated in response to BCR stimulation (Fig. 4.1a). The change in Foxo3 transcript levels was the most prominent. This was in contrast to mature recirculating B cells sorted from the same bone marrow. Like their splenic B cell counterparts, these cells downregulated Foxo1, Foxo3, and Foxo4 mRNA expression following stimulation through the BCR (Fig. 4.1b). These results suggest Foxos can play a role both in promoting apoptosis and maintaining cellular quiescence depending on the developmental state of the B cell.

IV.b.2. Foxo mRNA expression in immature B cells is controlled via the PI3K signaling pathway.

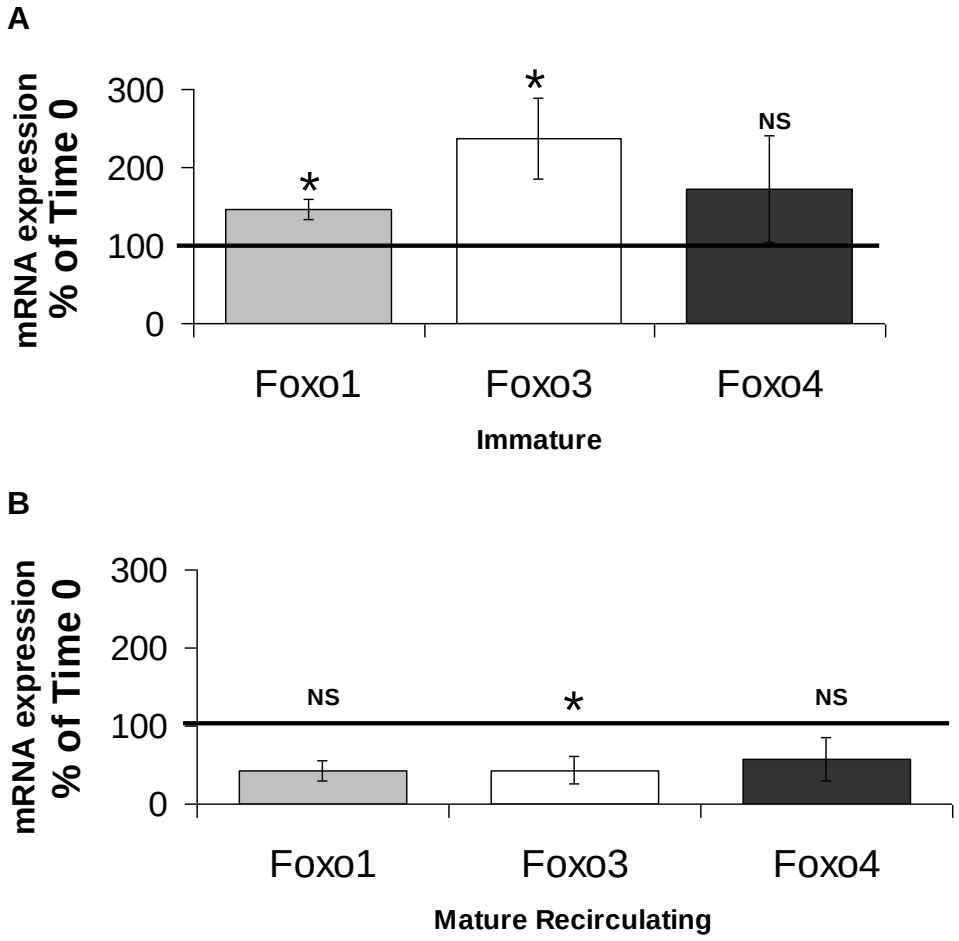


Figure 4.1: BCR-induced up-regulation of Foxo1, Foxo3, and Foxo4 mRNA in primary immature B cells

A) Purified immature B cells ($B220^+$, IgM^+, $AA4.1^+$) from the bone marrow of C57BL/6 mice were either harvested immediately (Time 0) or incubated for 14 h in the presence 10 μg/ml anti-IgM $F(ab')_2$. **B)** Purified mature recirculating B cells ($B220^+$, IgM^+, $AA4.1^-$) from the bone marrow of the same mice as A) were either harvested immediately (Time 0) or stimulated for 6 h with 10 μg/ml anti-IgM $F(ab')_2$. **A, B)** Independent RNA samples derived from three separate B cell preparations were analyzed by Q-PCR for expression of Foxo family members. Expression levels were normalized to GAPDH using the delta Ct method. Results from stimulated cells were then plotted as an average (±SD) percentage of the expression level at Time 0 in B cells of the appropriate maturational stage. To determine significance, P values in relation to Time 0 were calculated using a Student's t test and set to the following scale: not significant (NS) $p > 0.05$, * $p < 0.05$

As with primary immature B lymphocytes, cells from the WEHI 231 immature B cell line undergo cell cycle arrest and apoptosis following stimulation through the BCR (Benhamou et al. 1990, Hasbold and Klaus 1990). Previous studies have also shown that increased nuclear localization of Foxo3 occurs in WEHI 231 cells following anti-IgM stimulation (Chandramohan et al. 2004). Here, we show that these cells also upregulate Foxo, Cyclin G2, and Btg-1 mRNA expression following anti-IgM stimulation (Fig. 4.2). Thus, we chose to use this cell line in our further study of Foxo regulation at the immature stage of B cell development.

Genetic experiments have shown that there is a basal signal through the unligated BCR that is critical for positive selection in immature B cell development (Meffre and Nussenzweig 2002, Shaffer, and Schlissel 1997, Tze et al. 2005, Schram et al 2008). A steady-state of PI3K activity and PLCγ2 phosphorylation via this basal signal promotes the developmental transition through the immature B cell stage. Disruption of this signal by strong BCR crosslinking (i.e. by autoantigen) or by inhibiting the PI3K/PLCγ2 pathway increases Rag2 expression, promotes receptor editing, and halts cell cycle progression at the G0 to G1 transition (Verkoczy et al. 2007, Llorian et al 2007). Consistent with this role of basal signaling by the PI3K/PLCγ2 pathway in suppressing negative selection related events in immature B cells, the addition of PI3K and Erk inhibitors induced the upregulation of Foxo mRNA expression in the WEHI 231 cell line in the absence of BCR stimulation (Fig.4.3). These inhibitors did not block, and in some cases enhanced, BCR induced Foxo mRNA expression (Fig. 4.3).

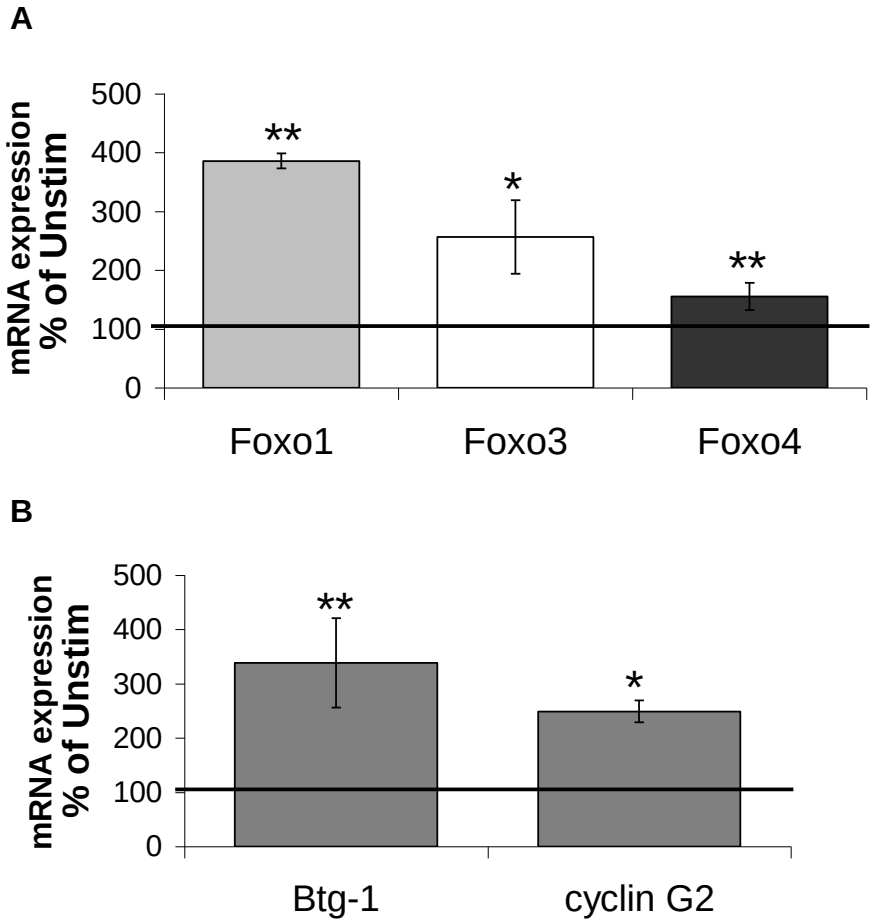
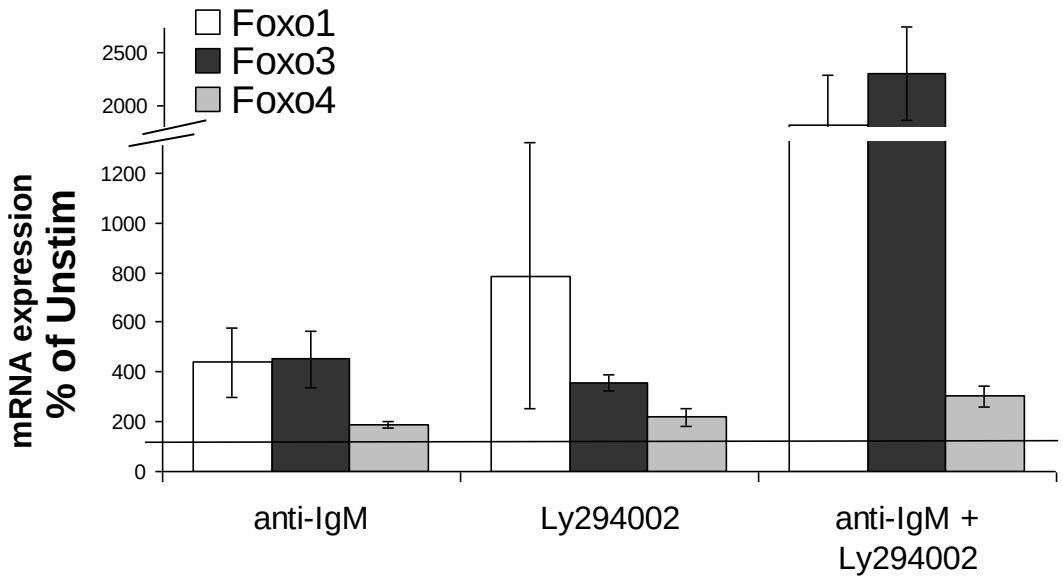


Figure 4.2: BCR-induced up-regulation of the mRNA expression of Foxos and their associated target genes in the WEHI 231 immature B line.
A, B) WEHI 231 cells were incubated for 24 h in either media alone (Unstim) or 10 μg/ml anti-IgM $F(ab')_2$. Independent RNA samples derived from three separate WEHI 231 preparations were analyzed by Q-PCR for expression of **A)** Foxo family members or **B)** Btg-1 and Cyclin G2. Expression levels were normalized to GAPDH using the delta Ct method. Results from the stimulated cells were then plotted as an average (±SD) percentage of the expression level in WEHI 231 cells incubated in media alone (Unstim). To determine significance, P values in relation to unstim were calculated using a Student's t test and set to the following scale: not significant (NS) $p > 0.05$, * $p < 0.05$, ** $p < 0.005$

A



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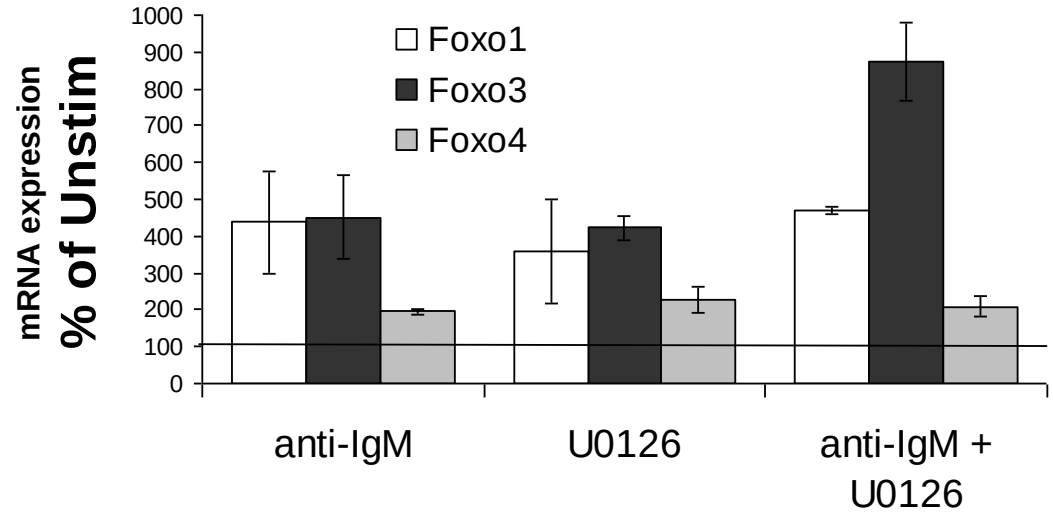


Figure 4.3: Up-regulation of the mRNA expression of Foxo1, Foxo3, and Foxo4 in the WEHI 231 immature B line in response to inhibitors of PI3K and Erk.
WEHI 231 cells were incubated for 24 h in either media alone (Unstim), 10 μg/ml anti-IgM $F(ab')_2$ fragments, **A)** the PI3K inhibitor LY294002 (10 μM) +/- 10 μg/ml anti-IgM $F(ab')_2$ fragments or **B)** the Erk inhibitor U0126 (10 μM) +/- 10 μg/ml anti-IgM $F(ab')_2$ fragments. Independent RNA samples derived from three separate WEHI 231 preparations were analyzed by Q-PCR for expression of Foxo family members Expression levels were normalized to GAPDH using the delta Ct method. Results from the stimulated cells were then plotted as an average (±SD) percentage of the expression level in WEHI 231 cells incubated in media alone (Unstim).

This suggests a model whereby basal PI3K signaling downregulates Foxo mRNA expression in immature B cells. Either blocking this basal signal or strongly engaging the BCR with antigen results in increased Foxo mRNA expression.

IV.b.3. Overexpression of Foxo3 promotes apoptosis in BCR-stimulated WEHI 231 cells

Of the three Foxo family members studied here, Foxo3 mRNA expression was increased the most in BCR stimulated primary immature B cells (Fig 4.1a). To determine the consequences of increased Foxo3 mRNA levels to negative selection related events, a Foxo3 cDNA was amplified by RT-PCR and cloned into the pMIG retroviral vector, which also expresses GFP. WEHI 231 cells were infected with the Foxo3 retrovirus. GFP positive cells were sorted and either incubated in media alone or stimulated with anti-IgM. WEHI 231 cells overexpressing Foxo3 demonstrated increased apoptosis (Fig. 4.4) in response to BCR crosslinking compared to cells expressing GFP alone. This supports the hypothesis that the upregulation of Foxo family members promotes the negative selection of immature B cells. It also emphasizes that altering Foxo mRNA expression alone can have functional consequence. Whereas other studies in B lymphocytes have used the overexpression of a constitutively active Foxo protein, which cannot be phosphorylated, to demonstrate a halt in cell cycle progression and/or increased apoptosis (Yusuf et al. 2004), our Foxo3 construct was not mutated. Control at the level of protein phosphorylation remained intact.

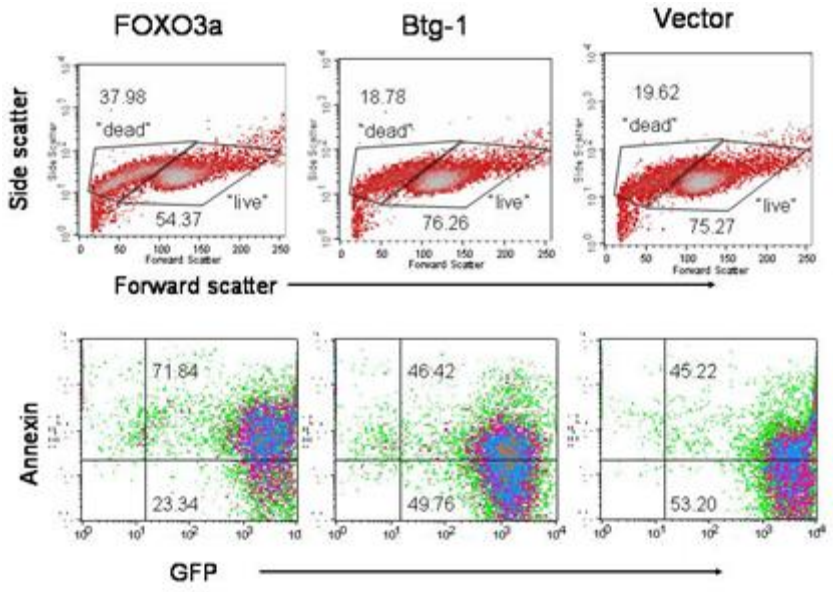


Figure 4.4: Foxo3, but not Btg-1, promotes BCR-stimulated apoptosis in WEHI 231 cells.

WEHI 231 cells were infected with retroviruses expressing GFP alone or GFP plus Foxo3 or Btg-1. GFP+ cells were sorted, expanded, and either incubated in media alone or stimulated for 48 hrs with 10 ug/ml anti-IgM F(ab)'2 fragments. Cells were stained with annexin V and analyzed by flow cytometry. There was no difference between sorts when cells were incubated in media alone (data not shown). Representative plots of anti-IgM stimulated cells are shown, with the frequency of cells in each population illustrated. All cells are pictured in the top panel, with "dead" and "live" gates defined by FSC and SSC. The bottom panel shows cells in the live gate only.

It is intriguing to note, however, that WEHI 231 cells overexpressing Foxo3 do not demonstrate increased expression of the known Foxo target genes Cyclin G2 or Btg-1, either basally or in response to anti-IgM (Fig. 4.5). Btg-1, like Cyclin G2, has been shown to negatively regulate cellular proliferation in a variety of cell types. It was originally isolated from the translocation break point region of a B cell chronic lymphocytic leukemia. The box C region of Btg-1 binds to PRMT1, promoting growth arrest through arginine methylation (Rimokh et al. 1991). In erythroid progenitors it has been shown to be a direct target of Foxo3 (Bakker et al. 2004). However, WEHI 231 cells retrovirally infected with Btg-1cDNA in the pMIG retroviral vector show reduced basal proliferation compared to cells expressing GFP alone (82,564 cpm for GFP vs. 27,615 cpm for Btg1 as measured by 3H-thymidine incorporation), but no difference in apoptosis (Hata et al. 2007, Fig. 4.4). This distinct phenotype from that of WEHI 231 cells retrovirally infected with Foxo3 suggests that the effect of Foxo3 on BCR-stimulated apoptosis is mediated via targets yet unexplored.

Preliminary studies with a retrovirus expressing Foxo4 suggest that the overexpression of each Foxo family member in WEHI 231 cells might have unique consequences. Whereas overexpression of Foxo3 had no effect on basal apoptosis, introduction of a Foxo4 retrovirus into WEHI 231 cells increased the frequency of apoptotic cells even in the absence of BCR engagement (Fig. 4.6). The effect of Foxo1 overexpression remains to be determined.

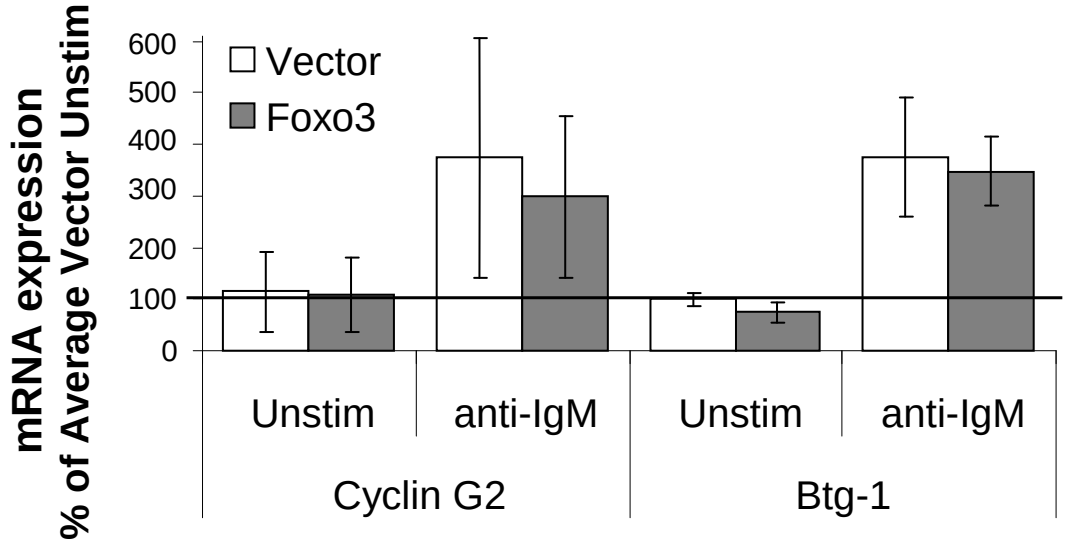


Figure 4.5: WEHI 231 cells overexpressing Foxo3 do not demonstrate increased expression of the known Fox target genes Btg-1 or Cyclin G2

WEHI 231 cells were infected with retroviruses expressing GFP alone or GFP plus Foxo3. GFP+ cells were sorted, expanded, and stimulated for 24 hrs with media alone or 10 ug/ml anti-IgM F(ab)'2 fragments. Independent RNA samples derived from 2-3 separate WEHI 231 preparations were analyzed by Q-PCR for expression of Cyclin G2 and Btg-1. Expression levels were normalized to GAPDH using the delta Ct method. Results from the stimulated cells were then plotted as an average (±SD) percentage of the expression level of wildtype cells incubated in media alone (unstim). There was no significant difference when P values for each Foxo3 condition were calculated in relation to the corresponding vector condition using a Student's t test ($p > 0.05$)

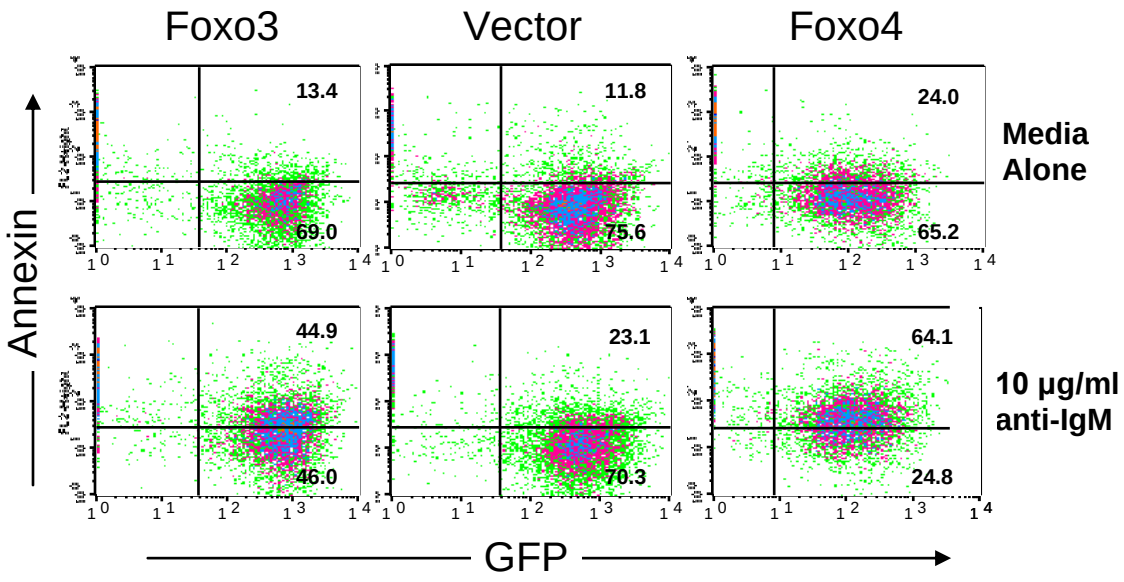


Figure 4.6: Foxo4, but not Foxo3, promotes higher basal levels of apoptosis in WEHI 231 cells.

WEHI 231 cells were infected with retroviruses expressing GFP alone or GFP plus Foxo3 or Foxo4. GFP+ cells were sorted, expanded, and stimulated for 24 hrs with media alone or 10 µg/ml anti-IgM F(ab)'2 fragments. Cells were stained with annexin V and analyzed by flow cytometry. Plots are representative of three separate trials, with the frequency of cells in each population illustrated. All cells pictured fall within the "live" gate defined by FSC and SSC.

IV.b.4. Immature B lymphocytes from Foxo3-/- mice display reduced levels of apoptosis following BCR stimulation.

The maintenance of B cell tolerance at the immature stage of development depends, in part, on the deletion of autoreactive B cells via apoptosis. Foxo family members can promote apoptosis (Burgering and Kops 2002, Jonsson et al. 2005, Modur et al. 2002, Tang et al 2002) and their expression is increased, both in primary immature B cells and WEHI 231 following BCR cross-linking (Fig. 4.1a, Fig. 4.2a). Given that overexpression of Foxo3 in WEHI 231 increased BCR-induced apoptosis, can absence of the transcription factor *in vivo* cause the opposing outcome? To answer this question, we isolated primary immature B cells from Foxo3-/- mice and their wildtype counterparts. Cells deficient in Foxo3 showed a marked decrease in BCR-induced apoptosis as measured by annexin staining 18 hrs post stimulation (Fig. 4.7).

This defect in the deletion of immature B cells, however, does not appear to coincide with significantly higher levels of serum autoantibodies. Antibodies against chromatin, as well as single (ss) and double (ds) stranded DNA in the serum of Foxo3-/- and wildtype mice were measured by ELISA. There was no difference in the expression of anti-chromatin (data not shown) or dsDNA antibodies. And while in Foxo3-/- mice there appeared to be a trend towards a higher level of anti-ssDNA antibodies at 6-8 months, this was not maintained when the same mice were allowed to age to 9-11 months (Fig. 4.8).

At the immature stage of development, the B cell rearranges a kappa or lambda light chain gene and produces a light chain which associates with the previously

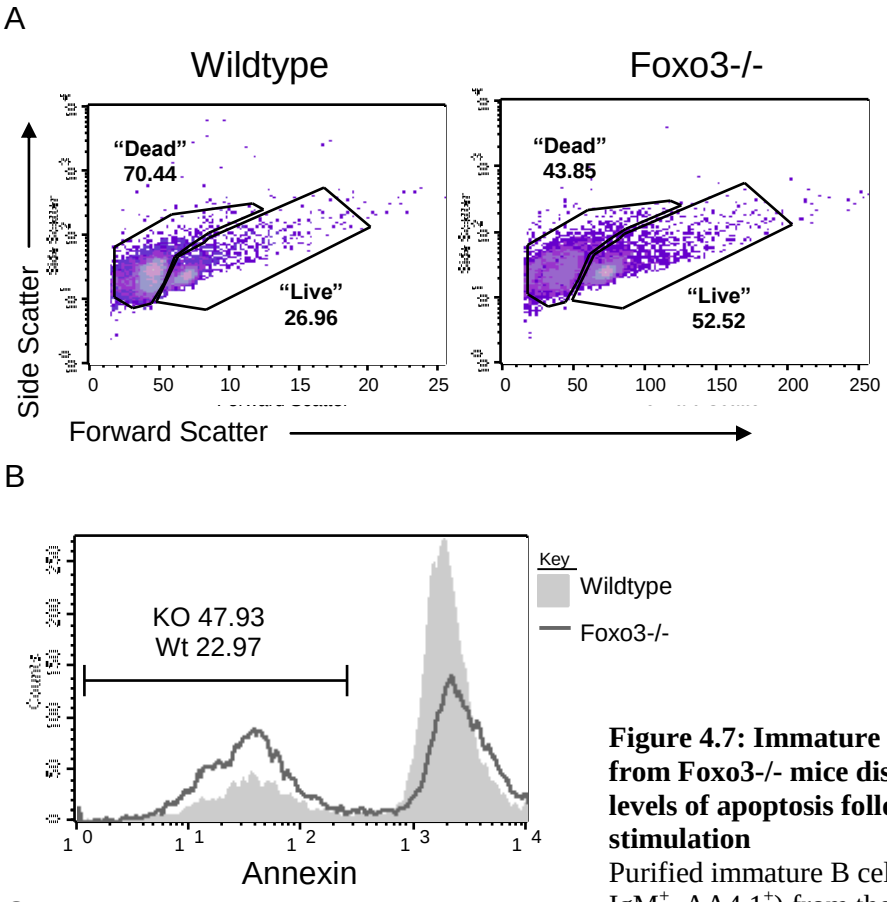


Figure 4.7: Immature B lymphocytes from Foxo3-/- mice display reduced levels of apoptosis following BCR stimulation

Purified immature B cells ($B220^+$, IgM^+, $AA4.1^+$) from the bone marrow of FVB mice of the indicated genotypes were either harvested immediately (time 0) or incubated for 18 h in the presence 10 μg/ml anti-IgM $F(ab')_2$. Cells were stained with annexin V and analyzed by flow cytometry. **A)** Representative plots of anti-IgM stimulated cells, with the frequency of cells in "dead" and "live" gates defined by FSC and SSC. **B)** Representative histogram of annexin staining for anti-IgM stimulated cells **C)** The percentage of live cells present following BCR stimulation for three separate experimental trials.

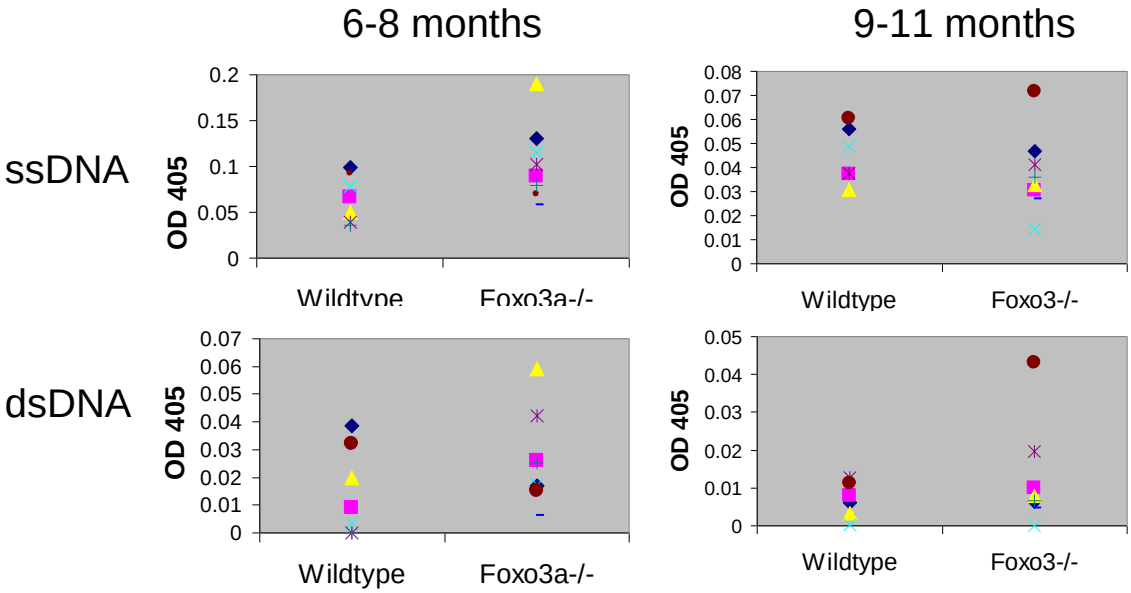
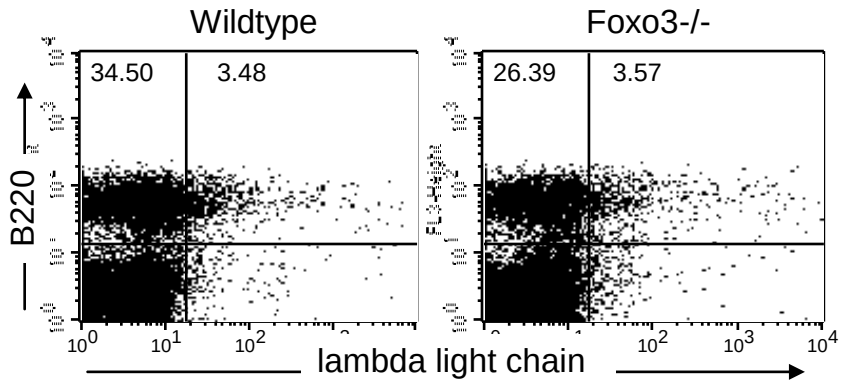


Figure 4.8: Foxo3-/- do not have significantly higher levels of serum autoantibodies in comparison to wildtype.
IgM antibodies against single (ss) and double (ds) stranded DNA in the serum of Foxo3- and wildtype mice were measured by ELISA. Each symbol represents an individual mouse. In Foxo3-/- mice there appeared to be a trend towards a higher level of anti-ssDNA antibodies at 6-8 months, however this was not maintained when the same mice were allowed to age to 9-11 months

synthesized mu heavy chain, creating a fully functional BCR. Kappa locus rearrangement occurs first. Production of a functional kappa light chain inhibits rearrangement of the lambda locus. Thus, the lambda locus will only undergo recombination if a functional kappa light chain cannot be produced, or if receptor editing occurs due to an autoreactive kappa light chain (Lang et al. 1997). In the Foxo3-/- mice, there was a defect in BCR-induced deletion, but no subsequent significant increase in serum autoantibodies. This maintenance of tolerance could be explained by an increase in receptor editing, which might be reflected in a higher frequency of B cell expressing the lambda light chain (Igλ) (Tiegs et al. 1993). We examined Igλ usage on the surface of splenic B cells via FACS staining. There was a trend towards greater Igλ usage in the Foxo3-/- mice (Fig. 4.9) which was subtle, but statistically significant. This does not, however, definitively demonstrate increased receptor editing in Foxo3-/- mice. Other assays to measure secondary recombination events in the light chain locus remain to be completed.

For example, a quantitative assay for recombining sequence (RS) rearrangement may be used estimate levels of antibody light chain receptor editing in various B cell populations. RS rearrangement is a recombination of a noncoding gene segment in the kappa antibody light chain locus. The segment is found at the 3' end of the locus. It may rearrange to inactivate the kappa antibody light chain locus in developing B cells. RS rearrangement levels are highest in the most highly edited B cells, and are inappropriately low in autoimmune mouse models of systemic lupus erythematosus (SLE) and type 1 diabetes (Panigrahi et al. 2008).

A



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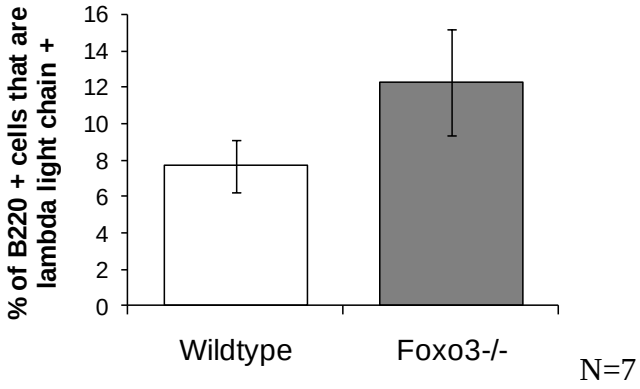

Figure 4.9: Trend towards greater lambda chain usage in the Foxo3-/- mice
A) Splenic cells were stained with antibodies against B220 and the lambda light chain. The frequency of B220+lambda- and B220+lambda+ cells in the lymphocyte gate is indicated. Data are representative of seven mice. **B)** The mean +/- SD of seven mice analyzed as in A) shown. p=0.0049 by Student's t-test.

IV.c. Discussion

In chapter three, mature B cells proliferating in response to anti-IgM were shown to downregulate Foxo mRNA expression. This was via the activation of the PI3K/Btk/BLNK/PLCγ2 pathway (Fig. 3.9). In this chapter, Foxo mRNA expression was upregulated in immature B cells, both when the tonic/basal signal through the BCR was disrupted, or the BCR is engaged with anti-IgM (Fig. 4.1a, Fig. 4.2a, Fig. 4.3). Inhibitor studies in the WEHI 231 immature B cell line further suggest that signals through PI3K and Erk are required to maintain the low basal levels of Foxo mRNA expression (Fig. 4.3). Whether this is via the same pathway(s) for Foxo1, Foxo3, and Foxo4 is yet to be determined. Whatever the case, the finding that overexpression of a wildtype Foxo mRNA in WEHI 231 is enough to increase apoptosis in response to BCR stimulation (Fig. 4.4, Fig. 4.6) emphasizes that control of Foxos at the mRNA level has functional consequence.

In our work, two known Foxo target genes, Cyclin G2 and Btg-1, were shown to be upregulated in the WEHI 231 cell line following BCR stimulation (Fig. 4.1b). The question of whether these targets are also upregulated when the tonic BCR signal is disrupted remains to be addressed. However, the expression of two additional Foxo targets, Cyclin D2 and $p27^{Kip1}$, were shown by Banerji et al. to be altered in each of these scenarios. The expression of Cyclin D2, a cell cycle progression protein, was downregulated and the pro-apoptotic gene $p27^{Kip1}$ upregulated following either strong BCR ligation or disruption of the tonic BCR signal via the PI3K inhibitor LY294002

(Banerji et al. 2001). These observations, attributed directly to changes in transcription, correlate with our study and suggest a tight regulation of Foxo mRNA is important at this critical checkpoint in B cell development. Further exploration of this hypothesis, as well as through analysis of Foxo protein phosphorylation in primary immature B cells, remains to be completed.

As in mature B cells, the question of redundancy among Foxo family members also arises at the immature stage of B cell development. For Foxo1 and Foxo3, inhibition of PI3K and stimulation through the BCR had a synergistic effect on the upregulation of mRNA expression. This was not the case for Foxo4 (Fig. 4.3), suggesting perhaps the regulation of Foxo4 mRNA expression is more tightly controlled. Overexpression of Foxo4 mRNA in the WEHI 231 cell line resulted in a basal increase in cellular apoptosis as measured by annexin staining. The same did not appear to occur with comparable levels of Foxo3 mRNA overexpression as measured by GFP expression. In this case, it was only following stimulation through the BCR that an increase in apoptosis relative to wildtype was observed (Fig. 4.6). In interpreting these results, it is important to keep in mind that we do not know what the basal Foxo4 protein levels are in the WEHI 231 cell line. We also do not know to what degree Foxo4 protein expression changes following Foxo4 retroviral infection. Are the levels of Foxo3 and Foxo4 protein comparable?

In addition, it is intriguing to note that overexpression of Btg-1 resulted in a phenotype unique from that induced by either Foxo3 or Foxo4. There was decreased proliferation as measured by thymidine incorporation in the absence of increased apoptosis (Fig. 4.4, Hata et al. 2007). It may be that in the immature B cells, Btg-1 is

primarily a Foxo1 target. In Jurkat T cells, ectopic expression of Foxo1 induced a similar block in proliferation, whereas Foxo3 induced apoptosis (Medema et al. 2000). Further study is warranted to address these questions.

How do these findings translate into a potential model for the control of Foxo1, Foxo3, and Foxo4, at the immature stage of B cell development? Changes in both protein phosphorylation and mRNA expression are likely to occur. Work by Verkoczy et al. suggests that at the pre to immature transition in B cell development, the absence of a functional light chain or the expression of an autoreactive BCR leads to the loss of Akt activity, increased glucose uptake, and metabolism. Subsequent progressive reduction in B cell size limits the number of attempts the cell can make at receptor editing before it undergoes apoptosis. Should successful editing occur, Akt activity is restored and the cell is rescued from death (Verkoczy et al. 2007). Amin et al. have recently shown that Foxo1, but not Foxo3 or Foxo4, directly activates RAG transcription, thus promoting receptor editing (Amin and Schlissel 2008). Our own experimental results suggest that in the absence of Foxo3 there is a defect in BCR-induced apoptosis. Together these studies support the following hypothesis for Foxo control in immature B cells.

In the immature B cell, preexisting stores of Foxo protein are largely phosphorylated and sequestered to the cytoplasm, where they may be degraded. Either disruption of tonic BCR signaling, or the engagement of the BCR with self-Ag, leads to the loss of Akt activity. In the absence of Akt singaling, Foxo proteins are dephosphorylated and enter the nucleus, halting cell cycle progression. Indeed, Amin and Schlissel have recently shown that treatment of primary immature B cells with anti-IgM

or inhibitors of PI3K results in a shift in Foxo1 localization from primarily cytoplasmice to primarily nuclear (Amin and Schlissel 2008). Foxo1 then activates RAG transcription, initiating light chain receptor editing. As this occurs, continued inhibition of PI3K signaling also results in an increased expression of Foxo mRNA and the subsequent translation of additional Foxo protein. Once Foxo protein expression, in particular Foxo3, reaches a high enough threshold, apoptosis will take place. However, in the event that receptor editing is successful, the restoration of Akt activity in response to tonic signaling by the newly generated BCR draws the Foxo1, Foxo3, and Foxo4 proteins out of the nucleus and cell death is averted (Fig. 4.10).

Many details of this hypothesis remain to be explored. Does Foxo4, for example, have a specific role in immature B cell development? Or, is this family member merely partially compensating for the loss of Foxo3? The latter would be an intriguing possibility given that the defect in BCR-induced apoptosis observed in Foxo3-/- mice is not complete (Fig. 4.7). We have shown that this defect alone is not associated with higher autoantibody titers (Fig. 4.8). There is, however, a trend towards higher usage of the lambda light chain, suggestive of increased receptor editing (Tiegs et al 1993) (Fig. 4.9). This may explain the prior observation. Perhaps the absence of Foxo3 delays the induction of apoptosis, extending the window for Foxo1 to successfully edit autoreactive receptors via its promotion of RAG expression.

It is important when drawing these conclusions to keep in mind that the genetic background of the mice, FVB, may have masked a more pronounced phenotype. Indeed, differences between this line of Foxo3-/- mice and a second line on a mixed background

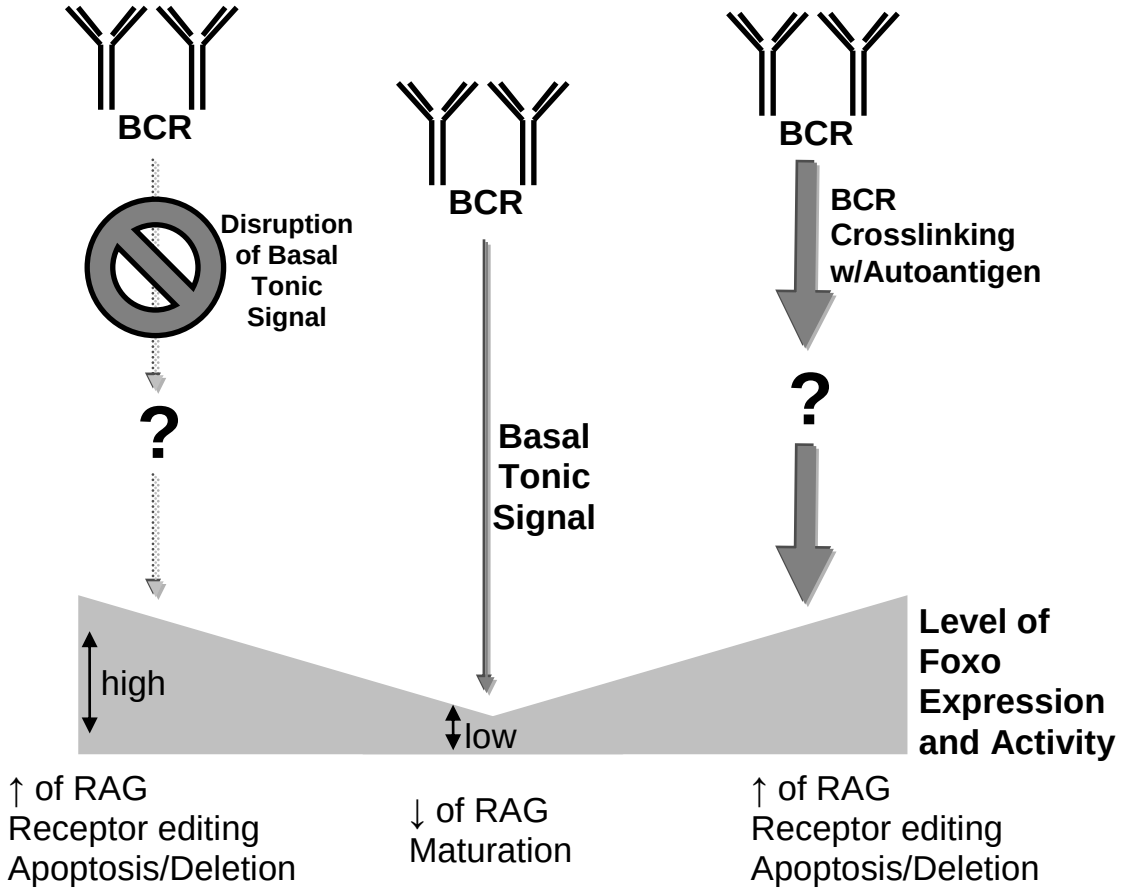

Figure 4.10: Schematic summary of our results examining Foxo regulation by immature B cell signaling through the BCR

In immature B cells, signals transmitted through the BCR have a different end results. A tonic or basal signal is necessary for downregulation of RAG and further B cell maturation. This is associated with a low level of Foxo expression/activity. Either disruption of the tonic signal through the unligated BCR or strong engagement of the BCR with anti-IgM leads to upregulation of Foxo expression/activity and a "negative" outcome of receptor editing, anergy, or clonal deletion. Whether this "negative" outcome occurs via the same or different mechanisms in each case has yet to be determined.

have already been described (Castrillon et al. 2003, Hedrick 2009, Lin et al. 2004). It is also possible that an actual breach in tolerance might not be noted unless Foxo3 deficiency is combined with other factors which increase autoimmune susceptibility. Further investigation is warranted.

CHAPTER V

FOXO3-/- MICE DEMONSTRATE REDUCED NUMBERS OF PRE-B AND RECIRCULATING B CELLS BUT NORMAL SPLENIC B CELL SUBPOPULATION DISTRIBUTION

Work presented in this chapter has been published in *International Immunology*, volume 21(7), pages 831-842. This work is reproduced with the permission of *International Immunology*.

V.a. Introduction

Foxo1, Foxo3, and Foxo4 share largely overlapping patterns of tissue expression and interact preferentially with the same DNA binding motif 5'-TTGTTTAC-3' (Furuyama et al 2000). Yet, individual disruption of each gene results in a distinct phenotype as outlined in Table 1.1. Thus, the Foxos are likely to have unique, non-overlapping roles in the immune system.

Work described in previous chapters establishes that in B cells there is an additional means of control for Foxos, aside from post-translational protein phosphorylation, at the level of mRNA expression. This has different functional consequences in immature versus mature B cells. In the WEHI 231 immature B cell line, overexpression of Foxo3 mRNA alone resulted in increased apoptosis in response to

BCR crosslinking (Fig. 4.4, Fig. 4.6). Foxo4 overexpression increased the frequency of apoptotic cells, even in the absence of BCR engagement (Fig. 4.6). In mature B cells, we have shown that the mRNA expression for each Foxo family member is controlled via a distinct set of pathways downstream of PI3K (Fig. 3.9). The results of these studies combined suggest that Foxo1, Foxo3, and Foxo4 have unique functions during B cell development and activation.

In the previous chapter, we examined the effects of Foxo3 deficiency at the immature stage of B cell development. Foxo3 was shown to have a unique effect on BCR-induced apoptosis in these cells (Fig. 4.7). In this chapter we extend our work to determine whether Foxo3-deficiency affects other aspects of B cell development. The choice of Foxo3-/- mice for these studies is based on the following observations. First, in primary immature B cells, Foxo3 mRNA expression was upregulated to the greatest degree following BCR stimulation (Fig.4.1). Second, the pattern of Foxo3 expression in inhibitor studies with splenic B cells was distinct from Foxo1 and Foxo4 (Fig. 3.6). Third, Foxo3 has been shown to have an effect on T cell activation. And though recent studies have shown this effect to be likely background dependent, it still suggests that Foxo3 could play a role in other lymphocytes (Lin et al. 2004). Finally, Foxo1-/- mice are embryonic lethal (Furuyama et al. 2004), while Foxo3-deficient mice are viable and readily examinable for B cell phenotypes (Castrillon et al. 2003, John et al. 2007).

Indeed, in this chapter we show that Foxo3-/- mice have reduced frequencies of pre-B cells in the bone marrow and recirculating B cells in the blood and bone marrow. These mice also demonstrate increased basal levels of IgG2a, IgG3, and IgA. However,

splenic B cell subpopulations are normal and the activation of mature resting B cells *in vitro* is unimpaired in the absence of Foxo3, suggesting that other Foxo family members may play a role in these processes.

V.b. Results

V.b.1. Foxo3 is dispensable for splenic B cell development and response to BCR engagement

Similar frequencies and total numbers of follicular, marginal zone, and transitional B cells were found in the spleens of wild type and Foxo3-/- mice (Fig. 5.1). Despite the anti-mitogenic and pro-apoptotic functions of Foxo3, splenic B cells lacking Foxo3 did not demonstrate altered proliferation (Fig. 5.2a) or survival (Fig. 5.2b) either basally or in response to anti-IgM. The lack of an effect of Foxo3-deficiency was not due to compensatory changes in the expression levels of Foxo1 or Foxo4 (Fig. 5.3a). mRNA expression of Cyclin G2 and Btg-1, cell cycle inhibitory genes which are known targets of Foxo3 (Bakker et al. 2004, Bakker et al. 2007, Bennin et al. 2002, Chen et al. 2006, Rouault et al. 1992), was comparable in wild type and Foxo3-/- B cells both basally and upon BCR engagement (Fig. 5.3).

V.b.2. Increased basal IgG2a, IgG3, and IgA levels in Foxo3-/- mice

Forced expression of a constitutively active form of Foxo1 in LPS-stimulated B cells inhibits the ability of these cells to differentiate into plasma cells (Omori et. al 2006). This finding led us to question whether plasma cell differentiation could be increased in the absence of other Foxo family members, including Foxo3. However, upregulation of the plasma cell marker CD138 was normal in cultures of Foxo3-/- B cells

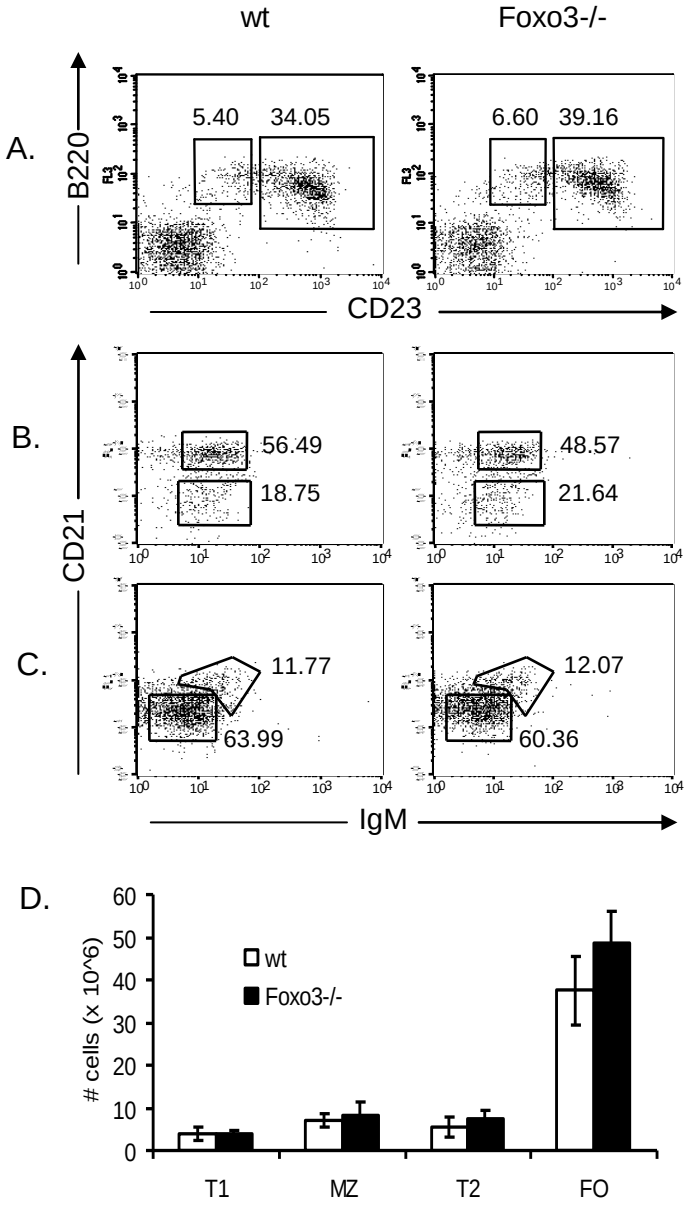

Figure 5.1: Splenic B cell subpopulations are normal in Foxo3-/- mice.
Splenocytes of the indicated mouse strain were stained with antibodies against B220, CD21, CD23, and IgM. Plots are representative of four mice. **A)** The percentage of total cells in each population is shown. **B)** The percentage of $B220^{+}$ $CD23^{low}$ cells in each population (T1 = CD21-IgM+, MZ = CD21+IgM+) is shown. **C)** The percentage of $B220^{+}$ $CD23^{high}$ cells in each population (T2 = $CD21^{high}$ IgM^{high}, FO = CD21+IgM+) is shown. **D)** The total number of cells in each population is shown as mean +/- SD, n = 4. There is no significant difference between wt and Foxo3-/- mice.

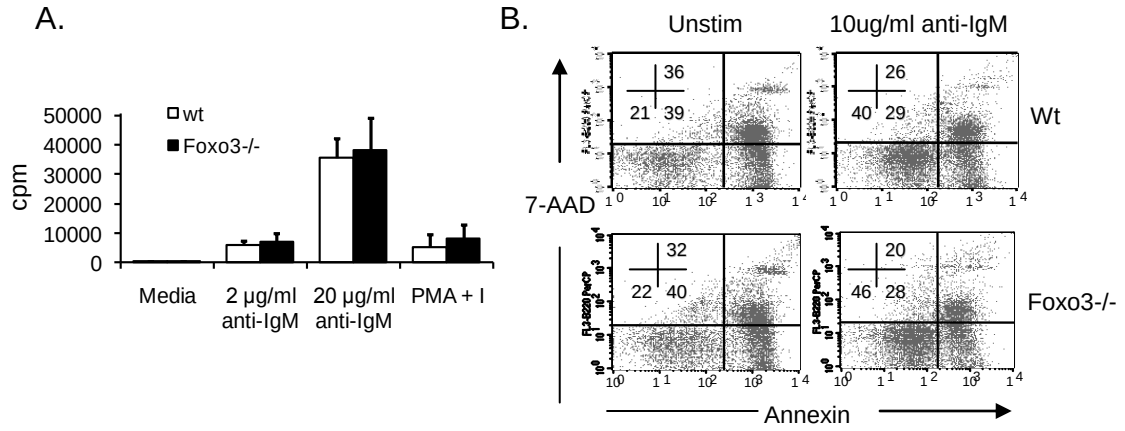

Figure 5.2: Normal splenic B cell response to BCR crosslinking in the absence of Foxo3.

A) Splenic B cells were cultured in media alone or stimulated for 48 h with 2 or 20 µg/mL of anti-IgM F(ab')$_2$ fragments, or 10 ng/mL PMA +1 µg/mL ionomycin. Cells were labeled with [^{3}H]thymidine for the final 6 hours of culture to measure proliferation. Data represent mean ± SD, n=3. **B)** Purified B cells were either harvested immediately (Unstim) or cultured in 10 µg/ml anti-IgM F(ab')$_2$ for 48 hrs and stained with annexin V and 7-AAD. The frequency of live (annexin V-, 7AAD-), early apoptotic (annexin V+, 7AAD-), and dead (annexin V+, 7AAD+) cells is shown for one of 3 representative mice per genotype.

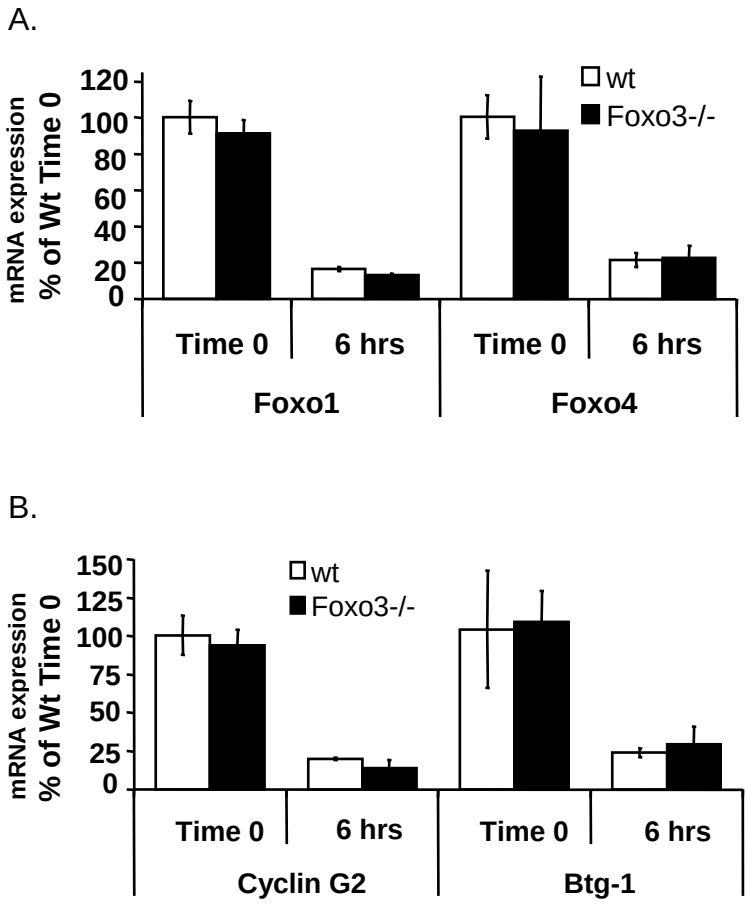

Figure 5.3: Foxo1 and Foxo4 mRNA expression in splenic B cells is not altered in the absence of Foxo3. Neither is the expression of two known Foxo targets, Cyclin G2 and Btg-1.

A, B) Purified B cells of the indicated mouse strain were either harvested immediately (Time 0) or incubated for 6 h in the presence 10 μg/ml anti-IgM $F(ab')_2$. Independent RNA samples derived from three separate B cell preparations were analyzed by Q-PCR for expression of **A)** Foxo1 and Foxo4 as well as **B)** Cyclin G2 and Btg-1. Expression levels for each gene were normalized to GAPDH using the delta Ct method and plotted as an average (±SD) percentage of the expression level at Time 0 for wildtype cells. There is no significant difference between wildtype and Foxo3-/- mice.

incubated with LPS or CD40 plus IL-4 (Fig. 5.4a). Wild type and Foxo3-/- B cells also had a similar proliferative response to these stimuli (Fig. 5.4b-c).

Although there was no general enhancement of plasma cell differentiation in vitro in the absence of Foxo3, it is possible that Foxo3 regulates B cell differentiation and antibody production in vivo. We therefore measured serum Ig levels, both basally and in response to immunization. Unimmunized Foxo3-/- mice had significantly increased amounts of total IgG2a, IgG3, and IgA relative to wild type controls, although levels of total IgM, IgG1, and IgG2b were unchanged (Fig. 5.5).

The ability of Foxo3-/- mice to mount a humoral immune response to specific antigens was also assessed. Responses to the T-independent antigen TNP-Ficoll (Fig. 5.6a) and the T-dependent antigen NP-CGG (Fig. 5.6b) were not significantly different between wild type and Foxo3-/- mice, although there was a trend toward a slightly increased response to NP-CGG in the absence of Foxo3-/- two weeks post immunization.

V.b.3. Decreased pre-B and recirculating B cells in Foxo3-/- mice

To determine whether Foxo3 contributes to other stages of B cell development, we examined the bone marrow of Foxo3-/- mice. Intriguingly, while both B220+CD43+ pro-B cells and B220+CD93+IgM+ immature B cells were present at normal numbers, there was a significant reduction in both the percentage and total number of B220+CD43-CD93+IgM- pre-B cells in the absence of Foxo3 (Fig. 5.7). IL-7 is an important growth and differentiation factor for B cell progenitors. In its absence, B cell development is blocked at the transition between pro- and pre-B cells (von Freeden-Jeffry et al. 1995).

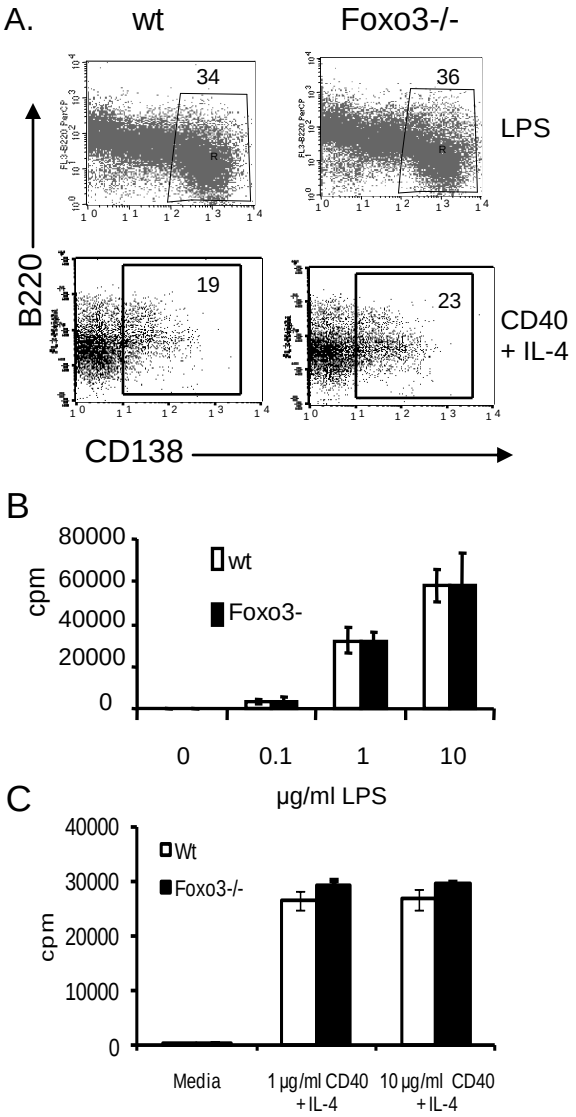

Figure 5.4: Normal in vitro proliferation and differentiation of Foxo3-/- splenic B cells in response to LPS and anti-CD40+IL-4.
A) Purified B cells were stimulated with 20 µg/ml LPS or 1 µg/ml anti-CD40 plus 10 ng/ml IL-4 for 72 hrs. and stained with anti-B220 and anti-CD138 antibodies. The frequency of $B220^{low}CD138+$ plasma cells is indicated for one of four representative mice per genotype**. B)** Purified B cells were stimulated with the indicated concentrations of LPS for 48 hrs. Cells were labeled with [^{3}H]thymidine for the final 6 hours of culture to measure proliferation. Data represent mean +/- SD of triplicate wells and are representative of two independent experiments. **C)** Purified B cells were stimulated with the indicated concentrations of CD40 + 10 ng/ml IL-4 for 48 hrs. Cells were labeled with [^{3}H]thymidine for the final 6 hours of culture to measure proliferation. Data represent mean +/- SD, n = 4.

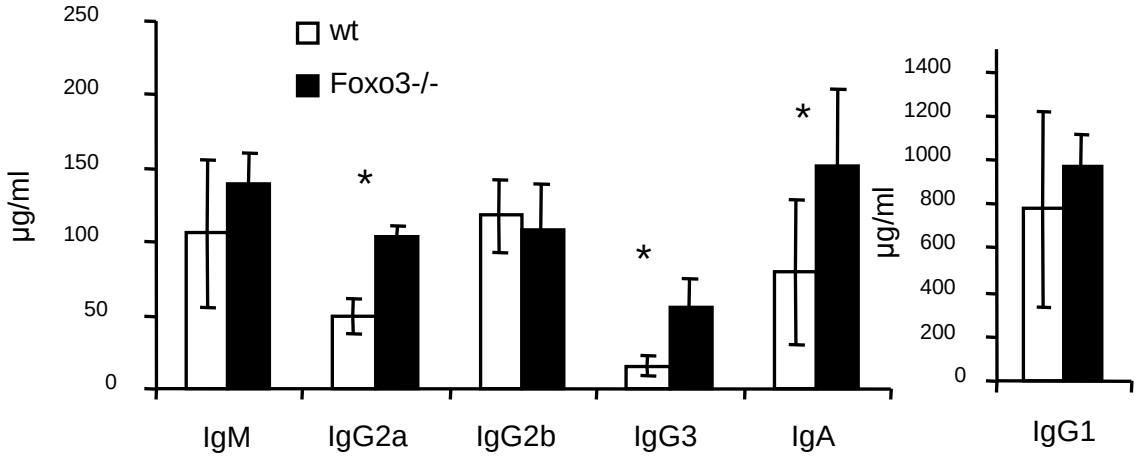

Figure 5.5: Increased basal levels of IgG2a, IgG3, and IgA in the serum of Foxo3-/- mice

Total amounts of the indicated Ig isotypes were measured by ELISA from the serum of 4-7 mice per genotype. Data represent mean +/- sd. To determine significance, P values for the Foxo3-/- in relation wildtype were calculated using a Student's t test and set to the following scale: * < 0.05.

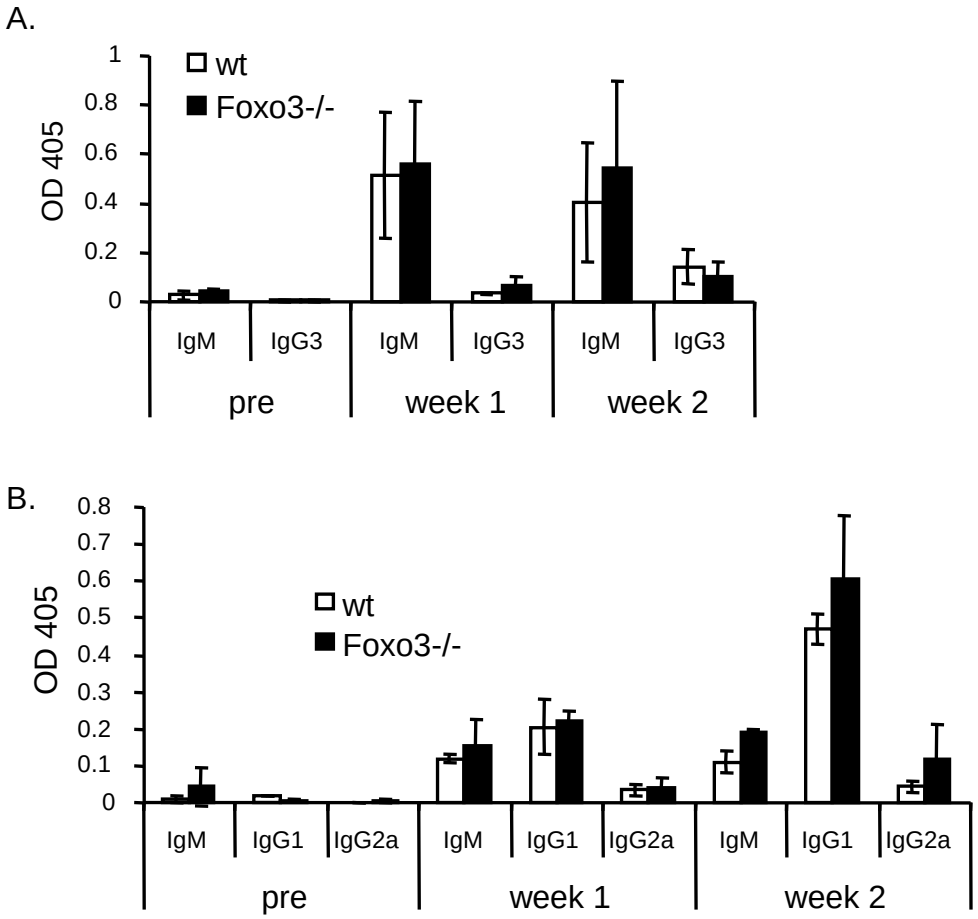

Fig. 5.6: Serum antibody responses to the T-independent antigen TNP-Ficoll and the T-dependent antigen NP-CGG are not significantly different between wildtype and Foxo3-/-.

A) Mice were immunized with 10 ug TNP-Ficoll. TNP-specific IgM and IgG3 in a 1:400 dilution of serum was measured by ELISA at day 0 (pre) and days 7 (week 1), and 14 (week 2) post immunization. Data represent mean +/- sd, n= 2 or 3 mice per genotype per time point. **B)** Mice were immunized with 100 ug NP-CGG in Alum. NP-specific IgM, IgG1, and IgG2a in a 1:400 dilution of serum were measured at day 0 (pre) and days 7 (week 1) and 14 (week 2) post immunization. Data represent mean +/- sd, n = 2 or 3 mice per genotype per time point. There is no significant difference between wildtype and Foxo3-/- mice.

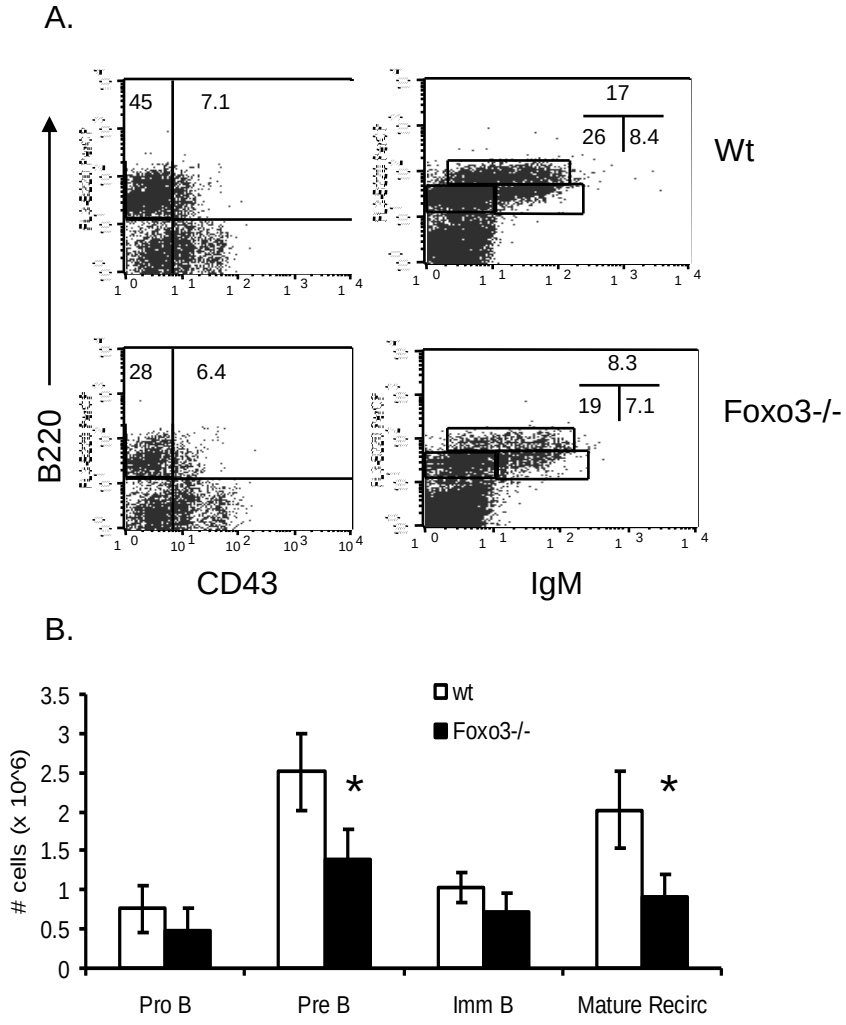

Figure 5.7: Alterations in the B cell compartment of Foxo3-/- bone marrow.
A) Bone marrow from wt and Foxo3-/- mice was stained with antibodies against B220, IgM, and CD43. The percentage of cells in each population within the lymphocyte gate is shown. Plots are representative of 4-5 individual mice. **B)** The total number of cells from two femurs and two tibias per mouse is shown for each population (Pro B = B220+CD43+, Pre B = B220+CD43-IgM-, immature = $B220^{lo}IgM+$, mature recirculating = $B220^{hi}IgM^{lo}$). Data represent mean +/- SD, n = 4-5. * $p < 0.05$ by Student's t-test. Similar results were obtained when cells were also stained with antibodies against AA4.1, which is expressed on Pro-B, Pre-B, and immature B cells but not recirculating B cells (data not shown).

However, we did not observe an impaired response to IL-7 in the absence of Foxo3. The IL-7 receptor alpha chain (CD127) was expressed at similar levels on pro- and pre-B cells from wild type and Foxo3-/- mice (Fig. 5.8a). B220+ cells from both wild type and Foxo3-deficient bone marrow expanded to a similar degree (two to three fold) upon culture with IL-7 for five days. IL-7 expanded cells from Foxo3-/- bone marrow proliferated normally (Fig. 5.8b) and had a cell cycle profile nearly identical to that of wild type cells (Fig. 5.8c). Rag2 is required for both heavy and light chain rearrangement and thus the development of B cells beyond the pro-B cell stage (Shinkai et al. 1992). However, IL-7 stimulated pre-B cultures from wild type and Foxo3-/- bone marrow expressed similar levels of Rag2 mRNA (Fig. 5.8d).

Analysis of Foxo3-/- bone marrow also revealed decreased frequencies and numbers of $B220^{hi}$ IgM+ CD93- recirculating B cells (Fig. 5.7). Since homing of mature B cells to the bone marrow is controlled in part by the interaction of CD22 on B cells with a sialic acid-containing ligand in the bone marrow microenvironment (Ghosh et al. 2006, Nitschke et al. 1999), we examined expression of CD22 on Foxo3-/- B cells (Fig. 5.9a). No difference was observed.

An alternative explanation for the decreased recirculating B cells in Foxo3-/- bone marrow is that these mice demonstrate a general impairment of B cell migration rather than a defect in homing specifically to the bone marrow. To address this issue, we analyzed B cells in the peripheral blood and peritoneal cavity. Foxo3-/- mice had a reduced frequency of B cells in the peripheral blood, and fewer mature B-2 cells in the peritoneal cavity than wild type mice (Fig. 5.9b-d).

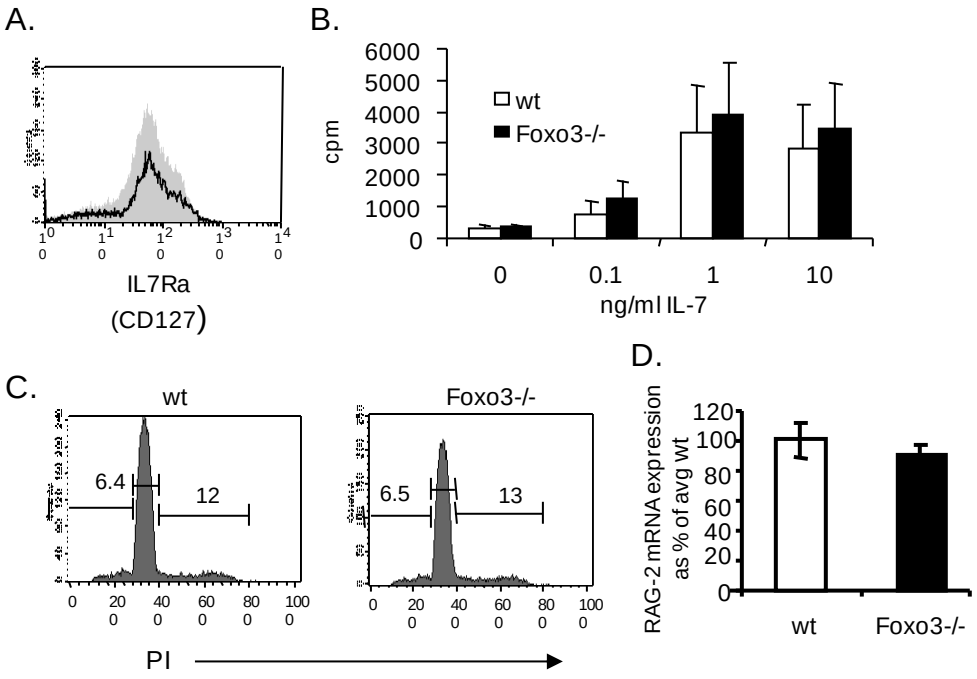

Figure 5.8: Foxo3-/- bone marrow cells respond normally to IL-7.
A) Bone marrow was stained with antibodies against IgM, B220, CD43, and IL-7Rα (CD127). The level of IL-7Rα on pro- and pre-B cells (B220+IgM-) is shown for wild type (shaded histogram) and Foxo3-/- (open histogram, dark line) mice. **B)** Bone marrow cells were plated in media alone or varying concentrations of IL-7 at 10^6/ml for 5 days. Proliferation was measured by ^{3}H-thymidine incorporation for an additional 24 hours. Data represent mean +/- SD, n = 3. **C)** Bone marrow cells were plated in 10 ng/ml IL-7 at 10^6/ml for 5 days and stained with PI to assess cell cycle status. The frequency of apoptotic (sub 2n), and S/G2/M cells (> 2n) is indicated. Plots are representative of n = 3-4. **D)** cDNA was prepared from day 5 IL-7 bone marrow cultures and subjected to Q-PCR for Rag2. Expression levels were normalized to GAPDH using the delta Ct method and plotted as a percentage of the average expression level in wild type B cells. Data represent mean +/- sd, n=2.

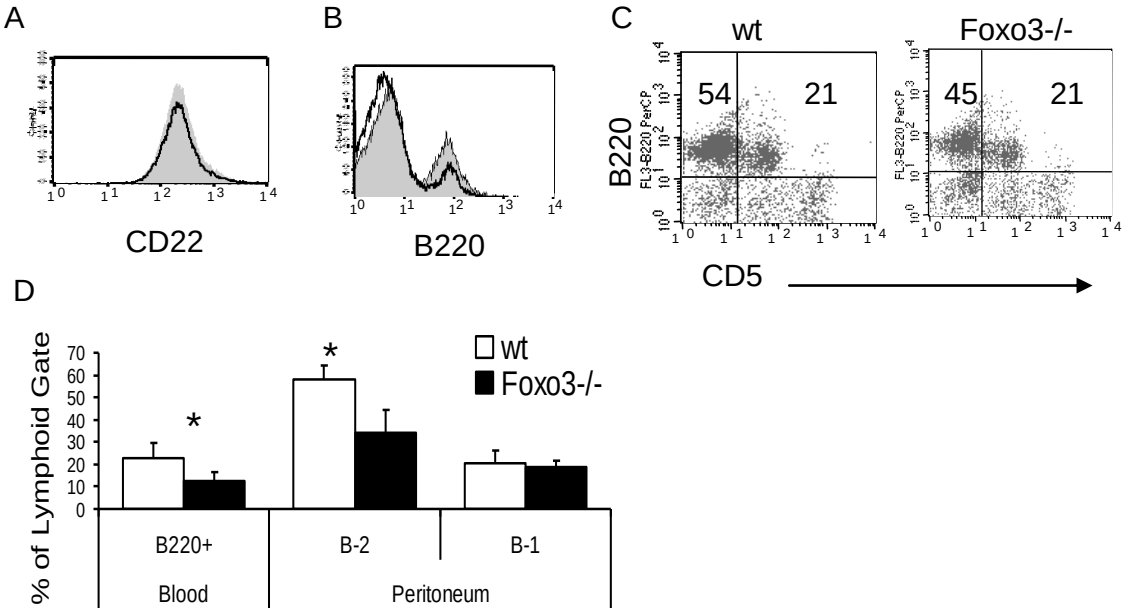

Figure 5.9: Reduced recirculating B cells in Foxo3-/- mice
A) Splenocytes were stained with antibodies against CD22, B220, CD21, and CD23. The level of CD22 expression on follicular B cells (B220+CD23+CD21+) from wild type (shaded histogram) and Foxo3-/- (open histogram, dark line) mice is shown. Data are representative of three mice. **B)** B220 expression is shown for cells in the lymphocyte gate of peripheral blood from wild type (shaded histogram) and Foxo3-/- (open histogram, dark line) mice. Data are representative of three mice. **C)** Peritoneal cells were stained with antibodies against B220 and CD5. The frequency of B-2 (B220+CD5-) and B-1 ($B220^{low}CD5+$) cells in the lymphocyte gate is indicated. Data are representative of three mice. **D)** The mean +/- SD of three mice analyzed as in B and C is shown. * $p \leq 0.05$ by paired Student's t-test.

Sphingosine-1 phosphate (S1P) contributes to the egress of lymphocytes from the peripheral lymphoid organs and their subsequent circulation (Matloubian et al 2004). Mice deficient in $S1P_1$, a receptor for S1P, have only a small reduction in splenic B cells but a dramatic decrease in recirculating B cells in the blood and bone marrow (Matloubian et al 2004). Inhibition of S1P signaling with FTY720 also reduces peritoneal B cell numbers (Kunisawa et al. 2007). PI3K has recently been shown to downregulate $S1P_1$ mRNA expression in T cells (Sinclair et al. 2008). Taken together, these results suggested that Foxo3 could control expression of $S1P_1$. There was a small, but consistent, reduction in $S1P_1$ mRNA in Foxo3-/- B cells to about seventy-five percent of wild type levels (Fig. 5.10). However, whether other Foxo3 targets in addition to $S1P_1$ also promote B cell egress, migration or homing remains to be determined.

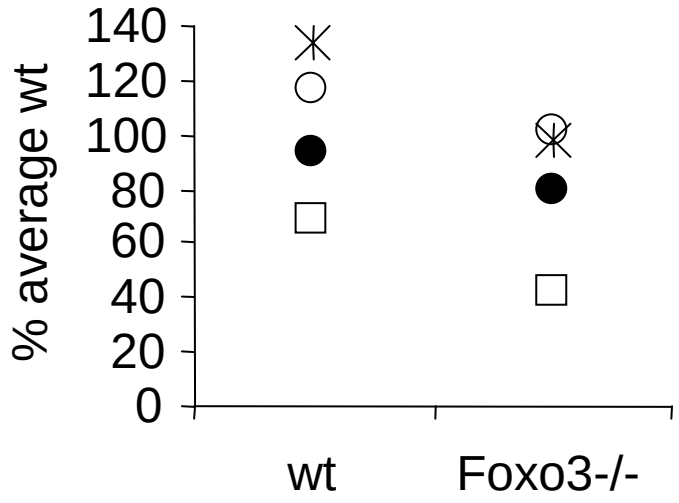

Figure 5.10: Reduced $S1P_1$ mRNA expression in splenic B cells of Foxo3-/- mice
Purified B cells were analyzed by Q-PCR for expression of $S1P_1$ (Edg1). Expression levels were normalized to GAPDH using the delta Ct method and plotted as a percentage of the average expression level in wild type B cells. Each symbol represents an independent experiment with one mouse of each genotype.

V.c. Discussion

Here, we show that Foxo3 plays a role in regulating pre-B cell numbers, B cell recirculation, and levels of total serum IgG2a, IgG3, and IgA. Foxo3 is dispensable, however, for the normal distribution of splenic B cell subpopulations and the response of splenic B cells to BCR crosslinking and LPS *in vitro*.

Foxo3 transactivates the cyclin G2 and Btg1 promoters in reporter assays in A20 B cells and NIH 3T3 cells, respectively (Bakker et al. 2007, Chen et al. 2006). However, we show that Foxo3 is not required for expression of these target genes in primary mature B cells (Fig. 5.3b). Nor is it necessary for the development or *in vitro* activation of normal splenic B cell subpopulations (Fig. 5.1, Fig. 5.2, Fig. 5.4). This suggests one of two possibilities. First, normal expression of Foxo1 and Foxo4 may compensate for the loss of Foxo3 in the control of Foxo target genes in splenic B cells. Addressing this question will likely require elimination of all three Foxo family members specifically in mature B cells. When deletion of Foxo1, Foxo3, and Foxo4 is induced simultaneously in all cells of adult animals, an extensive developmental block in the B cell lineage as well as a reduction in hematopoietic stem cells occurs (Tothova et al. 2007).

Alternatively, individual Foxo family members may have unique targets in B cells. In support of this model, the mRNA expression patterns for both cyclin G2 and Btg-1 were shown in a previous chapter to closely correlate with that of Foxo1, but not Foxo3 or Foxo4, in B cells treated with anti-IgM and various signaling inhibitors (Fig. 3.7). This suggests that Foxo1 may play a prominent role in regulating these and other

Foxo targets controlled by BCR engagement in splenic B cells. Indeed, a study by Dengler et al. demonstrates that Foxo1-deficient B cells have altered responses to BCR crosslinking (Dengler et al. 2008).

Although Foxo3 is dispensable for the normal distribution of splenic B cell subsets, we show here that it has a unique role in regulating pre-B cell numbers (Fig. 5.7). Despite this observation, no difference in IL-7R expression or expansion of B220+ cells in IL-7 cultures was observed between wild type and Foxo3-/- bone marrow (Fig. 5.8a-c). Rag2 expression was also normal in IL-7 expanded pre-B cells from Foxo3-/- mice (Fig. 5.8d). Impairment of earlier stages of B lymphopoiesis may play a role, however. While the frequency and number of pro-B cells was not significantly different between wild type and Foxo3-/- mice, there was a trend toward a slight reduction in this population in the absence of Foxo3. In addition, fewer cells fell into the lymphoid gate in Foxo3-/- bone marrow. Thus, some of the effect of loss of all three Foxo family members on hematopoietic stem cells and pro-B cells (Tothova et al. 2007) may be due specifically to Foxo3. Foxo3 may also act in the bone marrow microenvironment to support the development or survival of pre-B cells. Interestingly, mice lacking Foxo1 specifically in the B lineage were recently shown to also have a block in development at the pre-B stage (Dengler et al. 2008). However, unlike the case in Foxo3-/- mice, the absence of Foxo1 results in decreased expression of both Rag2 and IL-7R in early B lineage cells (Amin and Schlissel 2008, Dengler et al. 2008). Thus, Foxo3 and Foxo1 maintain pre-B cell numbers via distinct mechanisms.

It is intriguing to note that while Foxo3-/- mice show a decreased frequency of pre-B cells, the number of immature B cells in the bone marrow is comparable to wildtype mice (Fig. 5.7). What allows for normalization at this later stage of B cell development? Work introduced in the previous chapter suggests an answer. *In vitro*, the apoptotic response to BCR stimulation is decreased in immature B cells isolated from Foxo3-/- mice (Fig. 4.7). This is not a complete block in clonal deletion, but it would account for our observation of normal immature B cell numbers in spite of a smaller pre-B cell input.

Foxo3-deficiency also results in a decreased frequency of recirculating B cells in the bone marrow and blood as well as a reduction in B-2 cells in the peritoneum (Fig. 5.7, Fig. 5.9b-d). This function is likely to be B cell intrinsic, as a similar phenotype was observed in a recent study examining mice lacking Foxo3 specifically in B cells (Herzog et al. 2008). B cell expression of CD22, which mediates B cell homing to the bone marrow (Ghosh et al. 2006, Nitschke et al. 1999), was normal in the absence of Foxo3 (Fig. 5.9a). However, mRNA encoding $S1P_1$, which mediates S1P induced egress of B and T cells from lymphoid organs (Matloubian et al. 2004), was reduced in B cells from Foxo3-/- mice to approximately seventy-five percent of wildtype (Fig. 5.10). This is consistent with recent observations indicating that PI3K signaling downregulates $S1P_1$ expression via Foxo transcription factors in T cells (Fabre et al. 2008, Sinclair et al. 2008). B cells from $S1P_1$+/- mice demonstrate impaired exit from peripheral lymphoid organs, suggesting that the degree of change in $S1P_1$ expression seen in Foxo3-/- B cells could perhaps be physiologically relevant (Lo et al. 2005). S1P has recently been shown

to promote egress by counteracting other chemokines that retain lymphocytes in the spleen and lymph nodes (Pham et al. 2008). Foxo3 deficiency may disrupt the balance of these signals in favor of retention by altering responses to either S1P or other chemokines. However, the Foxo3-/- splenic B cell defect in $S1P_1$ mRNA expression is admittedly subtle. It is therefore likely that Foxo3 targets in addition to $S1P_1$ also promote B cell egress, migration or homing. It should also be noted that the block in B cell recirculation is incomplete in Foxo3-/- mice, indicating that other Foxo family members, or Foxo-independent mechanisms, likely contribute to this process as well.

Finally, Foxo3 has a unique role in regulating basal levels of serum IgG2a, IgG3, and IgA**.** This could result from either a heightened ability of B cells to respond to factors that induce switching to these isotypes (i.e. IFNγ, TGFβ) or an increased production of these factors by other cell types. The latter is a possibility given that generalized CD4+ T cell hyperactivity has been reported in an independently generated line of Foxo3-/- mice (Lin et al. 2004). In the same FVB line used for work presented in this thesis, Dejean et al. have also shown enhanced dendritic cell production of key inflammatory cytokines, including IL-6, which in turn results in greater T cell viability (Dejean et al. 2009). In our study, mice were able to respond and class switch upon immunization with TNP-Ficoll and NP-CGG in the absence of Foxo3 (Fig. 5.6). This is in contrast to mice lacking Foxo1 in mature B cells, which fail to class switch in response to either T-independent or T-dependent antigens (Dengler et al. 2008). Taken together, these results indicate that Foxo1 and Foxo3 regulate Ig production via distinct mechanisms. Though in the case of

Foxo3, it remains to be determined whether these mechanisms are B cell intrinsic or extrinsic.

Here we have shown that Foxo3-deficiency affects numerous aspects of B cell development. Based on these findings, possible roles for each Foxo family member and its associated target genes in the prevention and/or treatment of cancer, immunodeficiency, and autoimmunity will be discussed in the proceeding chapter.

CHAPTER VI

DISCUSSION

VI.a. Overall Conclusions

A functional immune system depends on a diverse B cell repertoire that develops in the bone marrow. Mature B cells, distributed throughout secondary lymphoid organs, respond to antigenic stimuli by dividing and differentiating into plasma cells. Signaling from the B cell receptor (BCR) plays a critical role at several points during these developmental processes. Cell survival, proliferation, differentiation, death, anergy, or receptor editing may occur in response to BCR stimulation. A variety of factors, including signal strength and duration, cytokine presence, and co-stimulation determine the ultimate B cell fate.

Work presented in this thesis demonstrates that the regulation of the Forkhead box class O (Foxo) family of transcription factors also has a role in determining the ultimate fate of the B cell. Regulation of these pro-apoptotic and anti-mitogenic genes occurs at both the level of mRNA expression and protein phosphorylation (Fig. 3.9). And although Foxo1, Foxo3, and Foxo4 are known to bind the same DNA target sequence (Furuyama et al. 2000), the mRNA expression of each family member is uniquely regulated downstream of PI3K (Fig. 3.6a, Fig. 3.9). Foxos also have distinct roles in the life of the B cell (Fig. 6.1, Amin and Schlissel 2008, Dengler et al. 2008, Herzog et al. 2008,

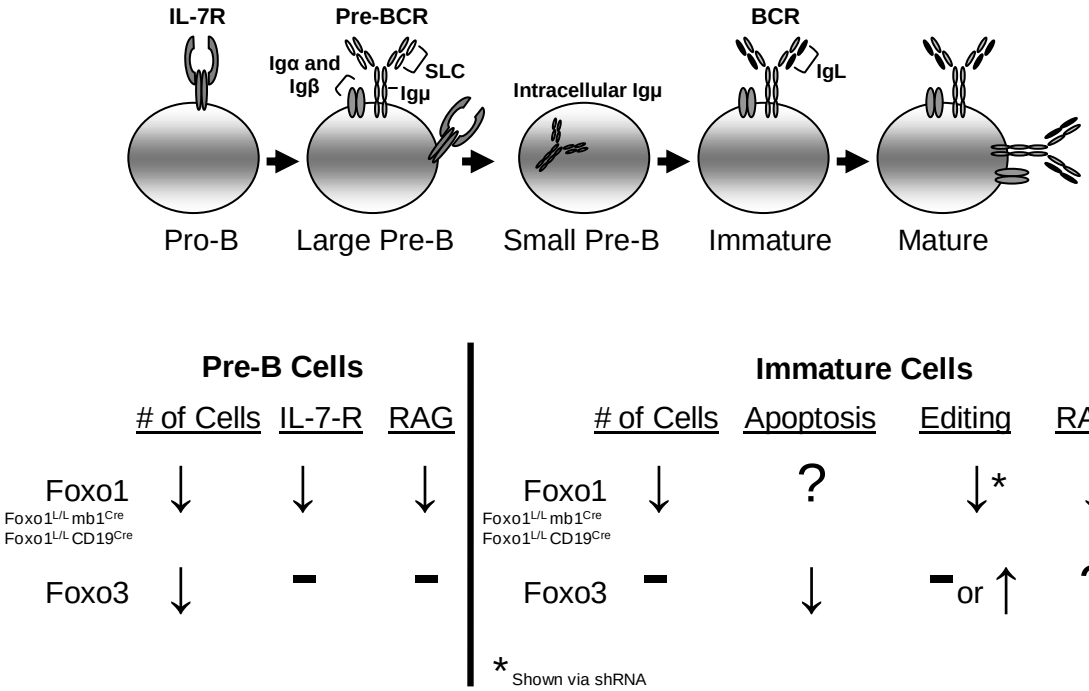

Figure 6.1: Comparing the effects of Foxo1 and Foxo3 deficiency at various stages of B cell development.
Compiled from Amin and Schlissel 2008, Dengler et al. 2008, Herzog et al. 2008, Herzog et al. 2009, and Hinman et al. 2009.

Hinman et al. 2009). Further exploration of these roles is important in understanding the maintenance of B cell homeostasis and its implications for cancer, immunodeficiency, and autoimmunity.

VI.b. BCR stimulation regulates Foxo protein phosphorylation and mRNA expression via distinct pathways.

In B lymphocytes the downregulation of Foxo mRNA expression is via the PI3K/Btk/BLNK/PLCγ2 pathway, whereas PI3K/Akt controls Foxo protein phosphorylation (Fig.3.9). Our results show that Btk-deficient or CsA treated B cells are capable of phosphorylating Foxo1 at serine-256, but upon BCR stimulation fail to downregulate Foxo mRNA expression (Fig. 3.3, Fig.3.5a, Fig. 3.6). This clearly demonstrates the distinct nature of the two pathways. However, the degree to which Foxo protein phosphorylation and mRNA expression are uncoupled may vary depending on cell type. Essaghir et al. have recently identified a positive feedback loop in human fibroblasts whereby, in the absence of growth factor, unphosphorylated Foxo3 protein drives the transcription of Foxo1 and Foxo4 mRNA (Essaghir et al. 2009). According to our experimental results, this feedback loop does not occur in the B lymphocyte. B cells from Foxo3-/- mice show normal basal levels of Foxo1 and Foxo4 mRNA. In response to BCR stimulation they downregulate these transcription factors to the same degree as observed in wildtype cells (Fig. 5.3a).

We have shown alterations in Foxo mRNA expression in response to a variety of stimuli (Fig. 3.8). However, it is not known whether Foxo mRNA expression in B lymphocytes is primarily controlled at the level of transcription or mRNA stability. Both may occur. The murine promoter regions for these genes have not been clearly identified. And given the number of recently published studies describing roles for miRNA in the immune system, the latter is an intriguing possibility. Overexpression of miR-150 in hematopoietic stem cells, for example, blocked B lymphopoiesis by inhibiting the transition from the pro-B to pre-B cell stage (Zhou et al. 2007). B cells have also been shown to require miR-155 for normal production of isotype-switched, high-affinity antibodies and for a memory response. The transcription factor Pu.1 has been shown to be direct target of miR-155. When Pu. 1 is overexpressed in wildtype B cells, fewer IgG1 cells are produced. This indicates that loss of Pu. 1 regulation is a contributory factor to the miR-155 phenotype (Vigorito et al. 2007). miR-181b impaired class switch recombination (CSR) when expressed in activated B cells. CSR is normally initiated by activation-induced cytidine deaminase (AID) through the deamination of cytosine residues on the immunoglobulin loci, which leads to the generation of DNA mutations or double-strand break intermediates. miR-181b directly targets AID, restricting its expression (de Yebenes et al. 2008). To date, none of these miRNAs has been studied for an effect on Foxo1, Foxo3, or Foxo4. However, Segura et al. have shown that miR-182 suppresses Foxo3 and in turn promotes the metastasis of a human melanoma cell line (Segura et al. 2009). Whether this miRNA plays a role in the B lymphocyte remains to be determined.

To date, understanding of the posttranslational control of Foxo proteins in the B lymphocyte has focused on Akt. However, Akt is not the only protein activated by PI3K that is capable of phosphorylating Foxo family members, leading to their sequestration from the nucleus. The serum and glucocorticoid-induced kinase (SGK), which is structurally related to Akt, can also play this role (Brunet et al. 2001). In other cell types, SGK has been shown to promote cell cycle progression in response to extracellular stimuli (Brunet et al. 2001, Buse et al. 1999, Kobayashi et al. 1999, Park et al. 1999). It is capable of phosphorylating the Foxo1, Foxo3 and Foxo4 proteins at the same sites as those targeted by Akt (Fig. 1.1). Although in mammals the biological consequences of SGK versus Akt phosphorylation have not been studied in detail, in *C. elegans* SGK is known to play a more prominent role in development, stress resistance and longevity, while Akt is critical to daur formation (Hertweck et al. 2004). The *C. elegans* enter the daur, or non-ageing larval stage, as a means of protection against unfavorable growth conditions. Given the complexity of Foxo control that we have described in B lymphocytes and the varying end outcomes, a role for SGK would not be hard to imagine. A probable role for IκB kinase (IKK) in Foxo protein phosphorylation has also been discussed in a previous chapter (Hu et al. 2004). Perhaps the interplay between these factors could be important in determining the timing of how Foxo proteins are drawn out of the nucleus and targeted for degradation. Further study is warranted.

Engagement of the BCR in mature B cells induces expression of a number of genes that promote cell cycle entry, cell cycle progression, and cell survival. The same is true when the T cell receptor (TCR) is engaged in mature T lymphocytes. Foxo

transcription factors would therefore be expected to play a similar role in these immune cells. Indeed when the TCR binds to its peptide:MHC ligand, Akt phosphorylates Foxo proteins leading to their nuclear exclusion (Fabre et al. 2005). In contrast, forced expression of a constitutively active Foxo protein in a T cell line promotes cell cycle arrest (Medema et al. 2000). The question then remains as to whether Foxo mRNA expression is regulated in these cells. If so, the PI3K/SLP76/Itk/PLCγ1 pathway downstream of the TCR may play a role (Smith-Garvin et al. 2009) analogous to the PI3K/BLNK/Btk/PLCγ2 pathway that acts in B cells.

VI.c. The stage of B cell development influences how BCR stimulation alters Foxo mRNA expression.

Several studies have shown preBCR signaling through PI3K/Akt to promote Foxo protein phosphorylation and allow for the expansion of the large pre-B cell population (Amin and Schlissel 2008, Herzog et al. 2008). The preBCR is then downregulated, proliferation ceases, and light chain rearrangement occurs starting at the large to small pre-B cell transition (Herzog et al. 2009, Reth et al. 1987). This coincides with the dephosphorylation of Foxo transcription factors (Herzog et al. 2008, Herzog et al. 2009). Whether there are subsequent changes in Foxo mRNA expression remains to be determined.

At later stages of B cell development, our work reveals a mechanism for control of Foxos at the level of mRNA expression via the PI3K pathway. In mature B cells, Foxo

mRNA expression was downregulated following BCR engagement (Fig.3.1a). In immature B cells, upregulation of Foxo mRNA was observed (Fig. 4.1a). This difference is not surprising, given that in the bone marrow immature B cells undergo cell cycle arrest and/or apoptosis following antigen stimulation. It does, however, raise questions as to how the BCR-induced regulation of Foxo1, Foxo3, and Foxo4 mRNA changes from one outcome to another as the B cell develops.

Once immature B cells leave the bone marrow and enter the spleen, they progress through a series of transitional stages subdivided on the basis of surface marker expression into T1(CD93+, IgM^{high}, CD23-), T2 (CD93+, IgM^{high}, CD23+) and T3 (CD93+, IgM^{low}, CD23+) (Allman et al. 2001, Carsetti et al. 1995, Chung et al. 2003). The earliest emigrants to the spleen, the T1 cells, remain sensitive to tolerance induction and antigen induced cell death. Thus, Foxo regulation in these cells would be expected to remain much the same as that for their predecessors. T1 cells give rise to T2, which are susceptible to anti-IgM-mediated apoptosis as well (Allman et al. 2001, Chung et al. 2002). There are, however, conflicting studies which also indicate some proliferative potential for T2 cells (Loder et al. 1999, Petro et al. 2002, Su and Rawlings 2002). The role of the T3 subset is also under debate. Recent work from several labs has suggested that these cells are anergic. Though they exhibit the same high basal levels of Ca^{2+} as in T1 and T2, T3 cells fail to flux Ca^{2+} and are hyporesponsive to stimulation through the BCR (Merrell et al. 2006, Teague et al. 2007). Foxo levels in these cells would therefore be expected to remain constant regardless of stimuli. Adoptive transfer experiments have demonstrated that it is the T2 subset, and not the T3, that is capable of acquiring a mature

phenotype *in vivo* (Merrell et al. 2006, Teague et al. 2007). This suggests that the T2 stage of development may be the critical point where BCR-induced regulation of Foxo protein phosphorylation and mRNA expression shifts outcomes to that observed in mature B lymphocytes.

It is at the T2 stage of development that B cells also acquire dependence on BAFF for continued survival. T2 cells undergo rapid apoptosis in the absence of this molecule. (Batten et al. 2000). This prompts the question as to how BAFF influences Foxo expression/activity. In microarray data generated through the Alliance for Cellular Signaling, primary murine B cells cultured with BAFF showed lower Foxo1 mRNA expression then those cultured in media alone (Zhu et al. 2004). These cells also had an increased rate of survival. Whether Foxo protein phosphorylation is also affected by BAFF remains to be determined, however, based on the above, further study is warranted.

The studies presented in this thesis have largely focused on the initiation of the immune response. We have shown that splenic B cells downregulate Foxo expression as they divide in response to BCR engagement (Fig. 3.1). Yet, what happens to Foxos beyond this initial burst of proliferation and differentiation? The termination of the immune response is critical for the maintenance of homeostasis. For example, FcγRIIb is a receptor for the constant region of IgG and is known to play a role in inhibiting continued B cell activation. When co-engaged with IgG, FcγRIIb ITIMs are phosphorylated by the Src kinase Lyn, creating a docking site for the inositol phosphatase SHIP. SHIP reduces the level of PIP3, the product of PI3K, thus reducing membrane

association and activation of PH domain containing proteins such as Btk and Akt (Aman et al. 1998, Bolland et al. 1998, Jacob et al. 1999, Scharenberg et al. 1998). Foxo mRNA expression would then be expected to rise and Foxo proteins would subsequently be generated and reenter the nucleus of the B cell. The outcome of this response, either apoptosis or cellular quiescence, might ultimately depend on the differentiated state of the cell; a short-lived plasmablast versus a mature B cell, long-lived plasma cell, or memory cell.

VI.d. Identifying distinct roles for Foxo1, Foxo3, and Foxo4 in the B lymphocyte

VI.d.1 Analysis of mouse models for Foxo1, Foxo3, and Foxo4 deficiency

Simultaneous deletion of Foxo1, Foxo3, and Foxo4 in hematopoietic stem cells leads to a considerable block in the generation of common lymphoid progenitors (CLPs). This is due, in part, to impaired protection of the cells from reactive oxygen species (Tothova et al. 2007). There is an increased rate of apoptosis. The severity of this phenotype in contrast to that of each individual Foxo knockout suggests that the Foxo transcription factors have both unique and redundant functions in the immune system.

Our characterization of the B cell compartment of Foxo3-/- mice establishes that this transcription factor plays a unique role in regulating pre-B cell numbers, clonal deletion of immature B cells, mature B cell recirculation, and levels of total IgG2a, IgG3, and IgA in the serum. Foxo3 is dispensable, however, for the normal distribution of splenic B cell subpopulations and the response of splenic B cells to BCR crosslinking and

LPS *in vitro*. It is intriguing to compare and contrast our findings to those obtained via B cell conditional knockouts of Foxo1 (Dengler et al. 2008). Both similarities and differences in the phenotypes can be observed (Fig. 6.1).

We have described a decrease in both the frequency and total number of pre-B cells in Foxo3-/- mice (Fig. 5.7). Dengler et al. have noted a similar and yet more severe defect in $Foxo1^{L/L}mb1^{Cre}$ mice, where Foxo1 is deleted at the earliest stage of pro-B cell development. They have attributed this defect to a requirement for Foxo1 in both IL-7Rα and RAG expression (Dengler et al. 2008). Amin et al. have likewise confirmed that it is Foxo1, and not Foxo3 or Foxo4 that regulates RAG expression in these cells (Amin and Schlissel 2008). In our knockout study, we have also shown no such requirement for Foxo3 (Fig. 5.8a, Fig. 5.8d). And while the pre-B cell defect in $Foxo1^{L/L}mb1^{Cre}$ mice coincides with a two-fold higher percentage of $B220^{+}CD43^{+}$ pro-B cells (Dengler et al. 2008), this population in the Foxo3-/- is comparable to wildtype (Fig. 5.7). Further study is therefore warranted and ongoing to tease out the unique mechanism by which Foxo3 affects pre-B cell numbers.

There is a decreased apoptotic response to BCR crosslinking in the immature B cell population of Foxo3-/- mice (Fig. 4.7). In a previous chapter, we speculated that Foxo3 plays a specific role in promoting clonal deletion. This may explain why, in spite of the reduced pre-B cell input described above, there are a normal number of immature B cells in Foxo3-/- mice (Fig. 5.7). Receptor editing, as reflected by Igλ usage (Tiegs et al. 1993), did not appear to be impaired in the absence of Foxo3, however (Fig 4.9), although more direct measurements of receptor editing are required to confirm this

observation. *In vivo*, the effect of Foxo1 deficiency in immature B cells has not been studied in detail, though Dengler et al. did note a smaller percentage of these cells relative to wildtype in both the $Foxo1^{L/L}mb1^{Cre}$ and $Foxo1^{L/L}Cd19^{Cre}$ models (Dengler et al. 2008). Amin et al. have also shown that a shRNA targeting Foxo1 reduces anti-IgM induced RAG expression in culture (Amin and Schlissel 2008). This supports a specific role for Foxo1 in receptor editing. In our studies, the defect in deletion of immature B cells in Foxo3-/- mice did not coincide with significantly higher levels of serum autoantibodies (Fig. 4.8). However if this phenotype was combined with reduced receptor editing due to limited Foxo1 expression, a measurable breech in tolerance would be more likely to occur. To test this hypothesis would require the generation of Foxo3-/- mice with a heterozygous mutation in Foxo1, as complete Foxo1 deficiency also affects responses of mature B cells, potentially obscuring an effect on autoantibody production (Dengler et al. 2008).

Splenic B cell subpopulations are normal in the absence of Foxo3. IgM expression is comparable to wildtype and the cells proliferate normally *in vitro* in response to BCR crosslinking (Fig. 5.1, Fig. 5.2). Due to prominent defects in early B cell development, the $Foxo1^{L/L}mb1^{Cre}$ and $Foxo1^{L/L}Cd19^{Cre}$ cannot give a clear picture as to the effects of Foxo1 deficiency in the periphery. In the $Foxo1^{L/L}Cd21^{Cre}$, however, deletion of Foxo1 does not occur until the transitional stage of B cell development. This is after IL-7 dependent differentiation, immunoglobulin gene rearrangement, and RAG-mediated receptor editing has taken place. Dengler et al. have shown that the splenic B cell subpopulations of $Foxo1^{L/L}Cd21^{Cre}$ mice are grossly normal, although there is a

lower overall expression of IgM. This, in turn, correlates with a dose dependent reduction in anti-IgM-induced survival (Dengler et al. 2008). It is interesting to note that neither Foxo1 nor Foxo3 deficiency affects LPS induced proliferation (Dengler et al. 2008, Fig. 5.3a-b). This observation raises an important question. Is the anti-IgM induced survival defect described in the splenic B cells of $Foxo1^{L/L}Cd21^{Cre}$ mice directly related to the absence of the transcription factor itself, or is it instead a side effect of reduced BCR expression?

Mature lymphocytes do not reside solely in the spleen. They move continuously through the blood stream and lymphatics from one peripheral lymphoid tissue to another in search of their cognate antigen. This movement is directed by both adhesion molecules and chemokines. Our analysis of Foxo3-/- bone marrow revealed a decrease in the frequency and total number of $B220^{hi}$ IgM+ CD93- recirculating B cells (Fig. 5.7). This observation was corroborated by Herzog et al. (Herzog et al. 2008). Foxo3-/- mice also had a reduced frequency of B cells in the peripheral blood, and fewer mature B-2 cells in the peritoneal cavity (Fig. 5.9b-d). In the previous chapter, we suggested that this general impairment of B cell migration could possibly be due to a small, but consistent, reduction in $S1P_1$ mRNA in Foxo3-/- B cells to about 75% of wild type levels (Fig. 5.10). $S1P_1$ is a receptor for S1P, which is responsible for lymphocyte egress from the peripheral lymphoid organs. The $Foxo1^{L/L}Cd21^{Cre}$ also appears to have a defect in B cell migration. The mice have 30% fewer B cells in the lymph nodes compared to wildtype. Dengler et al. have attributed this defect to a reduction in the adhesion molecule L-selectin (CD62L) (Dengler et al. 2008). When Foxo1 is conditionally deleted in T cells, a similar reduction

in L-selectin occurs. The T cells in turn do not effectively home to either the lymph nodes or spleen (Kerdiles et al. 2009). These findings are intriguing in that they show both Foxo1 and Foxo3 to play a role in lymphocyte migration. Neither transcription factor, however, can fully compensate for the loss of the other.

In the initial stages of B cell activation and subsequent plasma cell generation, the antibody response is dominated by IgM secretion. Over time, B cell clonal expansion and class switch recombination leads to the production of additional antibody isotypes. These isotypes, including IgG and IgA, are advantageous in that they have greater half-lives and tissue access, as well as distinct Fc-mediated effector functions. We have shown that Foxo3-/- mice are able to normally respond and class switch upon immunization with TNP-Ficoll and NP-CGG (Fig. 5.6). This is in contrast to $Foxo1^{L/L}Cd21^{Cre}$ mice, which can secrete IgM, but fail to class switch in response to either T-independent or T-dependent antigens (Dengler et al. 2008). In Foxo3-/- mice, we also noted higher basal levels of serum IgG2a, IgG3, and IgA (Fig. 5.5)**.** This could result from a heightened ability of the B cells to respond to external stimuli that induce switching to these isotypes. Indeed, we have preliminary evidence to suggest that isolated Foxo3-/- B cells are capable of producing greater amounts of IgG2a, IgG3, and IgA when cultured in either LPS alone or in a combination of LPS and IFNγ or TGFβ (data not shown). *In vivo,* an increased production of these factors by other cell types may also contribute to the observed phenotype. This is a likely scenario, given that Dejean et al. have shown enhanced dendritic cell production of inflammatory cytokines in Foxo3-/- mice (Dejean et al. 2009). And while an in depth analysis of the basal Ig levels in $Foxo1^{L/L}Cd21^{Cre}$

mice has not been completed, one would imagine based on the available challenge data (Dengler et al. 2008) that serum IgM would predominate. This contrast in phenotypes further emphasizes the unique effects of Foxo1 and Foxo3 deficiency in immune cell regulation.

The third member of the Foxo family of transcription factors, Foxo4, was for a time believed to have a null immune cell phenotype. However, Zhou et al have recently identified a novel role for this family member in the regulation of mucosal immunity. Foxo4-/- mice showed significantly higher mortality compared to wildtype following colonic injury. The colons of the mice which did survive were found to be severely inflamed with increased lymphocyte infiltration, goblet cell loss, and thickening of the vascular wall. This phenotype was attributed, in part, to higher basal expression of IFNγ, TNFα, CCL5 and CXCL9 in the colon, which was further exacerbated upon injury. These cytokines were not elevated elsewhere in the body. In culture, Zhou et al. have also shown increased IL-6 production by Foxo4-/- macrophages following LPS stimulation (Zhou et al. 2009). This is intriguing given that increased IL-6 production has also been noted in the dendritic cells of Foxo3-/- mice (Dejean et al. 2009). Could there be some degree of overlap in the function of these two Foxo family members? Indeed, Foxo3 deficiency has also been shown to affect mucosal immunity. In a study by Snoeks et al., Foxo3 deficient mice had more severe inflammation with an increased number of intraepithelial lymphocytes in response to colonic injury (Snoeks et al. 2009). There has not, however been an in depth analysis of the B cell compartment in Foxo4-/- mice. In light of the work presented here this is clearly warranted.

VI.d.2 Foxo target gene specificity

Given that Foxo1, Foxo3, and Foxo4 are capable of binding the same DNA consensus sequence, the target genes for these transcription factors have been largely grouped together in the literature. They are most often described collectively as Foxo targets, with no discrimination between family members (Table 1.2). Yet, the distinct phenotypes for each individual knockout suggest that Foxo1, Foxo3, and Foxo4 do have some degree of target gene specificity. Our work further supports this hypothesis.

Overexpression of Foxo3 mRNA has a significant functional impact on WEHI 231 immature B cells but does not affect the basal or BCR-induced expression of the known Foxo target genes Cyclin G2 and Btg-1 (Fig. 4.5). Furthermore, Cyclin G2 and Btg-1 expression are unaffected in mature B cells from Foxo3-/- mice (Fig. 5.3b). RAG and IL-7R expression are also shown to be normal in the absence of Foxo3 (Hinman et al. 2009, Fig. 5.8a, Fig. 5.8d), while they are clearly dependent on Foxo1 (Amin and Schlissel 2008, Dejean et al. 2009).

This suggests that Foxo3 does not play a major role in the control of the above genes. What targets, then, mediate the unique function of Foxo3 in immature B cell apoptosis? One candidate is $p27^{Kip1}$, a known Foxo target that mediates both cell cycle arrest and apoptosis in WEHI 231 cells (Banerji et al. 2001). Puma is another possibility. In T cells, Foxo3 was shown to directly upregulate Puma expression in response to cytokine and growth factor deprivation. This in turn led to increased apoptosis (You et al. 2006). We have preliminary evidence that would appear to rule out Bim as a Foxo3

specific target gene. Both the basal expression and anti-IgM-induced upregulation of mRNA encoding this pro-apoptotic molecule in WEHI 231 were unaffected by Foxo3 overexpression (data not shown). This is in contrast to the work of Mandal et al. in pre-T cells. With the expression of a constitutively active Foxo3 mutant, they showed significant upregulation of Bim gene expression (Mandal et al. 2008). It may be that Foxo target gene specificity varies depending on both cell type and context. It has also been proposed that certain specialized tasks may be controlled by only one Foxo family member, while other cellular functions are redundantly covered by two or three of the transcription factors. A comprehensive study of the gene expression profiles for immune cells isolated from Foxo1, Foxo3, and Foxo4 deficient animals would offer greater insight into this issue. CHIP analysis may also be helpful in identify differential binding of Foxo family members to specific target promoters under different conditions.

VI.e. Relevance of present study to human health and disease.

VI.e.1 How might other physiological processes known to alter Foxo activity affect the immune response?

Work presented in this thesis focuses on how signals transmitted through the BCR alter Foxo expression and ultimately B cell homeostasis. However, cytokines and other growth factors such as insulin and insulin-like growth factor-1 are also known to affect Foxo activity. It is intriguing to consider how these signals might integrate with those received through the BCR. For example, in a nutrient poor environment the level of

circulating insulin is reduced throughout the body. This in turn leads to a lower overall level of Foxo protein phosphorylation via the insulin/PI3K/Akt/Foxo pathway (Barthel et al. 2005). More active Foxo protein is present in the nuclei of the cells. Would it therefore be more difficult for mature B cells to be activated by their cognate antigen? Conversely, in a well fed animal would we see a more robust immune response?

VI.e.2 B cell maligancy

Both Foxo transcription factors themselves (Borkhardt et al. 1997) and their target Btg-1 have been associated with chromosomal translocations in leukemia (Rimokh et al. 1991). Resistance to cell death in B-chronic lymphocytic leukemia has been linked to inactivation of Foxo3 via protein phosphorylation (Ticchioni et al. 2004). Similarly, BCR-ABL, which causes both chronic myelogenous leukemia and acute lymphocytic leukemia, acts in part by downregulating Foxo3 (Essafi et al. 2005, Fernandez de Mattos et al. 2004). A dominant negative Foxo protein dramatically increases the frequency of B cell malignancy in Eu-myc transgenic mice (Bouchard et al. 2007). It has also been proposed that increased PKCβ signaling, which we showed in a previous chapter downregulates Foxo3 mRNA levels in normal B cells stimulated through the BCR, could contribute to lymphoma (Su et al. 2002). These findings are interesting in that they support the theory that both transcriptional and post-translational control of Foxo family members could have consequences for B cell malignancy.

VI.e.3 Immunodeficiency

Btg-1 mRNA expression is upregulated in EBV-transformed B cells from X-linked agammaglobulinemia (XLA) patients (Islam et al. 2002), suggesting aberrant expression of the Foxo target gene has consequences not only in cancer, but immunodeficiency as well. XLA results from the loss of Btk function (Satterthwaite and Witte 2000). We have shown that it is the PI3K/Btk pathway which controls Foxo mRNA expression. Therefore, could therapies which alter Foxo levels rescue this phenotype? Taken together with the fact that Foxo1 deficiency causes B cell immunodeficiency in mice (Dengler et al. 2008), these observations suggest that either too much or too little Foxo expression or activity can have detrimental effects on the development and activation of B cells.

VI.e.4 Autoimmunity

Foxo family members may also play a role in immune tolerance. Our work in a previous chapter showed that in the absence of Foxo3, BCR-induced apoptosis is reduced in immature B cells (Fig. 4.7). Foxo expression, or lack thereof, is therefore expected to alter the efficiency of clonal deletion in vivo. Foxo-induced Rag expression in immature B cells is also likely to promote elimination of autoreactive B cells via receptor editing (Amin and Schlissel 2008, Herzog et al. 2008). Preliminary studies have shown lower Foxo1 transcipt levels in the peripheral blood mononuclear cells of both systemic lupus erythematosus and rheumatoid arthritis patients (Kuo et al. 2007).

Thus, teasing out the roles for each Foxo family member and its associated target genes in the maintenance of B cell homeostasis has implications for prevention and/or treatment of a number of diseases.

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