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The E26 Transformation-Specific Transcription Factors PU.1, Spi-B, and Spi-C Regulate Transcriptional Activation and Repression of Nfkb1 to Control B Cell Development and Function

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

THE E26 TRANSFORMATION-SPECIFIC TRANSCRIPTION FACTORS PU.1, SPI-B, AND SPI-C REGULATE TRANSCRIPTIONAL ACTIVATION AND REPRESSION OF *NFKB1* TO CONTROL B CELL DEVELOPMENT AND FUNCTION

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by

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Abstract

Generating antibodies against T-independent and T-dependent antigens requires Toll-Like receptor (TLR) engagement on B cells for efficient responses. However, transcriptional regulation of TLR responses in B cells is not well understood. Transcription factors of the E26 transformation-specific family (ETS) are critically important for regulating hematopoiesis and cellular function. PU.1, Spi-B, and Spi-C are highly related ETS transcription factors that can bind nearly identical DNA sequences in the genome. PU.1 and Spi-B (encoded by *Spi1* and *Spib* respectively) are important for B cell development and function, but the function of Spi-C (encoded by *Spic*) in B cells is not clear. *The primary objective of this study was to determine the function of PU.1, Spi-B, and Spi-C during B cell development, and during TLR-mediated responses. It was hypothesized that PU.1 and Spi-B were required for positively regulating components of TLR responses, and Spi-C inhibited PU.1 and Spi-B targets.* $Spi1^{+/-}Spib^{-/-}$ (*PUB*) B cells proliferated poorly in response to TLR ligands compared to WT B cells. The NF-κB family member p50 (encoded by *Nfkb1*) is required for LPS responsiveness in mice, and *PUB* B cells expressed reduced *Nfkb1* mRNA transcripts and p50 protein. Forced expression of p50 in *PUB* B cells was capable of restoring TLR-mediated proliferation. It was determined that PU.1 and Spi-B directly regulated the *Nfkb1* promoter, and were required for proper TLR-mediated responses. Next, $Spib^{-/-}Spic^{+/-}$ mice were generated to determine if Spi-C was functionally redundant with Spi-B. $Spib^{-/-}Spic^{+/-}$ mice had restored number of B cells in the spleen, and had restored proliferative responses compared to $Spib^{-/-}$ mice. Steady-state *Nfkb1* levels were elevated in $Spib^{-/-}Spic^{+/-}$ B cells compared to $Spib^{-/-}$ B cells, and was the potential mechanism of the $Spib^{-/-}Spic^{+/-}$ phenotype. It was demonstrated that Spi-B directly activated the *Nfkb1* gene, while Spi-C directly repressed this gene. These results suggest that PU.1 and Spi-B are required for transcriptional activation of *Nfkb1*, and Spi-C has a negative regulatory role in B cell development and function. Determining the transcriptional regulation of B cell responses has important implications for understanding antibody forming responses, such as those seen in vaccinations.

Keywords

B cell, transcription factor, PU.1, Spi-B, Spi-C, *Spi1*, *Spib*, *Spic*, mouse, immunology, flow cytometry, RT-qPCR, chromatin immunoprecipitation, immunoblot, ELISA

Dedication

This thesis is dedicated to my mother and father, Kenneth and Emily Li. Their love and support have allowed me to succeed and achieve my academic goals, and will be sustained with me throughout life.

Co-Authorship Statement

Chapter 2 is adapted from Li SK, Abbas AK, Solomon LA, Groux GM, and DeKoter RP (2015) *Nfkb1* activation by the ETS transcription factors PU.1 and Spi-B promotes Toll-Like receptor-mediated splenic B cell proliferation. *Mol Cell Biol.* Text and images were reproduced with accordance to American Society for Microbiology Journals Statement of Authors' Rights (Appendix B). ChIP-seq experiments and analysis was performed by L Solomon. RT-qPCR experiments performed in LPS stimulated B cells were performed by G Groux. ChIP experiments were performed by A Abbas. All other experiments were performed by S Li in the laboratory of R Dekoter. The publication was written by S Li with suggestions from R DeKoter.

Chapter 3 is adapted from Li SK, Solomon LA, Fulkerson PC, and DeKoter RP (2015) Identification of a negative regulatory role for Spi-C in the murine B cell lineage. *J Immunol.* Doi: 10.4049/jimmunol.1402432. *Copyright 2015. The American Association of Immunologists, Inc.* Text and images were reproduced with permission from The American Association of Immunologists, Inc. (Appendix C). P Fulkerson generated the anti-Spi-C antibody. L Solomon performed immunoblot experiments. All other experiments were performed by S Li in the laboratory of R DeKoter. The publication was written by S Li with suggestions from R DeKoter.

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List of Abbreviations

Ab – antibody

APC – allophycocyanin

BAFF – B cell activating factor

BCR – B cell receptor

BLNK – B cell linker

BM – bone marrow

bp – base pairs

Breg – B regulatory cell

Btk – Bruton's tyrosine kinase

cDNA – complementary deoxyribonucleic acid

CFSE – carboxyfluorescein succinimidyl ester

ChIP – chromatin immunoprecipitation

ChIP-seq – chromatin immunoprecipitation sequencing

CLP – common lymphoid progenitor

CSR – class switch recombination

cpm – counts per minute

DC – dendritic cell

DNP-KLH – dinitrophenyl conjugated to keyhole limpet hemocyanin

DNP-LPS – dinitrophenyl conjugated to LPS

DJ – diversity and junction regions of the heavy and light Ig chains

ELISA – enzyme-linked immunosorbent assay

Eμ – immunoglobulin heavy chain intronic enhancer

ES – embryonic stem cell

ETS – E26 transformation-specific

FITC – fluorescein isothiocyanate

FO – follicular

GFP – green fluorescence protein

h – hours

HA – hemagglutinin

HSC – hematopoietic stem cell

Ig – immunoglobulin

IKK - IκB kinase

IL – interleukin

IRAK - interleukin-1 receptor-associated kinase

LMPP – lymphoid-primed multipotent progenitor

LPS – lipopolysaccharide

MFI – mean fluorescence intensity

MHCII – Major histocompatibility complex II

MPP – multipotent progenitor

mRNA – messenger RNA

MTT – 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide

MZ – marginal zone B cell

MZP – marginal zone precursor B cell

NF-κB – nuclear factor kappa B

OD – optical density

PAMP - pathogen associated molecular pattern

PE – phycoerythrin

PI3K – phosphoinositide 3-kinase

pro-B – progenitor B

PUB – PU.1$^{+/-}$Spi-B$^{-/-}$ (*Spi1*$^{+/-}$*Spib*$^{-/-}$)

pDC – plasmacytoid dendritic cell

qPCR – quantitative PCR

RP105 – radioprotective 105

RT-qPCR – reverse transcription quantitative PCR

SA – streptavidin

SD – standard deviation

SDS – sodium dodecyl sulfate

SEM – standard error mean

T1 – transitional-1

T2 – transitional-2

T3 – transitional-3

TD – T cell (thymus) dependent

TI – T cell (thymus) independent

TIR – Toll/IL-1R

TLR – Toll-like receptor

TSS – transcriptional start site

WT – wild-type

V – variable region of the Ig heavy chain

Chapter 1

1 Introduction

Humans are exposed to millions of potential pathogens on a daily basis. Therefore, protection from nearly unlimited pathogens is required over a lifetime. The ability of humans to avoid infection upon repeated exposure to pathogens is dependent on cellular components of the innate and adaptive immune system. Vaccines have eliminated many of the world's worst diseases such polio and smallpox, but there are currently no vaccines for many serious diseases such as HIV, cancers, and malaria. Generating vaccine-mediated protection in humans is complex; most current available vaccines have been developed with limited understanding of how they activate the immune system (Siegrist, 2008). Most effective vaccinations are mediated by antibodies produced by B cells. Antibodies function to activate complement, and neutralize or opsonize toxins and pathogenic microorganisms to aid in their destruction (Pone et al, 2010). Aside from the economic and commercial priorities of the vaccine development industry, the difficulty in generating protective vaccines is in maintaining long-lasting protection through the correct presentation of antigens (Plotkin, 2009). Therefore, understanding the regulation of B cell development, maintenance, and responses to antigens is important for determining the cellular mechanism of a vaccine.

1.1 B cell origin, function, and subsets

B cells are an essential component of the immune system. They can function as professional antigen presenting cells, modulate immune responses by cytokine production, and are the sole producers of antibodies (Pone et al, 2010). Each B cell produces a single unique antibody specificity, which is generated from germline recombination of the immunoglobulin (Ig) genes. Identical copies of unique Igs are embedded on the surface of a B cell, termed the B cell receptor (BCR), which distinguishes B cells from other lymphocytes. The BCR can be further diversified by Ig class switch recombination (CSR) and somatic hypermutation events. B cells initially

express and produce IgM and IgD isotypes, but can express IgG, IgA, or IgE following CSR. BCRs form clusters when bound by an antigen, thereby bringing together signaling molecules to contribute to B cell differentiation (Pone et al, 2010). In both mice and humans, B cells develop in the bone marrow (BM), and then migrate to the spleen to mature.

1.1.1 B cell development in the bone marrow

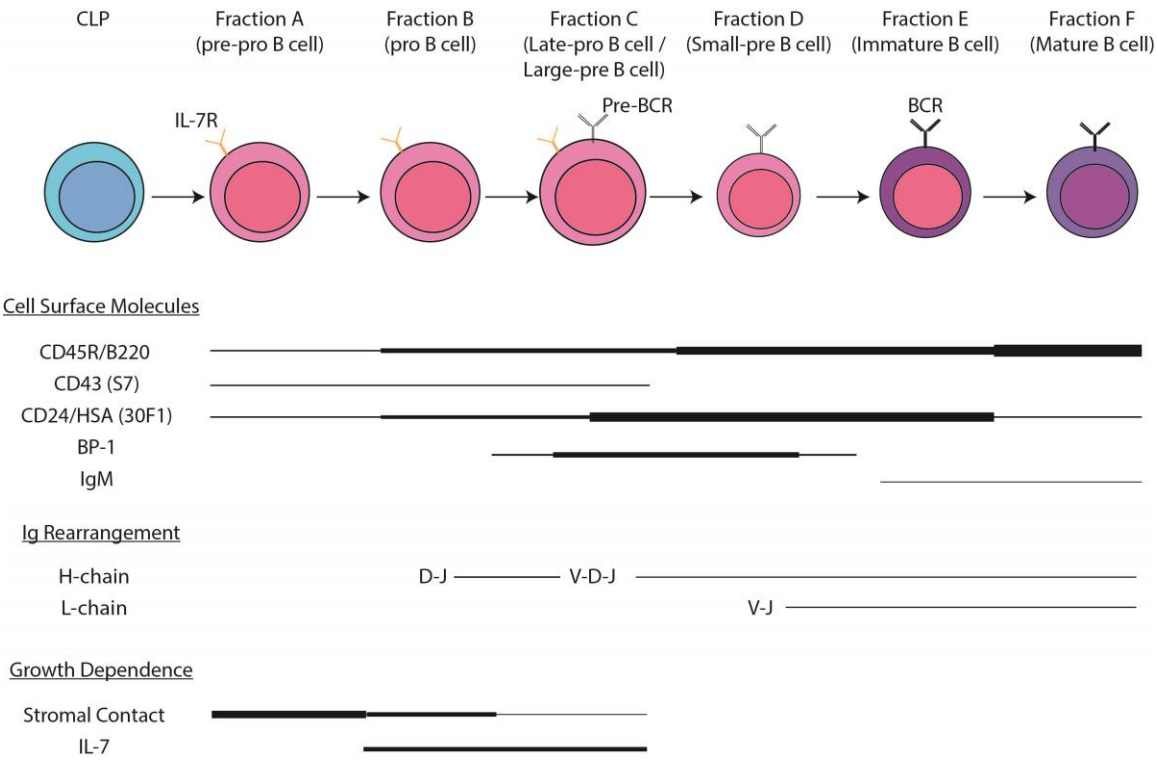
B cell development is a well-studied system in mice, and unlike many other somatic tissues, its development continues even in adults. There are many stages of B cell development, initiating with a pluripotent hematopoietic stem cell (HSC). HSCs differentiate into various progenitor stages, going in the order of a multipotent progenitor (MPP), lymphoid-primed multipotent progenitor (LMPP), and common lymphoid progenitor (CLP) (Nagasawa, 2006). Each progenitor stage is capable of generating a specific cell lineage or progressing to the next stage. CLPs can differentiate into T cells, dendritic cells, NK cells, and B cells. In 1991, *Hardy et al* defined a number of progenitor B cell stages in the bone marrow of mice, termed fractions A, B, C, D, E, and F (Hardy et al, 1991). The fractions progress in alphabetical order and are characterized based on cell-surface phenotype, differences in functionality, and Ig-gene rearrangement (Hardy et al, 1991) (Figure 1.1). B cells undergo Ig-gene rearrangement during differentiation, which is an important process for generating a repertoire of Ig receptors capable of recognizing any antigen.

Fraction A cells ($B220^{+}CD43^{+}CD24^{-}BP\text{-}1^{-}IgM^{-}$) are a heterogeneous population containing pre-pro B cells, which are the earliest population committed to the B cell lineage and require stromal contact for growth (Hardy et al, 1991; Li et al, 1996). Fraction A cells differentiate into fraction B ($B220^{+}CD43^{+}CD24^{+}BP\text{-}1^{-}IgM^{-}$) (pro B) cells, where rearrangement of the DJ (diversity and junction) regions of the Ig heavy chain begins. In addition, fraction B cells are less dependent on stromal cell contact for growth, but require IL-7 for growth (Hardy et al, 1991; Li et al, 1996). At the fraction C ($B220^{+}CD43^{+}CD24^{+}BP\text{-}1^{+}IgM^{-}$) (late-pro B cell) stage, most cells have variable (V)DJ Ig heavy chain rearrangements, and a small percentage of DJ rearranged Ig light chain can be detected (Ehlich et al, 1993). Rearranged heavy chain expression leads to surface

expression of the pre-BCR complex, which provides signaling for further differentiation. Within the fraction C population, a subpopulation of cells express higher levels of CD24 and are termed fraction C' (large-pre B) cells. Fraction C cells differentiate into fraction C' cells once receiving a signal through the pre-BCR, allowing the cells to enter cell cycle (Hardy et al, 1991). IL-7 is also required for growth at the fraction C cell stage, but stromal contact is not required (Hardy et al, 1991). B cell development proceeds to fraction D ($B220^{+}CD43^{-}CD24^{+}BP\text{-}1^{+}IgM^{-}$), consisting of small pre-B cells. A large portion of fraction D cells will have VJ rearrangement of the Ig light chain occur at this stage (Hardy et al, 1991). Cells become fraction E once the Ig light chain has fully rearranged VJ regions, and cells begin to express IgM. Fraction E cells are considered immature B cells, which migrate to the spleen to differentiate into mature B cells. Mature B cells capable of recirculation can be found in the bone marrow and are classified as fraction F cells, which express IgD, IgM, and higher levels of B220 compared to fraction E cells.

Figure 1.1. Defining Hardy Fractions of B cell development in the bone marrow.

Hardy fractions of B cell development are shown. Differences in cell surface molecules, immunoglobulin rearrangement, and growth dependence are indicated. Line presence and thickness represents the level of cell surface molecule expression or the dependence of growth conditions for the various stages of development. Figure is adapted from *Hardy et al.* (Hardy et al, 1991).


CLP
Fraction A (pre-pro B cell)
Fraction B (pro B cell)
Fraction C (Late-pro B cell / Large-pre B cell)
Fraction D (Small-pre B cell)
Fraction E (Immature B cell)
Fraction F (Mature B cell)
IL-7R
Pre-BCR
BCR
Cell Surface Molecules
CD45R/B220
CD43 (S7)
CD24/HSA (30F1)
BP-1
IgM
Ig Rearrangement
H-chain
D-J
V-D-J
L-chain
V-J
Growth Dependence
Stromal Contact
IL-7

1.1.2 Immature B cells

Transitional B cells are an important link between the BM and periphery during B cell development. Immature B cells express BCR on the cell surface, and B cells recognizing self-antigens will undergo deletion, receptor editing, or anergy (Chung et al, 2003). Non-self-reactive, immature B cells leave the bone marrow and progress through transient transitional (T1 and T2) B cell stages. Transitional B cells in the spleen are identified by CD93 (AA4.1) expression, a marker of immaturity. In addition to CD93, T1 and T2 B cells are characterized by the surface markers $IgM^{hi}IgD^{-}CD21^{-}CD23^{-}$ and $IgM^{hi}IgD^{+}CD21^{+}CD23^{+}$ respectively (Chung et al, 2003). T1 B cells do not have the ability to recirculate and are found in the bone marrow or spleen. T2 B cells, which develop from T1 B cells, enter the splenic follicles and have the ability to recirculate (Pillai & Cariappa, 2009). The differentiation from T1 to T2 B cells requires basal BCR signals, BAFF survival signals, and non-canonical NF-κB signals (Pillai & Cariappa, 2009). At the T2 B cell stage, cells then develop into mature B cells. The BM of a normal 6-8 week-old mouse will have ~$2x10^7$ immature B cells generated daily, but only ~10% will actually reach the periphery (Rolink et al, 1998).

1.1.3 Mature B cells

Mature B cells can recirculate through secondary lymphoid organs or join enriched static components in the spleen, peritoneal, and pleural cavities (Martin & Kearney, 2000). The spleen is the largest secondary lymphoid organ and contains about one-fourth of the body's lymphocytes (Cesta, 2006). Various B cell subsets can be found in the spleen including B-1, B-2, and B regulatory cells (Table 1.1).

1.1.3.1 B regulatory cells

The smallest populations of B cells in the spleen are B regulatory cells (Bregs), which consists of approximately 1-2% of the total B cells in the spleen, and have a unique phenotype of $CD19^{hi}CD1d^{hi}CD5^{+}$ (Yanaba et al, 2008). Bregs are also known as B10 cells since they produce the highest levels of interleukin-10 (IL-10) of all B cell subsets (Yanaba et al, 2008). IL-10 has anti-inflammatory and suppressive effects on most hematopoietic cells (Bouaziz et al, 2012), thus Bregs are capable of suppressing T

cell-mediated inflammation *in vivo* (Yanaba et al, 2008). Although the current developmental origin of Bregs is unknown, it is hypothesized that they are differentiated from either T2 marginal zone precursor (MZP) B cells or a separate Breg progenitor population (Vitale et al, 2010).

1.1.3.2 B-1 cells

B cells in the pleural and peritoneal cavities of adults are primarily B-1 cells, but they can also be found in the spleen in low frequencies (Baumgarth, 2011). B-1 cells are defined as $CD19^{hi}CD43^{+}CD23^{-}IgM^{hi}IgD^{low}$. They can be further subdivided into B-1a or B-1b cells according to the presence or absence of CD5 respectively (Baumgarth, 2011). B-1 cells generate natural IgM and IgA antibodies, which recognize self-antigens and pathogen-expressed molecules without prior antigen exposure (Racine & Winslow, 2009). Recent studies have also demonstrated that B-1 cells from the liver and peritoneal cavity have phagocytic capabilities (Nakashima et al, 2012; Parra et al, 2012), which suggests a previously unacknowledged mechanism of pathogen clearance by B cells. There has been controversy regarding the development of B-1 cells in relation to B-2 cells. However, there is increasing evidence suggesting that B-1 cells develop from a separate lineage from B-2 cell progenitors (Montecino-Rodriguez & Dorshkind, 2012). B-1 progenitor cells have been reported to arise as early as day 9 of gestation in the yolk sac of mice (Yoshimoto et al, 2011), but then develop from HSCs during later times (Montecino-Rodriguez & Dorshkind, 2012). Fetal liver and neonatal bone marrow HSCs are able of differentiating into B-1 or B-2 cells (Kikuchi & Kondo, 2006), but commitment to either lineage is already determined by the CLP stage of development (Barber et al, 2011).

1.1.3.3 B-2 cells: marginal zone and follicular B cells

The spleen is comprised of primarily B-2 cells called marginal zone (MZ) and follicular (FO) B cells. MZ B cells account for approximately 15% of the total B cells in the spleen, and FO B cells make up more than 70% (Baumgarth, 2011). Both MZ and FO B cells express high levels of B220 and CD19, and no longer express CD93. MZ B cells are characterized by high levels of surface receptors IgM, CD21, and CD1d, and low levels of IgD and CD23 (Pillai et al, 2005). Follicular B cells express lower levels of

IgM, CD21, and CD1d, and high levels of IgD and CD23 (Pillai et al, 2005). Aside from having different surface phenotypes, MZ and FO B cells also have different functions. There are differences in activation between MZ and FO B cells. Activation of B cells occurs either through a T-cell independent (TI) or T-cell dependent (TD) manner. TI responses occur in the absence of T cell help, and can be further subdivided into TI-type I (TI-I) or TI-type 2 (TI-II). MZ B cells reside between the marginal sinus and the red pulp. This is an ideal location to meet and respond rapidly to blood-borne pathogens, and thus MZ B cells play an important role in TI immune responses (Pillai & Cariappa, 2009; Pillai et al, 2005). Examples of TI-I antigens include lipopolysaccharide (LPS), an outer membrane component found in all Gram-negative bacteria, which binds to Toll-like receptor (TLR)4. An example of a TI-II antigen is dinitrophenylated ficoll (DNP-ficoll), which result in B cell activation through BCR engagement and clustering, independent of T cell help (Obukhanych & Nussenzweig, 2006). In contrast, FO B cells have an important role in TD immune responses to protein antigens (Pillai & Cariappa, 2009). TD antigens involve cross-linking BCRs and processing the antigens onto major histocompatibility complex class II (MHCII) molecules, and then are typically followed by CD40 co-stimulation by a T-helper cell. FO B cells are not only found in the spleen, but can also circulate in the blood. FO B cells are therefore better at generating effective antibody responses after interacting with T cells in response to blood-borne pathogens. MZ B cells express elevated T cell co-signaling molecules at basal and stimulated levels compared to FO B cells (Oliver et al, 1999). In addition, MZ B cells are also more readily activated and proliferative than FO B cells after stimulation with LPS, anti-IgM, or anti-CD40, and can become potent antigen presenting cells for T cells (Oliver et al, 1997; Oliver et al, 1999; Snapper et al, 1993; Snapper et al, 1996b).

A small fraction of MZ or FO B cells which are activated can become plasma cells or memory B cells. Plasma cells are terminally differentiated B cells committed to antibody secretion. Plasma cells constitutively secrete antibody against an antigen to provide protection during a primary response, but are unable to respond to secondary infections because of diminished surface Ig expression (Manz et al, 1998). In contrast, memory B cells express BCRs for an eliciting antigen and can rapidly secrete high amounts of antibody upon secondary exposure. Both memory B cells and plasma cells are

Table 1.1. Phenotype, frequency, and function of B cell subsets located in mouse spleens

	Breg	B-1(a/b)	MZ	FO
Phenotype	$B220^{+}$ $CD19^{hi}$ $CD5^{+}$ $CD1d^{hi}$	$B220^{+}$ $CD19^{+}$ $CD5^{(+/-)}$ $CD1d^{hi}$ $CD23^{-}$ $CD43^{+}$ IgM^{hi} IgD^{low}	$B220^{+}$ $CD19^{+}$ $CD5^{-}$ $CD1d^{hi}$ $CD21^{hi}$ $CD23^{low}$ $CD43^{-}$ IgM^{hi} IgD^{low}	$B220^{+}$ $CD19^{+}$ $CD5^{-}$ $CD1d^{low}$ $CD21^{low}$ $CD23^{hi}$ $CD43^{-}$ IgM^{low} IgD^{hi}
Frequency in spleen	~1%	~2%	~15%	>70%
Major Function in spleen	Anti-inflammatory roles; production of IL-10	Production of natural antibodies	Rapid response to TI antigens	Humoral response to TD antigens

important components for long-lasting antibody-mediated immunity as seen in vaccinations.

1.2 B cell related diseases

A fine balance between activation and inhibition of immune function is required. Loss of B cell function can make an individual more susceptible to infection, whereas gain of function during B cell development or activation can lead to autoimmunity. Furthermore, there are instances where either a gain of function or loss of function in B cells can result in a cancerous state.

1.2.1 B cell immunodeficiencies

Diseases related to loss of B cell function are classified into three major categories of B cell immunodeficiencies: 1) defects in early B cell development, 2) CSR defects, and 3) common variable immunodeficiency (CVID) (Conley et al, 2009). In all cases of B cell immunodeficiencies, there is a reduction in antibody production, which results in increased susceptibility to recurrent infections (Conley et al, 2009). Defects in early B cell development result in recurrent infections, hypogammaglobulinemia, reduced B cells in the periphery, and a block in early B cell differentiation (Conley et al, 2009). The most prevalent form of defects in early B cell development is X-linked agammagobulinemia (XLA), which accounts for approximately 85% of cases (Conley et al, 1998). XLA is caused primarily by mutations in the gene *Btk*, and is characterized by a marked decrease in mature B cells and lack of antibodies of all isotypes (Vetrie et al, 1993). A smaller proportion of XLA cases have been reported to be caused by mutations in genes encoding pre-BCR or BCR signaling components, such as Igα, Igβ, λ5, μ heavy chain, and BLNK (Conley et al, 2009; Minegishi et al, 1999a; Minegishi et al, 1998; Minegishi et al, 1999b; Yel et al, 1996).

Defects in CSR result in high levels of serum IgM, but little to no production of IgG, IgA, and IgE antibodies (Conley et al, 2009). CSR defects are primarily caused by mutations in Cd40lg (Allen et al, 1993). The loss of CD40-CD40L interaction results in

impaired T cell, B cell, and monocyte function, which can lead to opportunistic infections (Fuleihan, 1998). Mutations in Aicda, which encodes for activation-induced cytidine deaminase (AID), have also been attributed with defects in CSR (Revy et al, 2000). AID initiates CSR and somatic hypermutation during Ig gene rearrangement by deaminating cytosine residues in Ig heavy chain variable regions and switch regions (Muramatsu et al, 2000; Perlot et al, 2008).

CVID is characterized by reduced serum immunoglobulins and highly variable clinical features (Cunningham-Rundles & Bodian, 1999). In contrast to other B cell immunodeficiencies, CVID patients can have normal B cells, but low numbers of memory B cells (Wehr et al, 2008). The predisposing genetic factors associated with CVID are unknown, but mutations in the genes encoding ICOS, CD19, and TACI have been identified to small groups of patients (Grimbacher et al, 2003; van Zelm et al, 2006).

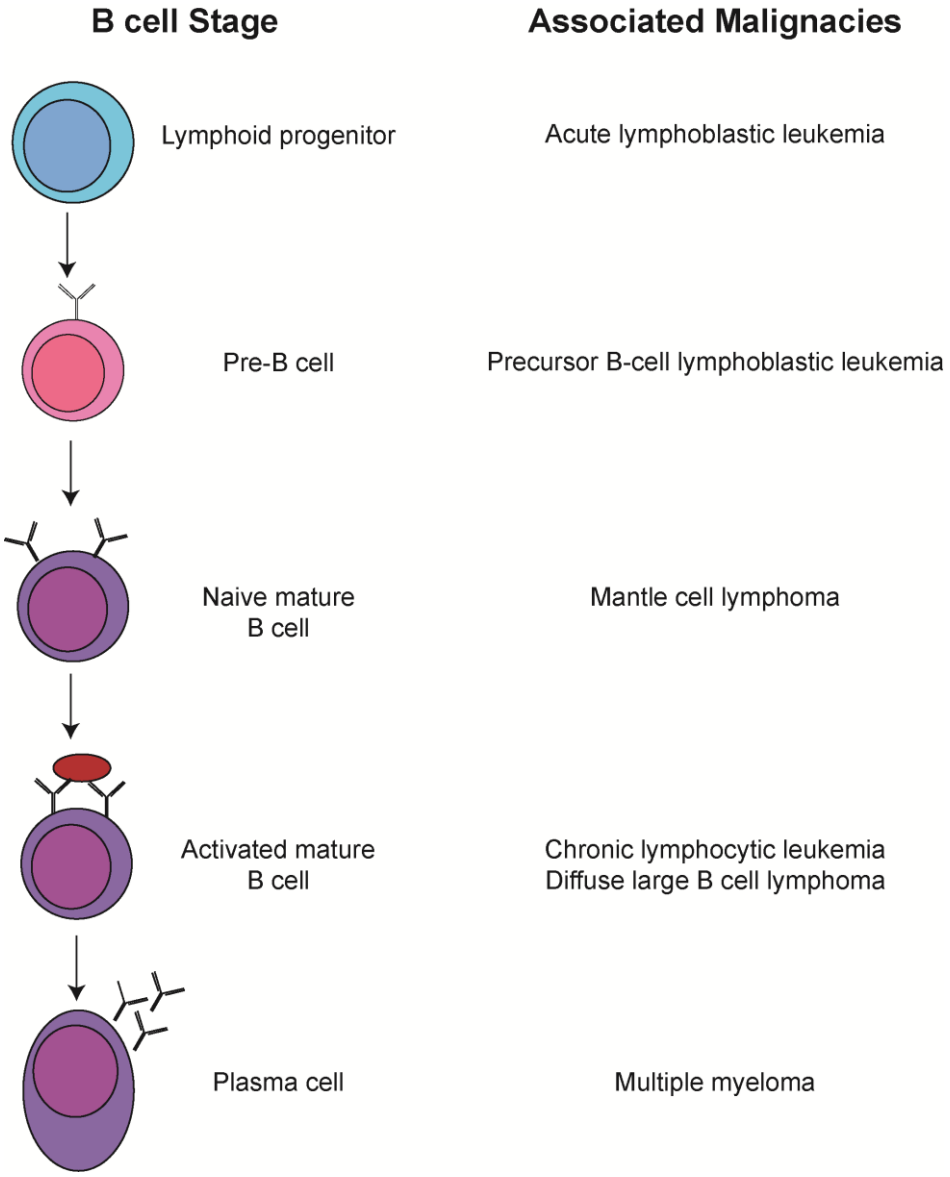
1.2.2 Autoimmunity

Several checkpoints occur during B cell development to ensure that B cells do not recognize host or self-antigens, collectively called central B cell tolerance. These mechanisms include BCR editing (Gay et al, 1993; Tiegs et al, 1993), deletion of self-reactive B cells (Nemazee & Buerki, 1989), and anergy (Nossal & Pike, 1980). Failure to regulate self-tolerance in B cells is associated with autoimmunity. Autoimmunity is characterized by immune activation in response to self-antigens leading to tissue destruction and organ failure (Alberghini et al, 2015). Some commonly studied autoimmune diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis, multiple sclerosis, and diabetes (Morel, 2004). B cells can contribute to autoimmune diseases through secretion of autoantibodies, presentation of autoantigens to T cells, and secretion of proinflammatory cytokines (Chan & Shlomchik, 1998; Harris et al, 2000; Wong et al, 2004). Therefore, understanding mechanisms of B cells development and function can provide therapeutic targets for preventing B cells from becoming auto-reactive.

1.2.3 B cell leukemia and lymphoma

If control of activation, proliferation or apoptosis is deregulated in B cells, various forms of cancers can arise depending on the initiating cell stage (Figure 1.2). Both leukemias and lymphomas have subtypes involving B cells. Leukemia is a cancer of bone marrow origin which results in abnormal growth of leukocytes. Lymphomas are characterized by uncontrolled lymphocyte growth which affects lymph nodes. Mutations in components of BCR signaling have been attributed to both of these diseases (Buchner et al, 2015; Burger & Chiorazzi, 2013; Davis et al, 2010). Chronic activation of BCR signaling can lead to proliferation and the activation of pro-survival genes, preventing malignant cells from undergoing apoptosis (Davis et al, 2001; Davis et al, 2010; Packham et al, 2014). As such, therapeutic strategies for treating leukemia and lymphomas have involved using drugs which inhibit BCR signaling kinases, such as Btk and Syk (Davis et al, 2010; Woyach et al, 2012). Understanding the regulation of B cell development and BCR signaling has provided key insights into how to treat some of these diseases.

Figure 1.2. Examples of B cell associated malignancies which can arise from different B cell subsets.


B cell Stage
Associated Malignacies
Lymphoid progenitor
Acute lymphoblastic leukemia
Pre-B cell
Precursor B-cell lymphoblastic leukemia
Naive mature
B cell
Mantle cell lymphoma
Activated mature
B cell
Chronic lymphocytic leukemia
Diffuse large B cell lymphoma
Plasma cell
Multiple myeloma

1.3 Toll-Like Receptors

Toll-Like receptors (TLR) are evolutionarily conserved, single-spanning transmembrane receptors expressed both intracellularly and on the surfaces of many cell types. TLRs belong to the Interleukin-1 receptor (IL-1R) family, as the receptors contain a C terminal intracellular Toll/IL-1R (TIR) signaling domain (West et al, 2006). TLRs function as hetero- or homo-dimers, and recognize conserved structures of viruses, bacteria, protozoa, and fungi, collectively called pathogen associated molecular patterns (PAMPs) (West et al, 2006). Recognition of ligands by TLRs is dependent on the N-terminal leucine-rich-repeat (LRR) motif, which contains 24 amino acids forming an α-helix and β-sheet joined by a loop (Kawai & Akira, 2007). Human TLRs contain 19-25 LRRs which together form a horseshoe-shaped structure (Bell et al, 2005). TLRs were initially identified in *Drosophila melanogaster* through a cDNA library screen for genes that regulated embryonic polarity during development (Hashimoto et al, 1988). TLR homologues in vertebrates, specifically TLR4, were discovered in mice and humans for being important during LPS-mediated inflammatory responses (Medzhitov et al, 1997; Poltorak et al, 1998). To date, thirteen members of TLRs have been identified in mice, and eleven in humans (Kawai & Akira, 2007). Each TLR recognizes one or more distinct categories of PAMPs.

LPS is the most widely studied ligand for TLR signaling induction. The mechanism for LPS recognition through TLRs is complex. The serum protein LPS-binding protein (LBP) first binds to LPS and transfers it to the molecule CD14. Delivery of LPS by CD14 is then transferred to TLR4, which forms a complex with MD-2 (Botos et al, 2011) . MD-2 is a coreceptor which binds to TLR4 and LPS (Visintin et al, 2006), and is essential for LPS recognition (Nagai et al, 2002a). Furthermore, immune cells including B cells, DCs, and macrophages express radioprotective 105 (RP105), an additional surface molecule which is also capable of LPS recognition (Fugier-Vivier et al, 1997; Miyake et al, 1995). RP105 interacts with the coreceptor MD-1 for LPS recognition and signaling transduction (Nagai et al, 2002b). Interestingly, the functional roles of RP105 differ between B cells, DCs, and macrophages. RP105/MD1 enhances LPS signaling through TLR4 in B cells (Ogata et al, 2000), but it functions as a negative

regulator in macrophages and DCs by directly inhibiting LPS binding to TLR4 (Divanovic et al, 2005).

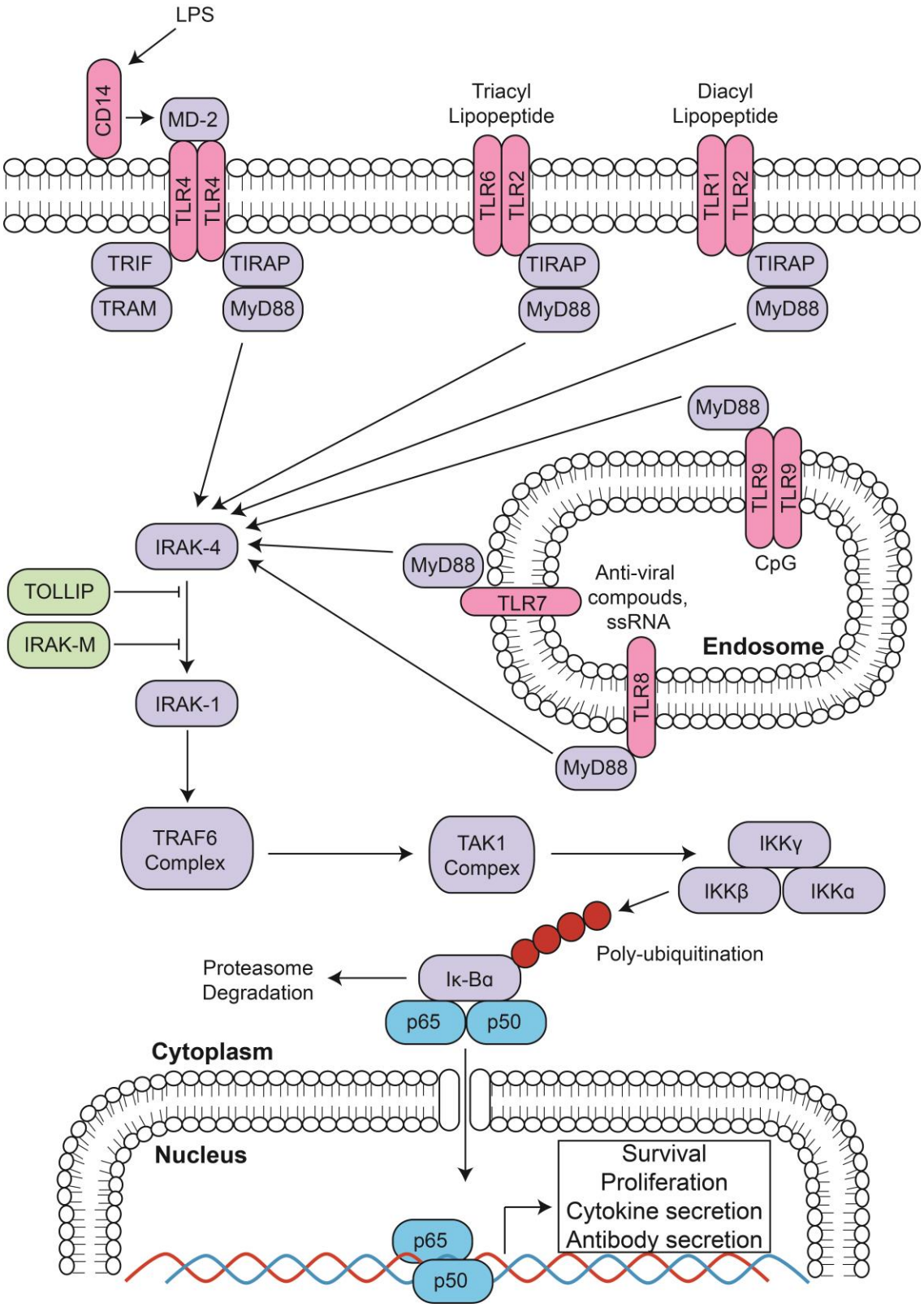
B cells express and respond to ligands of TLR1, TLR2, TLR4, TLR6, TLR7/8, and TLR9 (Dasari et al, 2005; Genestier et al, 2007; Gururajan et al, 2007; Hornung et al, 2002). B cells up-regulate cytokine synthesis, antibody secretion, and cell proliferation upon binding of TLR ligands onto their receptors, as a result of activation of the transcription factor nuclear factor (NF)-κB (Bekeredjian-Ding & Jego, 2009). The signaling events following TLR ligand binding have been well characterized (Figure 1.3). Activation of TLRs elicits a signaling response through a MyD88-dependent or TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway (Kawai & Akira, 2006). Except for TLR3, all TLRs can recruit the MyD88 adaptor protein for TLR signaling (Kawai & Akira, 2006; Kawai & Akira, 2007). On the other hand, TLR3 and TLR4 are capable of activating NF-κB through the recruitment of the adapter protein TRIF (Kawai & Akira, 2006). For the MyD88-dependent pathway, the association of TLRs and MyD88 stimulates and recruits members of the interleukin-1 receptor-associated kinase (IRAK) family, including IRAK-1, IRAK-2, IRAK-4, and IRAK-M (Kawai & Akira, 2006; Kawai & Akira, 2007). Once IRAK-4 and IRAK-1 are phosphorylated, they dissociate from MyD88 and activate tumor necrosis factor receptor-associated factor 6 (TRAF-6) (Kawai & Akira, 2006; Kawai & Akira, 2007). A complex of proteins is formed with TRAF-6 which in turn activates transforming growth factor-β-activated protein kinase 1 (TAK1). The TAK1 complex activates the IκB kinase (IKK) complex, consisting of IKKα, IKKβ, and IKKγ (Kawai & Akira, 2006; Kawai & Akira, 2007). The IKK complex phosphorylates IκBs for polyubiquitination and proteasomal degradation, which results in NF-κB subunits to be released into the nucleus and bind to DNA targets. Additionally, TLR ligands such as LPS are capable of activating phosphoinositide 3-kinase (PI3K) within minutes of B cell stimulation (Bone & Williams, 2001). TLR signaling can activate the PI3K pathway through the B cell adaptor for PI3K (BCAP). Phosphorylated BCAP accumulates at the cell membrane after LPS stimulation, and allows its associated PI3K to signal downstream (Ni et al, 2012). It has been demonstrated that sustained PI3K signaling and antigen receptor engagement by either LPS or BCR crosslinking is necessary for proliferation and survival of activated B

cells (Donahue & Fruman, 2003). Furthermore, it has been suggested that promoting activation of full TLR signaling requires MHCII interaction with Bruton's tyrosine kinase (Btk) and CD40 (Liu et al, 2011); therefore, TLR signaling is complex and its components may have redundant functions.

Understanding TLR activation in B cells has implications for human health and disease. TLR signaling in B cells is required for T-dependent antibody responses (Pasare & Medzhitov, 2005). It has been suggested that the production of effective neutralizing antibody responses, as seen in vaccinations, involves direct TLR ligand binding on B cells (Kasturi et al, 2011). Moreover, TLRs may have roles in B cell malignancies. One hypothesis is that chronic infections leading to TLR activation can induce excessive somatic hypermutation and CSR events, which can transform normal B cells into malignancies such as B cell lymphoma (Isaza-Correa et al, 2014). TLR stimulation can also lead to the proliferation of malignant B cells, and induce a cytokine profile which creates an immunosuppressive microenvironment (Isaza-Correa et al, 2014). Although much is known about TLRs and B cells, transcriptional regulation of genes involved for TLR expression on B cells remains unclear.

Figure 1.3. MyD88-dependent TLR signaling pathway in B cells.

A simplified schematic of the TLR signaling pathway is shown. TLR1/2, TLR 2/6, and TLR 4 are located at the cell surface, whereas TLR7, TLR8, and TLR9 are located in endosomes. Ligand binding results in a cascade of signaling events which eventually leads to polyubuitination of Iκ-Bα, which allows NF-κB (p65/p50 heterodimer) to translocate into the nucleus and upregulate genes involved in survival, proliferation, and cytokine and antibody secretion.

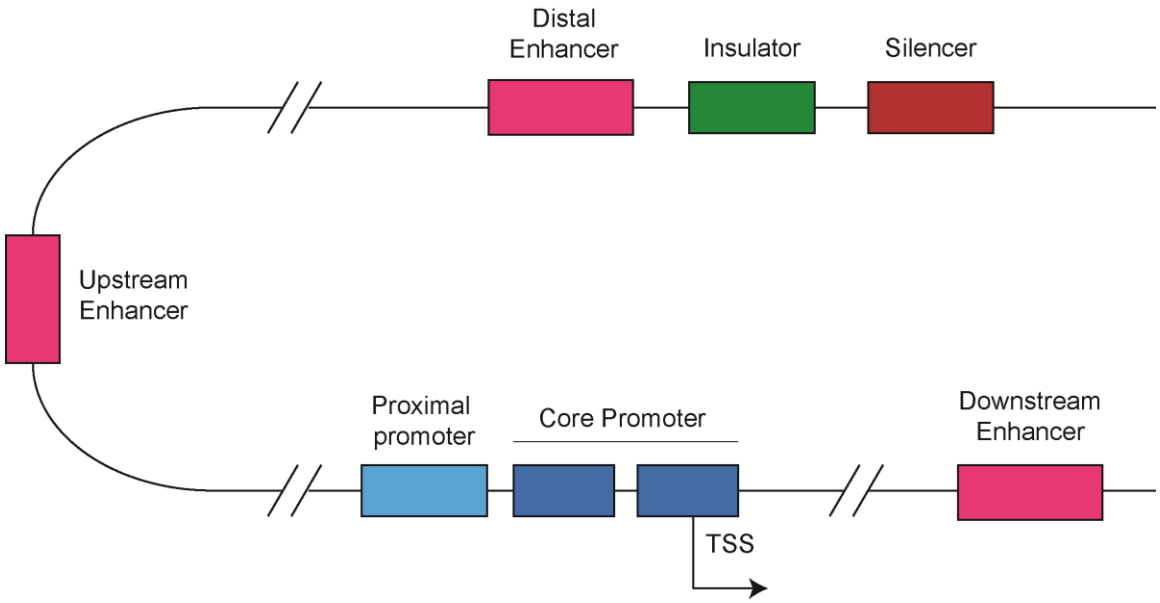

LPS
CD14
MD-2
TLR4
TLR4
Triacyl
Lipopeptide
TLR6
TLR2
Diacyl
Lipopeptide
TLR1
TLR2
TRIF
TRAM
TIRAP
MyD88
TIRAP
MyD88
TIRAP
MyD88
MyD88
TLR9
TLR9
CpG
IRAK-4
MyD88
TLR7
Anti-viral
compouds,
ssRNA
Endosome
TOLLIP
IRAK-M
IRAK-1
TLR8
MyD88
TRAF6
Complex
TAK1
Compex
IKKγ
IKKβ
IKKα
Poly-ubiquitination
Proteasome
Degradation
Ik-Bα
p65
p50
Cytoplasm
Nucleus
Survival
Proliferation
Cytokine secretion
Antibody secretion
p65
p50

1.4 Transcriptional Regulation

Cell fates and cellular function are regulated through complex and precise gene expression patterns (Spitz & Furlong, 2012). Regulation of gene expression is controlled by transcription factors, which are proteins that bind to cis-acting regulatory DNA sequences in order to recruit coactivators and RNA polymerase II (RNA Pol II) to target genes. RNA pol II is responsible for the transcription of DNA to mRNA in eukaryotes. Transcription factors are essential for the activation or repression of gene expression. Cis-regulatory DNA sequences include core promoters and promoter-proximal elements located near the transcription start site (TSS), and distal enhancers, silencers, and insulators (Figure 1.4) (Minegishi et al, 1999b; Spitz & Furlong, 2012). These cis-regulatory elements contain DNA sequences which transcription factors recognize and bind. Transcription factors can bind to promoters within a thousand base pairs of the TSS, or further away at distal enhancer, silencer, or insulator elements up to 1 million base pairs from the promoter (Maston et al, 2006). Furthermore, each regulatory element can contain multiple transcription factor binding sites, which drastically increases the ability to generate unique expression patterns. Therefore, understanding how gene expression profiles are regulated by regulatory sequences and transcription factors can give insight to development and function of cells and tissues.

Figure 1.4. Examples of cis-regulatory elements found in transcriptional regulation.

A simplified schematic of the transcriptional regulatory elements found in eukaryotes. Transcription factors can bind to proximal and core promoters, and distal enhancers, silencers, and insulators to regulate gene transcription. Figure is adapted from *Ong and Corces* (Ong & Corces, 2011).


Distal
Enhancer
Insulator
Silencer
Upstream
Enhancer
Proximal
promoter
Core Promoter
Downstream
Enhancer
TSS

1.5 Transcription factor NF-κB

The nuclear factor-κB (NF-κB) family of transcription factors was initially discovered in B cells by its ability to bind an enhancer element within the immunoglobulin-κ chain (Sen & Baltimore, 1986b). The expression of NF-κB was later determined to be inducible, and expressed in other lymphoid and non-lymphoid cell types (Sen & Baltimore, 1986a). NF-κB is present in the cytoplasm of most cells in an inactive complex with IκB inhibitor proteins (Baeuerle & Baltimore, 1988), which are identified by repeat 33 amino acid long ankyrin motifs (Li & Verma, 2002). Activation of NF-κB allows for its translocation into the nucleus, where it can bind to promoters or enhancers of genes containing the consensus sequence GGGRNYYYCC (R is any purine; Y is any pyrimidine; N is any nucleotide) to initiate transcription (Chen et al, 1998). The NF-κB family is composed of five subunits: NF-κB1 (p105/p50) , NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel, which all interact with DNA as hetero- or homodimers (Hayden & Ghosh, 2008). Both p100 and p105 are precursor proteins for p50 and p52 respectively, and contain ankyrin repeats which can function as IκB-like proteins (Li & Verma, 2002). Only RelA, RelB, and c-Rel contain C-terminal transactivation domains which allow transcriptional initiation (Hayden & Ghosh, 2012). All NF-κB family members share a Rel homology domain which mediates dimerization, interaction with its inhibitor, and DNA binding (Hayden & Ghosh, 2012). Since the initial discovery of NF-κB, its involvement during development and inflammation induction, response, and resolution have been researched in depth.

1.5.1 Classical and alternative NF-κB signaling

NF-κB signaling is generally defined as either the classical (canonical) or alternative (non-canonical) pathway. In the classical NF-κB pathway, NF-κB can be activated in B cells through various mechanisms such as through TLR, BCR, or proinflammatory cytokine stimulation (Li & Verma, 2002; Weil & Israel, 2004). A signaling cascade leads to phosphorylation of IκB by the IKK complex, which is recognized by ubiquitin ligase machinery, polyubiquitinated, and targeted for proteasomal degradation like previously described in section 1.3 *Toll-Like Receptor*. In

the alternative pathway, NF-κB is activated through TNF receptor (TNFR) family members such as B cell-activating factor (BAFF) receptor (Claudio et al, 2002) and CD40 (Homig-Holzel et al, 2008). Activation through TNFR family members leads to the activation of NF-κB-inducing kinase (NIK), which leads to the phosphorylation, ubiquitination, and processing of p100 to activate RelB/p52 NF-κB complexes (Sun, 2011). The heterodimerization of NF-κB subunits activated predominantly by each pathway allows crosstalk between classical and alternative NF-κB signaling.

1.5.2 Mouse models of NF-κB and the roles of NF-κB in B cells

Various NF-κB/Rel complexes are expressed during different developmental stages of B cells. In pre-B cells, p50/p65 is the primary inducible complex, p50/Rel is the predominant complex in mature B cells, and p52/RelB appears to be the dominant complex in plasma cells (Liou et al, 1994). Mice deficient for various NF-κB family members have been generated (Table 1.2), and have been used to elucidate the developmental and functional roles of each family member in B cells. With the exception of *Rela*, germline deletion of a single NF-κB family member does not result in embryonic lethality. Early death in germline *Rela* null embyros (~E15) is caused by excessive apoptosis of hepatocytes (Beg et al, 1995; Doi et al, 1999). Adoptive transfer of *Rela*$^{-/-}$ fetal liver cells into lethally irradiated *Rag1*$^{-/-}$ mice can lead to the generation of lymphocytes, but B cell development is significantly impaired as a result of sensitivity to TNF-α induced cell death (Prendes et al, 2003). *Relb* null mice have multiple immunological abnormalities, including impaired dendritic cell development, an expansion of myeloid cells in the spleen and bone marrow, and multi-organ inflammation caused by T cell infiltration (Weih et al, 1995; Weih et al, 1996). *Relb*$^{-/-}$ mice have normal Ig secretion abilities, but have impaired proliferation upon stimulation through TLR4, BCR, or CD40 (Snapper et al, 1996a). *Rel* deletion results in proliferative defects in response to LPS, anti-IgM or CD40L stimulation; in addition, there is an impairment in both basal and secondary antibody production (Kontgen et al, 1995). In *Nfkb1*$^{-/-}$ mice, there is impaired LPS mediated B cell proliferation and isotype switching (Sha et al, 1995). *Nfkb1*, *Rela*, *Relb* and *Rel*, are involved with MZ B cell, but not FO B cell development (Cariappa et al, 2000; Weih et al, 2001). Lastly, *Nkfb2* null mice have the most profound B cell phenotype. *Nfkb2*$^{-/-}$ mice have reduced MZ, FO, T1, and T2 B cells

in the spleen (Guo et al, 2007). Furthermore, there is a reduction of B cells in the bone marrow and lymph nodes, and *Nfkb2*$^{-/-}$ mice have impaired germinal center formation and T-dependent antibody responses (Caamano et al, 1998; Franzoso et al, 1998).

It is likely that there is functional compensation between NF-κB family members, since germline deletion of multiple transcription factors results in a more severe phenotype than deleting a single factor. For example, proliferation in response to LPS or anti-IgM, antibody production, and germinal center formation is further impaired in *Nfkb1*$^{-/-}$*Rel*$^{-/-}$mice, and B cells are more apoptotic when stimulated compared to either *Nfkb1*$^{-/-}$ or *Rel*$^{-/-}$ mice (Pohl et al, 2002). Moreover, B cells fail to progress past the T1 stage in *Nfkb1*$^{-/-}$*Nfkb2*$^{-/-}$ mice (Claudio et al, 2002), whereas differentiation past the T1 stage is possible in *Nfkb1*$^{-/-}$ or *Nfkb2*$^{-/-}$ mice. Overall, activation of NF-κB is an important component of B cell development and function.

Table 1.2. Phenotype of NF-κB family member null mutations in mice

Mutated gene	Phenotype in knockout mice	References
Rela	- Embryonic lethal (~E15)	(Beg et al, 1995)
	- TNF-α induced apoptosis of hepatocytes	
	- Required for MZ B cell development	(Cariappa et al, 2000)
Relb	- Impaired dendritic cell development	(Weih et al, 1995; Weih et al, 1996)
	- Expansion of myeloid cells in the spleen and bone marrow	
	- Multi-organ inflammation caused by T cell infiltration	
	- Impaired B cell proliferation upon stimulation through TLR4, BCR, or CD40	(Snapper et al, 1996a)
Rel	- B cell proliferative defects following LPS, anti-IgM or CD40L stimulation	(Kontgen et al, 1995)
	- Impaired antibody production at the basal level, and upon secondary immune response	
	- Required for MZ B cell development	(Cariappa et al, 2000)
Nfkb1	- Impaired LPS mediated B cell proliferation and isotype switching	(Sha et al, 1995)
	- Required for MZ B cell development	(Cariappa et al, 2000)
Nfkb2	- Reduced MZ, FO, T1, T2 B cell numbers in the spleen	(Guo et al, 2007)
	- Impaired germinal center formation	(Caamano et al, 1998; Franzoso et al, 1998)
	- Impaired T-dependent antibody responses	

1.6 E26 Transformation Specific (ETS) Transcription Factors

The ETS name is derived from the E26 avian erythroblastosis virus which carried the *v-ets* oncogene (Sharrocks, 2001). Proteins in the ETS family share an evolutionarily conserved DNA-binding domain, which forms a winged loop-helix-loop structure to bind purine rich consensus motifs containing the core sequence GGAA/T (Ciau-Uitz et al, 2013; Sharrocks, 2001). Members of the ETS transcription factor family are involved with the regulation of a variety of genes involved with growth, activation, and development in adults and embryos of many organisms (Sharrocks, 2001; Wasylyk et al, 1993). Adding to the complexity of transcriptional regulation, ETS proteins can function in collaboration with other transcription factors in the activation or repression of target genes (Sharrocks, 2001). This thesis focuses on the ETS transcription factors PU.1, Spi-B, and Spi-C, which belong to the Spi subfamily, and how each is involved with transcriptionally regulating genes involved with B cell development and function. Figure 1.5 demonstrates the structural similarities between PU.1, Spi-B, and Spi-C. All three transcription factors contain an N-terminal activation domain rich in acidic amino acids (ACID), and have similar amino acid homology within the DNA binding domain (DBD). PU.1 contains a Gln-rich (Q) region believed to be necessary for transactivation. The proline, glutamic acid, serine, and threonine (PEST) sequence contained within PU.1 and Spi-B are known to interact with interferon regulatory factor (IRF) family transcription factors, and is suggested to be involved with protein degradation (Brass et al, 1999; Rogers et al, 1986). Summarized in *Table 1.3* are the phenotypes of mice containing germline deletions of PU.1, Spi-B, or Spi-C.

1.6.1 *PU.1*

PU.1 was identified in 1988 as an oncogene from the proviral insertion of the Friend spleen focus forming virus into the *Spi-1* locus, which induced murine erythroleukemia (Moreau-Gachelin et al, 1988). In mice, PU.1 is encoded by the *Spi1* gene and is located on chromosome 2, whereas in humans, PU.1 is encoded by *SPI-1* and is located on chromosome 11. PU.1 is expressed in most hematopoietic cells, including macrophages, granulocytes, dendritic cells (DCs), B cells, and T cells (Carotta et al, 2010). With over 110 direct target genes identified to date (Turkistany & DeKoter, 2011),

PU.1 has been the most well-studied ETS transcription factor. The genes regulated by PU.1 include antibodies and their receptors, and cytokines and their receptors involving inflammation, leukocyte growth, and development (Turkistany & DeKoter, 2011). Of all the ETS transcription factors, *Spi1* defects have the most profound effects on normal hematopoiesis (Bartel et al, 2000). Targeted disruption of *Spi1* was first generated by *Scott et al.* in 1994 by deleting the PU.1 DNA binding region in exon 5 of the *Spi1* gene (Scott et al, 1994). Germline knock-out mice for the *Spi1* gene ($PU.1^{-/-}$) are unable to produce any lymphoid and myeloid cells during fetal hematopoiesis, and are also late-stage embryonic lethal (Scott et al, 1994). However, erythroid progenitors and megakaryocytes are still generated in $PU.1^{-/-}$ fetuses (Scott et al, 1994). Colony forming assays using fetal liver cells from embryonic day 14.5 (E14.5) $PU.1^{-/-}$ mice failed to generate macrophage, granulocyte, or B cell colonies *in vitro* (Scott et al, 1997). Furthermore, transplantation of fetal liver cells from $PU.1^{-/-}$ mice into lethally irradiated C57BL/6 mice failed to reconstitute cells from the lymphoid and myeloid lineages, further demonstrating the requirement of PU1 in these cell types (Scott et al, 1997). In 1996, *McKercher et al* generated a germline *Spi1* null mice using a targeted insertional disruption of the PU.1 DNA binding domain (McKercher et al, 1996). One major difference in these *Spi1* null mice was that they were viable at birth, but died within two days as a result of septicemia (McKercher et al, 1996). Mice could survive for up to 17 days if maintained on antibiotics, and T cell generation was observed 3-5 days after birth, suggesting a delay in T cell development (McKercher et al, 1996). Differences observed between *Spi1* null mice between *Scott et al.* and *McKercher et al.* are likely due to differences in their deletion strategy of the *Spi1* gene. Nonetheless, similar to the findings by *Scott et al., Spi1* null neonates generated by *McKercher et al.* produced no mature macrophages, neutrophils, B cells, and T cells (McKercher et al, 1996). Therefore, PU.1 is critical for the development and differentiation of cells of both the innate and adaptive immune system.

The expression level of PU.1 has effects on hematopoietic cell fate decisions. High concentrations of PU.1 promote macrophage differentiation, whereas low levels of PU.1 favor B cell differentiation (DeKoter & Singh, 2000). High expression of PU.1 occurs in HSCs and CLPs (Back et al, 2005; Nutt et al, 2005), but PU.1 expression is

Table 1.3. Phenotypes of mice containing germline deletions for ETS transcription factors

Mutated gene	Phenotype in knockout mice	References
Spi1 (*Scott et al. 1994*)	- Embryonic lethal (E17.5-18) - No commitment to B cells, monocytes, dendritic cells, neutrophils	(Scott et al, 1997; Scott et al, 1994)
Spi1 (*McKercher et al. 1996)*	- Post-natal viable for 48 h; viable up to 17 days if maintained on antibiotics - Delayed T cell development (when treated with antibiotics) - No mature macrophages, neutrophils, B cells, and T cells in neonatal mice	(McKercher et al, 1996)
Spib	- Viable, fertile - Fewer B cells; impaired BCR signaling, proliferative defect upon BCR stimulation, impaired secondary antibody responses	(Su et al, 1997; Su et al, 1996)
	- Defect in generation of intestinal microfold (M) cells; impaired uptake of pathogens	(Kanaya et al, 2012; Sato et al, 2013)
	- Impaired plasmacytoid dendritic cell development; impaired IFN production through TLR7 and TLR9 stimulation	(Sasaki et al, 2012)
Spic	- Viable - Defect in development of red pulp macrophages	(Kohyama et al, 2009)

reduced during commitment to the B cell lineage (Nutt et al, 2005). Although B cells express decreased levels of PU.1 upon differentiation, a certain threshold of expression is still required for proper B cell development. Mice generated with a hypomorphic allele of *Spi1* (*Spi1*$^{BN/BN}$), expressing a 5-fold reduction of PU.1 compared to WT in pro-B cells, were unable to generate B cells in the spleen or fetal liver (Houston et al, 2007). Furthermore, *Spi1*$^{BN/BN}$ fetal liver progenitor cells transplanted into sub-lethally irradiated *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice failed to generate B cells in the spleen and bone marrow of recipient mice (Houston et al, 2007). Conditional knockout mouse models for PU.1 were generated to further investigate the role of PU.1 on B cell development and function (Polli et al, 2005). PU.1 is essential specifically during the early stages of B cell development, since deletion of *Spi1* in committed B cell progenitors has no effect on B cell maturation, and has minimal effects on T-dependent responses (Polli et al, 2005). Overall, a sufficiently high level of PU.1 is required during HSC to pro-B cell differentiation, but PU.1 is not essential following B cell commitment.

1.6.2 Spi-B

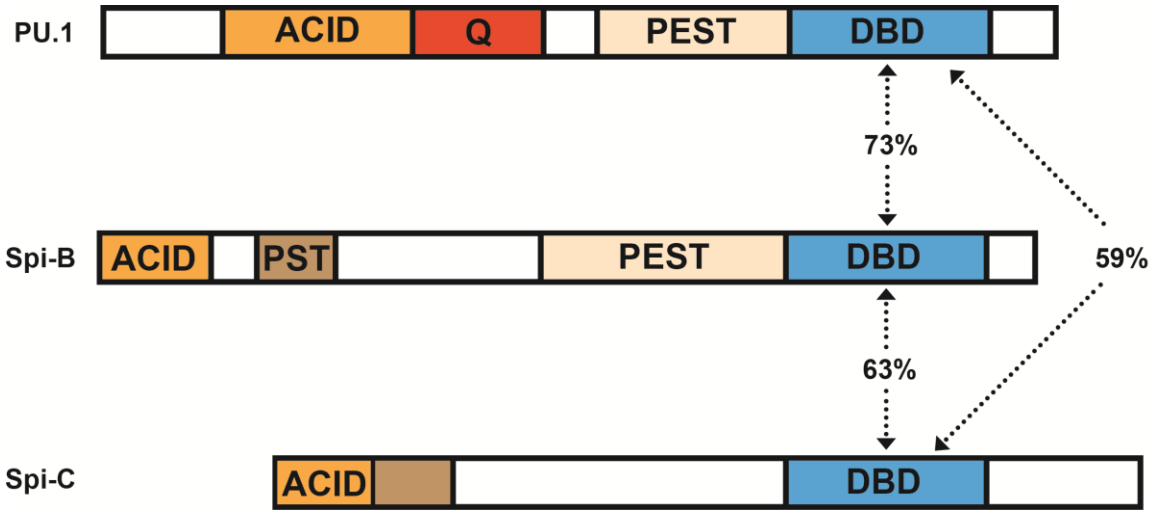
The second Spi subfamily member is Spi-B, which was identified by probing a cDNA library generated from Raji Burkitts lymphoma cells with the PU.1 ETS DNA-binding domain (Ray et al, 1992). Spi-B has been mapped to chromosome 19 in humans and to chromosome 7 in mice (Peters et al, 1997; Ray et al, 1992). Spi-B and PU.1 are highly conserved in humans, sharing 43% amino acid similarity with overall, and 67% similarity in its ETS domain (Ray et al, 1992) (Figure 1.5). Expression of Spi-B was initially believed to be exclusive to the B cells and T cells (Su et al, 1996), however, more recent studies have demonstrated a role for Spi-B in plasmacytoid dendritic cells (pDCs) (Sasaki et al, 2012) and intestinal microfold cells (Kanaya et al, 2012; Sato et al, 2013). Germline Spi-B knockout mice (*Spib*$^{-/-}$) are viable and possess mature B and T cells, potentially due to its degree of redundancy with PU.1. Although the functionality of *Spib*$^{-/-}$ T cells appears to be normal, *Spib*$^{-/-}$ B cells have defects in functional and humoral responses (Su et al, 1997). *Spib*$^{-/-}$ B cells which are stimulated *in vitro* with anti-IgM demonstrate poor proliferation with increased rates of apoptosis. Furthermore, *Spib*$^{-/-}$ mice immunized with dinitrophenol conjugated to keyhole limpet hemocyanin (DNP-

KLH) produce low levels of IgG1, IgG2a and IgG2b, and are unable to sustain germinal centers (Su et al, 1997). Therefore, Spi-B is required for BCR-mediated responses *in vitro* and *in vivo.*

Since Spi-B recognizes the same binding sites as PU.1 (Ray et al, 1992) and can activate the same reporter gene constructs *in vitro* (Muller et al, 1996; Ray et al, 1992; Su et al, 1996), it strengthened the hypothesis that both Spi-B and PU.1 have partial functional redundancy. To determine the function of both PU.1 and Spi-B in B cells, mice heterozygous for *Spi1* were crossed to $Spib^{-/-}$ to generate $Spi1^{+/-}Spib^{-/-}$ ($PU.1^{+/-}Spib^{-/-}$; *PUB*) mice (Garrett-Sinha et al, 1999), since complete PU.1 deletion resulted in embryonic lethality (Scott et al, 1994). Many of the deficiencies observed in the $Spib^{-/-}$ phenotype were further impaired by the additional reduction of PU.1. *PUB* mice had fewer B cells than $Spib^{-/-}$ mice, increased basal levels of apoptotic B cells, further impaired anti-IgM and LPS-mediated B cell proliferation, and reduced BCR signaling (Garrett-Sinha et al, 1999). Therefore, both PU.1 and Spi-B are required for normal BCR signal transduction, and function in a semi-redundant or complementary manner.

Figure 1.5. Major domains of the SPI-group ETS transcription factors.

The percentages indicate the amino acid sequence homology of the ETS DNA binding domain (DBD) between the three transcription factors. The acidic transactivation domains are denoted ACID. PU.1 and Spi-B has a glutamine (Q) rich and a proline/serine/threonine (PST) rich domain following the ACID domain respectively. PU.1 and Spi-B also have a proline/glutamic acid/serine/threonine (PEST) domain prior to the DBD.


PU.1
ACID
Q
PEST
DBD
73%
Spi-B
ACID
PST
PEST
DBD
59%
63%
Spi-C
ACID
DBD

1.6.3 Spi-C

Spi-C is encoded by the *Spic* gene located on chromosome 10 in mice, and 12 in humans, and was initially discovered via a yeast one-hybrid screening of a cDNA library made from LPS stimulated splenic B cells (Bemark et al, 1999; Carlsson et al, 2002). A second group discovered the same protein and published their findings months later, but identified it as PU.1-related factor (Prf) (Hashimoto et al, 1999). Spi-C is expressed in B cells, macrophage, and dendritic cell populations in the spleen, bone marrow, and lymph nodes according to Northern blot analysis (Bemark et al, 1999). Based on amino acid sequence homology in its DNA binding ETS domain, Spi-C is related to Spi-B and PU.1 (Figure 1.5). Spi-C is also capable of binding to the GGAA/T motif similar to PU.1 and Spi-B. However, there is a preference for an A in position -2 (two nucleotides before the GGAA core) for Spi-C (Bemark et al, 1999), whereas PU.1 prefers G at the -2 position (Ray-Gallet et al, 1995). Similar to PU.1 and Spi-B, Spi-C contains an acidic transactivation domain at the N-terminal which is conserved between species (Carlsson et al, 2003). However, Spi-C differs from PU.1 and Spi-B as it contains a much longer C-terminus relative to the ETS binding site (Carlsson et al, 2003). In addition, Spi-C does not interact with IRF-4 *in vitro,* suggesting that Spi-C has functionally distinct roles compared to PU.1 and Spi-B (Carlsson et al, 2003).

In B cells, Spi-C is highly expressed in the mature B cell populations, but is absent in the pre-B and plasma cell stages (Bemark et al, 1999). There are few target genes known for Spi-C in B cells. Spi-C has been reported to directly transcribe *Fcer2a* (DeKoter et al, 2010), and can cooperate with STAT6 to directly induce transcription of IgE (Carlsson et al, 2006). Ectopic expression experiments that overexpressed Spi-C in either cultured pro-B cells using a retroviral vector (Schweitzer et al, 2006), or in mice using a B cell specific transgene (Zhu et al, 2008), suggested that Spi-C functions as a negative regulator of transcription by opposing PU.1 and/or Spi-B activity. However, whether this opposition was due to direct competition for binding was not determined. Recently a *Spic* knockout mouse was generated making it possible to determine the effect of loss of function of Spi-C (Kohyama et al, 2009). Analysis of $Spic^{-/-}$ mice revealed that Spi-C is essential for the generation of red pulp macrophages in the spleen and is

inducible by Heme (Haldar et al, 2014). However, the role of Spi-C in B cell development and function was not examined closely in these mice.

1.7 Thesis Overview

This thesis characterizes the transcriptional regulation of BCR and TLR expression and signal transduction in B cells by the related ETS transcription factors PU.1, Spi-B, and Spi-C. Specifically, the mechanism by which PU.1, Spi-B, and Spi-C regulate the transcriptional activity of *Nfkb1* in B cells is elucidated. The primary objective of this study was to determine the function of PU.1, Spi-B, and Spi-C during B cell development, and during TLR-mediated responses. It was hypothesized that PU.1 and Spi-B were required for positively regulating components of TLR responses, and Spi-C inhibited PU.1 and Spi-B targets.

1.7.1 *Chapter 2: Nfkb1 activation by the ETS transcription factors PU.1 and Spi-B promotes Toll-Like receptor-mediated splenic B cell proliferation*

In this study, we found impairment in the ability of *PUB* B cells to proliferate in response to different TLR ligands. The impairment in proliferation was not due to a failure of activation or increased rates of apoptosis upon stimulation in *PUB* B cells. Transcript levels of genes associated to the TLR signaling pathway were measured, but despite decreased transcript levels of multiple genes of interest in *PUB* B cells, most were not sufficient to explain our phenotype when tested at the protein level. Nevertheless, decreased transcript levels of *Nfkb1*, and lower levels of its encoded protein p50 in *PUB* B cells were detected. P50 is activated upon TLR stimulation, and *Nfkb1*$^{-/-}$ B cells have a proliferative phenotype in response to TLR ligands which is strikingly similar to *PUB* B cells. It was hypothesized that PU.1 and Spi-B were involved with transcriptionally regulating the *Nfkb1* gene. It was determined that PU.1 and Spi-B directly activated *Nfkb1* transcription, and were essential for proper TLR-mediated responses. Our results demonstrated that PU.1 and Spi-B regulated TLR responses in B cells, which is an important auxiliary pathway involved in neutralizing antibody formation.

1.7.2 *Chapter 3: Identification of a negative regulatory role for Spi-C in the murine B cell lineage*

The role of Spi-C in B cell development and function was examined in this study. In order to determine whether Spi-C and Spi-B were functionally redundant, a novel mouse line was generated that was germline knockout for *Spib* and heterozygous for *Spic* (*Spib*$^{-/-}$*Spic*$^{+/-}$). *Spib*$^{-/-}$ B cells are defective in BCR signaling, and have reduced FO B cells compared to WT mice. Many aspects of the *Spib*$^{-/-}$ B cell phenotype were rescued in *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells, including a restoration in FO and T2 B cells, and restored proliferation in response to LPS and anti-IgM. The phenotypic rescue in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice was attributed to elevated transcript levels of *Nfkb1*. It was hypothesized that Spi-C was inhibiting Spi-B during transcriptional activation of *Nfkb1*. It was determined that Spi-C directly binds to the *Nfkb1* promoter to inhibit Spi-B's transcriptional activation of *Nfkb1*. Our results demonstrate a novel mechanism for Spi-C in opposing Spi-B in B cell development and function.

Chapter 2

2 *Nfkb1* activation by the ETS transcription factors PU.1 and Spi-B promotes Toll-Like receptor-mediated splenic B cell proliferation

Generation of antibodies against T-independent and T-dependent antigens requires Toll-Like receptor (TLR) engagement on B cells for efficient responses. However, regulation of TLR expression and responses in B cells is not well understood. PU.1 and Spi-B (encoded by *Spi1* and *Spib* respectively) are transcription factors of the E26-transformation-specific (ETS) family, and are important for B cell development and function. It was found that B cells from mice knockout for Spi-B and heterozygous for PU.1 (*Spi1*$^{+/-}$*Spib*$^{-/-}$, *PUB* mice) proliferated poorly in response to TLR ligands compared to WT B cells. The NF-κB family member p50 (encoded by *Nfkb1*) is required for LPS responsiveness in mice. *PUB* B cells expressed reduced *Nfkb1* mRNA transcripts and p50 protein. The *Nfkb1* promoter was regulated directly by PU.1 and Spi-B as shown by reporter assays and chromatin immunoprecipitation analysis. Occupancy of the *Nfkb1* promoter by PU.1 was reduced in *PUB* B cells compared to WT B cells. Finally, infection of *PUB* B cells with a retroviral vector encoding p50 substantially restored proliferation in response to LPS. We conclude that *Nfkb1* transcriptional activation by PU.1 and Spi-B promotes TLR-mediated B cell proliferation.

2.1 Introduction

Toll-Like receptors expressed by B cells recognize conserved microbial products. Engagement of TLR-ligands by B cells is required for thymus-independent responses that are sufficient to promote CSR, proliferation, and antigen presentation (Browne, 2012; Pone et al, 2010). Generation of optimal T-dependent antibody responses also requires TLR signaling in B cells (Pasare & Medzhitov, 2005; Ruprecht & Lanzavecchia, 2006). For example, efficient antibody responses to protein antigens after immunization with synthetic nanoparticles required engagement of TLRs on B cells (Kasturi et al, 2011).

Therefore, identifying factors controlling TLR expression and responses in B cells has important implications for the generation of neutralizing antibody responses.

Mature murine B cells express and respond to TLR1, TLR2, TLR4, TLR6, TLR7/8, and TLR9 ligands (Genestier et al, 2007; Gururajan et al, 2007; Hornung et al, 2002), resulting in NF-κB activation through MyD88 or TRIF-dependent pathways (Kawai & Akira, 2006). NF-κB activates genes involved in cytokine synthesis, antibody secretion, and cell proliferation (Bekeredjian-Ding & Jego, 2009). The NF-κB family includes p105 that is processed into p50 (encoded by *Nfkb1*), p100 that is processed into p52 (encoded by *Nfkb2*), RelA (p65), Rel (c-Rel), and RelB, which all interact with DNA as hetero- or homodimers (Hayden & Ghosh, 2008). Optimal TLR signaling in B cells may require MHC class II (MHCII) interaction with Bruton's tyrosine kinase (Btk) and CD40 (Liu et al, 2011). However, many aspects of gene regulation involving TLR expression and signal transduction in B cells remain unclear.

PU.1 and Spi-B are E26 transformation-specific (ETS)-family transcription factors encoded by *Spi1* and *Spib* respectively, and are important in B cell development and function (DeKoter et al, 2010). PU.1 is expressed in most hematopoietic cell types, directly regulating many genes involved in cellular communication (Turkistany & DeKoter, 2011). Spi-B is expressed in B cells, plasmacytoid dendritic cells, and at lower levels in T cells (Su et al, 1996). PU.1 and Spi-B share 67% amino acid homology in their DNA binding domain and can bind an identical consensus sequence containing the core motif 5'-GGAA-3' (Dahl et al, 2002; Pio et al, 1996; Ray-Gallet et al, 1995). Both transcription factors are expressed in B cells, regulate common target genes, and are functionally redundant (Sokalski et al, 2011; Xu et al, 2012). *Spi1*$^{-/-}$ mice die during embryogenesis and produce no B cells (Scott et al, 1994). In contrast, viable *Spib*$^{-/-}$ mice have fewer B cells, which are defective in BCR signaling and are unable to generate antibody responses to T-dependent antigens (Garrett-Sinha et al, 1999). *Spi1*$^{+/-}$ *Spib*$^{-/-}$ (*PUB*) mice have greater impairment in B cell numbers and BCR signaling compared to *Spib*$^{-/-}$ or *Spi1*$^{+/-}$ mice (Garrett-Sinha et al, 1999; Su et al, 1997), and also have reduced follicular (FO) and increased marginal zone (MZ) B cell frequencies compared to WT mice (DeKoter et al, 2010). Therefore, PU.1 and Spi-B are critically important for B cell

development and BCR signaling, but it remains unclear what these transcription factors regulate downstream to explain this phenotype.

In this study, it was determined whether PU.1 and Spi-B regulate innate immune responses in B cells. Impairment in TLR-mediated proliferation in *PUB* B cells was observed. Gene and protein expression analysis, luciferase reporter assays, and chromatin immunoprecipitation (ChIP) experiments demonstrated that PU.1 and Spi-B directly activate *Nfkb1* encoding p50. Infection of *PUB* B cells with a retroviral vector encoding p50 significantly increased proliferation in response to LPS. Therefore, decreased P50 expression is sufficient to explain many aspects of the *PUB* B cell phenotype. Our results suggest that PU.1 and Spi-B are important transcriptional regulators of TLR responses in B cells.

2.2 Results

2.2.1 *PUB* mice exhibit impaired TLR-mediated B cell proliferation

PUB B cells were previously reported to have reduced proliferation in response to anti-IgM or lipopolysaccharide (LPS) (Garrett-Sinha et al, 1999). To determine if impaired proliferation was limited to LPS (TLR4 ligand), splenic B cells were enriched by CD43 depletion and cultured with various TLR ligands. Compared to WT B cells, *PUB* B cells proliferated poorly at all tested concentrations of LPS (Fig. 2.1A) or CL097 (TLR7/8 ligand) (Fig. 2.1B), and proliferated poorly to Pam3CSK4 (TLR2/1), FSL1 (TLR6/2), ODN1826 (TLR9) (Fig. 2.1C), or anti-IgM (Fig. 2.1D-E) stimulation. Both WT and *PUB* B cells failed to respond to flagellin (TLR5) and Poly(I:C) LMW or HMW (TLR3) (Fig. 2.2B), but proliferated nearly equally well in response to anti-CD40 or LPS+anti-CD40 stimulation (Fig. 2.2C-D), which indicated no general proliferation impairment in *PUB* B cells. Anti-CD40 stimulation did not restore responsiveness of *PUB* B cells to anti-IgM (Fig. 2.2C-D). Interestingly, although *PUB* B cells proliferated poorly in response to anti-IgM or LPS stimulation alone, LPS+anti-IgM stimulation resulted in equivalent WT and *PUB* proliferation (Fig. 2.1F-G). Similar results were obtained using 3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyl-tetrazolium bromide (MTT)

proliferation assays and [^{3}H]-thymidine incorporation assays (Fig. 2.2D). Overall, these results suggested that proper PU.1 and Spi-B expression was required for TLR-mediated B cell proliferation.

Altered splenic B cell composition could explain proliferative defects in *PUB* B cells. However, *PUB* mouse spleens have increased ratios of MZ to FO B cell frequencies (DeKoter et al, 2010) and MZ B cells were shown to have greater TLR-mediated proliferative potential compared to FO B cells (Oliver et al, 1997). Former studies reported reduced B cell frequencies in *PUB* spleens (Garrett-Sinha et al, 1999), which was confirmed by our study (Fig. 2.3A-B). *PUB* spleens contained elevated frequencies of $CD11b^{+}GR1^{+}$ myeloid cells, but the absolute number of $CD11b^{+}GR1^{+}$ splenocytes were slightly reduced compared to WT mice (Fig. 2.3C-D). Flow cytometric analysis of $CD19^{hi}CD1d^{hi}CD5^{+}$ B regulatory cells (Yanaba et al, 2008) revealed no significant difference in frequency between *PUB* and WT spleens (Fig. 2.3E). Furthermore, spleens from *PUB* mice contained fewer cells than WT mice (Fig. 2.3F). Elevated B cell activating factor (BAFF) is common in B cell lymphopenia (Kreuzaler et al, 2012), and conceivably could impede TLR-mediated proliferation. Steady-state levels of serum BAFF were found to be elevated in *PUB* mice compared to WT (Fig. 2.4A). BAFF receptor (BAFF-R) expression was reduced on *PUB* B cells (Fig. 2.4B), consistent with what was expected from higher BAFF levels (Kreuzaler et al, 2012). To determine if increased BAFF could impair *PUB* B cell proliferation, splenic B cells were LPS stimulated with and without recombinant BAFF. Additional BAFF increased proliferation in both untreated or LPS treated groups in WT and *PUB* B cells (Fig. 2.4C). Therefore, impaired TLR-mediated *PUB* B cell proliferation was not explained by altered B cell composition or elevated BAFF levels.

Figure 2.1. Impaired TLR-mediated proliferation in *PUB* (*Spi1*$^{+/-}$*Spib*$^{-/-}$) B cells.

(A and B) B cells from *PUB* mice responded poorly compared to WT stimulated with different concentrations of LPS (A) and CL097 (B). *Y*-axis indicates OD_{570}, and *X*-axis indicates concentrations of agonists. (C) B cells from *PUB* mice responded poorly compared to WT stimulated with indicated TLR9, TLR6/2, TLR4, and TLR2/1 ligands. (D) Impaired *PUB* B cell proliferation in response to anti-IgM stimulation. (E) Quantitation of four independent experiments as done in D. Proliferation indices were calculated by normalizing OD_{570} of treatments to WT untreated. For A-E, B cell proliferation was assessed by MTT proliferation assay following 72 h stimulation. Data shows the mean ± SD for triplicate wells for A-D and are representative of at least two independent experiments. Values in E are shown as mean ±SEM (n=4). *p<0.05; **p<0.01; ***p<0.001.

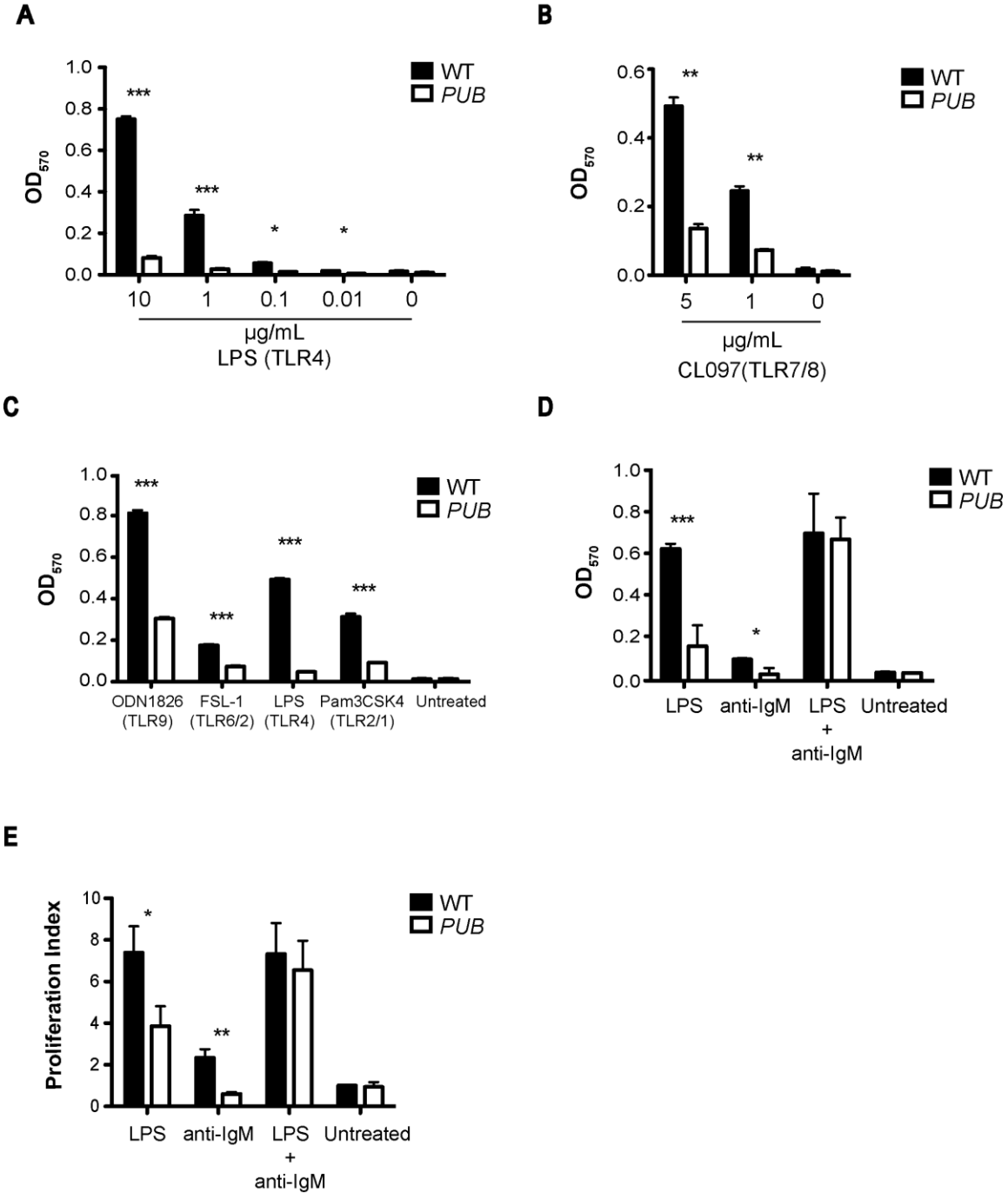

A
B
C
D
E
WT
PUB
OD570
***
**
*
10
1
0.1
0.01
0
5
µg/mL
LPS (TLR4)
CL097(TLR7/8)
ODN1826 (TLR9)
FSL-1 (TLR6/2)
LPS (TLR4)
Pam3CSK4 (TLR2/1)
Untreated
anti-IgM
LPS + anti-IgM
Proliferation Index

Figure 2.2. Proliferation of WT and *PUB* splenic B cells in response to various stimuli.

(A) B cells were enriched by magnetic depletion and purity was determined by $CD19^{+}B220^{+}$ flow cytometry analysis (box). (B) Neither WT nor *PUB* splenic B cells proliferated in response to TLR3, TLR5, and TLR7 ligands. Proliferation was assessed with the presence of different TLR ligands for 72 h in a 96-well plate using a MTT cell proliferation assay. Values are shown as mean ± standard deviation of triplicate wells. Data are representative of at least two independent experiments. (C and D) *Ex vivo* stimulation with LPS and anti-IgM, results in impaired B cell proliferation in *PUB* mice. Proliferation was measured in splenic *PUB* and WT B cells following 72 h stimulation with different combinations of LPS (10 μg/mL), anti-CD40 (10 μg/mL), and anti-IgM (50 μg/mL). Proliferation was assessed using a (C) MTT cell proliferation assay or (D) [^{3}H]-thymidine incorporation assay. [^{3}H]-thymidine (1 mCi/mL) was added to each well after 72 h stimulation, followed by scintillation counting 24 h after thymidine treatment. For B-D, values are shown as mean ± standard deviation of triplicate wells, and are representative of two independent experiments. Statistical analysis was performed using Student's t-test, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

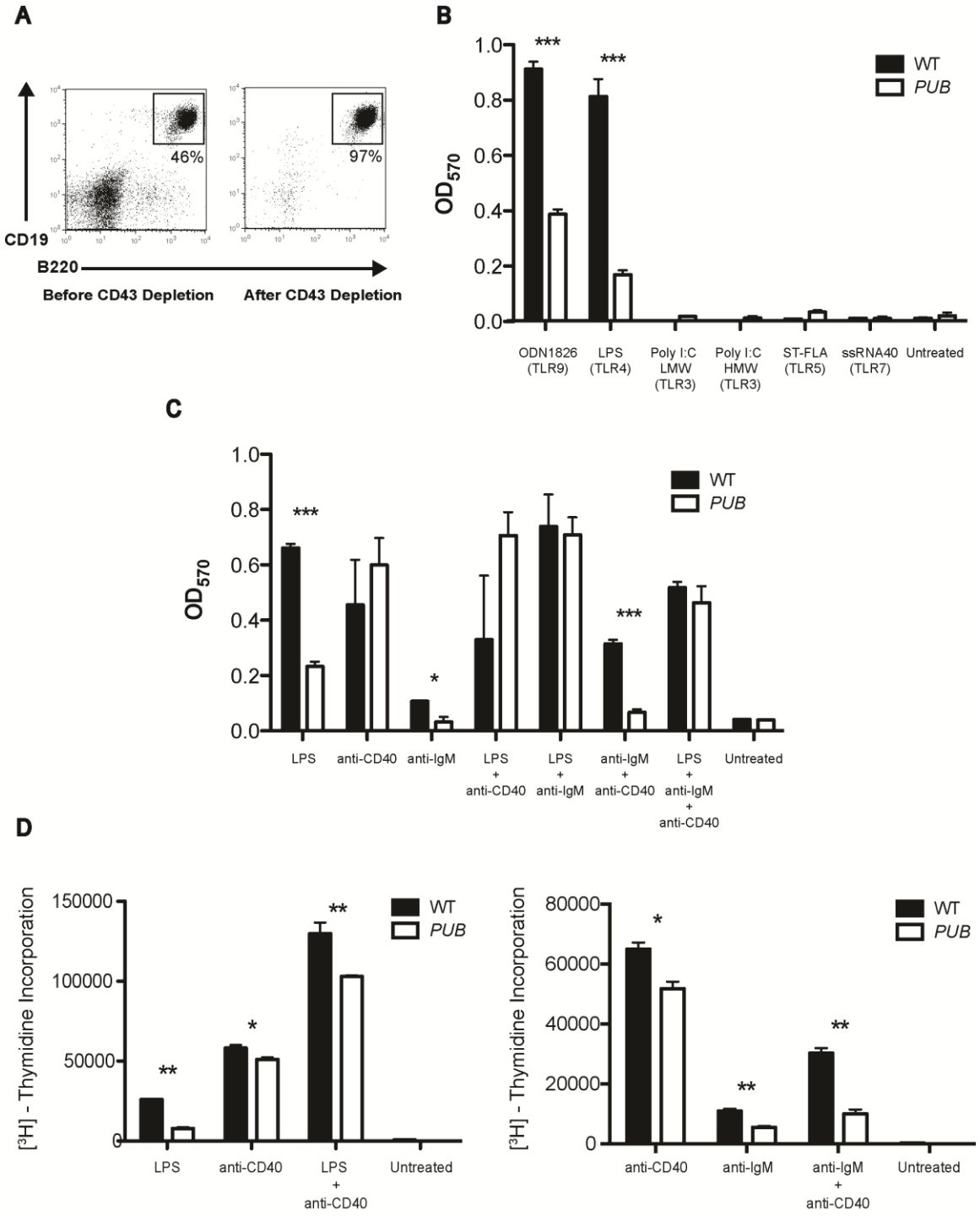

A
46%
97%
CD19
B220
Before CD43 Depletion
After CD43 Depletion
B
WT
PUB
OD570
ODN1826 (TLR9)
LPS (TLR4)
Poly I:C LMW (TLR3)
Poly I:C HMW (TLR3)
ST-FLA (TLR5)
ssRNA40 (TLR7)
Untreated
C
LPS
anti-CD40
anti-IgM
LPS + anti-CD40
LPS + anti-IgM
anti-IgM + anti-CD40
LPS + anti-IgM + anti-CD40
D
[3H] - Thymidine Incorporation

Figure 2.3. Splenic B and myeloid cell composition in *PUB* mice.

(A) Reduced B cell frequencies in the spleen of *PUB* mice. Flow cytometric analysis was performed to determine the frequency of B cells expressing CD19 and B220 in WT and *PUB* mice. Dashed box represents B cells in the spleen. Shown is a representative experiment of seven individual experiments. (B) Reduced $CD19^{+}$ and $B220^{+}$ cell frequencies and absolute numbers in the spleens of *PUB* mice. Quantitation of the frequency and absolute number of splenic B cells expressing CD19 and B220 (n=7). (C) Increased frequency of myeloid cells in the spleens of *PUB* mice. Flow cytometric analysis was performed to determine the frequency of myeloid cells expressing GR-1 and CD11b in WT and *PUB* mice. Shown is a representative experiment of seven independent experiments. (D) Increased frequency of myeloid cells, but decreased absolute number of myeloid cells in the spleens of *PUB* mice. Flow cytometric analysis was performed to determine the frequency and absolute numbers of splenic myeloid cells expressing GR-1 and CD11b for 7 mice. (E) There is no difference between the frequency of Breg (B10) cells between *PUB* and WT mice. Flow cytometric analysis was performed to determine the frequency of B10 cells. B10 cells were gated on $CD19^{hi}CD1d^{+}CD5^{+}$ (bolded quadrant). Shown is a representative experiment of two independent experiments. (F) Reduced absolute number of total splenocytes in *PUB* mice compared with C57BL/6 mice. Shown are total splenic cell counts WT and *PUB* mice (n=7). For all panels, *, $P<0.05$; **, $P<0.01$; ***. $P<0.001$. Error bars represent standard deviation.

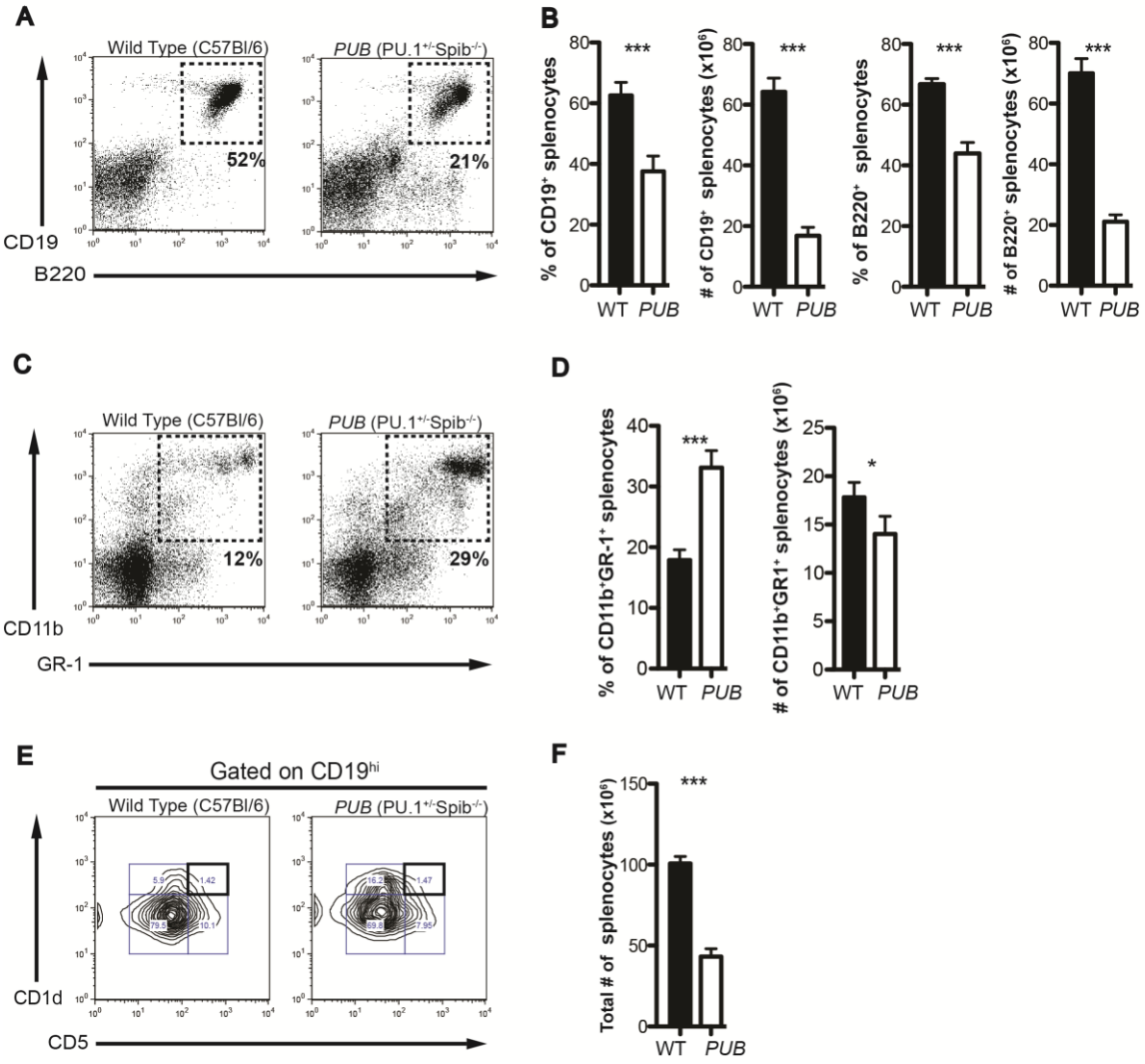
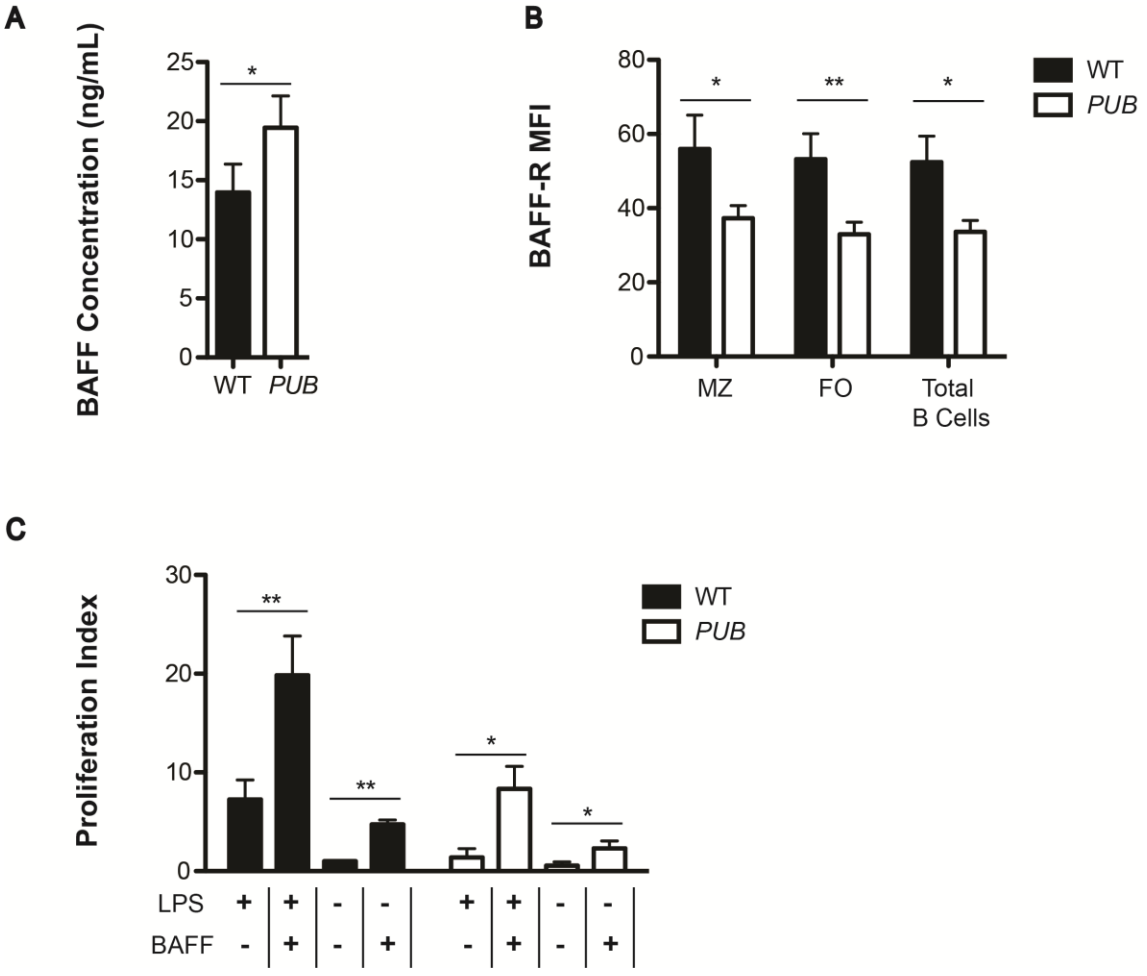

A
Wild Type (C57Bl/6)
PUB (PU.1+/-Spib-/-)
52%
21%
CD19
B220
B
% of CD19+ splenocytes
# of CD19+ splenocytes (x10^6)
% of B220+ splenocytes
# of B220+ splenocytes (x10^6)
***
WT PUB
C
Wild Type (C57Bl/6)
PUB (PU.1+/-Spib-/-)
12%
29%
CD11b
GR-1
D
% of CD11b+GR-1+ splenocytes
# of CD11b+GR1+ splenocytes (x10^6)
***
*
WT PUB
E
Gated on CD19hi
Wild Type (C57Bl/6)
PUB (PU.1+/-Spib-/-)
CD1d
CD5
F
Total # of splenocytes (x10^6)
***
WT PUB

Figure 2.4. Proliferation of splenic B cells in response to recombinant BAFF and analysis of BAFF-R and BAFF serum levels.

(A) Elevated BAFF levels were detected in sera from *PUB* compared to WT mice by ELISA. Values are shown as mean ± standard deviation from six individual mice. Data is representative of two independently performed experiments. (B) Reduced BAFF-R levels on the surface of splenic B cells in *PUB* mice compared to WT mice. Mean fluorescence intensities (MFIs) of BAFF-R was measured by flow cytometry on total $CD19^{+}$ cells, gated $CD21^{hi}CD19^{+}$ (MZ) B cells, or gated $CD21^{int}CD19^{+}$ (FO) B cells from WT and *PUB* spleens. Data is shown as mean ± standard error mean of six independent experiments. (C) Addition of recombinant BAFF (100 ng/mL) increased proliferation in splenic WT and *PUB* B cells when treated with LPS, as compared with treatments without BAFF. Proliferation was assessed following 72 h stimulation in a 96-well plate using a MTT cell proliferation assay. Values are shown as mean ± standard error mean of five independent experiments. For all panels, *, $P<0.05$; **, $P<0.01$.

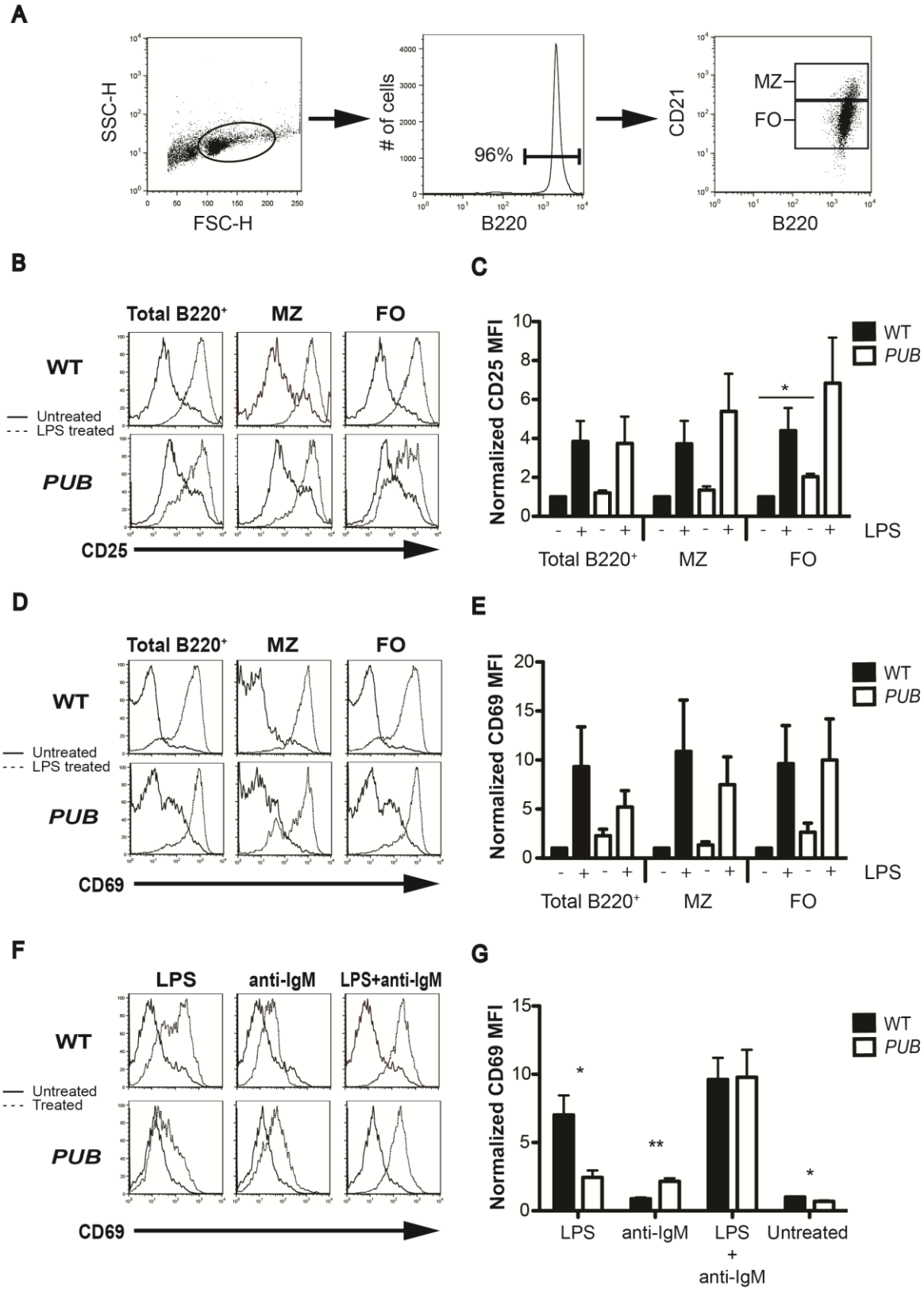

A
BAFF Concentration (ng/mL)
25
20
15
10
5
0
*
WT
PUB
B
BAFF-R MFI
80
60
40
20
0
*
**
*
MZ
FO
Total B Cells
WT
PUB
C
Proliferation Index
30
20
10
0
**
**
*
*
WT
PUB
LPS + + - - + + - -
BAFF - + - + - + - +

2.2.2 *PUB* B cells are activated following stimulation

We wanted to determine the mechanism of impaired TLR-mediated B cell proliferation in *PUB* mice. To determine whether *PUB* B cells were capable of activation by TLR ligands, MFI of CD25 or CD69 activation markers were measured on total $B220^{+}$ cells, or B cells with MZ ($CD21^{hi}B220^{+}$), or FO ($CD21^{int}B220^{+}$) phenotypes after 24 h LPS stimulation (Fig. 2.5A). All WT and *PUB* B cell subsets upregulated CD25 following LPS stimulation compared to untreated cells (Fig. 2.5B-C). Untreated FO-phenotype *PUB* B cells expressed more CD25 than WT (Fig. 2.5C). Next, CD69 expression was measured 24 h after LPS stimulation (Fig. 2.5D-E). Both WT and *PUB* B cells increased CD69 expression following LPS stimulation for all subsets (Fig. 2E). CD69 was also measured 72 h post stimulation in total $B220^{+}$ cells. *PUB* B cells failed to upregulate CD69 to WT levels following 72 h LPS stimulation (Fig. 2.5F-G). In contrast, *PUB* B cells expressed higher levels of CD69 following anti-IgM stimulation, and LPS + anti-IgM stimulation resulted in equivalent expression between WT and *PUB* B cells (Fig. 2.5F-G). In summary, *PUB* B cells were activated following LPS stimulation, but CD69 upregulation was reduced compared to WT when measured at 72 h.

Figure 2.5. Splenic *PUB* B cells are activated following LPS stimulation.

(A) Flow cytometry gating strategy on total $B220^{+}$, MZ ($CD21^{hi}B220^{+}$), or FO ($CD21^{int}B220^{+}$) B cells. (B) Histograms show increased CD25 expression following 24 h LPS stimulation in WT and *PUB* B cells. (C) Quantitation of data shown in B. (D) Histograms show increased CD69 expression following 24 h LPS stimulation in WT and *PUB* mice. (E) Quantitation of data shown in D. (F) CD69 expression is lower in *PUB* total B cells than WT total B cells at 72 h post stimulation with LPS. CD69 expression is also shown for anti-IgM and LPS+anti-IgM conditions. (G) Quantitation of data shown in F. Values in C, E, and G show the normalized CD25 or CD69 MFI ±SEM for five independent experiments. Normalized MFI was calculated by dividing MFI values by WT untreated MFI for each given subset. *$p<0.05$; **$p<0.01$.

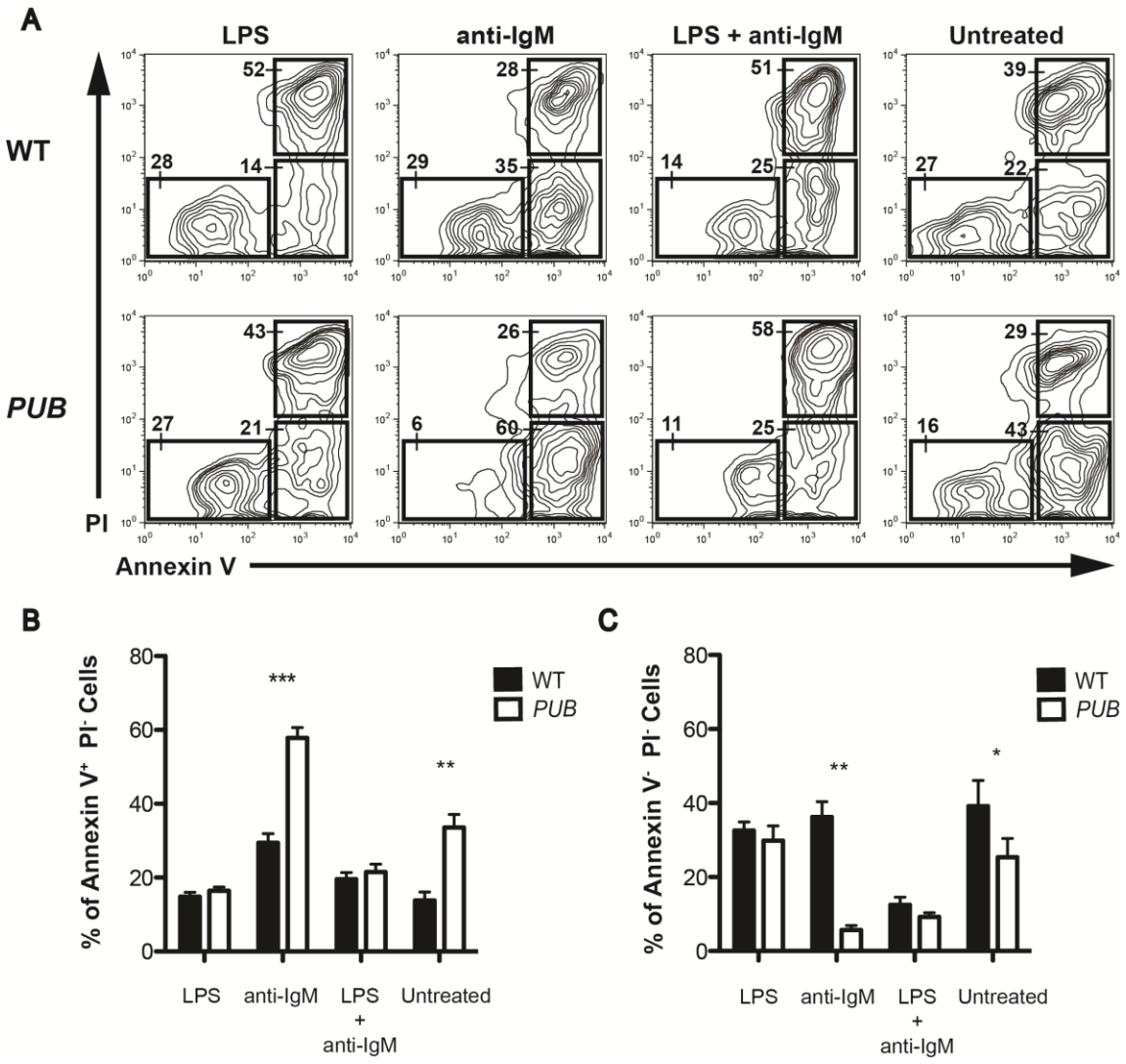

A
SSC-H
FSC-H
# of cells
96%
B220
CD21
MZ
FO
B220
B
Total B220+
MZ
FO
WT
Untreated
LPS treated
PUB
CD25
C
Normalized CD25 MFI
WT
PUB
LPS
Total B220+
MZ
FO
D
Total B220+
MZ
FO
WT
Untreated
LPS treated
PUB
CD69
E
Normalized CD69 MFI
WT
PUB
LPS
Total B220+
MZ
FO
F
LPS
anti-IgM
LPS+anti-IgM
WT
Untreated
Treated
PUB
CD69
G
Normalized CD69 MFI
WT
PUB
LPS
anti-IgM
LPS + anti-IgM
Untreated

2.2.3 Apoptosis in *PUB* splenic B cells is not increased following LPS stimulation

PUB B cells were previously reported to exhibit increased steady-state levels of apoptosis (Garrett-Sinha et al, 1999). Therefore, impaired TLR-mediated *PUB* B cell proliferation could be due to increased apoptotic cell death. To test this hypothesis, we stained B cells for Annexin V and PI after 72 h incubation with or without LPS and/or anti-IgM (Fig. 2.6A). Under the culture conditions used, a mean of 36% of WT B cells were low/negative for Annexin V staining after anti-IgM stimulation, while a mean of 6% of *PUB* B cells were low/negative for Annexin V staining after anti-IgM stimulation (Fig. 2.6A, 2.6C). Therefore few *PUB* B cells survived anti-IgM stimulation, in accord with proliferation results shown in Fig. 2.1E. Frequencies of apoptotic (Annexin $V^{hi}PI^{-}$) *PUB* B cells untreated or anti-IgM stimulated were significantly higher than WT controls; and frequencies of live (Annexin $V^{-}PI^{-}$) *PUB* B cells untreated or anti-IgM stimulated were significantly decreased compared to WT controls. However, frequencies of apoptotic cells between LPS or LPS+anti-IgM treated WT and *PUB* B cells were similar (Fig. 2.6B). No significant difference was observed between live WT and *PUB* B cells stimulated with LPS or LPS+anti-IgM. (Fig. 2.6C). Therefore, these results suggest that reduced proliferation of *PUB* B cells in response to LPS was not primarily due to increased apoptosis.

Figure 2.6. Apoptosis in *PUB* B cells is not elevated following LPS stimulation.

(A) Apoptosis was analyzed by flow cytometry using Annexin V and PI staining in enriched B cells that were untreated or treated with indicated stimuli. (B) Quantitation of apoptotic (Annexin V^{hi} PI^{-}) cells. (C) Quantitation of live (Annexin V^{-} PI^{-}) cells. Data in B and C show the mean ± SEM of four independent experiments. *p<0.05; **p<0.01; ***p<0.001.


A
LPS
anti-IgM
LPS + anti-IgM
Untreated
WT
PUB
52
28
14
28
29
35
51
14
25
39
27
22
43
27
21
26
6
60
58
11
25
29
16
43
PI
Annexin V
B
% of Annexin V+ PI- Cells
WT
PUB
***
**
LPS
anti-IgM
LPS + anti-IgM
Untreated
C
% of Annexin V- PI- Cells
WT
PUB
**
*
LPS
anti-IgM
LPS + anti-IgM
Untreated

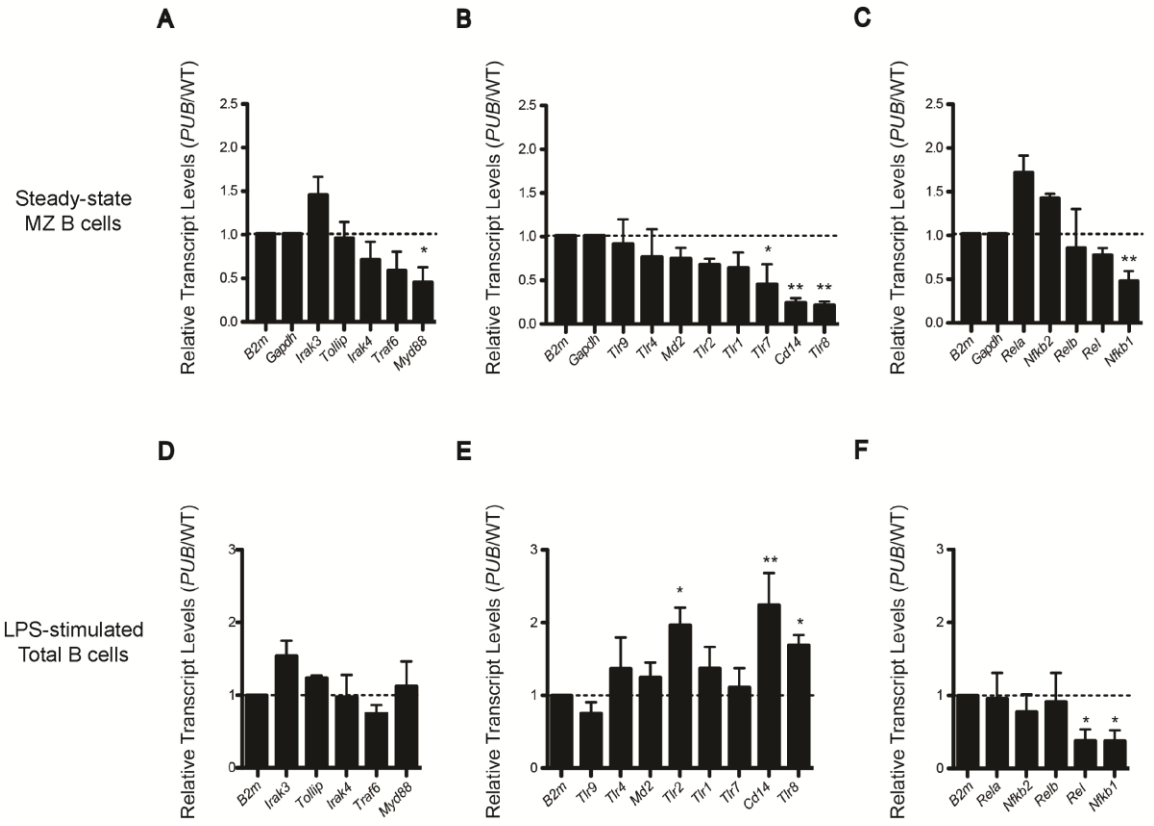
2.2.4 *PUB* mice have altered transcript and protein levels of genes involved in TLR signaling

MZ B cells are more readily activated and proliferative than FO B cells after LPS stimulation (Oliver et al, 1997; Snapper et al, 1993). Despite increased MZ B cells in *PUB* mice (DeKoter et al, 2010), impaired proliferation occurred in splenic *PUB* B cells for all MyD88 dependent TLR ligands compared to WT (Fig. 2.1B-C). Therefore, components of the MyD88-dependent signaling pathway might be regulated by PU.1 and Spi-B. To test this hypothesis, RNA was prepared from sorted MZ B cells from WT and *PUB* mice. Steady-state levels of mRNA transcripts encoding components of the MyD88-dependent signaling were measured by RT-qPCR analysis. We examined transcript levels of downstream signaling components (Fig. 2.7A), TLRs and related receptors (Fig. 2.7B), and NF-κB/Rel subunits (Fig. 2.7C). Significant reductions in *Myd88*, *Cd14*, *Tlr8*, *Tlr7* and *Nfkb1* transcripts were measured in *PUB* MZ B cells (Fig. 2.7A-C). Next, we stimulated enriched total B cells with LPS for 16 h and assessed gene expression profiles by RT-qPCR. Transcript levels of downstream TLR signaling genes were not significantly different in *PUB* B cells compared to WT (Fig. 2.7D). Although steady-state transcript levels of TLR and related receptors were reduced in *PUB* MZ B cells, transcript levels of *Tlr2*, *Cd14,* and *Tlr8* were higher in total LPS stimulated *PUB* B cells (Fig. 2.7E). Lastly, LPS stimulated *PUB* B cells expressed lower transcript levels of *Rel* and *Nfkb1* (Fig. 2.7F).

MyD88 is the adapter protein utilized by all TLRs to which B cells responded in our hands (Fig. 2.1). However, immunoblotting analysis showed MyD88 levels were unchanged between *PUB* and WT enriched B cells (Fig. 2.8A). Despite elevated *Cd14* transcripts in LPS stimulated *PUB* B cells, surface CD14 expression was equal to WT 72 h post LPS or anti-IgM stimulation, and was elevated when LPS+anti-IgM stimulated (Fig. 2.8B). Therefore, our results suggest that altered levels of MyD88 and CD14 did not account for defective TLR responses in *PUB* B cells.

Figure 2.7. Measurement of steady-state and LPS stimulated transcript levels of TLR signaling genes.

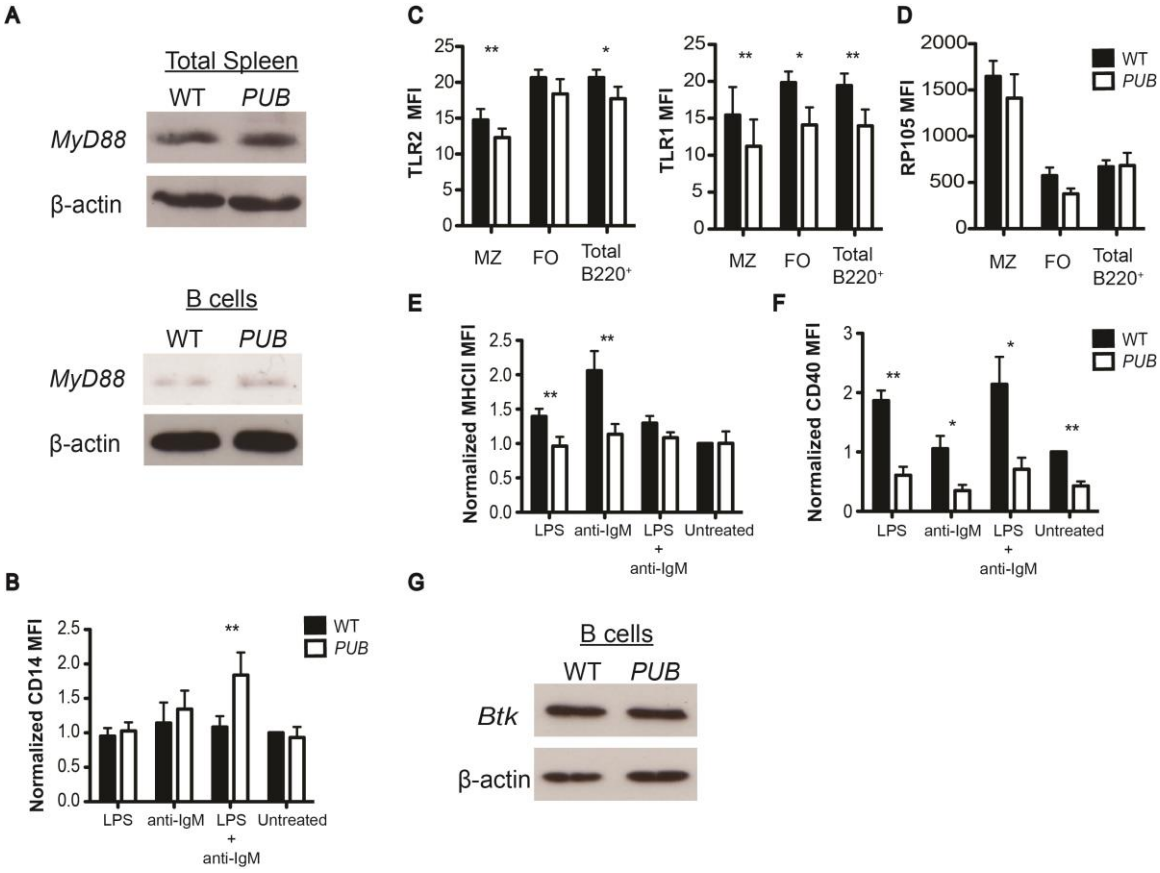
(A-C) Steady-state transcript levels of genes related to downstream TLR signaling (A), TLRs and related receptors (B), and NF-κB subunits (C) were measured in MZ B cells of *PUB* and WT mice. (D-F) Transcript levels in total B cells following 16 h LPS stimulation of genes related to downstream (D) TLR signaling, (E) TLRs and related receptors, and (F) NF-κB subunits. Analysis was performed on RNA prepared from MZ B cells enriched by cell sorting, or total enriched B cells. RT-qPCR was used to determine relative mRNA transcript levels in *PUB* B cells compared to WT B cells, after normalizing to *Gapdh* or *B2m*. *Y*-axis indicates mean fold change ±SEM relative to WT levels for three individual mice. Values of 1 (dashed line) indicate no difference in transcript levels between *PUB* and WT B cells. *$p<0.05$; **$p<0.01$; ***$p<0.001$.


A
B
C
D
E
F
Steady-state
MZ B cells
LPS-stimulated
Total B cells
Relative Transcript Levels (PUB/WT)
B2m
Gapdh
Irak3
Tollip
Irak4
Traf6
Myd88
Tlr9
Tlr4
Md2
Tlr2
Tlr1
Tlr7
Cd14
Tlr8
Rela
Nfkb2
Relb
Rel
Nfkb1

Next, surface expression of TLRs and related receptors were examined by flow cytometry. Reduced TLR2 and TLR1 was measured on freshly isolated MZ, FO, and total B cells of *PUB* mice (Fig. 2.8C). LPS can also activate B cells through binding RP105 (Nagai et al, 2012), but levels between WT and *PUB* B cells were equal (Fig. 2.8D). TLR signaling in B cells has been reported to involve MHCII, CD40, and Btk interaction (Liu et al, 2011), and PU.1 activates the *Btk* promoter (Himmelmann et al, 1996). PU.1 also directly regulates the transcriptional coactivator CIITA, which regulates MHCII genes (Yoon & Boss, 2010). Since PU.1 transcriptionally regulates genes involved with TLR signaling, TLR-mediated B cell proliferation might be regulated by PU.1 and/or Spi-B through these auxiliary pathways and interactions. Decreased gene transcription in *PUB* mice could result in decreased protein, and consequently fewer protein interactions required for TLR signaling. To test this hypothesis, we measured protein levels of MHCII, CD40, and Btk. *PUB* B cells failed to upregulate surface MHCII (Fig. 2.8E) or CD40 (Fig. 2.8F) following 72 h LPS or anti-IgM stimulation. Immunoblotting analysis showed equal Btk protein expression between *PUB* and WT B cells (Fig. 2.8G). Overall, decreased TLR responsiveness in *PUB* B cells was accompanied by reduced TLR1 and TLR2 expression, and a failure to upregulate MHCII or CD40. However, these observations were not sufficient to explain the *PUB* phenotype.

Figure 2.8. *PUB* B cells have decreased basal surface expression of TLR1 and TLR2 and cannot upregulate MHCII and CD40 expression following stimulation.

(A) Immunoblotting for MyD88 and β-actin was performed using total spleen cells (top panel) or enriched B cell lysates (lower panel) from WT and *PUB* mice. (B) CD14 expression is increased in *PUB* B cells following LPS+anti-IgM stimulation, but not increased when stimulated with LPS or anti-IgM. (C) *PUB* B cells have decreased TLR1 and TLR2 expression. TLR1 and TLR2 MFI was measured for gated MZ ($CD21^{hi}B220^{+}$), FO ($CD21^{int}B220^{hi}$), and total $B220^{+}$ B cells using flow cytometry. Data shows the mean ±SEM (n=5). (D) RP105 surface levels are equivalent between WT and *PUB* B cells. RP105 MFI was measured for MZ, FO, and total $B220^{+}$ cells using flow cytometry. Data shows the mean ±SEM (n=3). *PUB* B cells fail to upregulate (E) MHCII expression or (F) CD40 expression following LPS, anti-IgM, or LPS+anti-IgM stimulation. (G) Immunoblotting for Btk, and β-actin was performed using enriched B cell lysates from WT and *PUB* mice. Immunoblotting experiments are shown as a representative of two individual mice. For B, E, and F, enriched B cells were stimulated for 72 h before analysis of CD14, MHCII, and CD40 expression by flow cytometry. Values represent MFIs of total B cells expressing the indicated markers, normalized to untreated WT B cells. Normalized MFIs were calculated by dividing MFI values by the WT untreated MFI. Data shows the mean ±SEM of four independent experiments. *$p<0.05$; **$p<0.01$.


A
Total Spleen
WT
PUB
MyD88
β-actin
B cells
WT
PUB
MyD88
β-actin
B
Normalized CD14 MFI
WT
PUB
LPS
anti-IgM
LPS + anti-IgM
Untreated
C
TLR2 MFI
TLR1 MFI
MZ
FO
Total B220+
D
RP105 MFI
E
Normalized MHCII MFI
F
Normalized CD40 MFI
G
B cells
Btk
β-actin

2.2.5 Direct *Nfkb1* promoter activation by PU.1 and Spi-B

Steady-state levels of *Rel* and *Nfkb1* mRNA transcripts were expressed at reduced levels in *PUB* B cells stimulated with LPS compared to WT B cells (Fig. 2.7F). It was previously shown that *Rel* is regulated by PU.1 and/or Spi-B in B cells (Hu et al, 2001). *Nfkb1* encodes for p50 and is required for murine B cell proliferation in response to LPS or anti-IgM (Sha et al, 1995). Reduced p50 expression could sufficiently explain many aspects of the *PUB* phenotype. Immunoblotting analysis confirmed lower p50 levels in splenic *PUB* B cells (Fig. 2.9A). We therefore investigated the role of reduced *Nfkb1* in *PUB* B cell proliferation.

PU.1 and Spi-B can interchangeably bind sites of target genes (Ray-Gallet et al, 1995). To determine locations of binding similarity between PU.1 and Spi-B, genome-wide ChIP-sequencing (ChIP-seq) was performed using anti-FLAG Ab on WEHI-279 B cell lymphoma cells overexpressing either 3X-FLAG tagged PU.1 or Spi-B. Both PU.1 and Spi-B were enriched at the *Nfkb1* promoter (Fig. 2.9B). To determine whether PU.1 and Spi-B regulate *Nfkb1* directly, we aligned the murine *Nfkb1* promoter sequence from multiple species, using a published transcription start site (TSS) for reference (Cogswell et al, 1993). At the promoter, a 213 bp region was found to be 87% conserved between multiple species, with exception to the 67% conservation of the Tasmanian Devil sequence (Fig. 2.9C). Potential PU.1/Spi-B transcription factor binding sites were predicted using MatInspector software. Three conserved binding sites with a matrix similarity score >0.9 were predicted among all species, and a lower scoring fourth site was identified near the TSS (Fig. 2.9C). Next, luciferase assays were used to determine PU.1 and Spi-B's contribution to *Nfkb1* transcriptional activation. The conserved *Nfkb1* mouse promoter region was cloned and tested by transient transfection in WEHI-279 cells. Site directed mutagenesis was performed on the predicted PU.1/Spi-B binding sites of the two closest sites of the TSS of the promoter, by substituting GGAA (TTCC on complement strand) binding site to GGAC (GTCC) (Fig. 2.9D), which was previously reported to abolish PU.1 binding (DeKoter et al, 2010; Xu et al, 2012). Transcriptional activation was detected at the *Nfkb1* promoter, and mutation of PU.1/Spi-B sites significantly reduced transcriptional activation (Fig. 2.9E). To determine if PU.1 interacts

directly with the *Nfkb1* promoter, ChIP analysis was performed. Reanalysis of published ChIP-seq data (Heinz et al, 2010), and with our own ChIP-seq results (Fig. 2.9B), revealed PU.1 interacting at the *Nfkb1* promoter. Chromatin was prepared from freshly isolated untreated and LPS stimulated B cells, and immunoprecipitated with anti-PU.1 or control Abs. WT and *PUB* B cells were LPS stimulated for 16 h, as this time-point provided increased p50 expression upon LPS exposure (Souvannavong et al, 2007). Relative amounts of immunoprecipitated DNA was determined by qPCR from the *Nfkb1* promoter region, the positive control *Mef2c* gene enhancer (DeKoter et al, 2010), and the negative control *Hprt*. ChIP analysis confirmed less PU.1 occupancy at the *Nfkb1* promoter of both freshly isolated untreated and LPS treated *PUB* B cells, whereas no significant difference occurred at *Hprt* (Fig. 2.9F-G). Together, these data indicate that both PU.1 and Spi-B directly activate *Nfkb1* transcription in mice, suggesting that reduced p50 expression in *PUB* B cells is the result of reduced PU.1 and Spi-B.

Enrichment of PU.1 and Spi-B binding did not occur most highly at the promoter of *Nkfb1*. Several intronic regions of *Nfkb1* contained even larger PU.1 and Spi-B enrichment sites, suggesting that PU.1 or Spi-B may have higher affinity at enhancer regulatory elements within the gene body which could act as enhancers (Fig. 2.10A). Using the mouse intron as a template, an alignment was performed across multiple species on the intron outlined in Fig. 2.10A. It was found that there was a high degree of sequence similarity and a conserved ETS binding site within the intronic region (Fig. 2.10B). The conserved region containing the ETS binding site was cloned into a pGL3-SV40 vector, which contained the SV40 promoter upstream of the luciferase gene. When luciferase activity was measured in the pGL3-SV40 vector with the *Nfkb1* intron inserted, there was no increase in luciferase activity (Figure 2.10C). Therefore, the PU.1/Spi-B binding region in intron 6-7 of *Nfkb1* did not increase activation of the SV40 promoter.

Figure 2.9. *Nfkb1* is directly activated by PU.1 and Spi-B.

(A) *PUB* B cells express decreased p50 protein levels. Immunoblotting for p50 and β-actin was performed using enriched B cell lysates from WT and *PUB* mice. (B) Chromatin immunoprecipitation-sequencing (ChIP-seq) analysis of PU.1 and Spi-B interaction with the *Nfkb1* gene. Murine WEHI-279 B cell lymphoma cells expressing 3XFLAG-tagged PU.1 (top) or Spi-B (bottom) were analyzed using anti-FLAG ChIP-seq. Peaks located within and near the *Nfkb1* gene are shown, and the dashed box represents PU.1 and Spi-B binding at the *Nfkb1* promoter. (C) Schematic showing alignment of the annotated *Nfkb1* promoter between multiple species. Grey text represents conserved nucleotides, and boxes indicate predicted PU.1 binding sites with similarity score and strand location (+/-) listed. Circled nucleotide represents known TSS in the human *Nfkb1* promoter. Dots represent TSS upstream or downstream of the annotated sequence. (D) Luciferase reporter vector schematic with an arrow representing the murine *Nfkb1* TSS. The *Nfkb1* promoter sequence was cloned by PCR and ligated into pGL3-basic. Predicted ETS binding sites (wt) were mutated (mut) using site-directed mutagenesis. (E) Mutated ETS binding sites reduced promoter activity in WEHI-279 B cells. Cells were transfected with plasmids indicated on the *x*-axis. The *y*-axis indicates fold-induction of luciferase activity relative to pGL3-basic. Luciferase activity was normalized by transfection with *Renilla* luciferase expression vector. Data shows the mean ±SEM (n=5). (F) Interaction of PU.1 with the *Nfkb1* promoter is reduced in *PUB* B cells. ChIP data shows % enrichment relative to input for PU.1 interaction with *Nfkb1* and *Hprt* promoters, and is representative of three independent experiments. (G) Mean fold enrichment of PU.1 binding in *PUB* B cells normalized to WT B cells for indicated genes. Values of 1 (dashed line) indicate no difference in PU.1 binding between *PUB* and WT B cells as compared to IgG. ChIP was performed on chromatin prepared from untreated or LPS stimulated (16 h) freshly isolated splenic WT and *PUB* B cells using anti-PU.1 and control IgG Abs. qPCR was used to measure the amount of immunoprecipitated DNA after purification. Primers were designed to recognize promoters of *Nfkb1, Hprt,* and *Mef2c* enhancer. Data shows the mean ±SEM of three independent experiments. *$p<0.05$.

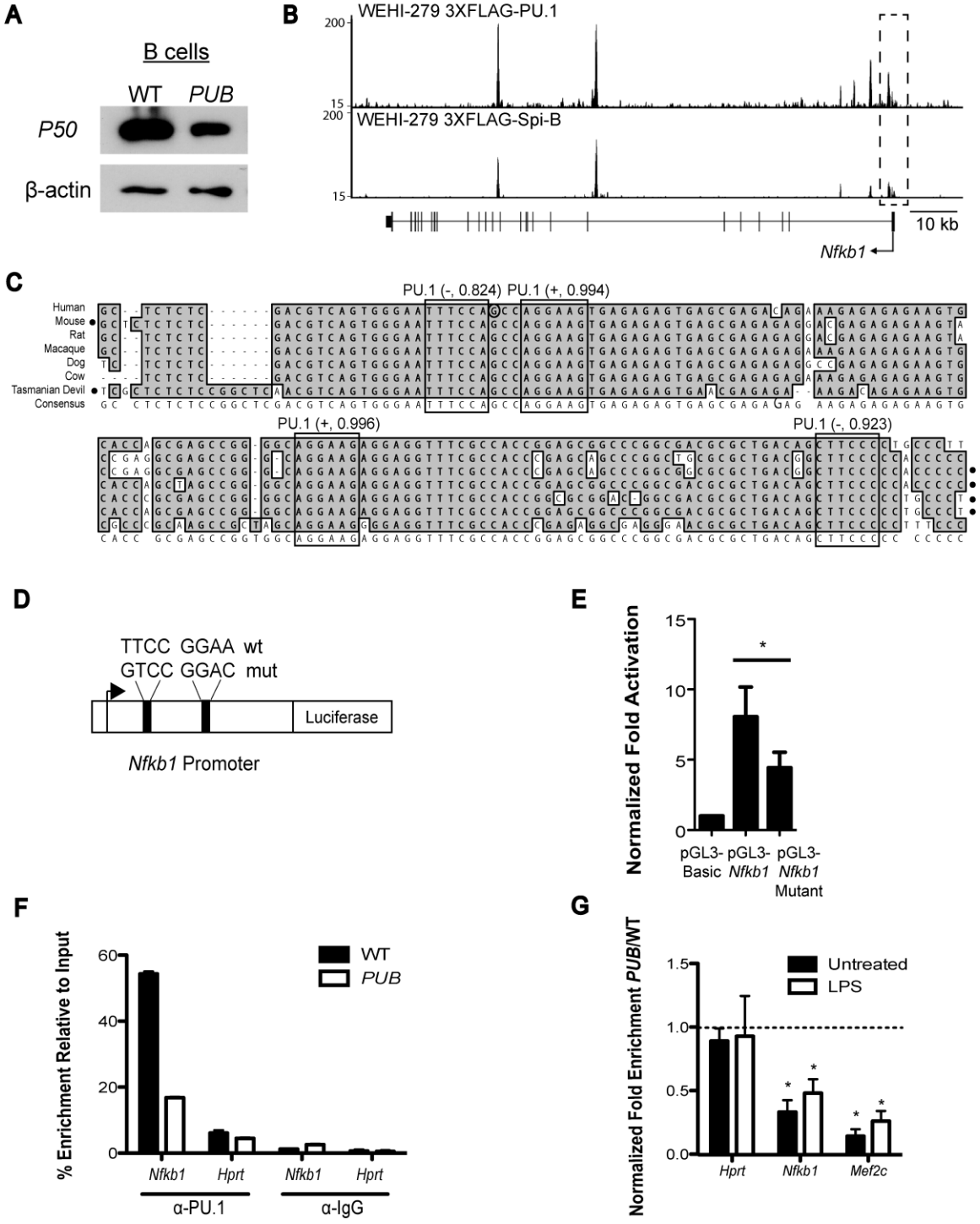
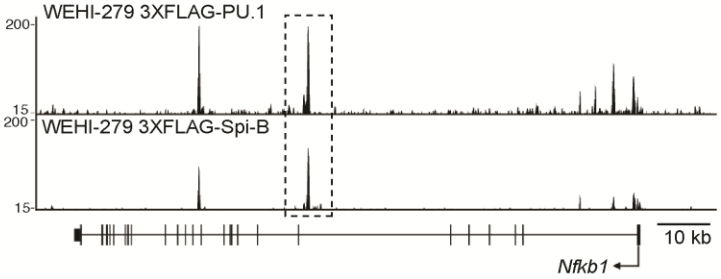

A
B cells
WT
PUB
P50
β-actin
B
WEHI-279 3XFLAG-PU.1
WEHI-279 3XFLAG-Spi-B
10 kb
Nfkb1
C
PU.1 (-, 0.824)
PU.1 (+, 0.994)
PU.1 (+, 0.996)
PU.1 (-, 0.923)
Human
Mouse
Rat
Macaque
Dog
Cow
Tasmanian Devil
Consensus
D
TTCC GGAA wt
GTCC GGAC mut
Luciferase
Nfkb1 Promoter
E
Normalized Fold Activation
pGL3-Basic
pGL3-Nfkb1
pGL3-Nfkb1 Mutant
F
% Enrichment Relative to Input
WT
PUB
Nfkb1
Hprt
α-PU.1
α-IgG
G
Normalized Fold Enrichment PUB/WT
Untreated
LPS
Hprt
Nfkb1
Mef2c

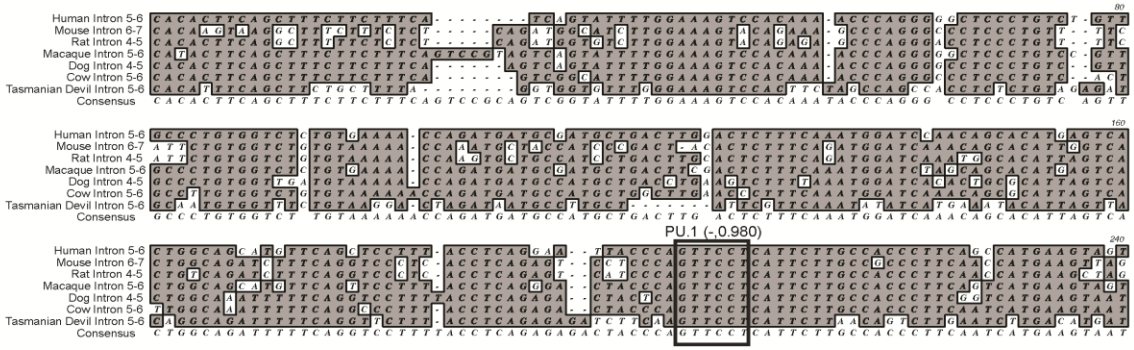
Figure 2.10. Analysis of PU.1/Spi-B binding in the *Nfkb1* intron.

(A) PU.1 and Spi-B bind to the intron of *Nfkb1*. ChIP-seq analysis was performed in WEHI-279 3XFLAG-PU.1 and WEHI-279 3XFLAG-Spi-B cells, and immunoprecipitated with anti-FLAG antibody. The dashed box indicates the intron region of *Nfkb1* where PU.1 and Spi-B bind. (B) Alignment of the *Nfkb1* intron across multiple species where PU.1 and Spi-B bind. The region has a high degree of similarity shown by the grey highlighted nucleotides. The sequence in the box is the ETS binding site predicted by MatInspector. (C) The conserved intron sequence (400 bp) was cloned into the pGL3-SV40 vector, and luciferase activity was measured in transfected WEHI-279 cells.

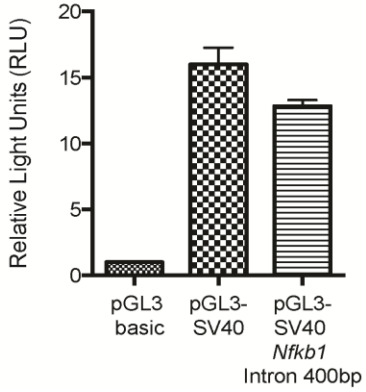
A


WEHI-279 3XFLAG-PU.1
200
15
WEHI-279 3XFLAG-Spi-B
200
15
10 kb
Nfkb1

B


Human Intron 5-6
Mouse Intron 6-7
Rat Intron 4-5
Macaque Intron 5-6
Dog Intron 4-5
Cow Intron 5-6
Tasmanian Devil Intron 5-6
Consensus
PU.1 (-,0.980)

C


Relative Light Units (RLU)
20
15
10
5
0
pGL3 basic
pGL3-SV40
pGL3-SV40 Nfkb1 Intron 400bp

2.2.6 Forced expression of p50 or c-Rel in *PUB* B cells increases TLR-mediated proliferation

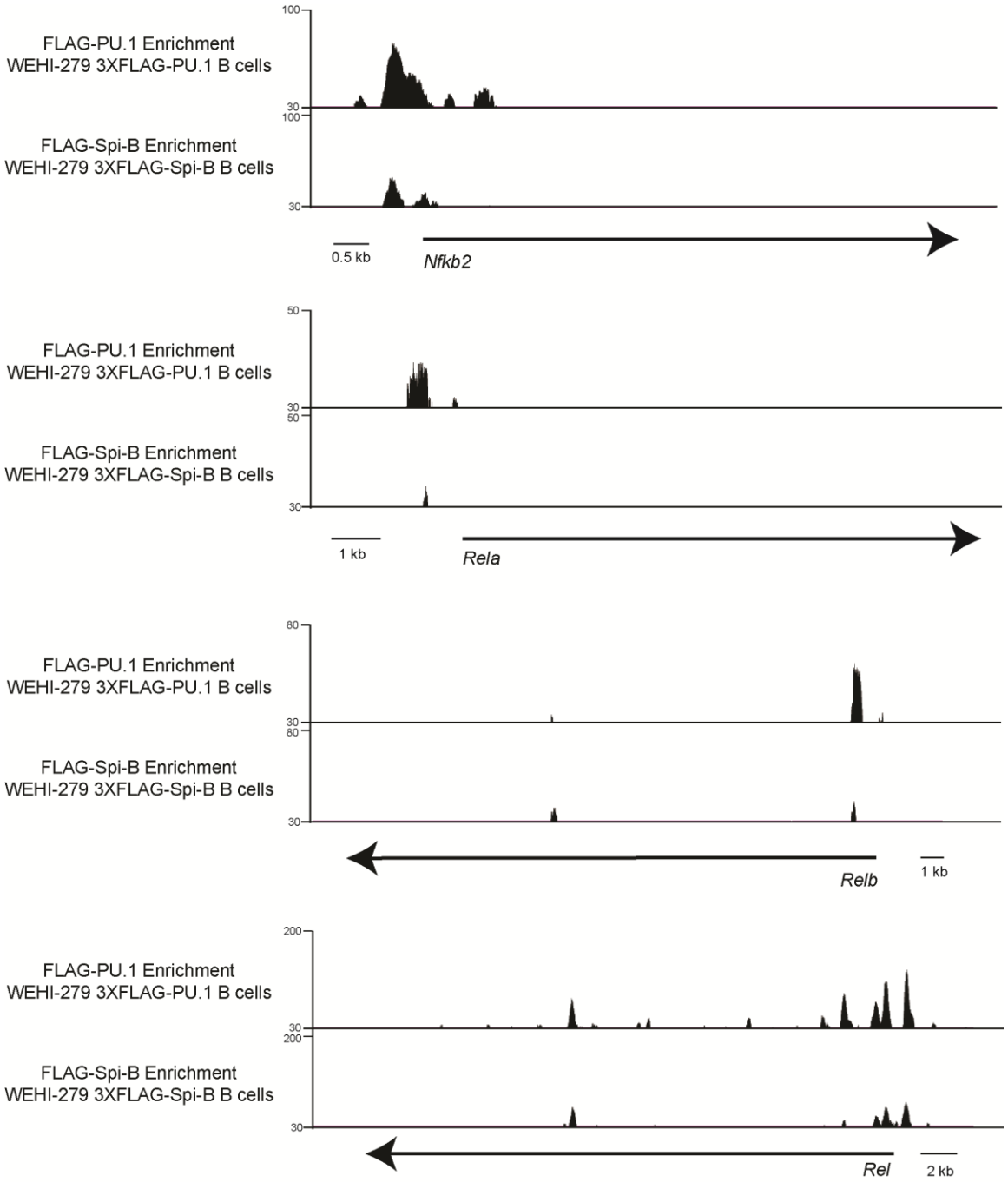
PU.1 and Spi-B have been previously reported to directly regulate *Rel* transcription (Hu et al, 2001). The *Rel* gene encodes for the NF-κB family member c-Rel, which is required for survival and proliferation of B cells (Hu et al, 2001; Kontgen et al, 1995). Lower transcript levels of both *Rel* and *Nfkb1* were detected in LPS stimulated *PUB* B cells (Fig. 2.7F). Therefore it is conceivable that forced expression of c-Rel or p50 could increase TLR-mediated proliferation in *PUB* B cells.

To assess whether PU.1 and Spi-B could directly bind near promoters of other NF-κB family member, re-analysis of ChIP-seq data in WEHI-279 3XFLAG-PU.1 and WEHI-279 3XFLAG-Spi-B cell was performed. It was confirmed that PU.1 and Spi-B can bind proximal to the TSS of *Nfkb2, Rela, Relb,* and *Rel* (Figure 2.11). Therefore, PU.1 and Spi-B may be involved with the transcriptional regulation of genes for all NF-κB family members in B cells.

To determine if the *PUB* phenotype could be complemented by forced p50 or c-Rel expression, a retroviral vector was constructed encoding FLAG-tagged p50 (MIG-P50) (Fig. 2.12A). A previously described retroviral vector containing c-Rel in the MIGR1 vector was used to generate MIG-cRel virus (Sanjabi et al, 2005). FLAG-tagged p50 protein was confirmed to be expressed in retroviral packaging cells transfected with MIG-P50 using immunoblot (Fig. 2.12B). WT and *PUB* splenic B cells were enriched by magnetic separation and stimulated with LPS and BAFF for 24 h to improve B cell survival, followed by infection with MIG-P50 or MIGR1 virus as a control. Green fluorescence, indicating B cell infection, was determined by flow cytometry (Fig. 2.12C). Next, proliferation in response to LPS was assessed 24 hours post infection using MTT assay (Fig. 2.12D). Despite low infection frequencies, both WT and *PUB* splenic B cells infected with MIG-P50 virus had significantly increased proliferation compared to MIGR1 infected cells (Fig. 2.12E). These data therefore suggest that forced p50 expression restores proliferation in *PUB* B cells.

Figure 2.11. PU.1 and Spi-B bind near TSS of all NF-κB family members in WEHI-279 B cells.

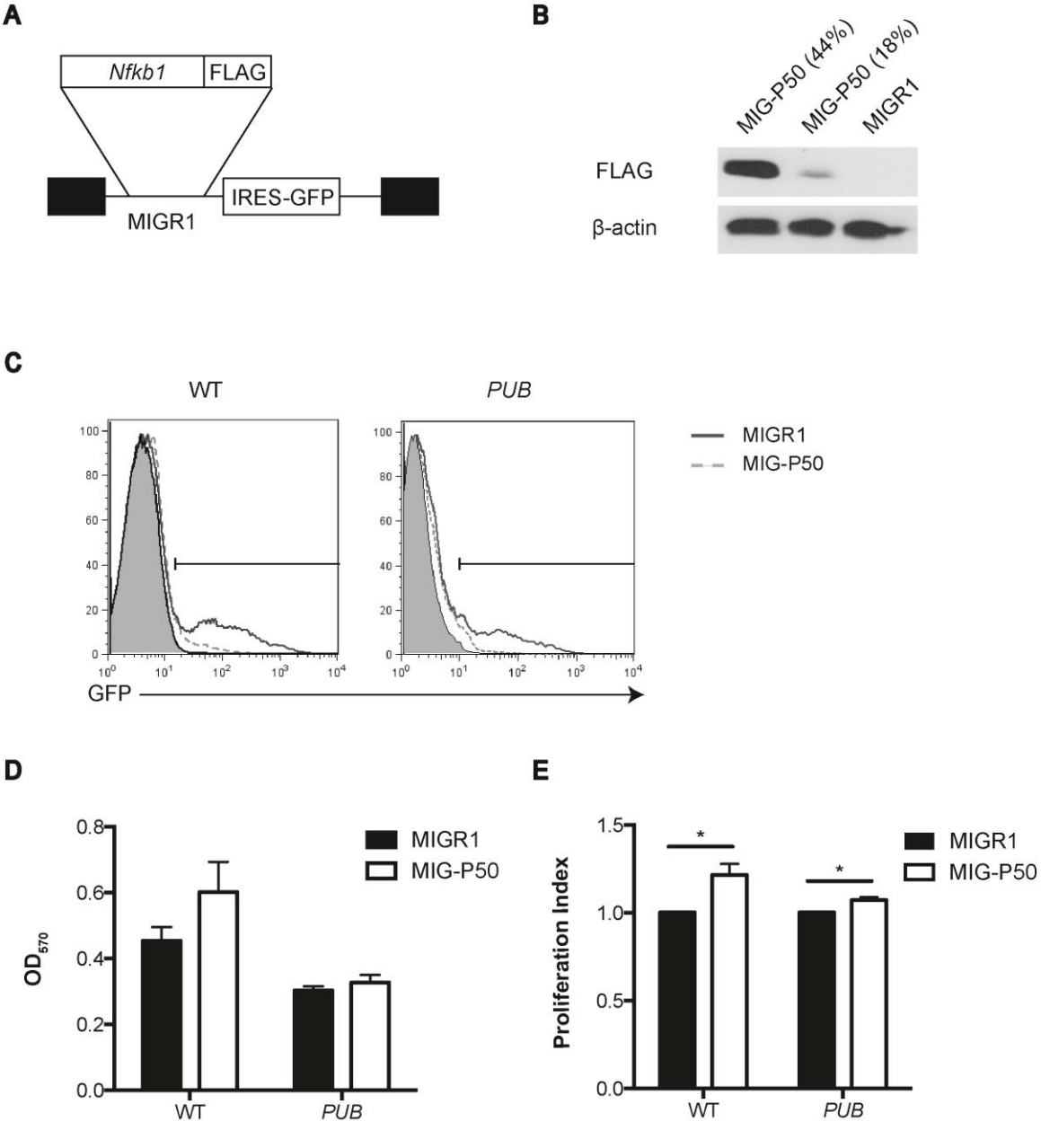
FLAG-tagged PU.1 and Spi-B enrichment at all NF-κB family members is demonstrated by enriched sequence tags. ChIP-seq was performed using anti-FLAG antibody on WEHI-279 3XFLAG-PU.1 and WEHI-279 3XFLAG-Spi-B cells.


FLAG-PU.1 Enrichment
WEHI-279 3XFLAG-PU.1 B cells
100
30
FLAG-Spi-B Enrichment
WEHI-279 3XFLAG-Spi-B B cells
100
30
0.5 kb
Nfkb2
FLAG-PU.1 Enrichment
WEHI-279 3XFLAG-PU.1 B cells
50
30
FLAG-Spi-B Enrichment
WEHI-279 3XFLAG-Spi-B B cells
50
30
1 kb
Rela
FLAG-PU.1 Enrichment
WEHI-279 3XFLAG-PU.1 B cells
80
30
FLAG-Spi-B Enrichment
WEHI-279 3XFLAG-Spi-B B cells
80
30
Relb
1 kb
FLAG-PU.1 Enrichment
WEHI-279 3XFLAG-PU.1 B cells
200
30
FLAG-Spi-B Enrichment
WEHI-279 3XFLAG-Spi-B B cells
200
30
Rel
2 kb

For efficient retroviral integration, B cells needed to be in a state of division. Since *PUB* B cells proliferated poorly in response to LPS alone (Fig. 2.6A, C), WT or *PUB* B cells were stimulated with LPS, IL-2, IL-4, and IL-5 (IL-2+4+5), conditions previously reported to improve survival (Grumont et al, 1998). LPS+IL-2+4+5 had minimal effects on proliferation/survival in unstimulated or LPS stimulated WT B cells measured using the MTT assay (Fig. 2.13A). However, the addition of IL-2+4+5 increased the frequency of *PUB* B cells that proliferated/survived compared to LPS alone (Fig. 2.13A). To differentiate between a proliferation or survival effect of IL-2+4+5 on *PUB* B cells, cells were stained with the proliferation dye eFluor® 450 prior to stimulation, and the dilution of eFluor® 450 was assessed 72 h later by flow cytometry. WT and *PUB* B cells responded minimally to IL-2+4+5 alone, based on the dilution of eFluor® 450 (Fig. 2.13B). In WT B cells, addition of IL-2+4+5 did not affect LPS-stimulated dilution of eFluor® 450 dye. In contrast, the frequency of cells that diluted eFluor® 450 dye was dramatically increased in *PUB* B cells stimulated with LPS and IL-2+4+5 compared to LPS alone (Fig. 2.13B). These results suggested that IL-2+4+5 increased survival in LPS stimulated *PUB* B cells.

Figure 2.12. Forced expression of p50 significantly restores proliferation in *PUB* B cells.

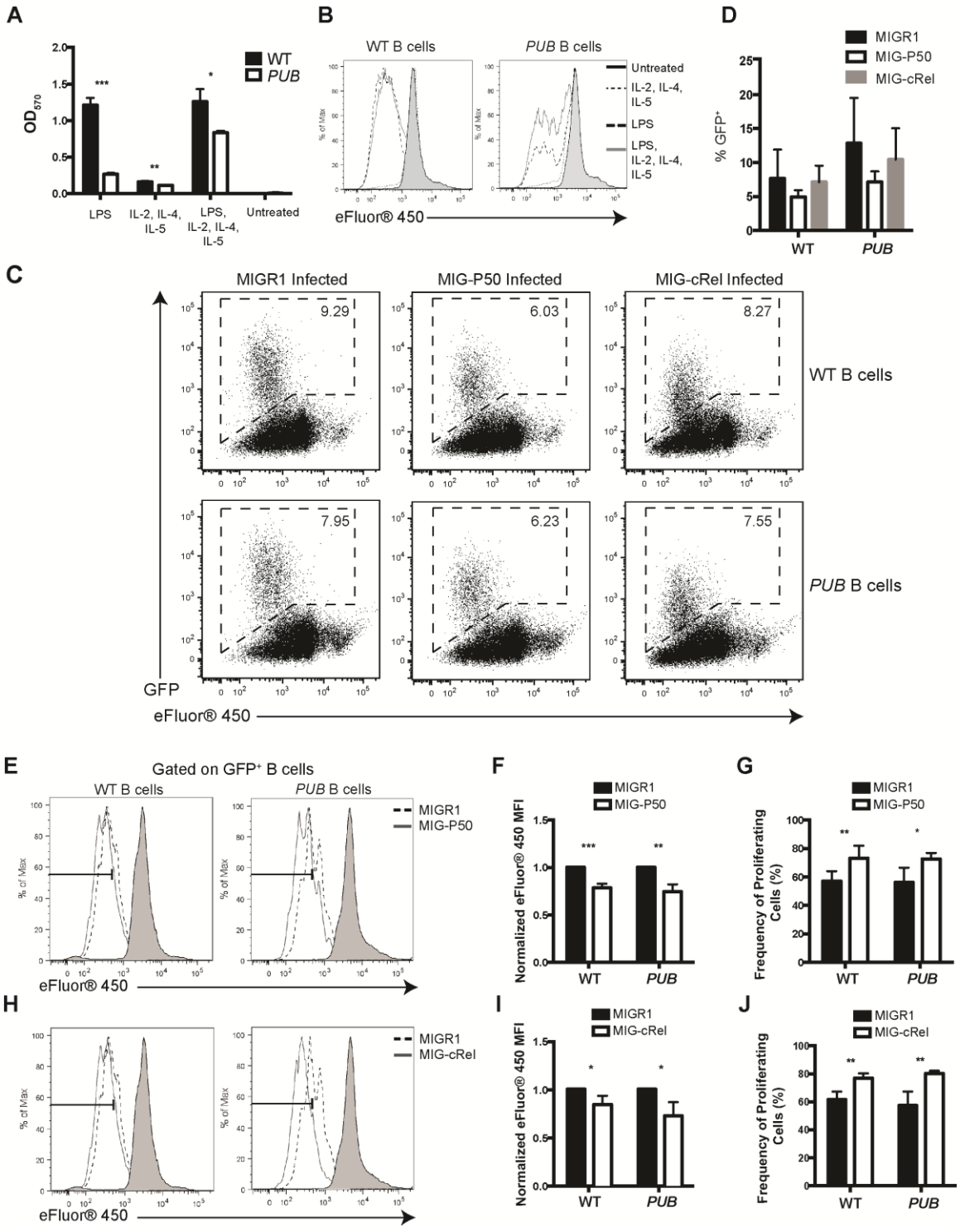
(A) Schematic of the retroviral vector encoding FLAG-tagged *Nfkb1* and GFP. (B) P50 expression in transfected Plat-E cells. Immunoblot analysis was performed using anti-FLAG and anti-β-actin antibodies. Cell lysates were generated from Plat-E lysates transfected with MIGR1 and MIG-P50 at high (44%) and low (18%) transfection efficiencies. (C) Infection frequencies as determined by flow cytometry of green fluorescence. WT splenic B cells were infected with MIGR1 (solid line, 18%) and MIG-P50 (dashed line, 5%) virus. *PUB* splenic B cells were infected with MIGR1 (solid line, 16%) and MIG-P50 (dashed line, 4%) virus. Filled histograms indicated uninfected cells. (D) Proliferation of WT and *PUB* B cells 24 h post-infection with MIGR1 and MIG-P50 virus. Cells were stimulated 24 h with LPS + BAFF prior to infection. Proliferation was assessed via a MTT proliferation assay. Data shows the mean ±SD of duplicate measurements, and is representative of 5 individual experiments. (E) Quantitation of five individual experiments as performed in D. Data shows the mean ±SEM. Proliferation index was calculated by normalizing OD_{570} of MIG-P50 infected cells to MIGR1 infected cells in WT and *PUB*. *$p<0.05$.


A
Nfkb1
FLAG
IRES-GFP
MIGR1
B
MIG-P50 (44%)
MIG-P50 (18%)
MIGR1
FLAG
β-actin
C
WT
PUB
MIGR1
MIG-P50
GFP
D
OD570
MIGR1
MIG-P50
WT
PUB
E
Proliferation Index
MIGR1
MIG-P50
WT
PUB

Next, to determine whether increased expression of p50 or c-Rel could increase proliferation in infected WT or *PUB* cells, B cells were stimulated with LPS and IL-2+4+5 for 24 h, then infected with MIGR1 (control), MIG-P50, or MIG-cRel retrovirus. GFP expression was used as a marker for p50 or c-Rel infection, and a GFP^+ population was gated for proliferation analysis (Fig. 2.13C). WT and *PUB* B cells were infected with MIGR1, MIG-P50, and MIG-cRel retroviruses with similar frequencies (Fig. 2.13D). Next, proliferation was assessed for gated GFP^+ p50-infected cells using eFluor® 450 staining and flow cytometry 48 h post infection. All cells that expressed GFP had also diluted eFluor® 450, compared to GFP negative cells, which was expected since retroviral infection requires cell division (Fig. 2.13E). For both WT and *PUB* GFP^+ B cells infected with MIG-P50, there was an increase in the frequency of cells that had diluted eFluor® 450 compared to cells infected with MIGR1, suggesting one or more extra rounds of cell division (Fig. 2.13E). This result was statistically significant based on five independent experiments, and expressed either as a decrease in the eFluor® 450 mean fluorescence intensity (MFI) gated on GFP^+ cells (Fig. 2.13F), or as the relative frequency of cells diluting eFluor® 450 dye past the gate shown in Fig. 7G (Fig. 2.13G). Finally, proliferation was assessed on MIG-c-Rel infected B cells (gated GFP^+ cells) by eFluor® 450 staining using flow cytometry 48 h post infection (Fig. 2.13H). Similar to MIG-P50 infection, MIG-cRel infection in WT and *PUB* B cells resulted in a greater degree of proliferation compared to MIGR1 infected cells (Fig. 2.13I). In addition, a greater frequency of B cells proliferated when infected with MIG-cRel, compared to MIGR1 infection (Fig. 2.13J). Overall, these results demonstrated that infection with retroviral vectors encoding p50 or c-Rel expression was capable of increasing TLR-mediated proliferation in WT and *PUB* B cells.

Figure 2.13. Forced expression of p50 or c-Rel increases proliferation in *PUB* B cells.

(A) Proliferation of WT and *PUB* B cells in response to LPS and IL-2+4+5. Proliferation was assessed 72 h post stimulation using an MTT proliferation assay. Data shows the mean ±SD of triplicate measurements, and was representative of 2 individual experiments. (B) *PUB* B cells have enhanced survival when stimulated with IL-2+4+5 in combination with LPS (grey line), compared to LPS (dashed line) or IL-2+4+5 (dotted line) alone. Shaded histogram represents untreated B cells. Proliferation was assessed by the dilution of the proliferation dye eFluor® 450 using flow cytometry 72 h post stimulation. Data shown are representative of three independent experiments. (C) MIGR1, MIG-P50, and MIG-cRel infected B cells were identified by GFP expression (dashed gate) using flow cytometry. B cells were labeled with proliferation dye eFluor® 450 and stimulated with LPS and IL-2+4+5 for 24 h prior to infection with virus. (D) Similar infection frequencies between MIGR1, MIG-P50, and MIG-cRel infected WT and *PUB* B cells based on gated population in C. (E) GFP^+ B cells were gated and assessed for dilution of eFluor® 450 to compare proliferation between MIG-P50 (dashed line) and MIGR1 (solid line) infected B cells. Shaded histogram represents unstimulated control B cells. (F) WT and *PUB* B cells infected with MIG-P50 proliferate and have increased dilution of eFluor® 450 compared to MIGR1-infected cells. Results are expressed as the mean fluorescence intensity (MFI) of gated GFP^+ cells. (G) Increased frequency of proliferating cells observed in MIG-P50 infected cells compared to MIGR1 infected cells. Results are expressed as the mean frequency of cells that diluted eFluor® 450 past the gate shown in panel E. (H) GFP^+ B cells were gated and assessed for dilution of eFluor® 450 to compare proliferation between MIG-c-Rel (dashed line) and MIGR1 (solid line) infected B cells. Shaded histogram represents unstimulated control B cells. (I) WT and *PUB* B cells infected with MIG-c-Rel proliferate more than MIGR1 infected cells, based on dilution of eFluor® 450. (J) Increased frequency of proliferating cells observed in MIG-c-Rel infected cells compared to MIGR1 infected cells. Results are expressed as the mean frequency of cells that diluted eFluor® 450 past the gate shown in panel J. For F-G and I-J, data is a quantitation of five individual experiments as performed in E and J respectively. Data shows the mean ±SD. Normalized MFIs in F and I were calculated by dividing MFI values of MIG-P50/MIG-cRel infected cells to MFI values of MIGR1 infected cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$.


A
B
C
D
E
F
G
H
I
J
WT
PUB
LPS
IL-2, IL-4, IL-5
LPS, IL-2, IL-4, IL-5
Untreated
WT B cells
PUB B cells
eFluor® 450
MIGR1
MIG-P50
MIG-cRel
% GFP+
MIGR1 Infected
MIG-P50 Infected
MIG-cRel Infected
9.29
6.03
8.27
7.95
6.23
7.55
GFP
Gated on GFP+ B cells
Normalized eFluor® 450 MFI
Frequency of Proliferating Cells (%)

2.3 Discussion

The goal of this study was to understand how TLR-initiated signaling in B cells is regulated. B cells from *PUB* mice proliferated poorly in response to TLR ligands. LPS stimulated *PUB* B cells were activated and did not have increased apoptosis relative to WT cells. The mechanism for the *PUB* phenotype revealed reduction in steady-state and LPS stimulated levels of *Nfkb1* transcripts, and accordingly lower p50 protein levels. Mutation of two of the three ETS binding sites within the cloned *Nfkb1* promoter reduced its activation, suggesting that PU.1 and Spi-B regulate its transcription. PU.1 and Spi-B interacted with the *Nfkb1* promoter as shown by ChIP analysis, and PU.1 occupancy at the *Nfkb1* promoter was decreased in *PUB* B cells. Proliferation of *PUB* B cells was increased upon retroviral complementation with p50. In summary, these results suggest that activation of *Nfkb1* by PU.1 and/or Spi-B promotes TLR-mediated splenic B cell proliferation.

PU.1 and Spi-B are thought to be able to bind identical DNA binding sites (Ray-Gallet et al, 1995). This idea was supported by our ChIP-seq results showing that PU.1 and Spi-B directly bind to the *Nfkb1* promoter. Multiple TLR signaling genes have been characterized as direct PU.1 targets, including *Tlr4*, *Tlr9*, *Btk*, and *Rel* (Hu et al, 2001; Muller et al, 1996; Roger et al, 2005; Schroder et al, 2007). It is likely that altered protein levels of these genes may contribute to the *PUB* B cell phenotype in addition to reduced p50. Notably, transcript levels of *Rel* were reduced in *PUB* B cells stimulated with LPS. The *Rel* gene encodes the NF-κB family member c-Rel, which is important for survival and proliferation in B cells (Hu et al, 2001; Kontgen et al, 1995). Reduced c-Rel expression impairs B cell proliferation, as $Rel^{+/-}$ and $Rel^{-/-}$ B cells proliferate poorly in response to LPS or anti-IgM (Kontgen et al, 1995). Therefore, it is likely that impaired proliferation of *PUB* B cells in response to LPS or anti-IgM is explained by reduced p50 and c-Rel (Hu et al, 2001). Proliferation in $Rel^{-/-}$ B cells is reported to be more impaired than $Nfkb1^{-/-}$ B cells in response to either LPS or anti-IgM (Grumont et al, 1998; Kontgen et al, 1995; Pohl et al, 2002). In addition, $Rel^{+/-}$ B cells have reduced proliferation in response to LPS or anti-IgM compared to WT B cells, but there is no difference in levels of proliferation between $Nfkb1^{+/-}$ and WT B cells for those same mitogens (Sha et al,

1995; Snapper et al, 1996b). LPS stimulated *Nfkb1*$^{-/-}$ B cells fail to proliferate, anti-IgM stimulation results in only a small reduction in proliferation, and anti-CD40/CD40L stimulation results in normal proliferation (Garrett-Sinha et al, 1999; Sha et al, 1995; Snapper et al, 1993). Deletion of both NF-κB1 and c-Rel (*Nfkb1*$^{-/-}$*Rel*$^{-/-}$) in B cells results in a further impaired response to LPS or anti-IgM compared to *Nfkb1*$^{-/-}$ or *Rel*$^{-/-}$ B cells. Future studies will need to be done to determine whether restoration of both p50 and c-Rel together can further improve proliferation responses in *PUB* B cells.

PUB B cells were unable to increase MHCII or CD40 expression upon LPS or anti-IgM stimulation. The impairment of MHCII and CD40 upregulation upon anti-IgM expression could be explained by increased apoptosis in *PUB* B cells. CD40 was functional in *PUB* B cells since anti-CD40 stimulation appeared normal despite decreased levels of CD40 on *PUB* B cells, furthermore, LPS+anti-CD40 stimulation was able to rescue defective LPS-mediated proliferation. Failure to upregulate MHCII and CD40 upon stimulation could result in decreased protein interaction necessary for TLR activation, which could be a consequence of reduced p50 signaling. *PUB* B cells were activated by LPS as measured by CD25 and CD69, but CD69 was not upregulated as high as WT B cells. The *Cd69* gene promoter has multiple PU.1 binding sites, and is transcriptionally regulated by NF-κB complexes (Castellanos et al, 1997; Lopez-Cabrera et al, 1995). Therefore, reduced MHCII, CD40, and CD69 upregulation upon LPS stimulation may be a consequence of reduced p50 expression.

Although proliferation in *PUB* B cells was impaired upon TLR or IgM stimulation alone, the combination of LPS+anti-IgM synergistically restored proliferation and activation to WT levels in *PUB* B cells. The molecular basis of this observation is unclear, but a comparable finding was made using *Nfkb1*$^{-/-}$, *Rel*$^{-/-}$, or *Nfkb1*$^{-/-}$*Rel*$^{-/-}$ B cells, where LPS synergized with anti-IgM when inducing proliferation (Pohl et al, 2002). Furthermore, LPS and IL-2+4+5 were used to maximize cell survival to allow B cells into early-log phase growth for viral infection. The combination of IL-2+4+5 was previously reported to not induce proliferation on its own, but synergized with proliferative responses to mitogens in WT, *Rel*$^{-/-}$, and *Nfkb1*$^{-/-}$ B cells (Grumont et al, 1998). Similarly, *PUB* B cells did not proliferate highly in response to IL-2+4+5 alone,

but the proliferation response was increased in response to LPS and IL-2+4+5. This synergistic proliferation observed in *PUB* B cells is likely due to multiple or alternate signaling pathways becoming activated, that are not regulated by PU.1 and Spi-B. For example, LPS can also activate PI3K (Bone & Williams, 2001) or mitogen-activated protein kinase (MAPK) pathways (Gerondakis et al, 2007). Therefore, there are likely alternative pathways that can compensate in the event of having loss of PU.1 and Spi-B function. Further analysis of PU.1 and Spi-B involvement on the activation of these pathways through TLR ligands is required.

PUB mice have reduced frequencies of B cells with an increased ratio of MZ to FO B cells, indicating PU.1 and Spi-B may regulate peripheral B cell differentiation (DeKoter et al, 2010). FO and MZ B cell differentiation both require canonical NF-κB signaling. Weak BCR signaling promotes MZ B cell development, whereas strong BCR signaling drives FO B cell development (Pillai & Cariappa, 2009). *Nfkb1*$^{-/-}$ mice have MZ B cells, although in decreased numbers (Cariappa et al, 2000), and MZ B cells accumulate over time in spleens of these mice (Ferguson & Corley, 2005). Therefore, increased MZ B cell and decreased FO B cell frequencies in *PUB* spleens might be caused by a combination of altered NF-κB signaling, weakened BCR signaling, and increased apoptosis upon BCR stimulation.

In summary, our experiments establish the importance of PU.1 and Spi-B for innate immune responses in B cells. Decreased p50 and reduced *Nfkb1* gene activation in *PUB* mice is associated with poor TLR-mediated B cell proliferation. Understanding TLR regulation in B cells has implications for generation of antibody responses, since effective thymus-independent responses and production of antibodies in vaccinations are dependent on direct TLR triggering on B cells (Kasturi et al, 2011). Further analysis of mice lacking Spi-B and reduced PU.1 is expected to provide additional insight into other genes involved in TLR signaling and antibody forming responses.

2.4 Materials and Methods

2.4.1 Generation and breeding of mice

Mice were housed at Western University's Health Sciences animal facility (London, Ontario, Canada) and monitored under an approved animal use subcommittee protocol in accord with Western University Council on Animal Care. C57BL/6 (WT) mice were purchased from Charles River Laboratories (Pointe-Claire, Quebec, Canada). *PUB* mice were generated by mating male and female *PUB* mice, and genotyping was performed by PCR as previously described (Garrett-Sinha et al, 1999; Su et al, 1997). Experiments were performed on mice aged 6-16 wks.

2.4.2 B cell enrichment and proliferation analysis

RBCs were removed from spleen cell suspensions by hypotonic lysis with ammonium chloride solution. B cells were enriched by negative selection using biotin-conjugated anti-CD43 (S7) Ab, streptavidin (SA) microbeads, LD depletion columns, and a VarioMACS™ separation unit (Miltenyi Biotec, Germany). Enrichment of B cells provided a >95% pure B cell population as determined by flow cytometry. B cells ($2x10^5$/well) were plated in 96-well flat bottom plates and stimulated with LPS (10 μg/mL, List Biological Laboratories), anti-IgM Ab (50 μg/mL, affinity pure F(ab')2 fragment, goat anti-mouse IgM, μ chain specific; Jackson ImmunoResearch Laboratories), Pam3CSK4 (1 μg/mL), HKLM (10^8 cells/mL), Poly(I:C) LMW or HMW (10 μg/mL), ST-FLA (10 μg/mL). FSL1 (1 μg/mL), ODN1826 (5 uM, InvivoGen), BAFF (100 ng/mL, Peprotech, New Jersey, USA), or LEAF™ purified anti-mouse CD40 (10 μg/mL; IC10, BioLegend) in complete DMEM. Proliferation was assessed after 72 h incubation at 37°C with a TACS® MTT Cell Proliferation assay (Trevigen) used according to the manufacturer's instructions. For [^{3}H]-thymidine incorporation assays, [^{3}H]-thymidine (1 mCi/mL/well) was added after 72 h stimulation, followed by scintillation counting 18 h later.

2.4.3 Flow cytometry

Antibodies purchased from eBioscience (San Diego, CA) or BioLegend (San Diego, CA) included allophycocyanin (APC)-conjugated anti-B220 (RA3-6B2), anti-MHCII [I-A/I-E (M5.144.15.2)], anti-CD40 (3/23), BAFF-R (eBio7H22-E16), phycoerythrin (PE)-conjugated anti-CD19 (1D3), IgG Isotype Control (eBio299Arm), anti-CD69 (H1.2F3), anti-CD281/TLR1 (eBioTR23), anti-CD282/TLR2 (T2.5), IgG2a κ Isotype Control (eBM2a), anti-CD14 (Sa14-2), anti-CD180/RP105 (RP/14), fluorescein isothiocyanate (FITC)-conjugated anti-CD21/CD35 (eBio8D9), Alexa Fluor®488-conjugated anti-CD1d (1B1), Biotin-conjugated anti-CD25 (7D4), anti-CD5 (53-7.3), or SA conjugated-PE. For proliferation analyses, cells were stained with the proliferation dye eFluor® 450 (eBioscience). Antibody-stained cell analysis and sorting was performed using a FACSCalibur and FACSAriaIII system respectively (BD Biosciences, San Jose, CA). Sorted cells were determined to be >98% purity. Data analysis was performed using FlowJo software (FlowJo LLC, Ashland, OR).

2.4.4 Reverse transcription-quantitative PCR

RNA was isolated using Trizol® reagent (Life Technologies Inc. Burlington ON). cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, Ontario, Canada), and qPCR was performed with a Rotor-Gene 6000 instrument (Corbett Life Sciences, Valencia, CA). Relative mRNA transcript levels were normalized to GAPDH or β2m and compared between samples using the comparative threshold cycle method. Calculations were performed using REST© 2009 software (Pfaffl et al, 2002). Primer sequences are listed in Table 2.1.

2.4.5 Immunoblot analysis

Lysates were prepared using the Laemelli method and applied to 8%-10% SDS polyacrylamide gels for electrophoresis. Proteins were transferred to nitrocellulose membranes using a Trans-Blot Semi-Dry system (Bio-Rad), and membranes were blocked for 1 h in 5% milk in TBST. Membranes were probed with polyclonal anti-MyD88 (eBioscience), anti-Btk (B3H5, Cell Signaling Technology, Whitby ON), anti-

Nfkb1 (Poly6197, BioLegend), or anti-β-Actin (I-19, Santa Cruz Biotechnology) Ab, and diluted to concentrations recommended by their manufacturers in 1% milk/TBST overnight at 4°C. A secondary horseradish peroxidase-conjugated anti-goat or anti-mouse Ab was incubated in 1% milk/TBST. Membranes were washed and visualized with SuperSignal West Pico reagent (Thermo-Fisher Scientific, Waltham MA).

2.4.6 Bioinformatic analysis

A TSS was previously described for the human *NFKB1* promoter (Cogswell et al, 1993). DNA sequences from human, mouse, rat, macaque, dog, cow, and Tasmanian devil were obtained from the Ensembl database and aligned using MacVector (Accelrys, San Diego, CA). Predicted transcription factor binding sites were analyzed using MatInspector (Genomatix, Ann Arbor, MI), with scores >0.9 selected for further analysis.

2.4.7 Plasmids and cloning

The *Nfkb1* promoter was amplified from C57BL/6 genomic DNA by PCR using LA-TAQ (TaKaRa Clontech Laboratories Inc, Mountain View, CA), purified, and cloned into pSC-A vector using a PCR cloning kit (Agilent Technologies, La Jolla, CA). A 468bp *Nfkb1* promoter fragment was PCR amplified using a forward primer containing a HindIII site and subcloned. The promoter was HindIII digested from pSC-A and ligated into the HindIII site of the pGL3-basic (Promega) luciferase reporter vector. Predicted ETS binding sites were mutated using the QuickChange lightning site-directed mutagenesis kit according to the manufacturer's instructions (Agilent Technologies). The murine stem cell virus-internal ribosomal entry site-green fluorescent protein (MSCV-IRES-EGFP; MIGR1) retroviral vector has been described (Pear et al, 1998). MSCV-P50-IRES-EGFP (MIG-P50) was generated by cloning p50-FLAG from the p50 cFlag pcDNA3 vector purchased from Addgene (Plasmid 20018; Cambridge, MA) by XhoI and EcoRI digestion. P50-FLAG was cloned into the psC-A vector, excised with EcoRI, and ligated into the MIGR1 vector. MIG-cRel (Plasmid 26984) was obtained from Addgene (Cambridge, MA) (Sanjabi et al, 2005). All constructs were verified by DNA sequencing. Transfection-quality plasmid DNA was prepared by growth in DH5α bacteria and purified with plasmid maxi prep kit (Qiagen). All restriction enzymes were purchased

from New England Biolabs (Ipswich, MA). Primer sequences used for cloning are listed in Table 2.1.

2.4.8 Generation of retrovirus

MIG-P50, MIG-cRel, and MIGR1 retroviral vectors were generated by transient transfection using PEIpro™ (Polyplus-transfection, Illkirch, France) into Platinum-E (Plat-E) packaging cells (Morita et al, 2000). Plat-E cells were maintained in complete DMEM (1.5 g/mL glucose) with blasticidin (10 μg/mL) and puromycin (1 μg/mL). Cells were passaged in antibiotic free media 72 h prior to transfection, then plated at $3x10^6$ cells per 100mm culture dish. A 2:1 PEI/DNA (20 μL PEIpro™ and 10 μg plasmid DNA) ratio was used for transfections, with a final working concentration for PEI at 0.5 μg/mL. Transfections were performed using DMEM without FBS or antibiotics for 4 h at 37°C. Cells were washed with fresh complete DMEM following transfection and incubated for 48h at 37°C. Virus-containing supernatants were collected following 48 h, and virus production was confirmed by green fluorescence and FLAG-tagged protein using flow cytometry and immunoblot respectively.

2.4.9 Retroviral infection of primary B cells and proliferation analysis

To assess proliferation in total B cells, B cells were isolated from spleens by magnetic enrichment as mentioned in 2.4.2 and stimulated for 24 h with BAFF (100 ng/mL) and LPS (10 μg/mL). Next, cells were infected by spinoculation with virus by centrifugation at 800 x g for 2 h at 30°C in the presence of polybrene at a final concentration of 4 μg/mL. After centrifugation, cells were cultured for another 24 h. Infection frequencies were detected by flow cytometric analysis for green fluorescence expression. Proliferation of total cells was assessed by MTT proliferation assay.

To assess proliferation specifically in infected B cells, B cells were isolated from spleens by magnetic enrichment as mentioned in 2.4.2 and stained with the cell proliferation dye eFluor® 450 (10 μM; eBioscience). B cells were stimulated for 24 h with LPS, IL-2 (10 ng/mL), IL-4 (10 ng/mL), and IL-5 (10 ng/mL). Next, cells were infected by spinoculation with virus by centrifugation at 800 x g for 2 h at 30°C in

Table 2.1. Forward and reverse primers used for RT-qPCR analysis, cloning, and site directed mutagenesis.

Primer Name	DNA Sequence (5’ -> 3’)	Product size
B2m fwd	5’-TGG CTC ACA CTG AAT TCA CCC CCA-3’	100 bp
rev	5’-TCT CGA TCC CAG TAG ACG GTC TTG-3’	
Cd14 fwd	5’-CCC AAG CAC ACT CGC TCA ACT TT-3’	106 bp
rev	5’-ATC AGT CCT CTC TCG CCC AAT-3’	
Rel fwd	5’-ACC CAA TTT ATG ACA ACC GTG CCC-3’	170 bp
rev	5’-ACC TCT GGC TTC CCA GTC ATT CAA-3’	
Gapdh fwd	5’-GAA CAT CAT CCC TGC ATC CA-3’	78 bp
rev	5’-CCA GTG AGC TTC CCG TTC A-3’	
Hprt ChIP fwd	5’-GGC CCA CCT AGT CAG ATA AGA GT-3’	193 bp
rev	5’-GAA AGC AGT GAG GTA AGC CCA AC-3’	
Myd88 fwd	5’-TAA GTT GTG TGT GTC CGA CCG TGA-3’	192 bp
rev	5’-ATC AGT CGC TTC TGT TGG ACA CCT-3’	
Traf6 fwd	5’-TTT ACG GGA AGC AGT GCA AAC ACC-3’	181 bp
rev	5’-ATT TGG GCA CTT TAC CGT CAG GGA-3’	
Irak3 fwd	5’-AGA GCT GGC TGC ATA TTT CAC GGA-3’	107 bp
rev	5’-GGG TTG TGC CAT TTG TGC ACT GTA-3’	
Irak4 fwd	5’-ACG GGC TTC GGC AAG GCT A-3’	192 bp
rev	5’-ATC CAG CAG TAG TTG AGG TTC ACG-3’	
Mef2c ChIP fwd	5’-GAA GGA AGC ACC TTT ACA CC-3’	125 bp
rev	5’-TTC AGC AAA TCC CTC CTA GT-3’	
Md2 fwd	5’-TAG AGT TGC CGA AGC GTA AGG AAG-3’	116 bp
rev	5’-TCC CTC GAA AGA GAA TGG TAT TGA-3’	
*Nfkb1 f*wd	5’-GCT GAG TCC TGC TCC TTC TAA A-3’	104 bp
rev	5’-CCT CTG TGT AGC CCA TCT GTT GC-3’	
Nfkb1 ChIP fwd	5’-TCC GTC TGT CTG CTC TCT-3’	123 bp

Rev	5’-GGT GGC GAA ACC TCC TC-3’	
Nfkb2 fwd	5’-CGG TGG AGA CGA AGT TTA TTT GCT C-3’	169 bp
rev	5’-ATC TTG TGA TAG GGC GGT GT-3’	
Rela fwd	5’-CGG GAT GGC TAC TAT GAG GCT GAC-3’	134 bp
rev	5’-GGG TTA TTG TTG GTC TGG ATT CGC-3’	
Relb fwd	5’-ACA CCC ACA TAG CCT CGT-3’	126 bp
rev	5’-ATT TCC TTC TTC CTA ACA CAC TGGA-3’	
Tlr1 fwd	5’-ACA GTC AGC CTC AAG CAT TTG GAC-3’	117 bp
rev	5’-TAC CCG AGA ACC GCT CAA CC-3’	
Tlr2 fwd	5’-ATG CTT CGT TGT TCC CTG TGT TGC-3’	125 bp
rev	5’-AAA GTG GTT GTC GCC TGC TTC CA-3’	
Tlr4 fwd	5’-ACT GTT CTT CTC CTG CCT GA-3’	102 bp
rev	5’-GGG ACT TTG CTG AGT TTC TGA TCC-3’	
Tlr7 fwd	5’-GTG ATG CTG TGT GGT TTG TCT GGT-3’	108 bp
rev	5’-CAC TTT GAC CTT TGT GTG CTC CTG-3’	
Tlr8 fwd	5’-TGG TTA TGT TGG CTG CTC TGG TTC-3’	110 bp
rev	5’-TTG GGA TGT GGA TGA AGT CCT GTA GC-3’	
Tlr9 fwd	5’-TGG ACG GGA ACT GCT ACT ACA AGA AC-3’	101 bp
rev	5’-CTT CAG AGA CAG ATG GGT GAG ATT GC-3’	
Tollip fwd	5’-GGT CGC CTC AGC ATC ACT-3’	104 bp
rev	5’-TCA TAA ACA GCA TAG CCC AGA CGCA-3’	
Nfkb1 Promoter fwd	5’-CCC AAA GAA TAA AGA CGC TCA AAT GC-3’	1745 bp
rev	5’-TCA GAA GAC CGA AGG AAG CCT ACA-3’	
Nfkb1 Sublone w/ HindIII	5’-CAA GCT TAC CTC CCG CCC CC-3’	468 bp
Rev	5’-CCT GGA GAT GCG GCG CGG-3’	
pGL3-*Nfkb1* Mutation	5’-TCT CGA CGT CAG TGG GAA TGT CCA GC-3’	N/A
pGL3-*Nfkb1* Mutation anti	5’-TCG CTC ACT CTC TCA CGT CCT GGC TGG-3’	

the presence of polybrene at a final concentration of 4 μg/mL. Infection frequencies were detected by flow cytometric analysis for green fluorescence expression. Proliferation of infected cells was assessed by the dilution of eFluor® 450 using flow cytometry 48h post infection.

2.4.10 Transient transfection by electroporation

WEHI-279 cells in mid to early log phase growth were washed three times with serum-free DMEM (4.5g/L, Lonza). Cells were incubated for 10 min at room temperature with 10 μg of each luciferase reporter plasmid and 1 μg of pRL-TK (Promega). Cell-DNA mixtures were electroporated at 220 V and 950 mF using 4-mm gap cuvettes and a GenePulser II with Capacitance Extender (Bio-Rad), incubated at room temperature for 10 min, then transferred to 6-well culture plates in complete DMEM and incubated at 37°C for 24 h. A Dual-Luciferase Reporter Assay System (Promega) was performed on cell lysates. Light production was measured using a Lumat LB 9507 tube luminometer (Berthold Technologies, Oak Ridge, TN).

2.4.11 ChIP-Seq Analysis

ChIP-seq experiments were performed using mouse WEHI-279 cells. Cells were spin-infected with a MIGR1 vector for expression of 3XFLAG-PU.1 or 3XFLAG-Spi-B. Immunoprecipitation was performed using anti-FLAG microbeads (M2, Sigma-Aldrich, St. Louis, MO). Purified chromatin was sequenced using an Illumina HiSeq instrument. Peak finding and data analysis was performed using Galaxy Suite (Blankenberg et al, 2010; Giardine et al, 2005; Goecks et al, 2010). Reads were aligned to the reference genome (NCBI37/mm9), using BOWTIE (Langmead et al, 2009). Peaks were called using MACS version 1.0.1 (Zhang et al, 2008) with default parameters, a P-value cutoff for peak detection of 1e-05 and an effective genome size of 2.7e+9 bp. Functional analysis of cis-regulatory regions bound by PU.1 and Spi-B were identified using GREAT V.2.0.2 (McLean et al, 2010). Data are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE58128.

2.4.12 ChIP experiments

Chromatin was prepared from enriched B cells as previously described (DeKoter et al, 2010). In brief, cells were cross-linked and lysed followed by sonication. Sonicated chromatin was incubated overnight at 4°C with anti-PU.1 or control mouse anti-IgG Ab (Santa Cruz Biotechnology) conjugated to protein G DynaBeads (Invitrogen, Burlington, Ontario, Canada). Magnetic bead complexes were enriched using a MagneSphere® Technology Magnetic Separation Stand (Promega) and washed. Immunocomplexes were eluted and cross-linked chromatin was reversed overnight at 65°C. DNA was purified using a QIAquick PCR purification kit (Qiagen). Enrichment was measured using qPCR of immunoprecipitated DNA using primers indicated in supplemental Table 2.1.

2.4.13 ELISA analysis

Serum was collected and quantified using a Quantikine ELISA Mouse BAFF/BLyS/TNFSF13B Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Plates were analyzed using an EON Microplate Spectrophotometer with Gen5 software (BioTek, Winooski, VT).

2.4.14 Statistical analysis

Statistical significance was determined using Student's *t* test unless otherwise indicated. P values ≤ 0.05 were considered significant. For all figures, *P ≤ 0.05, **P ≤ 0.01, and ***$\leq$P 0.001. Statistical analysis was performed using Prism 5.0 (Graph-Pad Software).

Chapter 3

3 Identification of a negative regulatory role for Spi-C in the murine B cell lineage

Spi-C is an E26 transformation-specific family (ETS) transcription factor that is most highly related to PU.1 and Spi-B. Spi-C (encoded by *Spic*) is expressed in developing B cells, but its function in B cell development and function is not well characterized. To determine if Spi-C functions as a negative regulator of Spi-B (encoded by *Spib*), mice were generated that were germline knockout for *Spib* and heterozygous for *Spic* (*Spib*$^{-/-}$*Spic*$^{+/-}$). Interestingly, loss of one allele of *Spic* substantially rescued B cell frequencies and absolute numbers in the spleens of *Spib*$^{-/-}$ mice. *Spib*$^{-/-}$*Spic*$^{+/-}$ mice had restored proliferation compared to *Spib*$^{-/-}$ mice in response to anti-IgM or LPS stimulation. *Spib*$^{-/-}$ B cells had reduced expression of genes required for B cell receptor signalling including *Nfkb1* encoding P50, and *Nfkb1* transcript levels were restored by loss of one allele of *Spic*. Finally, Spi-B was shown to directly activate the *Nfkb1* gene while Spi-C was shown to repress this gene. These results indicate a novel role for Spi-C as a negative regulator of B cell development and function.

3.1 Introduction

B cell development occurs in the bone marrow of mammals, where committed progenitor B (pro-B) cells are generated from lymphoid progenitor cells. Pro-B cells develop into mature B cells through a series of defined stages marked by rearrangement of immunoglobulin heavy chain (IgH) and immunoglobulin light chain genes. Immature B cells leaving the bone marrow are termed transitional-1 (T1) B cells, which migrate to the spleen and differentiate into T2 B cells (Pillai & Cariappa, 2009). T2 B cells can mature into either follicular (FO) or marginal zone (MZ) B cells that go on to participate in antibody formation and immune regulation (Pillai & Cariappa, 2009).

Transcription factors function as either activators or repressors of gene expression to govern B cell development. E26-transformation-specific (ETS) transcription factors

PU.1 (encoded by *Spi1*) and Spi-B (encoded by *Spib*) function as transcriptional activators during B cell development. PU.1 is required to generate B cell progenitors, as *Spi1*$^{-/-}$ mice have a profound block in B cell development (Houston et al, 2007; Scott et al, 1994). *Spib*$^{-/-}$ mice have reduced numbers of B cells that are defective in BCR signalling and are unable to sustain antibody responses to T-dependent antigens (Garrett-Sinha et al, 1999; Su et al, 1997). PU.1 and Spi-B activate transcription by interacting with the consensus core motif 5'-GGAA-3' at specific sites within the genome (Ray-Gallet et al, 1995). Due to the similarities in DNA binding specificities and overlapping expression patterns, many gene targets are functionally redundant between PU.1 and Spi-B, although there are also exclusive roles for each (DeKoter et al, 2010; Sokalski et al, 2011; Su et al, 1997).

Transcriptional networks that control B cell differentiation include both transcriptional activators and repressors (Rothenberg, 2014). Interestingly, few published examples of negative regulators of ETS transcription factor function exist. Spi-C is an ETS transcription factor that is a potential negative regulator. Spi-C was originally described as a PU.1-related protein containing an N-terminal acid activation domain (Bemark et al, 1999; Carlsson et al, 2003; Carlsson et al, 2006). Ectopic overexpression of Spi-C in either cultured pro-B cells using a retroviral vector (Schweitzer et al, 2006) or in mice using a B cell specific transgene (Eμ-Spi-C) (Zhu et al, 2008) has suggested that Spi-C functions as a negative regulator by opposing PU.1 activity. Transgenic Spi-C expression under the control of the B cell-specific *IgH* intronic enhancer resulted in reduced absolute numbers of T1, T2, MZ, and FO B cells in Eμ-Spi-C mice compared to WT mice (Zhu et al, 2008). Recently a *Spic* knockout mouse was generated, allowing for Spi-C loss-of-function analysis (Kohyama et al, 2009). Spi-C was revealed to be essential for generating red pulp macrophages in the spleen and is inducible by Heme (Haldar et al, 2014). Spi-C is upregulated in B cells lacking the transcriptional repressors Bach1/Bach2, and results in altered gene expression patterns in B cells (Itoh-Nakadai et al, 2014). Therefore, Spi-C may have a regulatory role in B cells for development and function.

The goal of this study was to determine if Spi-C plays a negative regulatory role in B cell development and function. A loss-of-function approach was used by crossing a

Spic null allele onto a *Spib* knockout background (*Spib*$^{-/-}$*Spic*$^{+/-}$ mice). The effect of *Spic* heterozygosity in *Spib*$^{-/-}$ mice might 1) have no effect on the phenotype, 2) further impair B cell development, or 3) rescue the *Spib*$^{-/-}$ phenotype. It was observed that *Spic* heterozygosity substantially rescued both development and function of B cells in *Spib*$^{-/-}$ mice. Taken together, our results identify a negative regulatory role for Spi-C in B cells.

3.2 Results

3.2.1 Restored splenic B cells numbers in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice compared to *Spib*$^{-/-}$ *Spic*$^{+/+}$ mice

To determine roles of Spi-C in B cell development, mice homozygous for germline null alleles of *Spib* (Su et al, 1997) and heterozygous *Spic* (Kohyama et al, 2009) were mated. *Spib*$^{-/-}$*Spic*$^{+/-}$ mice were generated near Mendelian ratios (Table 3.1) and were healthy and fertile. However, few *Spib*$^{-/-}$*Spic*$^{-/-}$ mice were generated (Table 3.1). Therefore, these studies focussed on analysis of *Spib*$^{-/-}$*Spic*$^{+/+}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice.

To determine that the haploid state of Spi-C is insufficient in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice, immunoblot analysis was performed on WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ spleen lysates to detect protein levels of Spi-C. A reduction in Spi-C was measured in *Spib*$^{-/-}$*Spic*$^{+/-}$ spleen lysates compared to WT or *Spib*$^{-/-}$ (Fig. 3.1A). As previously noted, *Spib*$^{-/-}$ mice had reduced total numbers of splenocytes compared to WT (Fig. 3.1B). However, total numbers of splenocytes were substantially rescued in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice compared to *Spib*$^{-/-}$ mice (Fig. 1B). In order to assess the population of mature B cells, frequencies of B220^{+}CD93^{-} cells were determined using flow cytometry (Fig. 3.1C). *Spib*$^{-/-}$ spleens contained decreased frequencies of mature B cells and decreased absolute numbers of mature B cells. In contrast, *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens had substantially rescued frequencies and absolute numbers of mature B cells compared to *Spib*$^{-/-}$ spleens (Fig. 3.1D). Overall, these results showed that knocking out one allele of *Spic* on a *Spib*$^{-/-}$ background rescued absolute number of mature B cells in *Spib*$^{-/-}$ spleens. These results suggest that Spi-C levels are involved in regulating B cell differentiation.

Table 3.1. Expected and actual frequencies of mouse genotypes from offspring generated by mating male and female *Spib*$^{-/-}$*Spic*$^{+/-}$.

Genotype	Number of mice produced	Expected Frequency[a] (%)	Actual Frequency[b] (%)
Spib$^{-/-}$*Spic*$^{+/+}$	101	25	36.5
Spib$^{-/-}$*Spic*$^{+/-}$	174	50	62.8
Spib$^{-/-}$*Spic*$^{-/-}$	2	25	0.7

[a]Expected frequencies are based on Mendelian ratios.

[b]Actual frequencies are based on the genotyping of 277 pups.

3.2.2 Rescued absolute number of FO B cells in *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens compared to *Spib*$^{-/-}$ spleens

Spib$^{-/-}$ mice were reported to have reduced frequencies of FO B cells (DeKoter et al, 2010). To determine whether mature MZ and FO B cell populations were restored in *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens, flow cytometry was performed on splenocytes from WT, *Spib*$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. Mature B cells were gated on B220^{+}CD93^{-}, followed by analysis of CD21 and IgM surface expression to identify FO (CD21^{low}IgM^{int}) and MZ (CD21^{hi}IgM^{hi}) B cells (Fig. 3.1E). Absolute numbers of MZ B cells were unaffected by either reduced Spi-B or Spi-C expression. However, FO B cells were decreased in both frequency and absolute number in *Spib*$^{-/-}$ spleens compared to WT (Fig. 3.1F). *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens contained restored absolute numbers of FO B cells compared to *Spib*$^{-/-}$ spleens (Fig. 3.1F). Next, MZ and FO B cell populations were quantified by CD23 and IgM surface expression (Fig. 3.1G). Absolute numbers of MZ B cells (IgM^{hi}CD23$^{-/low}$) were unchanged between WT, *Spib*$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens. However, frequencies of FO B cells (IgM^{int}CD23^{+}) were reduced in *Spib*$^{-/-}$ spleens, and this reduction was rescued in *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens (Fig. 3.1H). Taken together, these results suggest that the rescue in absolute numbers of mature B cells in *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens was primarily a result of increased FO B cells.

It has been previously reported that there is differential Spi-C transcript expression between B-1a, B-1b, and splenic B2 cells (Kretschmer et al, 2003). To assess if there were any differences in B-1 cell populations in the peritoneal cavity of *Spib*$^{-/-}$ *Spic*$^{+/-}$, peritoneal fluid isolated from WT, *Spib*$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice were analyzed by flow cytometry (Figure 3.2A). No differences in CD19^{+}CD5^{+} (B-1a) cell frequency were detected between WT, *Spib*$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice (Figure 3.2B). *Spib*$^{-/-}$*Spic*$^{+/-}$ B-1a cells from the peritoneal cavity expressed elevated CD19 levels compared to WT mice (Figure 3.2C). Therefore, loss of Spi-C expression in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice altered CD19 expression in B-1a cells.

Figure 3.1. Spi-C heterozygosity rescues absolute numbers of B cells in the spleens of *Spib*$^{-/-}$ mice.

(A) Spi-C protein expression measured in WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ spleen lysates. Immunoblot was performed using anti-Spi-C antibody and anti-β-Actin as a loading control. Data shown is a representative of 3 individual experiments. (B) Cell counts based on total cells isolated from the spleens of WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. (C) Mature B cells were quantified by flow cytometry based on B220 and CD93. The dashed box represents the mature B cell population. (D) Quantitation of the frequency and absolute number of B220^{+}CD93^{-} splenocytes identified in WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. (E) Mature FO and MZ B cell populations were quantified based on CD21 and IgM surface expression. (F) Quantitation of the frequency and absolute number of MZ (CD21^{hi}IgM^{hi}) and FO (CD21^{low}IgM^{int}) mature B cells (B220^{+}CD93^{-}) in the spleen. (G) Mature FO and MZ B cell populations were quantified based on CD23 and IgM surface expression. Cells were isolated from WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens and analyzed by flow cytometry. (H) Quantitation of the frequency and absolute number of MZ (CD23^{low}IgM^{hi}) and FO (CD23^{hi}IgM^{int}) mature B cells (B220^{+}CD93^{-}) in the spleen. For B, and D, data is shown as mean and SD of 14 individual mice. For F and H, data is shown as mean and SD of 9 individual mice. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

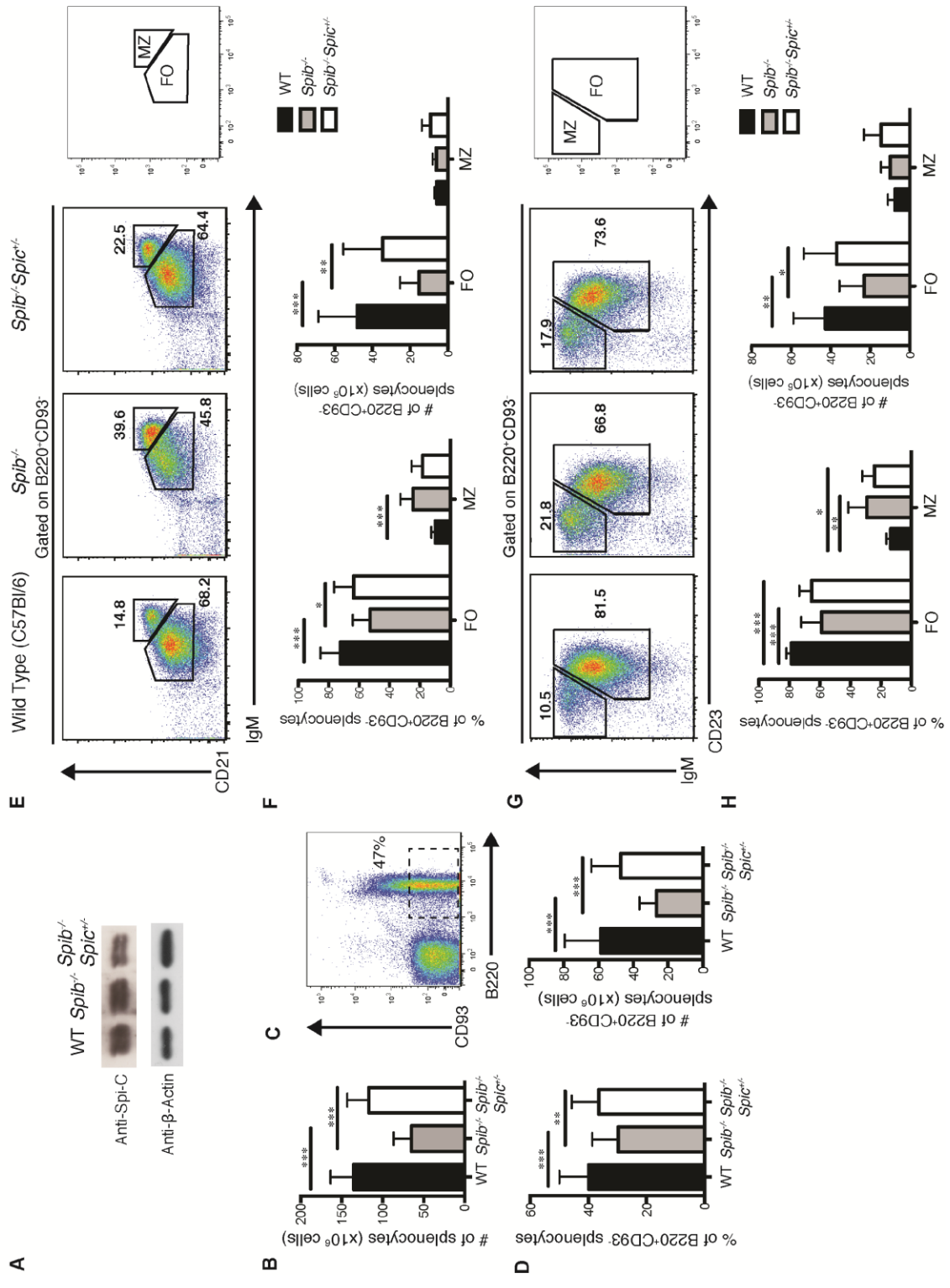
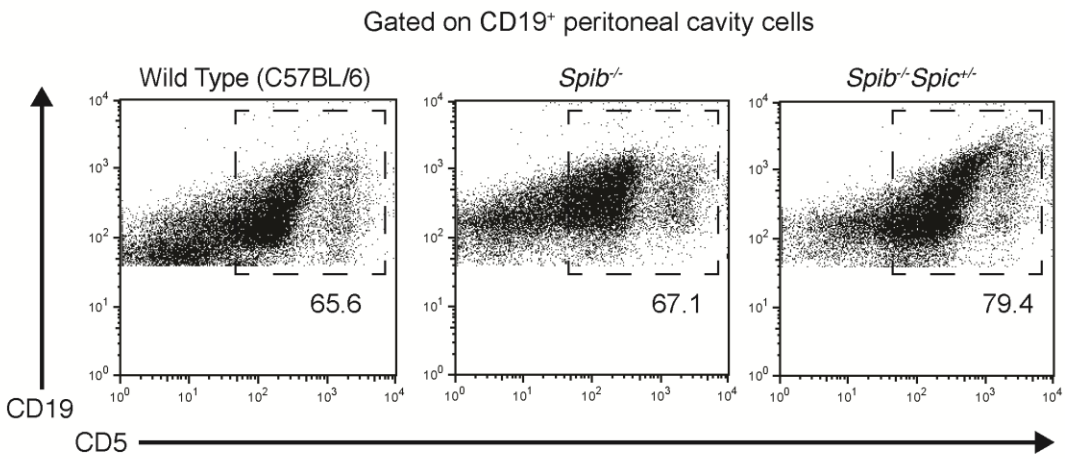

A
WT
Spib-/-
Spic+/-
Anti-Spi-C
Anti-β-Actin
B
# of splenocytes (x10^6 cells)
WT Spib-/- Spic+/-
C
CD93
B220
47%
D
% of B220+CD93- splenocytes
# of B220+CD93- splenocytes (x10^6 cells)
E
Wild Type (C57Bl/6)
Spib-/-
Gated on B220+CD93-
Spib-/-Spic+/-
CD21
IgM
14.8
68.2
39.6
45.8
22.5
64.4
FO
MZ
F
WT
Spib-/-
Spib-/-Spic+/-
% of B220+CD93- splenocytes
# of B220+CD93- splenocytes (x10^6 cells)
G
Gated on B220+CD93
IgM
CD23
10.5
81.5
21.8
66.8
17.9
73.6
H

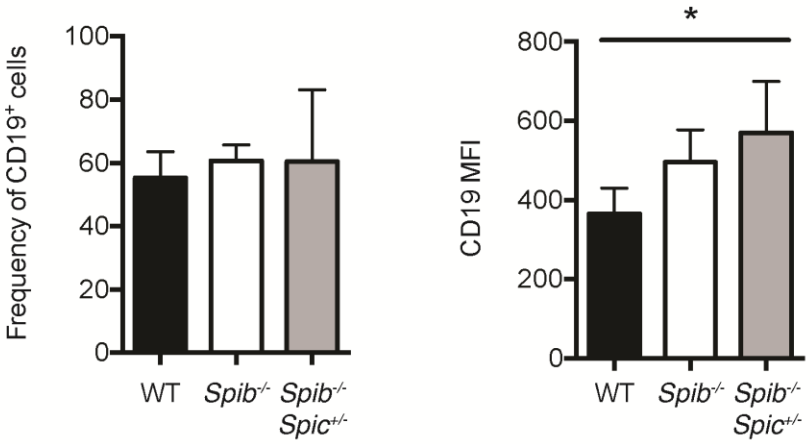
Figure 3.2. B-1a cells from the peritoneal cavity of *Spib*$^{-/-}$*Spic*$^{+/-}$ mice express elevated CD19 levels.

(A) Comparison of the frequency of CD19^{+}CD5^{+} cells in the peritoneal cavity of WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. Dashed box represents the B-1a population. Data is a representative of three independent experiments. (B) Quantitation of A showing the mean frequency of B-1a cells as a percentage of total CD19^{+} cells. (C) Levels of CD19 determined by the mean fluorescence intensity (MFI). For B and C, data shows mean and SD of three individual mice.

A


Gated on CD19+ peritoneal cavity cells
Wild Type (C57BL/6)
Spib-/-
Spib-/-Spic+/-
65.6
67.1
79.4
CD19
CD5

B


Frequency of CD19+ cells
100
80
60
40
20
0
WT
Spib-/-
Spib-/- Spic+/-
CD19 MFI
800
600
400
200
0
*
WT
Spib-/-
Spib-/- Spic+/-

3.2.3 Increased transitional B cells in *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens compared to *Spib*$^{-/-}$ spleens

Since *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens had increased numbers of FO B cells compared to *Spib*$^{-/-}$, it was hypothesized that there were increased immature B cells developing in the bone marrow of these mice. To test this, flow cytometry analysis was performed using the "Hardy" staining scheme for bone marrow cells (Hardy et al, 1991) (Fig. 3.3A). Although *Spib*$^{-/-}$ mice had increased fraction D and reduced fraction F populations compared to WT B cells, no significant differences in frequencies of fractions A through F were observed between *Spib*$^{-/-}$ and *Spib*$^{-/-}$ *Spic*$^{+/-}$ mice (Fig. 3.3B-C). These results suggested that restored FO B cell numbers were not due to an increase in frequency of B cell progenitors derived from the bone marrow.

To assess whether rates of apoptosis were altered in *Spib*$^{-/-}$ *Spic*$^{+/-}$ B cells, flow cytometry was performed on WT, *Spib*$^{-/-}$ and *Spib*$^{-/-}$ *Spic*$^{+/-}$ splenocytes. Cells were gated into T1, T2, MZ, and FO B cell populations based on B220, CD93, IgM, and CD23 staining, and apoptotic cells were gated for Annexin V^{+}7-AAD^{-} (Fig. 3.4A). No differences were detected in apoptotic frequency in either total B cells (Fig. 3.4B) or gated T1, T2, MZ, or FO B cells (Fig. 3.3C) between WT, *Spib*$^{-/-}$ and *Spib*$^{-/-}$ *Spic*$^{+/-}$ mice. These results suggest that reduced Spi-C expression does not significantly alter steady-state levels of apoptosis in *Spib*$^{-/-}$ mice.

To determine the source of rescued numbers of FO B cells in *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens compared to *Spib*$^{-/-}$, immature transitional B cell populations were examined. Immature B cells were gated on expression of both B220 and CD93 (Fig. 3.5A). There were increased frequencies of B220^{+}CD93^{+} immature B cells in both *Spib*$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens compared to WT, but no significant difference in frequency of these cells between *Spib*$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens. In contrast, absolute numbers of immature B cells were nearly doubled in *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens compared to WT or *Spib*$^{-/-}$ spleens (Fig. 3.5B). To determine which immature B cell subset was accountable for overall increased numbers, analysis of transitional T1, T2, and T3 B cell populations was performed by gating immature B220^{+}CD93^{+} B cells for differential expression of IgM and CD23 (Fig. 3.5C).

Figure 3.3. No differences in B cell composition between *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice in the bone marrow.

(A) Bone marrow analysis was performed on WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. Cells were analyzed by flow cytometry and gated using the Hardy scheme for quantifying B cell populations during development. Data shown is a representative of 8 individual mice. (B) Quantitation of individual fractions A-F located in the bone marrow calculated as a percentage of total B220^{+} cells in the bone marrow. (C) Quantitation of individual fractions A-F located in the bone marrow calculated as a percentage of total cells in the bone marrow. Fractions were gated using the Hardy scheme. For B-C, data is shown as mean and SD of 8 individual mice. **p<0.01; ***p<0.001.

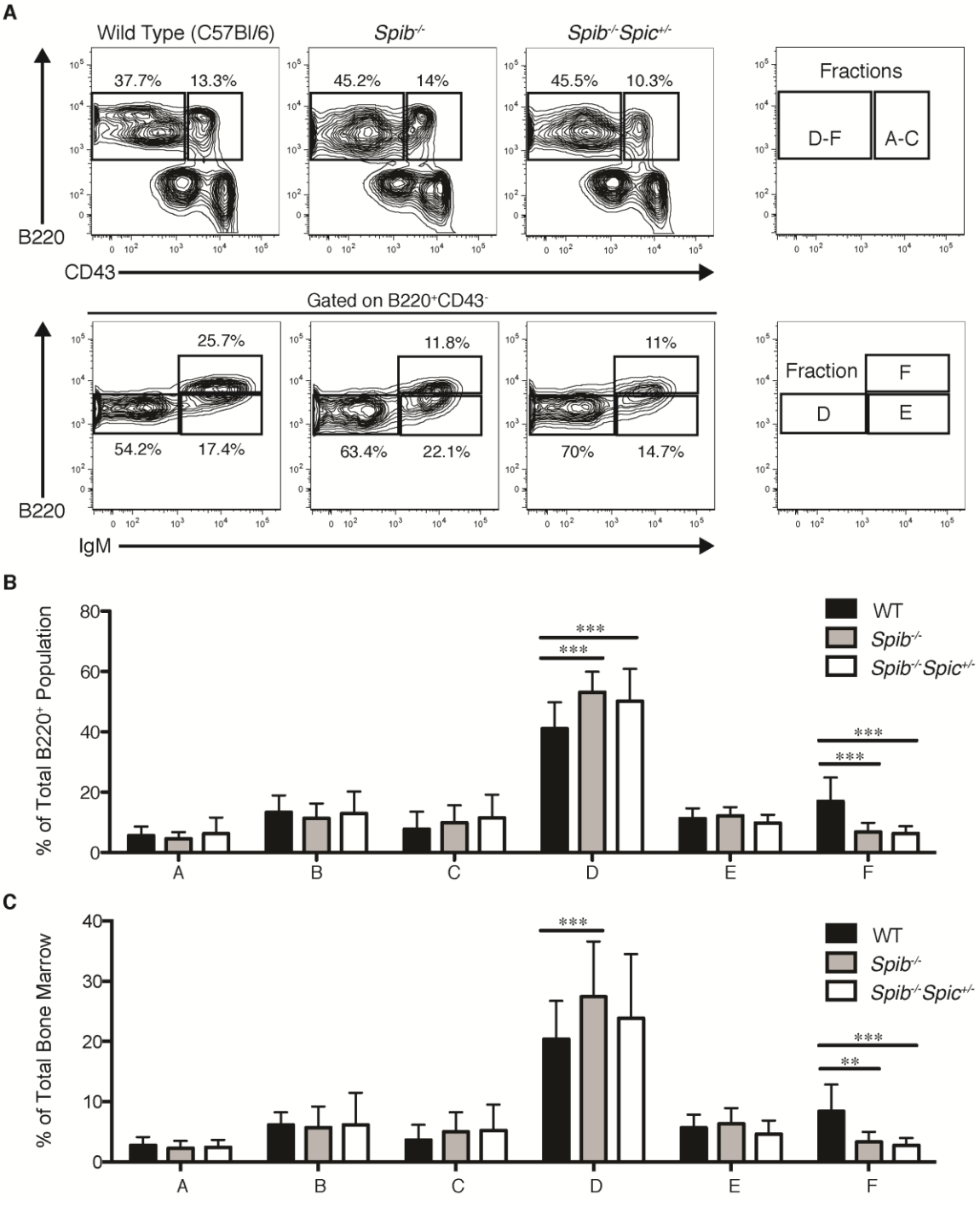
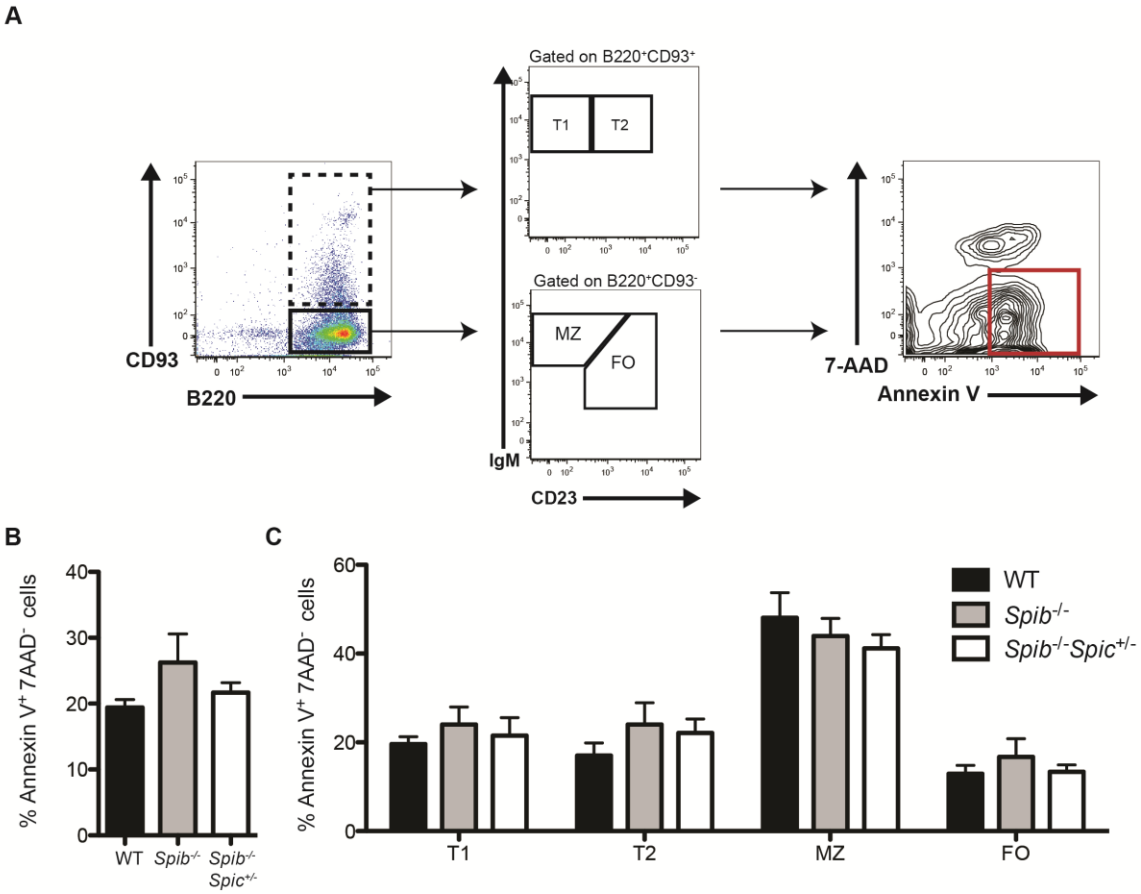

A
Wild Type (C57Bl/6)
Spib-/-
Spib-/-Spic+/-
37.7%
13.3%
45.2%
14%
45.5%
10.3%
Fractions
D-F
A-C
B220
CD43
Gated on B220+CD43-
25.7%
11.8%
11%
54.2%
17.4%
63.4%
22.1%
70%
14.7%
Fraction
F
D
E
B220
IgM
B
% of Total B220+ Population
WT
Spib-/-
Spib-/-Spic+/-
***
***
***
***
A
B
C
D
E
F
C
% of Total Bone Marrow
WT
Spib-/-
Spib-/-Spic+/-
***
***
**
A
B
C
D
E
F

Figure 3.4. No differences in the frequency of apoptosis between WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$ *Spic*$^{+/-}$ B cells.

(A) Rates of apoptosis were analyzed by flow cytometry using Annexin V and 7-AAD staining on WT, *Spib*$^{-/-,}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ splenocytes. Prior to Ab staining, spleens were enriched for B cells by magnetic CD43^{+} cell depletion. Cells were stained for B220, CD93, IgM, CD23, Annexin V, and 7-AAD to allow for gating on transitional T1, T2, and mature FO and MZ B cell populations. Apoptotic frequencies (Annexin V^{+} 7-AAD^{-}; red box) were assessed for each gated subset. (B) Quantitation of the frequency of apoptosis in total B cells. (C) Quantitation of apoptotic transitional T1, T2, FO and MZ B cells. For B-C, data is shown as mean and SD of 4 individual mice.


A
Gated on B220+CD93+
T1
T2
Gated on B220+CD93-
MZ
FO
CD93
B220
IgM
CD23
7-AAD
Annexin V
B
% Annexin V+ 7AAD- cells
WT
Spib-/-
Spib-/- Spic+/-
C
% Annexin V+ 7AAD- cells
WT
Spib-/-
Spib-/-Spic+/-
T1
T2
MZ
FO

No differences in T3 B cells were observed between WT, *Spib*$^{-/-}$ and *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens. In contrast, *Spib*$^{-/-}$ and *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens contained elevated frequencies and absolute numbers of T1 B cells compared to WT spleens, although there was no quantitative difference in the T1 population between *Spib*$^{-/-}$ and *Spib*$^{-/-}$ *Spic*$^{+/-}$ mice. For T2 populations, *Spib*$^{-/-}$ spleens contained fewer cells than WT, and both frequency and absolute numbers of T2 cells were increased in *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens (Fig. 3.5D). To confirm that T2 B cell numbers were increased in *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens compared to *Spib*$^{-/-}$, splenic B cells were examined using an alternative staining scheme based on CD21 and IgM expression (Fig. 3.6A). Absolute numbers of T2 B cells were significantly reduced in *Spib*$^{-/-}$ spleens compared to WT, and were rescued in *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens using the alternative staining scheme (Fig. 3.6B). In summary, these results showed that reduced Spi-C in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice resulted in a substantial rescue of the frequency of T2 cells in the spleen of *Spib*$^{-/-}$ mice.

3.2.4 LPS and anti-IgM mediated proliferation is rescued in *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells compared to *Spib*$^{-/-}$ B cells

B cells lacking Spi-B have been previously reported to have impaired proliferative responses to either LPS or anti-IgM (Garrett-Sinha et al, 1999; Su et al, 1997). To determine whether *Spic* heterozygosity could rescue functional defects of *Spib* knockout B cells, proliferation of *Spib*$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ splenic B cells in response to LPS or anti-IgM was assessed using a MTT proliferation assay. Compared to WT B cells, *Spib*$^{-/-}$ B cells proliferated poorly in response to LPS or anti-IgM (Fig. 3.7A). In contrast, *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells were substantially rescued for LPS or anti-IgM-mediated proliferation compared to *Spib*$^{-/-}$ B cells (Fig. 3.7A-B). To confirm the proliferation rescue, LPS-mediated proliferation was measured by flow cytometry following staining B cells with carboxyfluorescein succinimidyl ester (CFSE) (Fig. 3.7C). Fewer cells underwent cell division in *Spib*$^{-/-}$ B cells stimulated with LPS compared to WT B cells. However, more proliferating cells were detected in *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells compared to *Spib*$^{-/-}$ B cells (Fig. 3.7D). Therefore, these results suggested that B cell proliferation in response to LPS or anti-IgM is positively regulated by Spi-B, but negatively regulated by Spi-C.

Figure 3.5. *Spib*$^{-/-}$*Spic*$^{+/-}$ mice have elevated transitional B cells and restored transitional-2 B cells compared to *Spib*$^{-/-}$ mice.

(A) Total transitional B cell populations were quantified in WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. Splenocytes were stained with CD93 and B220 and analyzed by flow cytometry. The dashed-box represents total transitional B cells. (B) Increased frequencies and absolute number of total transitional B cells in spleens of *Spib*$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. (C) Transitional B cell subsets T1 (Top left box, IgM^{hi}CD23^{-}), T2 (Top right box, IgM^{hi}CD23^{+}), and T3 (Bottom right box, IgM^{low}CD23^{+}) were quantified in WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. Cells were gated on B220 and CD93 and analyzed for IgM and CD23 staining by flow cytometry. (D) Increased T2 subset in *Spib*$^{-/-}$*Spic*$^{+/-}$ compared to *Spib*$^{-/-}$ mice. Quantitation of results shown in (C). Data is shown as mean frequency and absolute numbers of gated B220^{+}CD93^{+} splenocytes. For B and D, data is shown as the mean and SD of 9 individual mice. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

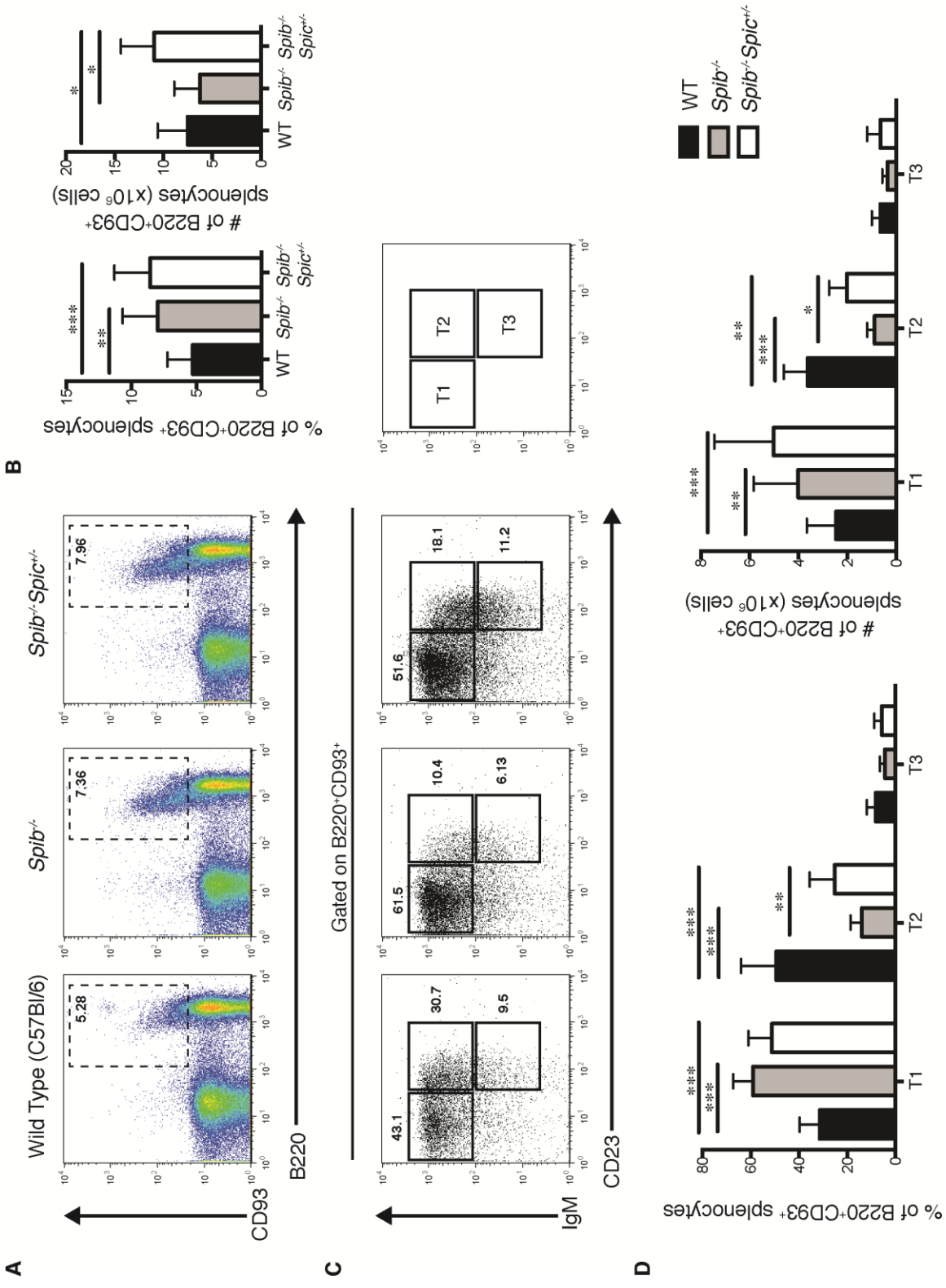
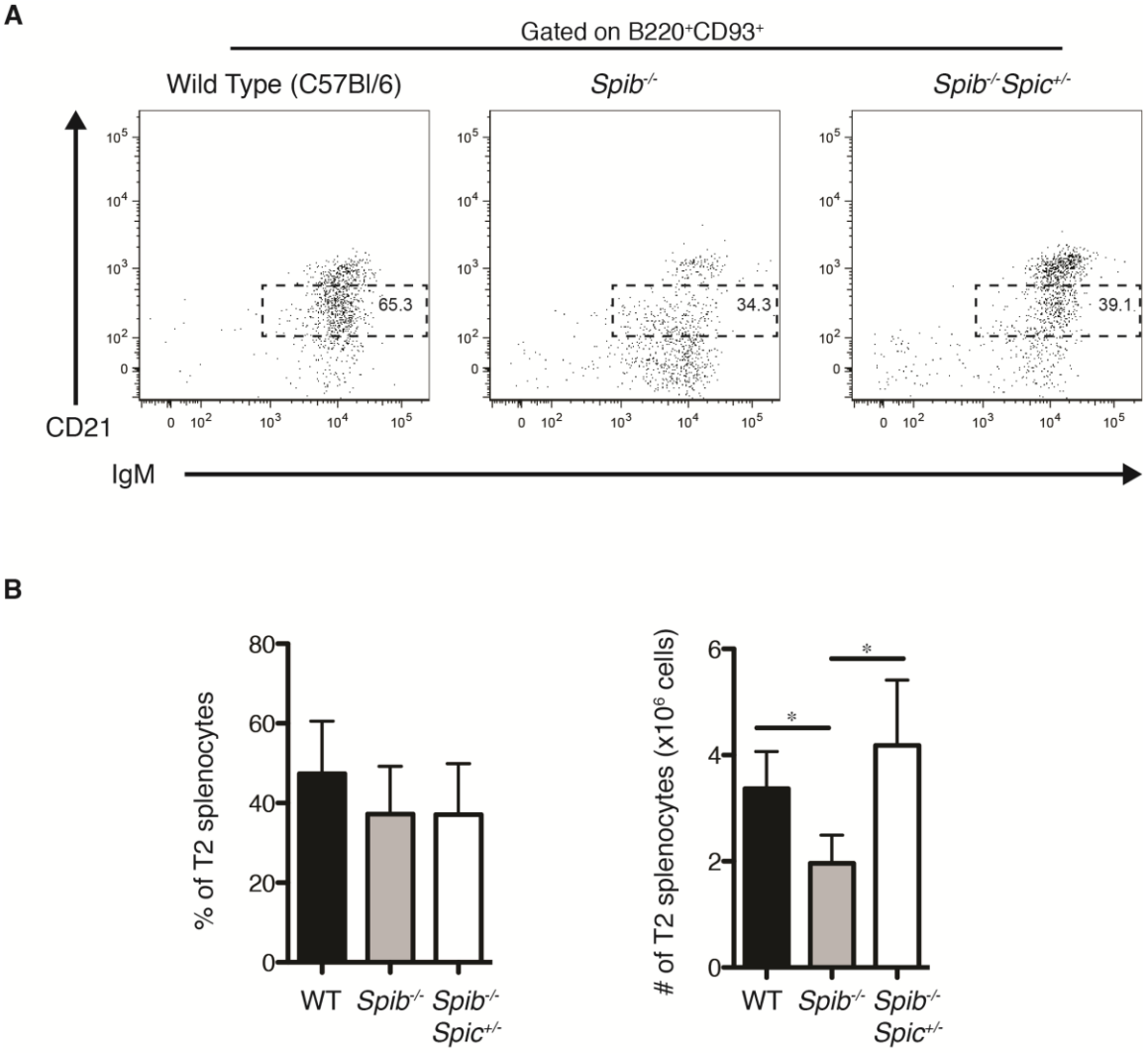

A
Wild Type (C57Bl/6)
Spib-/-
Spib-/-Spic+/-
5.28
7.36
7.96
CD93
B220
B
% of B220+CD93+ splenocytes
# of B220+CD93+ splenocytes (x10^6 cells)
WT
Spib-/-
Spib-/- Spic+/-
C
Gated on B220+CD93+
43.1
30.7
9.5
61.5
10.4
6.13
51.6
18.1
11.2
IgM
CD23
T1
T2
T3
D
% of B220+CD93+ splenocytes
# of B220+CD93+ splenocytes (x10^6 cells)
WT
Spib-/-
Spib-/-Spic+/-

Figure 3.6. *Spib*$^{-/-}$*Spic*$^{+/-}$ mice have restored transitional-2 B cells compared to *Spib*$^{-/-}$ mice.

(A) T2 B cells (IgM^{+}CD21^{int}; dashed box) were quantified in WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$ *Spic*$^{+/-}$ mice. Cells were gated on B220 and CD93 and analyzed for IgM and CD21 staining by flow cytometry. (B) Restored T2 subset in *Spib*$^{-/-}$*Spic*$^{+/-}$ compared to *Spib*$^{-/-}$ mice. Quantitation of results shown in (A). Data is shown as mean and SD for frequency and absolute numbers of gated B220^{+}CD93^{+} splenocytes from 7 individual mice. *$p<0.05$.


A
Gated on B220+CD93+
Wild Type (C57Bl/6)
Spib-/-
Spib-/-Spic+/-
65.3
34.3
39.1
CD21
IgM
B
% of T2 splenocytes
# of T2 splenocytes (x10^6 cells)
WT
Spib-/-
Spib-/- Spic+/-
*
*

To determine if the *Spib*$^{-/-}$*Spic*$^{+/-}$ B cell phenotype could be reversed by ectopic expression of Spi-C, *Spib*$^{-/-}$*Spic*$^{+/-}$ mice were crossed to Eμ-Spi-C transgenic mice (Zhu et al, 2008). Spleens from Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ mice contained fewer cells than *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens (Fig. 3.7E). Flow cytometry analysis was performed on Eμ-Spi-C *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens to assess for differences in B cell populations. Frequencies of MZ and FO B cells in Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens were unchanged compared to *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens, however, there was a significant reduction in the absolute number of MZ and FO B cells in Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ mice (Fig. 3.7F). Proliferation of Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells following LPS stimulation was assessed by CFSE staining. Compared to *Spib*$^{-/-}$ *Spic*$^{+/-}$ B cells, frequencies of proliferating Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells decreased (Fig. 3.7G). This data suggests that reintroducing Spi-C using a lymphocyte-specific transgene reverses the *Spib*$^{-/-}$*Spic*$^{+/-}$ B cell proliferation phenotype.

Since anti-IgM mediated proliferation was restored in *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells compared to *Spib*$^{-/-}$, it was possible that *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells could have restored BCR signaling. To test for this, phosphorylated levels of Syk were measured in B cells using flow cytometry (Fig. 3.7H). There was a detectable increase in phospho-Syk levels following anti-IgM stimulation; however, there was no difference in the increase between WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells (Fig. 3.7I). Next, total Syk was measured in B cells using immunoblot analysis. There was a noticeable increase in total Syk protein in *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells compared to WT B cells (Fig. 3.7J). Therefore, the restoration in BCR signaling in *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells is likely independent of Syk.

It was previously reported that *Spib*$^{-/-}$ mice had normal basal levels of all Ig isotypes (Su et al, 1997). To determine whether reduced Spi-C had an effect on isotype switching in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice, basal serum levels of different immunoglobulin were measured by ELISA. No significant differences in basal levels of IgM (Fig. 3.8A) or IgG2b (Fig. 3.8B) were measured between WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. Therefore, the data suggests that Spi-C does not alter basal levels of Ig isotypes in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice.

Figure 3.7. B cell proliferation is rescued in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice compared to *Spib*$^{-/-}$ mice.

(A) *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells have significantly increased proliferation compared to *Spib*$^{-/-}$ B cells. Proliferation was measured using an MTT proliferation assay. Data shown is the mean and SD of triplicate wells and is a representative of 5 experiments. (B) Quantitation of 5 experiments from (A) showing proliferation index, which is calculated by normalizing corrected OD_{570} values to the untreated WT control. Data is shown as mean and SEM. (C) Proliferation assessed by flow cytometry following CFSE staining on WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells. Gate shown represents the frequency of proliferating cells. Data shown is a representative of 4 individual experiments (D) Quantitation of CFSE experiments as performed in (C) showing frequency of cells that underwent cell division. (E) Cell counts based on total cells isolated from the spleens of *Spib*$^{-/-}$*Spic*$^{+/-}$ *and* Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. (F) Quantitation of the frequency and absolute number of MZ and FO mature B cells ($B220^{+}CD93^{-}$) in the spleens of *Spib*$^{-/-}$*Spic*$^{+/-}$ *and* Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. FO and MZ B cell populations were quantified based on CD23 and IgM surface expression measured by flow cytometry. For E and F, data is shown as mean and SD of 5 individual mice. (G) Proliferation assessed by flow cytometry following CFSE staining on *Spib*$^{-/-}$*Spic*$^{+/-}$ and Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells. Data is shown as mean and SD for 4 individual experiments. (H) Phospho-Syk levels were assessed in WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells using flow cytometry. Splenocytes were stained with B220, stimulated with anti-IgM for 2 minutes or untreated, and then stained with phospho-Syk prior to analysis. (I) Quantitation of phospho-Syk levels in $B220^{+}$ splenocytes following anti-IgM stimulation. Phospho-Syk levels are shown as normalized phospho-Syk MFI of anti-IgM to untreated. Data is shown as the mean and SD of 5 individual experiments. (J) Syk protein expression measured in WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$ *Spic*$^{+/-}$ B cell lysates. Immunoblot was performed using anti-Syk antibody and anti-β-Actin as a loading control. Data shown is a representative of 3 individual experiments. For A-D and G, total B cells were enriched by magnetic separation and stimulated with LPS or anti-IgM for 72 hours. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

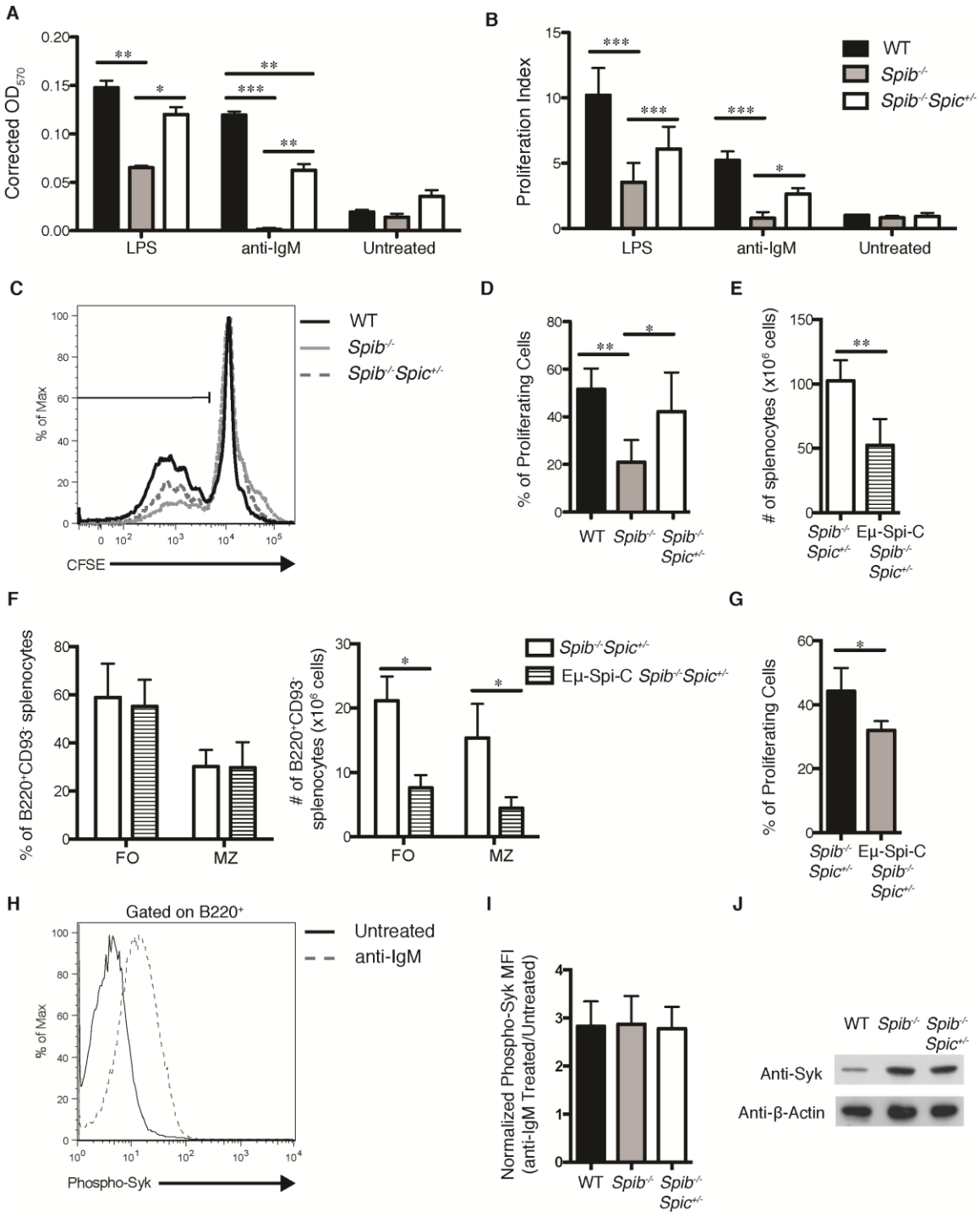
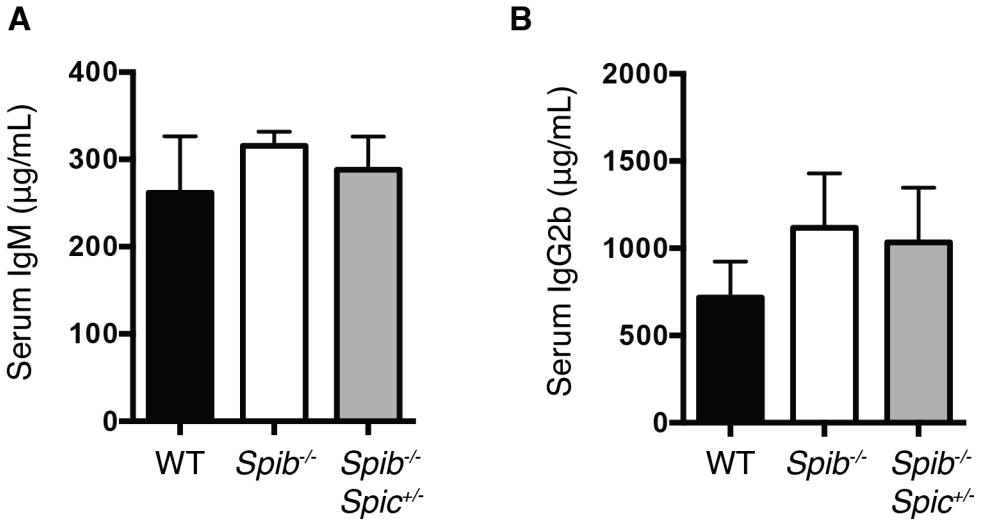

A
Corrected OD570
LPS
anti-IgM
Untreated
B
Proliferation Index
WT
Spib-/-
Spib-/-Spic+/-
C
% of Max
CFSE
D
% of Proliferating Cells
E
# of splenocytes (x10^6 cells)
F
% of B220+CD93- splenocytes
# of B220+CD93- splenocytes (x10^6 cells)
FO
MZ
Eμ-Spi-C Spib-/-Spic+/-
G
H
Gated on B220+
Untreated
anti-IgM
Phospho-Syk
I
Normalized Phospho-Syk MFI (anti-IgM Treated/Untreated)
J
Anti-Syk
Anti-β-Actin

Figure 3.8. Equivalent basal serum antibody levels between WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice.

(A) IgM and (B) IgG2b serum levels measured by ELISA. Data shows mean and standard deviation (n=6).


A
400
300
200
100
0
Serum IgM (µg/mL)
WT
Spib-/-
Spib-/-
Spic+/-
B
2000
1500
1000
500
0
Serum IgG2b (µg/mL)
WT
Spib-/-
Spib-/-
Spic+/-

3.2.5 Spi-C represses *Nfkb1* promoter activation by Spi-B

Finally, a potential mechanism for Spi-C's regulation of B cell proliferation was investigated. Stimulation of B cells with LPS or anti-IgM results in NF-κB transcription factor activation (Pone et al, 2010). P50 (encoded by *Nfkb1*) is required for murine B cell proliferation in response to LPS or anti-IgM (Sha et al, 1995). We recently showed that PU.1 and Spi-B are required for *Nfkb1* transcription in B cells, and retroviral transduction with p50 substantially rescues LPS-induced proliferation of $Spi1^{+/-}Spib^{-/-}$ B cells (Li et al, 2015). Therefore, it was determined whether restored proliferation in $Spib^{-/-}Spic^{+/-}$ B cells was associated with increased mRNA transcript levels of *Nfkb1* compared to $Spib^{-/-}$ B cells. RT-qPCR was performed on cDNA synthesized from MZ and FO B cells sorted from $Spib^{-/-}$ and $Spib^{-/-}Spic^{+/-}$ spleens. Steady-state *Nfkb1* mRNA transcript levels were increased in MZ and FO B cells of $Spib^{-/-}Spic^{+/-}$ mice compared to $Spib^{-/-}$ mice (Fig. 3.9A), suggesting that Spi-C may negatively regulate *Nfkb1* in B cells.

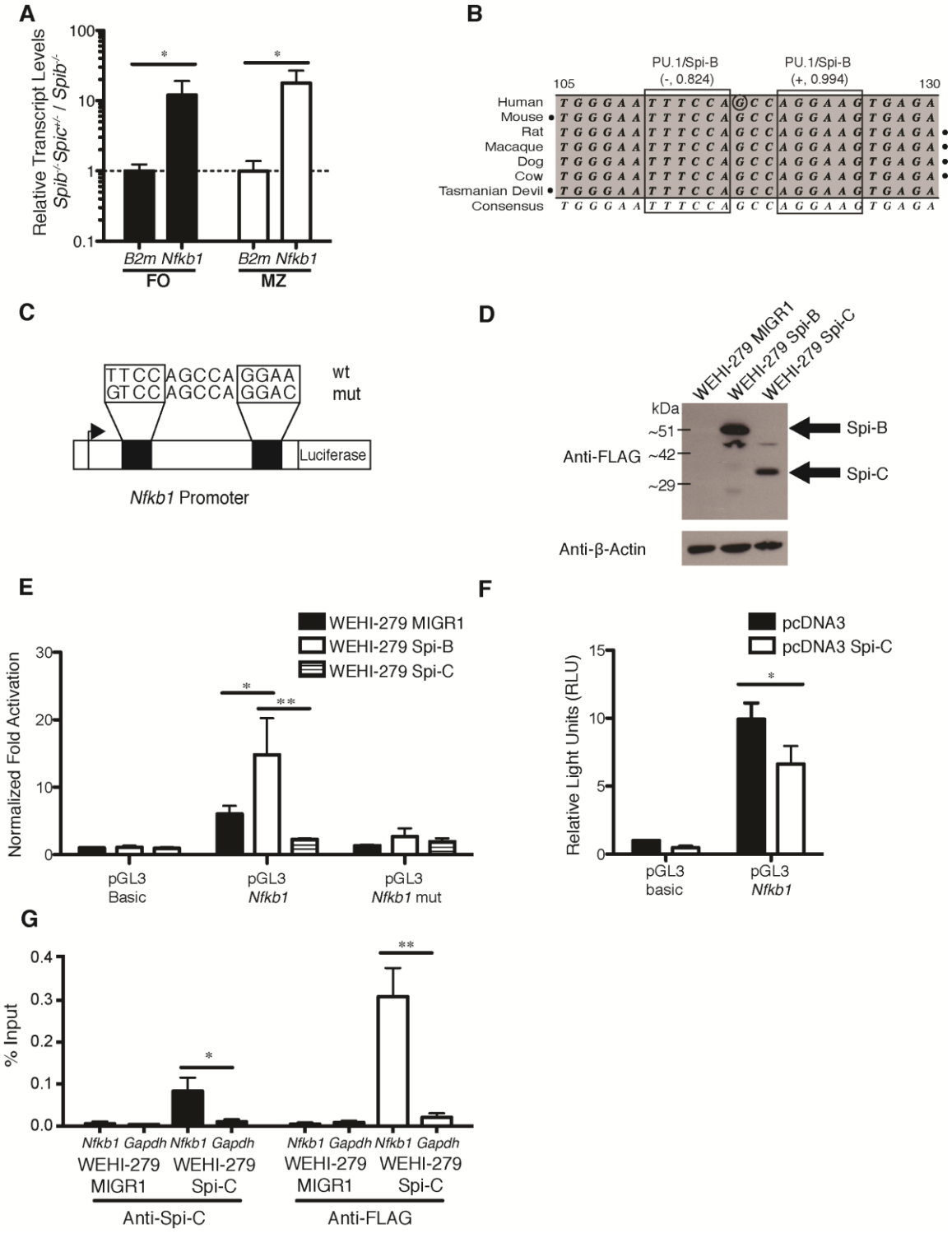
To assess whether Spi-B and Spi-C regulate *Nfkb1* directly, the murine *Nfkb1* promoter sequence was aligned with multiple species using a published transcription start site (TSS) for reference (Cogswell et al, 1993). The *Nfkb1* 5' UTR contained two highly conserved ETS binding sites predicted among all species near the TSS using MatInspector software (Fig. 3.9B). Next, the mouse *Nfkb1* proximal promoter was cloned as described in Materials & Methods and ligated into the pGL3-basic luciferase reporter vector. Site directed mutagenesis was performed to mutate two predicted ETS binding sites closest to the transcriptional start site by substituting GGAA (TTCC on complement strand) with GGAC (GTCC), which has been previously reported to abolish Spi-B binding (DeKoter et al, 2010; Xu et al, 2012) (Fig. 3.9C). To determine whether Spi-B and Spi-C regulate *Nfkb1* transcription in opposing fashion, luciferase assays were performed using transient transfection of WEHI-279 B lymphoma cells that ectopically express 3XFLAG tagged Spi-B or Spi-C (DeKoter et al, 2010; Xu et al, 2012). WEHI-279 cells infected with an empty MIGR1 virus were used as a control (WEHI-279 MIGR1). Expression of 3XFLAG Spi-B and Spi-C protein was confirmed by anti-FLAG immunoblot analysis in infected cell lines (Fig. 3.9D). The *Nfkb1* promoter was active in WEHI-279 MIGR1 cells, and mutation of the ETS sites impaired its activation (Fig.

3.9E). WEHI-279 Spi-B cells displayed a higher level of activation compared to WEHI-279 MIGR1 cells, whereas mutation of the ETS sites reduced *Nfkb1* activation. Interestingly, the activity of the *Nfkb1* promoter in WEHI-279 Spi-C cells was lower than both WEHI-279 MIGR1 and WEHI-279 Spi-B cells, and mutation of the ETS site resulted in no change of activity (Fig. 3.9E). To confirm that Spi-C expression decreased *Nfkb1* transcriptional activation, luciferase activity was measured following co-transfection of a Spi-C expression vector (pcDNA3 Spi-C) and the pGL3-*Nfkb1* vector into WEHI-279 Spi-B cells. Co-transfection with pcDNA3 Spi-C resulted in decreased *Nfkb1* activation compared to co-transfection with the vector control (pcDNA3) (Fig. 3.9F). These results suggest that Spi-B transcriptionally activates *Nfkb1,* and Spi-C inhibits *Nfkb1* transcription.

We recently showed that PU.1 and Spi-B directly interact with the *Nfkb1* promoter to activate its transcription (Li et al, 2015). To determine if Spi-C directly interacts with the *Nfkb1* promoter, ChIP analysis was performed using anti-Spi-C and anti-FLAG Ab on chromatin isolated from WEHI-279 Spi-C and WEHI-279 MIGR1 cells. Relative amounts of immunoprecipitated DNA were determined by qPCR from the *Nfkb1* promoter region, and the *Gapdh* promoter as a negative control. ChIP analysis confirmed that Spi-C was significantly enriched at the *Nfkb1* promoter compared to the *Gapdh* promoter in WEHI-279 Spi-C cells, but not WEHI-279 MIGR1 cells (Fig. 3.9G). Together, these data indicate that Spi-C directly interacts with the *Nfkb1* promoter, suggesting that it opposes Spi-B-mediated activation of *Nfkb1* transcription in B cells.

Figure 3.9. Spi-C inhibits activation of the *Nfkb1* promoter by Spi-B.

(A) Measurement of transcript levels of *Nfkb1* in *Spib*$^{-/-}$*Spic*$^{+/-}$ compared to *Spib*$^{-/-}$ B cells. RT-qPCR analysis was performed on RNA prepared from unstimulated sorted FO and MZ B cells. mRNA transcript levels of genes indicated on the *X-axis* were quantified after normalizing to β2 microglobulin (*B2m*). Data is shown as mean and SD, and is a representative of 3 individual mice. (B) Alignment of the annotated *Nfkb1* promoter between multiple species, showing nucleotides 105-130 of the conserved 213 bp region. Grey text represents conserved nucleotides, and boxes indicate predicted PU.1/Spi-B binding sites with similarity score and strand location (+/-) listed. Circled nucleotide represents known TSS in the human *Nfkb1* promoter. Dots represent TSS upstream or downstream of the annotated sequence. (C) Schematic of luciferase reporter vector. The *Nfkb1* promoter was cloned by PCR and ligated into pGL3-basic. ETS binding sites (wt) were mutated (mut) using site-directed mutagenesis. (D) WEHI-279 B cell lymphoma cell lines were generated that overexpress 3XFLAG-Spi-B or Spi-C. Immunoblot analysis demonstrates the overexpression levels of Spi-B or Spi-C in these cell lines. (E) Overexpression of Spi-C inhibits activation of the *Nfkb1* promoter. Mutation of the predicted ETS binding sites reduces promoter activity in WEHI-279 MIGR1 and WEHI-279 Spi-B cells. (F) Spi-C expression in WEHI-279 Spi-B cells decreases *Nfkb1* activation. WEHI-279 Spi-B cells were co-transfected with a Spi-C expression vector (pcDNA3 Spi-C) along with the luciferase vectors. In E and F, cells were transfected with the plasmids indicated on the x-axis. The y-axis indicates fold-induction of luciferase activity relative to pGL3-basic. Luciferase activity was normalized by transfection with Renilla luciferase expression vector. Data shown is the mean and SEM of three independent experiments. (G) Spi-C directly binds to the *Nfkb1* promoter. ChIP analysis was performed on WEHI-279 MIGR1 and WEHI-279 Spi-C cells immunoprecipitated with anti-FLAG or anti-Spi-C Ab. Data shows percent enrichment relative to input for Spi-C interaction with *Nfkb1* and *Gapdh* promoters. Statistics was performed using a paired Student's t-test. Error bars represent SEM from 6 independent experiments. **$p<0.01$; *$p<0.05$.


A
Relative Transcript Levels
Spib-/-Spic+/- / Spib-/-
100
10
1
0.1
*
*
B2m Nfkb1
FO
B2m Nfkb1
MZ
B
PU.1/Spi-B
(-, 0.824)
PU.1/Spi-B
(+, 0.994)
105
130
Human
Mouse
Rat
Macaque
Dog
Cow
Tasmanian Devil
Consensus
T G G G A A T T T C C A G C C A G G A A G T G A G A
C
TTCC AGCCA GGAA
GTCC AGCCA GGAC
wt
mut
Luciferase
Nfkb1 Promoter
D
WEHI-279 MIGR1
WEHI-279 Spi-B
WEHI-279 Spi-C
kDa
~51
~42
~29
Anti-FLAG
Spi-B
Spi-C
Anti-β-Actin
E
WEHI-279 MIGR1
WEHI-279 Spi-B
WEHI-279 Spi-C
Normalized Fold Activation
30
20
10
0
*
**
pGL3
Basic
pGL3
Nfkb1
pGL3
Nfkb1 mut
F
pcDNA3
pcDNA3 Spi-C
Relative Light Units (RLU)
15
10
5
0
*
pGL3
basic
pGL3
Nfkb1
G
% Input
0.4
0.3
0.2
0.1
0.0
*
**
Nfkb1 Gapdh Nfkb1 Gapdh
WEHI-279 MIGR1
WEHI-279 Spi-C
Anti-Spi-C
Nfkb1 Gapdh Nfkb1 Gapdh
WEHI-279 MIGR1
WEHI-279 Spi-C
Anti-FLAG

3.3 Discussion

The purpose of these experiments was to understand how Spi-C contributes to B cell development and function. Spi-C heterozygosity restored many aspects of the *Spib*$^{-/-}$ phenotype. *Spib*$^{-/-}$*Spic*$^{+/-}$ mice had restored numbers of FO and T2 B cells in their spleens relative to *Spib*$^{-/-}$ mice. Furthermore, proliferation in response to LPS and anti-IgM-mediated stimulation was restored in *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells compared to *Spib*$^{-/-}$ B cells. Investigation of a potential mechanism for the *Spib*$^{-/-}$*Spic*$^{+/-}$ phenotypic rescue revealed that steady-state levels of *Nfkb1* were elevated in sorted FO and MZ B cells from *Spib*$^{-/-}$ *Spic*$^{+/-}$ spleens compared to *Spib*$^{-/-}$ spleens. Ectopic expression of Spi-B in B cells resulted in increased *Nfkb1* promoter activity, which was dependent on the ETS binding site. Conversely, overexpression of Spi-C inhibited *Nfkb1* activation by Spi-B. ChIP and ChIP-seq analysis demonstrated that both Spi-B and Spi-C are capable of directly binding to the *Nfkb1* promoter. In summary, these results suggest that Spi-C opposes Spi-B in B cell transcriptional regulation.

Intercrosses of *Spib*$^{-/-}$*Spic*$^{+/-}$ mice generated *Spib*$^{-/-}$*Spic*$^{-/-}$ mice at a frequency less than 1% of live births. Similar findings were reported where intercrosses of *Spic*$^{+/-}$ mice generated only 9% *Spic*$^{-/-}$ (Kohyama et al, 2009). Previous studies have demonstrated that embryos injected with *Spic*-siRNA resulted in reduced rate of blastocyst development (Kageyama et al, 2006). It is possible that Spi-B and Spi-C may have overlapping roles in prenatal development, and therefore embryonic death may be occurring at the blastocyst stage in *Spib*$^{-/-}$*Spic*$^{-/-}$ mice. Therefore, low birth frequency of *Spib*$^{-/-}$*Spic*$^{-/-}$ mice is likely due to reduced embryonic or fetal viability.

Few target genes for Spi-C have been identified in B cells, and Spi-C was reported to function either as an activator or as a repressor of gene transcription. It is possible that altered expression of other unidentified target genes may contribute to the *Spib*$^{-/-}$*Spic*$^{+/-}$ B cell phenotype. Spi-C ectopically expressed in pro-B cells can directly bind and oppose transcription of the *Fcgr2b* gene mediated by PU.1 (Schweitzer et al, 2006). In contrast, transcription of the gene *Fcer2a,* encoding CD23, was reported to be directly activated by Spi-C in WEHI-279 Spi-C cells (DeKoter et al, 2010). Earlier

reports showed that Spi-C cooperated with STAT6 to directly induce transcription of IgE under the control of IL-4 (Carlsson et al, 2006). In macrophages, Spi-C was reported to directly activate *Vcam1* transcription (Kohyama et al, 2009). Our data demonstrated that Spi-C directly opposes transcriptional activation of *Nfkb1* by preventing Spi-B and possibly PU.1 from binding to the *Nfkb1* promoter in B cells. In summary, Spi-C is capable of both activation and repression of its target genes, and can also transcriptionally regulate target genes by preventing other transcription factors from binding.

Previous studies have demonstrated that the transcription factors PU.1 and Spi-B are required to maintain proper levels of *Nfkb1* in B cells (Li et al, 2015). Spi-B directly binds to the *Nfkb1* promoter and activates transcription. In contrast, Spi-C directly binds to the *Nfkb1* promoter, but does not activate transcription. Therefore, decreased Spi-C expression may reduce its occupancy of the *Nkfb1* promoter in $Spib^{-/-}Spic^{+/-}$ B cells, permitting increased activation of *Nfkb1* by PU.1. Elevated *Nfkb1* transcript levels in $Spib^{-/-}Spic^{+/-}$ B cells compared to $Spib^{-/-}$ B cells suggest that Spi-C and Spi-B oppose each other to regulate appropriate *Nfkb1* levels. $Spib^{-/-}$ and $Nfkb1^{-/-}$ B cells proliferate similarly to LPS and anti-IgM. LPS stimulated $Nfkb1^{-/-}$ B cells fail to proliferate, and anti-IgM stimulation results in reduced proliferation (Sha et al, 1995). Overall, elevated *Nfkb1* expression in $Spib^{-/-}Spic^{+/-}$ B cells compared to $Spib^{-/-}$ B cells may sufficiently explain the rescue in proliferation.

$Spib^{-/-}Spic^{+/-}$ mice have restored FO and T2 B cell numbers compared to $Spib^{-/-}$ mice, indicating that Spi-B and Spi-C regulate peripheral B cell differentiation. FO and T2 cell differentiation both require NF-κB signaling and BCR signaling. Transitioning from a T1 to T2 B cell requires non-canonical NF-κB signals and basal BCR signals, and maturation of a T2 to a FO B cell requires canonical NF-κB signals and strong BCR signaling (Pillai & Cariappa, 2009). $Nfkb1^{-/-}Nfkb2^{-/-}$ mice have a developmental block at the T2 stage and fail to generate any mature B cells (Claudio et al, 2002). Therefore, elevated *Nfkb1* expression in developing $Spib^{-/-}Spic^{+/-}$ could be sufficient to explain the restored FO B cell population. It is also conceivable that Spi-C may also be a negative regulator of c-Rel expression, as PU.1 and Spi-B have been previously reported to directly activate *Rel* transcription (Hu et al, 2001). Alternatively, it is possible that FO

and T2 B cells in *Spib*$^{-/-}$*Spic*$^{+/-}$ have a longer life-span than *Spib*$^{-/-}$ B cells, which could contribute to its restoration. *Spib*$^{-/-}$ B cells have impaired BCR signaling and thus proliferate poorly in response to anti-IgM stimulation (Garrett-Sinha et al, 1999; Su et al, 1997). Transgenic Spi-C expression in B cells results in reduced transcript levels of the BCR signaling genes *Btk* and *Blnk* in FO B cells (Zhu et al, 2008). Restored proliferative response to anti-IgM in *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells compared to *Spib*$^{-/-}$ B cells suggests that BCR signaling is possibly restored due to reduced Spi-C expression. Since WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells are capable of phosphorylating Syk at equivalent levels following anti-IgM stimulation, the rescue in BCR signaling is likely downstream of Syk. Therefore, rescued FO and T2 B cells in *Spib*$^{-/-}$*Spic*$^{+/-}$ spleens might be caused by a combination of restored NF-κB and BCR signalling.

It is possible that aspects of the B cell phenotype of *Spib*$^{-/-}$*Spic*$^{+/-}$ mice are not cell-intrinsic. However, evidence was provided for a cell-intrinsic effect of Spi-C on B cell proliferation using the Eμ-Spi-C transgenic mouse. In this mouse model the Spi-C transgene is under the control of a lymphocyte-specific Eμ intronic enhancer. We found that crossing this transgenic mouse to *Spib*$^{-/-}$*Spic*$^{+/-}$ mice (Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$) resulted in a reduction in proliferation from isolated B cells, suggesting Spi-C has a cell-intrinsic effect on B cell function. Furthermore, overexpression of Spi-C in Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ mice reduced the number of FO and MZ B cells, suggesting a cell-intrinsic role for Spi-C in B cell development.

In summary, our results demonstrate a novel mechanism for Spi-C as a negative regulator of B cell development and function. Understanding transcriptional regulation in B cells has human health implications, since dysfunctional B cell development can lead to leukemia of the B cell lineage (Sokalski et al, 2011; Xu et al, 2012). Furthermore, antibody formation in B cells is transcriptionally regulated, so understanding potential activators and repressors of this process can improve interventions for vaccine development. Further analysis on mice lacking Spi-B and reduced Spi-C can provide additional insight into other genes involved in B cell development, function, and disease.

3.4 Materials and Methods

3.4.1 Generation and breeding of mice

Mice were housed at Western University's Health Sciences animal facility (London, Ontario, Canada) and monitored under an approved animal use subcommittee protocol in accord with Western University Council on Animal Care. C57BL/6 (WT) mice were purchased from Charles River Laboratories (Pointe-Claire, Quebec, Canada). *Spib*$^{+/-}$ *Spic*$^{+/-}$ mice were backcrossed to C57BL/6 mice for 5 generations prior to the generation of *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. *Spib*$^{-/-}$*Spic*$^{+/-}$ mice were generated by mating male and female *Spib*$^{-/-}$ *Spic*$^{+/-}$ mice. Eμ-Spi-C *Spib*$^{-/-}$*Spic*$^{+/-}$ mice were generated by mating Eμ-Spi-C^{+} male mice to *Spib*$^{-/-}$*Spic*$^{+/-}$ females. Genotyping was performed by PCR as previously described (DeKoter et al, 2010; Kohyama et al, 2009). All experiments were performed on mice aged 6-12 wks.

3.4.2 B cell enrichment and proliferation analysis

RBCs were removed from spleen cell suspensions by hypotonic lysis with ammonium chloride solution. B cells were enriched by negative selection using biotin-conjugated anti-CD43 (S7) Ab, streptavidin (SA) microbeads, LD depletion columns, and a QuadroMACS™ separation unit (Miltenyi Biotec, Germany). B cells ($2x10^5$/well) were plated in 96-well flat bottom plates and stimulated with LPS (10 μg/mL, List Biological Laboratories), anti-IgM Ab (50 μg/mL, affinity pure F(ab')2 fragment, or goat anti-mouse IgM, μ chain specific; Jackson ImmunoResearch Laboratories), in complete IMDM. Proliferation was assessed after 72 h incubation at 37°C with a TACS® MTT Cell Proliferation assay (Trevigen, Gaithersburg, MD) used according to the manufacturer's instructions. For CFSE analysis, $10x10^6$ B cells were stained with 5 μM of CFSE (Biolegend, San Diego, CA) for 5 min at room temperature prior to plating. Frequency of stained cells was assessed after 72 h incubation at 37°C by flow cytometry.

3.4.3 Flow cytometry

Antibodies and reagents purchased from eBioscience (San Diego, CA), BD Bioscience (Franklin Lakes, NJ), or BioLegend included 7-AAD, CFSE, brilliant violet 421 conjugated anti-CD45R/B220 (Ra3-6B2), allophycocyanin (APC)-conjugated IgM (II/41), APC-conjugated Annexin V, biotin-conjugated anti-CD43 (S7), phycoerythrin (PE) conjugated CD93 (AA4.1), PE conjugated anti-BP-1 (BP-1), PE-conjugated anti-CD19 (1D3), PE-conjugated phospho-Syk (moch1ct), fluorescein isothiocynate (FITC)-conjugated CD24 (30-F1), FITC-conjugated anti-CD21/CD35 (eBio8D9), FITC-conjugated anti-CD23 (B3B4), and PE-Cy5-conjugated streptavidin. Cells were blocked with purified anti-CD16/CD32 (Mouse BD Fc Block^{TM}). Intracellular staining was performed using a two-step protocol and intracellular fixation and permeabilization buffer set obtained from eBioscience. Antibody-stained cell analysis was performed using FACSCalibur and LSRII systems, and sorting was performed using a FACSAriaIII system (BD Biosciences). Sorted cells were determined to be >98% purity. Data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR).

3.4.4 Reverse transcription-quantitative PCR

RNA was isolated with Trizol reagent (Invitrogen, Burlington, Ontario, Canada). cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, Ontario, Canada), and qPCR was performed with a Rotor-Gene 6000 instrument (Corbett Life Sciences, Valencia, CA). Relative mRNA transcript levels were normalized to β2m and compared between samples using the comparative threshold cycle method. Calculations were performed using REST© 2009 software (Pfaffl et al, 2002). Primer sequences are listed in Table 3.2.

3.4.5 Immunoblot analysis

Lysates were prepared using Laemelli buffer and applied to 8%-10% SDS polyacrylamide gels for electrophoresis. Proteins were transferred to nitrocellulose membranes using a Trans-Blot Semi-Dry system (Bio-Rad), and membranes were blocked for 1 hour in 5% milk in TBST. Membranes were probed with anti-Spi-C (Aviva

Systems Biology, San Diego, CA), anti-Flag (M2, Sigma Aldrich, St. Louis, MO), anti-Syk (C-20, Santa Cruz Biotechnology, Dallas, Texas), or anti-β-Actin (I-19, Santa Cruz Biotechnology) Ab, and diluted to manufacturer recommended concentrations in 1% milk/TBST overnight at 4°C. A secondary horseradish peroxidase-conjugated anti-goat Ab was incubated in 1% milk/TBST. Membranes were washed and visualized with SuperSignal West Pico reagent (Thermo-Fisher Scientific).

3.4.6 ELISAs

Serum was collected and quantified for IgG2b and IgM. ELISA kits were purchased from eBioscience and performed according to the manufacturer's instructions. ELISA plates were analyzed using an Epoch microplate spectrophotometer with Gen5 software (BioTek, Winooski, VT).

3.4.7 Plasmids and cloning

Spi-C cDNA was cloned into the hemagglutinin (HA) tag-containing pcDNA3 vector as previously described (Schweitzer et al, 2006). The *Nfkb1* promoter was amplified from C57BL/6 genomic DNA by PCR using LA-TAQ (TaKaRa; Clontech Laboratories Inc, Mountain View, CA), purified, and cloned into the pSC-A vector using a PCR cloning kit (Agilent Technologies, La Jolla, CA). A 468bp *Nfkb1* promoter was PCR amplified using a forward primer containing a HindIII site and subcloned. The promoter was HindIII digested from pSC-A and ligated into the HindIII site of the pGL3-basic (Promega, Madison, WI) luciferase reporter vector. ETS binding sites were mutated using the QuickChange lightning site-directed mutagenesis kit according to the manufacturer's instructions (Agilent Technologies). Constructs were verified by DNA sequencing. Transfection-quality plasmid DNA was prepared by growth in DH5α bacteria and purified with plasmid maxi prep kit (Geneaid, New Taipei City, Taiwan). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Primer sequences used for cloning are listed in Table 3.2.

Table 3.2. Forward and reverse primers used for RT-qPCR analysis, cloning, and site directed mutagenesis.

Primer Name	DNA Sequence (5’ -> 3’)
B2m fwd	5’-TGG CTC ACA CTG AAT TCA CCC CCA-3’
rev	5’-TCT CGA TCC CAG TAG ACG GTC TTG-3’
Gapdh promoter ChIP fwd	5’-GTCTGTTATATGGGCGTCCTGTCA-3’
rev	5’-CGTTCACACCGACCTTCACCATTT-3’
*Nfkb1 f*wd	5’-GCT GAG TCC TGC TCC TTC TAA A-3’
rev	5’-CCT CTG TGT AGC CCA TCT GTT GC-3’
Nfkb1 promoter ChIP fwd	5’-TCC GTC TGT CTG CTC TCT-3’
rev	5’-GAA AGC AGT GAG GTA AGC CCA AC-3’
Nfkb1 Promoter fwd	5’-CCC AAA GAA TAA AGA CGC TCA AAT GC-3’
rev	5’-TCA GAA GAC CGA AGG AAG CCT ACA-3’
Nfkb1 Sublone w/ HindIII fwd	5’-CAA GCT TAC CTC CCG CCC CC-3’
Rev	5’-CCT GGA GAT GCG GCG CGG-3’
pGL3-*Nfkb1* Mutation	5’-TCT CGA CGT CAG TGG GAA TGT CCA GC-3’
pGL3-*Nfkb1* Mutation anti	5’-TCG CTC ACT CTC TCA CGT CCT GGC TGG-3’

3.4.8 Transient transfection

WEHI-279 MIGR1, MIG-3XFLAG-Spi-B, and MIG-3XFLAG-Spi-C cells were generated as previously described (DeKoter et al, 2010; Xu et al, 2012). Cells in mid to early log phase growth were washed three times with serum-free DMEM (4.5g/L, Wisent Inc, St-Bruno, Quebec) and incubated for 10 minutes at room temperature with 10 μg of each luciferase reporter plasmid and 1 μg of pRL-TK (Promega). Cell-DNA mixtures were electroporated at 220 V and 950 mF using 4-mm gap cuvettes with a GenePulser II with Capacitance Extender (Bio-Rad), incubated at room temperature for 10 min, then transferred to 6-well culture plates in complete DMEM and incubated at 37°C for 24 hours. A Dual-Luciferase Reporter Assay System (Promega) was performed on cell lysates. Light production was measured using a Lumat LB 9507 tube luminometer (Berthold Technologies, Oak Ridge, TN).

3.4.9 ChIP experiments

Chromatin was prepared from enriched B cells as previously described (Sokalski et al, 2011; Xu et al, 2012). In brief, cells were cross-linked and lysed followed by sonication. Sonicated chromatin was incubated overnight at 4°C with anti-Spi-C (generated by Fulkerson lab) and anti-FLAG (M2, Sigma-Aldrich) conjugated to protein G DynaBeads (Invitrogen). Magnetic bead complexes were enriched using a MagneSphere® Technology Magnetic Separation Stand (Promega) and washed. Immunocomplexes were eluted and cross-linked chromatin was reversed overnight at 65°C. DNA was purified using a QIAquick PCR purification kit (Qiagen, Limburg, Netherlands). Enrichment was measured using qPCR of immunoprecipitated DNA using primers indicated in Table EI.

3.4.10 ChIP-Seq Analysis

ChIP-seq experiments were performed using mouse WEHI-279 cells. Cells were spin-infected with a MIGR1 vector for expression of 3XFLAG-PU.1 or 3XFLAG-Spi-B. Immunoprecipitation was performed using anti-FLAG microbeads (M2, Sigma-Aldrich). Purified chromatin was sequenced on an Ilimuna HiSeq at the BC cancer research

agency. Peak finding and data analysis was performed using Galaxy Suite (Blankenberg et al, 2010; Giardine et al, 2005; Goecks et al, 2010). Reads were aligned to the reference genome (NCBI37/mm9), using BOWTIE (Langmead et al, 2009). Peaks were called using MACS version 1.0.1 (Zhang et al, 2008) with default parameters, a P-value cutoff for peak detection of 1e-05 and an effective genome size of 2.7e+9 bp. The ChIP-seq data from this publication have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) and assigned the identifier GSE48891 and GSE58128.

3.4.11 Statistical analysis

Statistical significance was determined using one-way or two-way ANOVA analysis with a Bonferroni post-test unless otherwise indicated. P values ≤ 0.05 were considered significant. For all figures, *P ≤ 0.05, **P ≤ 0.01, and ***$\leq$P 0.001. Statistical analysis was performed using Prism 5.0 (Graph-Pad Software).

Chapter 4

4 Overall discussion and future directions

This thesis describes the ability of the transcription factors PU.1, Spi-B, and Spi-C to control B cell development and proliferative function. The hypothesis that PU.1 and Spi-B were required for positively regulating components of TLR responses was tested. In chapter 2, B cells from *PUB* (*Spi1*$^{+/-}$*Spib*$^{-/-}$) mice were shown to be impaired for TLR-mediated proliferation responses. The impairment in *PUB* B cells was attributed to reduced levels of p50 protein, which is encoded by the *Nfkb1* gene. It was demonstrated that both PU.1 and Spi-B are capable of directly binding to the *Nfkb1* promoter, and the ETS binding sites within the promoter were required for efficient transcription of the gene. Impaired TLR-mediated proliferation was restored upon retroviral reinsertion of p50 into *PUB* B cells. These results suggest that *Nfkb1* activation by PU.1 and Spi-B is important for TLR-initiated B cell responses.

Chapter 3 characterized the role of Spi-C in B cells. The hypothesis that Spi-C inhibited target genes of PU.1 and Spi-B was tested. By generating the novel mouse model *Spib*$^{-/-}$*Spic*$^{+/-}$ and comparing it to *Spib*$^{-/-}$ mice, it was determined that Spi-C has a functionally distinct role compared to Spi-B. Spi-C heterozygosity restored many aspects of the *Spib*$^{-/-}$ phenotype, including a restoration in FO and T2 B cell numbers in the spleen, and restored LPS and anti-IgM-mediated B cell proliferation. *Nfkb1* was hypothesized to participate in the mechanism for the *Spib*$^{-/-}$*Spic*$^{+/-}$ phenotype. Transcript levels of *Nfkb1* were elevated in *Spib*$^{-/-}$*Spic*$^{+/-}$ B cells compared to *Spib*$^{-/-}$ B cells. In B cell lines overexpressing Spi-B, *Nfkb1* promoter activity was substantially elevated; however, *Nfkb1* promoter activity was significantly reduced in B cell lines overexpressing Spi-C. It was demonstrated that Spi-C directly bound to the same sites as PU.1 and Spi-B at the *Nfkb1* promoter. Therefore, Spi-C directly opposes Spi-B transcriptional regulation in B cells.

These studies have identified a mechanism by which PU.1, Spi-B, and Spi-C regulate development and proliferative function in B cells. PU.1 and Spi-B are capable of each individually binding directly to the *Nfkb1* promoter to activate its expression (Figure

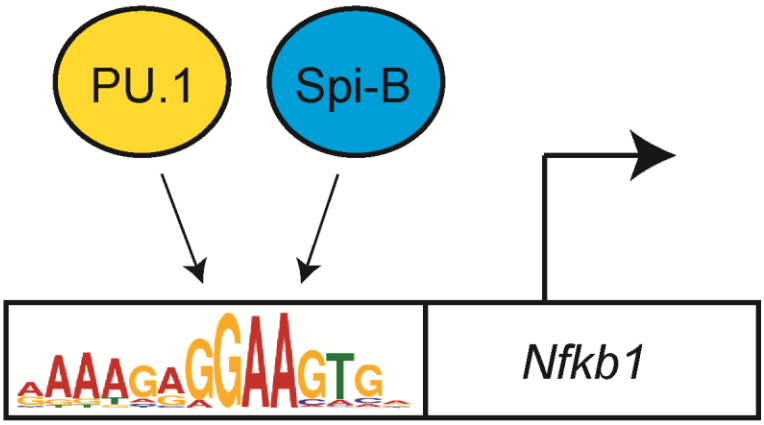
4.1A). In contrast, Spi-C directly binds to the same *Nfkb1* promoter region and represses *Nfkb1* activation (Figure 4.1B). Since all three transcription factors can bind to the same regions, we hypothesize that Spi-C can function to compete with PU.1 and Spi-B for binding at the same sites within the *Nfkb1* promoter. However, the mechanism of activation/repression through direct competition for binding can be complex.

There are many aspects of transcriptional regulation which can be addressed in the future. Repression of *Nfkb1* by Spi-C could be mediated through recruitment of other transcription factors or histone modifying proteins which affect the accessibility of the *Nfkb1* promoter. Likewise, the activation of *Nfkb1* by PU.1 and Spi-B can involve interactions with distal enhancer sites to recruit additional transcription factors to activate gene promoters or modulate chromatin accessibility. A high-throughput method of ChIP-mass spectrometry could identify the plethora of interacting proteins recruited by PU.1, Spi-B, or Spi-C (Wang et al, 2013). Furthermore, using chromosome conformation capture (3C) assays would elucidate the interactions between distal regulatory enhancers or insulators (Hagege et al, 2007). Overall, determining what transcription factors or associated proteins are recruited by PU.1, Spi-B, and Spi-C could improve our understanding of transcriptional regulatory networks.

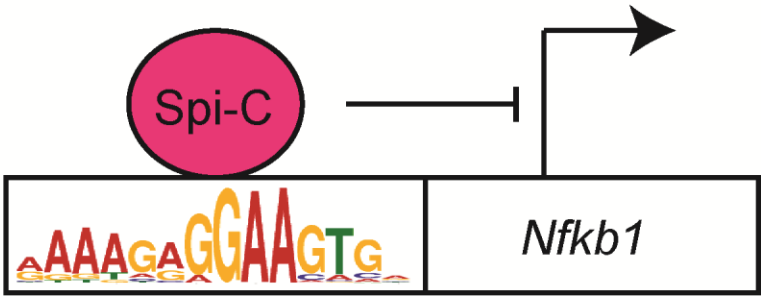
Figure 4.1. A model of transcriptional regulation of *Nfkb1* by PU.1, Spi-B, and Spi-C.

(A) PU.1 and Spi-B directly bind to the *Nkfb1* promoter to activate transcription. (B) Spi-C can directly bind to the same *Nkfb1* promoter region to inhibit its transcription.

A



B



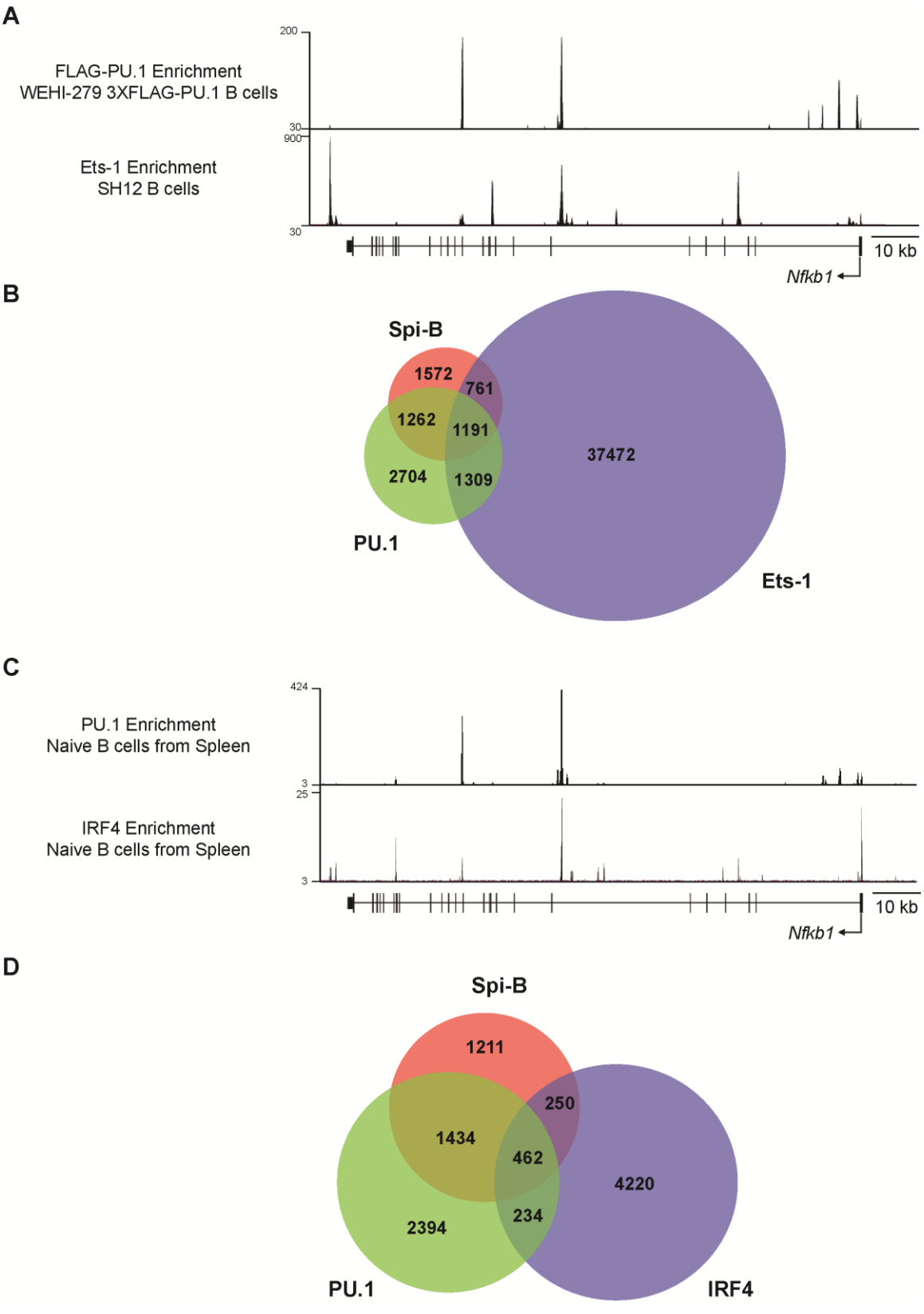
4.1 Transcriptional *Nfkb1* regulation

This thesis described transcriptional regulation of *Nfkb1* by PU.1, Spi-B, and Spi-C in B cells. Using the *PUB* mouse model, it was demonstrated that PU.1 and Spi-B directly regulated *Nfkb1* transcription. Spi-C was shown to be directly responsible for repressing *Nfkb1* transcription by interacting with the same sites in the promoter. There are multiple ETS binding sites within the *Nfkb1* promoter, and it has now been demonstrated that at least the first two sites are required for *Nfkb1* activation. While both ChIP and ChIP-seq analysis have validated direct PU.1, Spi-B, and Spi-C binding to the *Nfkb1* promoter, there are still many unknown aspects of its regulation. Other ETS family transcription factors are likely involved with *Nfkb1* activation in B cells. The ETS transcription factor Ets-1 was previously reported to be required for activating *Nfkb1* in human Jurkat T cells at a ETS binding site further downstream of the TSS (Lambert et al, 1997). Since Ets-1 is also expressed in B cells and is required for the generation of mature B cells (Eyquem et al, 2004; Garrett-Sinha, 2013), it is likely also involved with regulating *Nfkb1* expression. Comparing enrichment of FLAG-tagged PU.1 in WEHI-279 3XFLAG-PU.1 B cell lymphoma cells with Ets-1 binding in SH12 B cell lymphoma cell lines, there are regions where both transcription factors can be found (Figure 4.2A). The *Nfkb1* promoter is likely one of the common regions where PU.1, Spi-B, and Ets-1 can each bind, despite the large number of unique sites bound by Ets-1 (Figure 4.2B). It is currently unclear which other proteins are recruited by PU.1, Spi-B, and Spi-C to the promoter to initiate or repress *Nfkb1* transcription. Since PU.1 and Spi-B have been reported to interact with IRF4 (Care et al, 2014; Escalante et al, 2002), it is possible that PU.1 or Spi-B recruits IRF4 to regulate *Nkfb1* transcription. Based on publicly available ChIP-seq data from splenic B cells, there is a high degree of similarity in the binding patterns seen at *Nfkb1* between PU.1 and IRF4 in naïve splenic B cells (Figure 4.2C). Moreover, there are multiple overlapping sites of transcription factor binding across the mouse genome, when comparing ChIP-seq peaks between PU.1, Spi-B, and IRF-4 B cell datasets (Figure 4.2D). Therefore, it is conceivable that *Nfkb1* transcription can be regulated through recruiting interacting transcription factors and related ETS transcription factors in B cells.

Enrichment of PU.1 and Spi-B binding did not occur most highly at the promoter of *Nkfb1*. Several intronic regions of *Nfkb1* contained even larger PU.1 and Spi-B enrichment sites, suggesting that PU.1 or Spi-B may have higher affinity at enhancer regulatory elements within the gene body. Preliminary luciferase experiments suggested that the PU.1/Spi-B binding region in intron 6-7 of *Nfkb1* was not acting as an enhancer. Therefore, it is unclear whether the ETS binding site within the intron 6-7 of *Nkfb1* is essential for maximal expression. Analysis using luciferase constructs containing both non-mutated and mutated forms of the intron and promoter could determine the importance of the ETS binding site within the *Nkfb1* intron. Although the intron region may not be an enhancer, it is possible that the site is important for regulating *Nfkb1* expression. PU.1 and Spi-B could be binding to the region with other transcription factors to recruit proper chromatin remodeling complexes, or could be acting on proximal genes. Interestingly, IκB-γ inhibits DNA binding activity of p50 (Bell et al, 1996) and is encoded within the *Nfkb1* gene (Heron et al, 1995). Therefore, PU.1 and Spi-B could also be regulating IκB-γ expression for the purpose of a feedback-loop mechanism for regulating NF-κB activity.

Figure 4.2. Potential regulation of the *Nfkb1* gene by other ETS and ETS-interacting transcription factors.

(A) UCSC genome browser depiction of ChIP-seq data from WEHI-279 3XFLAG-PU.1 cells and SH12 cells showing PU.1 and Ets-1 binding at the *Nfkb1* gene respectively. (B) Venn diagram for regions of significant ChIP binding between PU.1, Spi-B, and Ets-1 from WEHI-279 3XFLAG-PU.1, WEHI-279 3XFLAG-Spi-B, and SH12 lymphoma cell lines respectively. (C) UCSC genome browser depiction of ChIP-seq data from naïve B cells showing PU.1 and IRF4 binding at the *Nfkb1* gene respectively. (D) Venn diagram for regions of significant ChIP binding between PU.1, Spi-B, and Ets-1 from WEHI-279 3XFLAG-PU.1, WEHI-279 3XFLAG-Spi-B, and naïve splenic B cells respectively. Overlapping binding regions in B and D were determined by subtraction of intervals. ChIP-seq data on Ets-1 binding in SH12 cells, PU.1 binding in naïve B cells, and IRF4 binding in naïve B cells is listed as GSM1003774, GSM537989, and GSM978747 in GEO.


A
FLAG-PU.1 Enrichment
WEHI-279 3XFLAG-PU.1 B cells
200
30
Ets-1 Enrichment
SH12 B cells
900
30
10 kb
Nfkb1
B
Spi-B
1572
761
1262
1191
37472
2704
1309
PU.1
Ets-1
C
PU.1 Enrichment
Naive B cells from Spleen
424
3
IRF4 Enrichment
Naive B cells from Spleen
25
3
10 kb
Nfkb1
D
Spi-B
1211
250
1434
462
4220
2394
234
PU.1
IRF4

4.2 Regulation of other NF-κB family members by PU.1, Spi-B, and Spi-C

It has been previously reported that PU.1 and Spi-B are involved in the transcriptional regulation of *Rel*, and it is required to maintain survival in B cells and allow proper development (Hu et al, 2001). Therefore, it is predicted that PU.1, Spi-B, and Spi-C are involved in regulating the transcription of other NF-κB family members aside from *Nfkb1* and *Rel*. Re-analysis of the ChIP-seq data in WEHI-279 3XFLAG-PU.1 and WEHI-279 3XFLAG-Spi-B cell lines confirmed that PU.1 and Spi-B bound proximal to the TSS of *Nfkb2, Rela, Relb,* and *Rel* (Figure 2.11). Therefore, PU.1 and Spi-B may be involved with the transcriptional regulation of those genes in B cells. It is hypothesized that Spi-C would inhibit transcriptional activation of *Rel*, similar to its effect on *Nfkb1* transcription.

Constitutive NF-κB activation is commonly seen in lymphomas (Davis et al, 2001; Jost & Ruland, 2007). Since WEHI-279 B cells do not express Spi-C (DeKoter et al, 2010), down regulation of Spi-C may be one method for tumor cells maintaining high levels of NF-κB expression. To test if Spi-C inhibits *Rel* transcription, studies could be performed by transfecting *Rel* promoter-containing luciferase constructs into WEHI-279 cell overexpressing Spi-B or Spi-C cells to determine *Rel* activation. Although PU.1 and Spi-B bind near the promoters for the NF-κB genes in WEHI-279 B cells, their importance in transcriptional regulation would need to be validated using expression constructs containing mutated ETS binding sites. Moreover, the transcriptional regulation of the different NF-κB members is stage specific, since different NF-κB members are predominantly activated depending on the cell type (Liou et al, 1994). ChIP-seq analysis could be performed using different types of B cell subsets to compare the differences in binding enrichment at various cell stages.

4.3 Spi-C as a repressor of PU.1 and Spi-B activity

Interactions between IRF-4 and PU.1 or Spi-B are important for regulating promoters and enhancers for genes involved in B cell function (Pongubala & Atchison, 1997; Rao et al, 1999). Spi-C was proposed as a negative control mediator due to its inability to interact with IRF-4 *in vitro* at the λB enhancer (Carlsson et al, 2003). Subsequent gain-of-function studies suggested that Spi-C functions as a negative regulator of transcription by opposing PU.1 activity. Overexpression of Spi-C in cultured pro-B cells resulted in decreased FcγRIIb expression and opposed transcription of the *Fcgr2b* gene mediated by PU.1 (Schweitzer et al, 2006). Furthermore, mice overexpressing Spi-C under control of the IgH intronic enhancer (Eμ-Spi-C) contained pre-B cells and FO B cells with reduced transcript levels of BLNK, Btk, and B220 compared to WT B cells (Zhu et al, 2008). Eμ-Spi-C B cells also proliferated poorly in response to anti-IgM or anti-CD40 (Zhu et al, 2008). Therefore, it was suggested that Spi-C impairs B cell function by affecting genes associated with BCR signaling.

The generation of a Spi-C deficient mouse ($Spic^{-/-}$) has allowed for loss-of-function analysis for Spi-C. However, $Spic^{-/-}$ mice were initially reported to have no abnormalities in B cell development (Kohyama et al, 2009). It is possible that B cells from $Spic^{-/-}$ mice could have functional differences, such as increased proliferation to TLR ligands or BCR signaling. By using $Spib^{-/-}Spic^{+/-}$ mice, it was demonstrated for the first time that reduced Spi-C expression can affect B cell proliferation and development. Furthermore, Spi-C expression may be normally repressed in B cells. For example, when the transcription factors Bach1 and Bach2 are deleted in mice, Spi-C is upregulated in B cells and results in altered gene expression patterns (Itoh-Nakadai et al, 2014). The authors concluded that expression levels of Spi-C may determine the responsiveness of B cells, and may be required for the differentiation and function of mature B cells (Itoh-Nakadai et al, 2014).

Overexpression of Spi-C in $Spib^{-/-}Spic^{+/-}$ B cells reversed the phenotype comparable to $Spib^{-/-}$. LPS-mediated proliferation in Eμ-Spi-C $Spib^{-/-}Spic^{+/-}$ mice was impaired compared to $Spib^{-/-}Spic^{+/-}$, and there was also a significant reduction in total,

MZ, and FO B cells in mice overexpressing Spi-C. Therefore, the data suggests that relatively low levels of Spi-C expression are required for proper development and function in B cells. In previous studies, Eμ-Spi-C mice were crossed to *PUB* mice (Eμ-Spi-C *PUB*) in order to determine if Spi-C could rescue the *PUB* phenotype. Although the Eμ-Spi-C *PUB* mice had restored frequencies of FO B cells based on CD23 expression, it remains unclear whether B cell function is restored in these mice. During the start of the cumulative studies described in this thesis, some experiments were performed on Eμ-Spi-C *PUB* mice as well. Proliferation studies were performed using WT, *PUB*, and Eμ-Spi-C *PUB* B cells. In preliminary studies, it was found that Eμ-Spi-C *PUB* B cells proliferated more poorly than *PUB* B cells in response to various TLR ligands (Figure 4.3A-B). Furthermore, transcript expression levels of genes in the TLR signaling pathway, with the exception of *Myd88* and *Irak4*, were further reduced in Eμ-Spi-C *PUB* MZ B cells compared to *PUB* MZ B cells (Figure 4.3). At the time, it was unclear how overexpression of Spi-C was affecting the *PUB* phenotype, so those experiments were excluded from the manuscript described in Chapter 2. However, since there is now additional evidence demonstrating Spi-C's involvement at being a repressor in B cells, these studies now coincide with this idea. Therefore, the data suggests that overexpression of Spi-C impairs the ability of B cells to function properly in response to TLR ligands, likely due to repression of genes within the TLR signaling pathway.

Future experiments demonstrating competition between PU.1/Spi-B and Spi-C for ETS binding sites would further elucidate the role of Spi-C in gene repression. ChIP could be performed in Eμ-Spi-C B cells and WT B cells using anti-PU.1antibody, and then PU.1 binding enrichment could be determined at the *Nfkb1* promoter by using qPCR. By comparing PU.1 enrichment in cells overexpressing Spi-C to those with normal Spi-C levels, it would be possible to demonstrate decreased PU.1 enrichment in Spi-C overexpressing cells. The same experiment would be performed with Spi-B, but there is currently no commercially available anti-mouse Spi-B antibody for effective ChIP applications. ChIP-seq has not been performed for Spi-B or Spi-C enrichment in naïve B cells. In Chapter 3, a Spi-C antibody was used for ChIP in WEHI-279 3XFLAG-Spi-C cells; however, the affinity of the anti-Spi-C antibody was weaker than anti-FLAG.

ChIP-seq analysis performed on splenic B cells using anti-Spi-C antibody would help in determining potential direct targets of Spi-C in primary B cells.

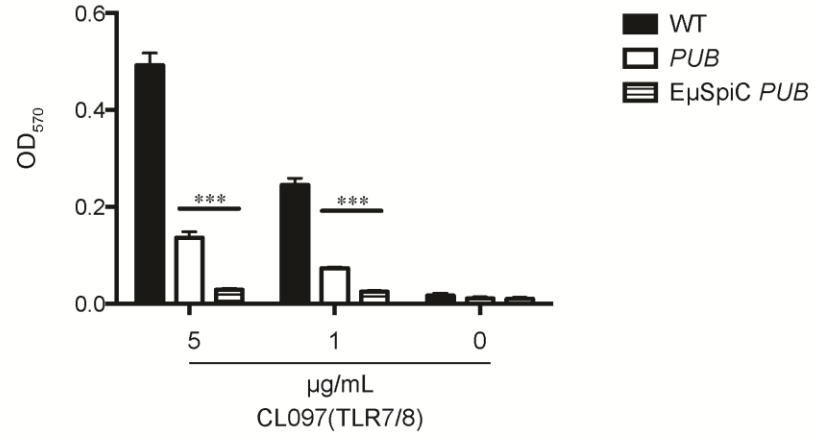
4.4 Spi-C as a transcriptional activator

Few target genes have been demonstrated to be directly activated by Spi-C. In B cells, Spi-C has been reported to directly activate *Fcer2a,* which encodes for the IgE low affinity Fc receptor (DeKoter et al, 2010), Furthermore, pro-B cells ectopically expressing Spi-C have demonstrated direct binding of Spi-C to the *IgH* intronic enhancer to initiate transcription of the Ig heavy chain (Schweitzer et al, 2006). In macrophages, Spi-C was reported to directly regulate *Vcam1* expression, which encodes for the cell adhesion molecule vascular cell adhesion molecule 1 (VCAM-1) (Kohyama et al, 2009). Another potential target gene of Spi-C may be *Grap2*, a SH2 and SH3 domain-containing adaptor protein, shown previously to require PU.1 and Spi-B in B cells (Garrett-Sinha et al, 2005). Interestingly, Eμ-Spi-C mice expressed elevated levels of *Grap2* in FO B cells and pre-B cells (Zhu et al, 2008), suggesting that Spi-C could directly activate *Grap2* transcription. *Myd88* may be a gene activated by Spi-C, since Eμ-Spi-C *PUB* MZ B cells express elevated transcript levels of *Myd88* compared to *PUB* MZ B cells (Figure 4.3C). However, further analysis would need to be done to validate this hypothesis. The list of target Spi-C genes could be vastly expanded by performing RNA-seq analyses. RNA from sorted B cell subsets from *Spib*$^{-/-}$*Spic*$^{+/-}$ and *Spib*$^{-/-}$ mice would be sequenced, aligned, and compared for differences in splicing, promoter use, and transcript abundance. Transcript levels of genes decreased in *Spib*$^{-/-}$*Spic*$^{+/-}$ compared to *Spib*$^{-/-}$ would suggest that Spi-C is involved in its activation. Alternatively, forced Spi-C expression could be conducted in the pro-B cell line 38B9 using a MIG-Spi-C retrovirus. RNA-seq analysis could be performed on Spi-C overexpressing 38B9 cells and compared to control cells to determine target genes of interest.

Figure 4.3. Spi-C overexpression in *PUB* B cells results in further impairment in TLR-mediated proliferation and altered gene profiles.

(A) B cells from Eμ-Spi-C *PUB* mice respond more poorly than *PUB* B cells stimulated with different concentrations of CL097 (TLR7 agonist). (B) B cells from Eμ-Spi-C *PUB* mice responded more poorly than *PUB* B cells stimulated with indicated TLR9, TLR6/2, TLR4, and TLR2/1 ligands. Data shows the mean ± SD for triplicate wells for A-B, and is a representative of two independent experiments. ***$p<0.001$. (C) Altered transcript levels of genes related to TLR signaling. RT-qPCR was performed using cDNA isolated from WT, *PUB*, and Eμ-Spi-C *PUB* MZ B cells. Expression levels shown are relative to WT levels.

A


0.6
0.4
0.2
0.0
OD570
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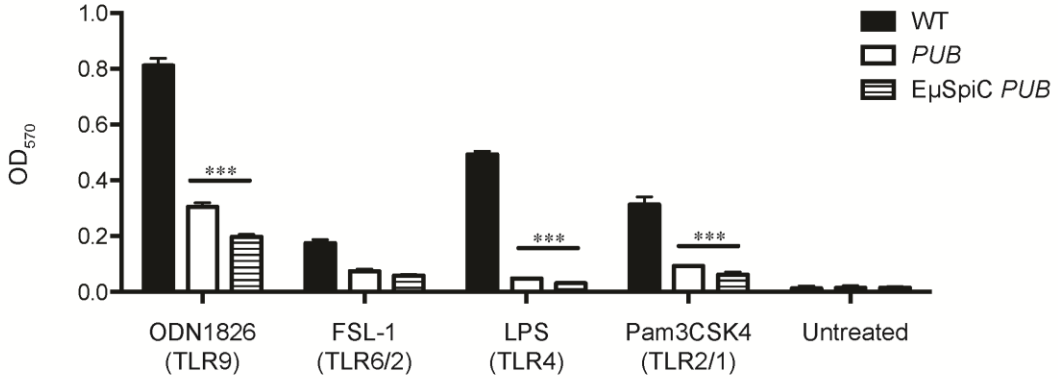
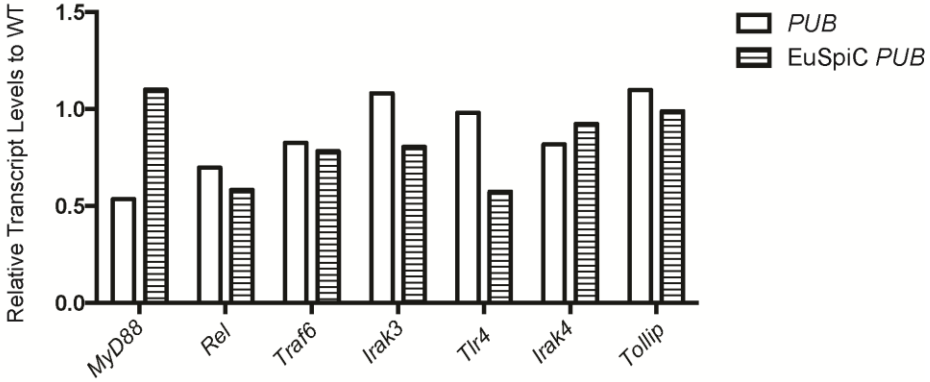
***
5
1
0
µg/mL
CL097(TLR7/8)
WT
PUB
EµSpiC PUB

B


1.0
0.8
0.6
0.4
0.2
0.0
OD570
***
***
***
ODN1826
(TLR9)
FSL-1
(TLR6/2)
LPS
(TLR4)
Pam3CSK4
(TLR2/1)
Untreated
WT
PUB
EµSpiC PUB

C


Relative Transcript Levels to WT
1.5
1.0
0.5
0.0
MyD88
Rel
Traf6
Irak3
Tlr4
Irak4
Tollip
PUB
EuSpiC PUB

4.5 Intrinsic vs extrinsic effects of PU.1, Spi-B, and Spi-C – potential uses of adoptive transfer models

A caveat to germline knockout models of mice is that the deletion affects all cells, leading to both direct and indirect effects of gene loss in the target tissue. Therefore, a developmental or function phenotype could be due to defects in multiple cell types. For example, Spi-B is expressed in pDCs, and is critical for its development and function (Sasaki et al, 2012). Upon TLR7 or TLR9 ligand stimulation, *Spib*$^{-/-}$ pDCs have impaired cytokine production (Sasaki et al, 2012). Therefore, it is possible that the limited production of cytokines by pDCs in *Spib*$^{-/-}$ mice could alter development and function of B cells. The possibility of extrinsic effects caused by Spi-C on B cells was partially addressed by crossing the Eμ-Spi-C mice to *Spib*$^{-/-}$*Spic*$^{+/-}$ mice. Since overexpression of Spi-C occurs through the B cell-specific *IgH* intronic enhancer (Zhu et al, 2008), the developmental and proliferation impairment in c mice must have been a cell- intrinsic effect of Spi-C in B cells. However, there could still be cell-extrinsic effects on the B cell lineage as a result of whole-body loss of Spi-B. To fully address these limitations without generating a conditional knockout mouse, adoptive transfer models could be used. Fetal liver cells from WT, *PUB*, *Spib*$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice would be isolated and injected into irradiated B cell deficient (μMT) mice, and cells would be allowed to reconstitute for four weeks. Flow cytometry analysis could be performed on the blood, spleen, and bone marrow to assess B cell development. Defects detected upon knockout cell reconstitution would suggest intrinsic effects caused by genes deleted in transferred cells.

4.6 Generation of a conditional knockout model for Spi-C

While conditional knockout models exist for PU.1 in B cells (Polli et al, 2005; Sokalski et al, 2011), similar models have not been generated for Spi-C. Generating a B cell conditional knockout mouse for Spi-C using the Cre/LoxP system would be highly beneficial. The conditional knockout would bypass the issues of embryonic lethality and confounding factors caused by all cells lacking Spi-C. Germline *Spic* knockout mice appear to cause at least partial embryonic lethality (Kohyama et al, 2009), and this

observation becomes more apparent when crossing the *Spic* null allele to mice which are germline *Spib* knockout.

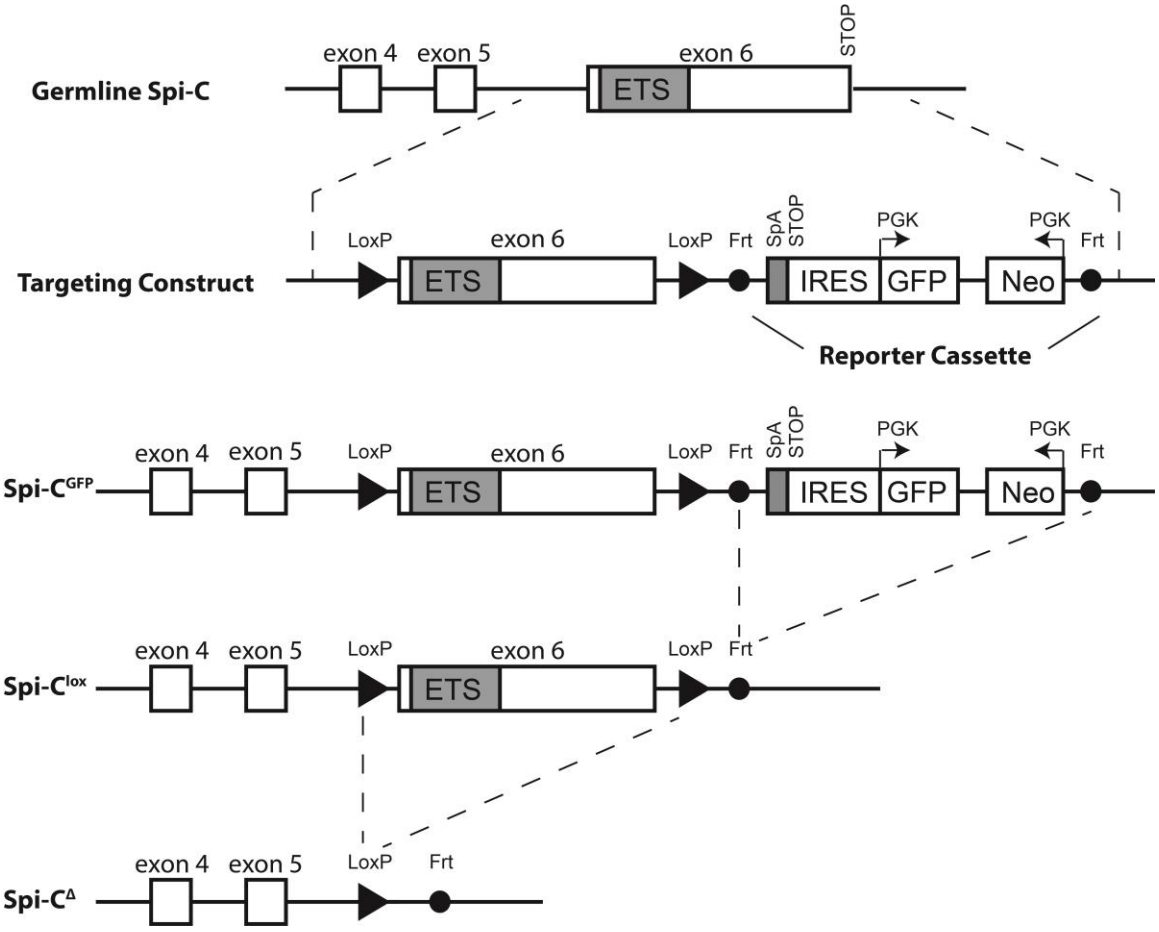
In order to utilize the Cre/LoxP system, the *Spic* gene would need to be flanked by LoxP sites for targeted deletion. One strategy would be to target exon 6 of the *Spic* gene which contains the ETS DNA binding domain, a similar approach performed for *Spi1* gene (Polli et al, 2005). The targeting vector would contain LoxP sites flanking (floxed) the coding region of exon 6, and a reporter cassette containing IRES-GFP and a selection marker neomycin (neo) flanked by Frt sites prior to the 3' UTR (Figure 4.4). Once the vector has been homologously recombined into mouse embryonic stem cells, clones could be selected by GFP expression and neomycin resistance. Chimeric mice generated would contain GFP in the *Spic* locus (Spi-C^{GFP}), which would allow for measuring Spi-C levels by flow cytometry. In order to remove the GFP reporter cassette, chimeric mice generated could be crossed to mice expressing flp recombinanse to generate Spi-C^{lox} mice. A B cell-specific Cre mouse which could be used is the Mb1-Cre mouse, where Cre is expressed under the control of the Ig-α subunit of the BCR. The benefit of using the Mb1-Cre mouse rather than the CD19-Cre mouse is that the efficiency of targeted deletion is greater (Hobeika et al, 2006). Crossing Spi-C^{lox} mice to Mb1-Cre mice would delete the floxed exon 6 of Spi-C to generate Spi-C^{Δ} mice, which lack Spi-C expression in the B cell lineage only. In addition, mice with total Spi-C deletion in all cells could be generated as controls, by crossing Spi-C^{lox} mice to deleter-Cre mice (Schwenk et al, 1995).

It is unclear at which stages of B cell development levels of Spi-C are required for proper function or development. Spi-C levels have previously been reported to be lowest at fractions A-C in the bone marrow, and highest in immature B cells in the spleen (Zhu et al, 2008). The use of Spi-C^{GFP} mice would be useful in comparing the levels of Spi-C between different stages of B cell development through the convenience of flow cytometry, since GFP expression would correlate with the levels of Spi-C. Since Spi-C is involved with blastocyst development (Kageyama et al, 2006), Spi-C^{GFP} mice would be a useful system to determine Spi-C expression during embryogenesis. Aside from analyzing development in the bone marrow and spleen, B cells from other tissues such as

the peritoneal cavity and lymph nodes could be analyzed. It is hypothesized that Spi-C has a role in B-1 cell function. In one study, microarray analysis was performed on splenic and peritoneal B-1 cells to compare gene expression profiles, and it was found that splenic B-1a cells expressed higher Spi-C transcript expression levels compared to peritoneal B-1a, B-1b, or splenic B2 cells (Kretschmer et al, 2003). Interestingly, $CD19^{+}CD5^{+}$ B-1a cells from the peritoneal cavity of $Spib^{-/-}Spic^{+/-}$ mice express elevated levels of CD19 compared to WT B-1a cells (Figure 3.2C), suggesting that *Cd19* expression may be a repressor target gene of Spi-C. Therefore, levels of Spi-C expression may be involved with proper B-1a cell function.

Figure 4.4. Conditional deletion of Spi-C.

Spi-C targeting construct containing exon 6 of *Spic* flanked by LoxP sites (►). The reporter cassette contains an internal ribosome entry site (IRES), green fluorescent protein (GFP) and a neomycin (Neo) selection marker flanked by Frt sites (●). GFP and Neo are driven by the phosphoglycerate kinase (PGK) promoter. The targeting construct is introduced into the *Spic* locus by homologous recombination to generate Spi-C^{GFP} mice. The reporter cassette is removed by crossing Spi-C^{GFP} mice to mice expressing flp recombinase, generating Spi-C^{lox} mice. Exon 6 of *Spic* is deleted by crossing Spi-C^{lox} mice with mice expressing Cre recombinase, creating Spi-C^{Δ} mice.

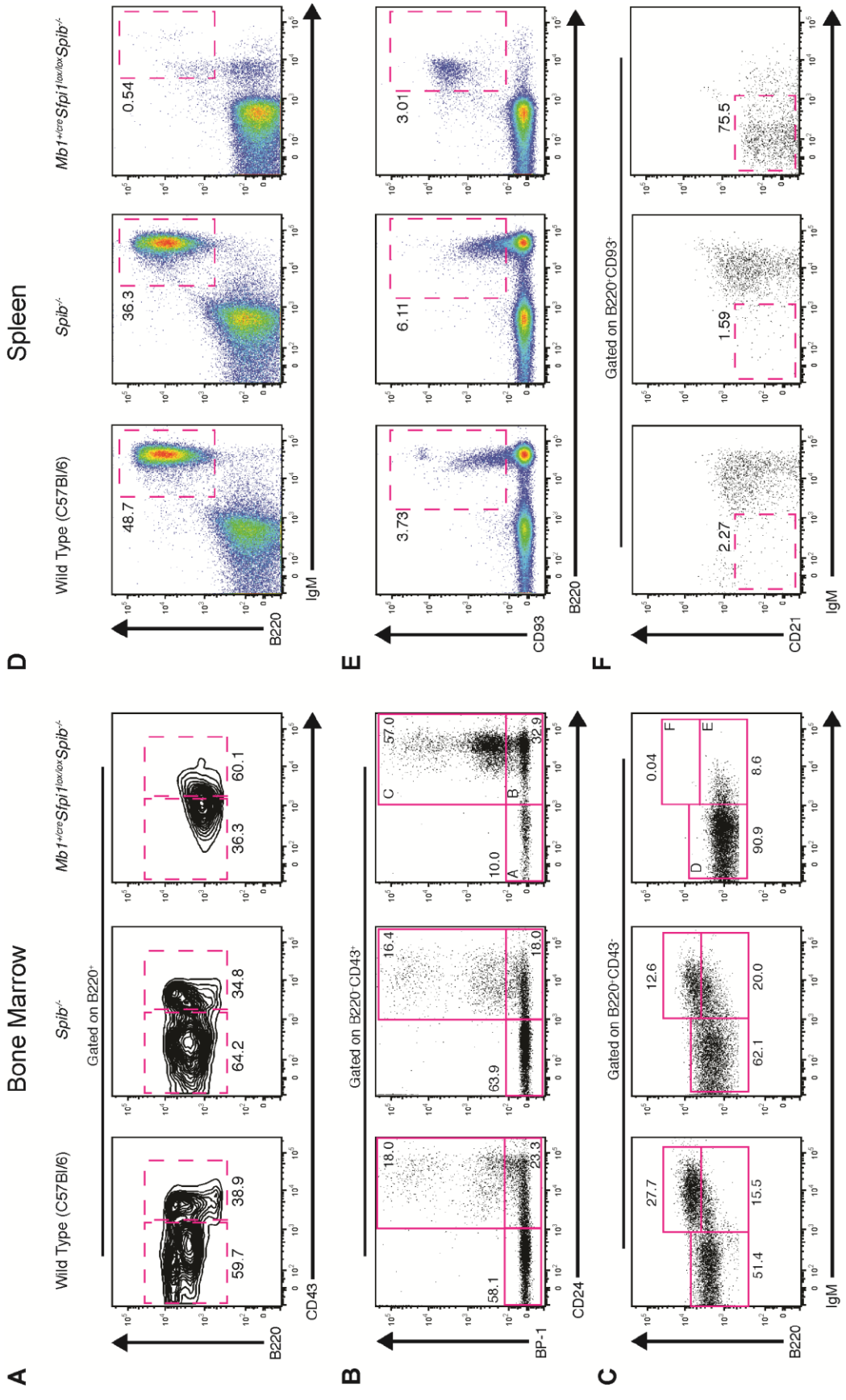

Germline Spi-C
exon 4
exon 5
exon 6
ETS
STOP
Targeting Construct
LoxP
exon 6
ETS
LoxP
Frt
SpA
STOP
PGK
IRES
GFP
PGK
Neo
Frt
Reporter Cassette
Spi-C^GFP
exon 4
exon 5
LoxP
exon 6
ETS
LoxP
Frt
SpA
STOP
PGK
IRES
GFP
PGK
Neo
Frt
Spi-C^lox
exon 4
exon 5
LoxP
exon 6
ETS
LoxP
Frt
Spi-C^Δ
exon 4
exon 5
LoxP
Frt

4.7 Existing conditional knockout models for PU.1 and Spi-B

Our laboratory has generated mice with deleted *Spi1* under the control of the B cell-specific *Cd19* locus on a *Spib*$^{-/-}$ background (*CD19*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$) (Sokalski et al, 2011). These mice have few CD19^{+}B220^{+} B cells within the spleen. Moreover, these mice develop B cell acute lymphoblastic leukemia with 100% incidence by 21 weeks of age (Sokalski et al, 2011). Therefore, B cell deficiency in these mice combined with leukemia precludes the use of this model to study mature B cell function. Another conditional knockout model which was generated by our lab utilizes the *Mb1* gene to drive cre-recombinase to delete floxed *Spi1* on a *Spib*$^{-/-}$ background (*Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$). *Mb1* encodes for CD79a, a membrane glycoprotein which is part of the BCR. The *Mb1* driven *Spi1* deletion resulted in an elevated fraction C cell population and a developmental block at the fraction D stage in the bone marrow (Figure 4.5A-C). Spleens of *Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ mice contained no mature B220^{+}IgM^{+} B cells in the spleen (Figure 4.5D), but contained an abnormal immature B220^{+}CD93^{+} B cell population which was negative for both CD21 and IgM (Figure 4.5E-F). The B220^{+}CD93^{-}CD21^{-}IgM^{-} B cell population in spleens of *Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ mice is similar to the leukemic population previously described in *CD19*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ (Sokalski et al, 2011). Therefore, *Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ are likely prone to developing leukemia with age, as observed in *CD19*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ mice (Sokalski et al, 2011). An advantage of the *Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ mouse is that the developmental block is more defined than in the *CD19* condition knockout. It would be possible to determine the effects of Spi-C at these early stages of development, since transcript levels of Spi-C rise during the transition from CD43^{+} (Fraction A-C) to CD43^{-} (Fraction D-F) stage (Zhu et al, 2008). Expression of Spi-C could have a role in the leukemia mouse models. Spi-C expression could be either increased or decreased by crossing leukemia prone mice to Eμ-Spi-C or *Spib*$^{-/-}$*Spic*$^{+/-}$ mice respectively. Since reduction of Spi-C increased proliferation in *Spib*$^{-/-}$ mice, it is conceivable that decreased Spi-C levels in *CD19*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ mice would exacerbate the disease. In contrast, overexpression of Spi-C could alleviate the disease, since overexpression of Spi-C in Eμ-Spi-C *PUB* mice decreases B cell proliferation.

Figure 4.5. Impaired B cell development in mice deficient in PU.1 and Spi-B.

(A) Bone marrow cells gated on B220 and CD43 for identifying "Hardy" B cell fractions in bone marrow. (B) Increased Fraction C population (BP-1^{+}CD24^{+}B220^{+}CD43^{+}) in *Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ bone marrow. (C) Developmental block at Fraction D (B220^{+}IgM^{-}CD43^{-}) in *Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ bone marrow. (D) Absence of mature B220^{+}IgM^{+} cells in the spleen of *Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ mice. (E) An abnormal CD93^{hi} immature B cell population exists in the *Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ spleen. (F) The immature B cells in *Mb1*$^{+/cre}$*Spi1*$^{lox/lox}$*Spib*$^{-/-}$ spleen are primarily CD21 and IgM negative. Analysis for A-F was performed by flow cytometry and are a representative of 3 independent experiments.


A
Bone Marrow
Wild Type (C57Bl/6)
Spib-/-
Mb1+/creSfpi1lox/loxSpib-/-
Gated on B220+
B220
CD43
59.7
38.9
64.2
34.8
36.3
60.1
B
Gated on B220+CD43+
BP-1
CD24
58.1
18.0
23.3
63.9
16.4
18.0
10.0
57.0
32.9
A
B
C
C
Gated on B220+CD43-
B220
IgM
27.7
51.4
15.5
62.1
12.6
20.0
90.9
0.04
8.6
D
Spleen
Wild Type (C57Bl/6)
Spib-/-
Mb1+/creSfpi1lox/loxSpib-/-
B220
IgM
48.7
36.3
0.54
E
CD93
B220
3.73
6.11
3.01
F
Gated on B220+CD93+
CD21
IgM
2.27
1.59
75.5

4.8 In Vivo Studies

Proliferation studies performed in this thesis were *ex vivo* models. Primary B cells were isolated from the spleens of mice and cultured in media with TLR-ligands. The measured response in an *in vitro* culture system is a simplification a cell's response. Since the B cells are no longer in their native environment, they are not in contact with other cell types within the confines of the structural organ, or with cytokines produced by other cells which could influence the response in B cells. Therefore, *in vivo* proliferation studies could be performed to further enhance the current results of this thesis. A common method to measure proliferation *in vivo* is based on 5-Bromo-2'-Deoxyuridine (BrdU) incorporation and detection by flow cytometry (Carayon & Bord, 1992). Proliferation analysis by BrdU incorporation would allow multiple B cell subsets to be simultaneously measured between different strains of mice, following inoculation with specific antigens such as DNP-KLH or DNP-LPS.

Unfortunately for the described studies in chapter 2 and chapter 3, *in vivo* response studies could not be effectively performed since the mice were housed in a conventional facility. For future studies, the mice would need to be re-derived in a specific pathogen free animal facility for proper *in vivo* studies. WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$ *Spic*$^{+/-}$ mice immunized for DNP-KLH or DNP-LPS would be used to differentiate differences in TD and TI immune responses respectively. Flow cytometry and immunohistochemistry analysis could be performed on the spleen and lymph nodes to assess differences in germinal center formation. Anti-DNP-KLH or anti-DNP-LPS ELISAs could determine differences in antibody production using serum collected from mice. Immunization studies performed in *Spib*$^{-/-}$*Spic*$^{+/-}$ mice would determine whether Spi-C has an effect on germinal center formation, or antibody production.

4.9 Significance of these studies

ETS transcription factors are likely involved with transcriptionally regulating BCR and TLR signaling genes. Since TLR and BCR signaling are both required for optimal antibody responses, PU.1, Spi-B, and Spi-C can potentially regulate antibody formation. Studying the regulatory interactions between TLR and BCR signaling will allow improved predictions of B cell responses during immunization approaches. Determining the genes essential for antibody formation could provide therapeutic interventions for immunodeficiencies, or improve development of effective vaccines. Determining how B cells respond to different antigens can lead to novel developments of vaccine adjuvants or synthetic vaccines designed to activate appropriate amounts of TLR and BCR signaling.

Proper levels of Spi-C likely need to be regulated during B cell development. I hypothesize that reduced Spi-C levels can alter B cell function during development through the transitional B cell stages. Peripheral tolerance of B cells may be regulated by Spi-C through BCR signaling genes. Spi-C may negatively regulate genes involved in BCR signaling, which can affect the differentiation of T1 to T2 B cells. The developmental block at the T2 stage in *Spib*$^{-/-}$ mice could result from a combination of Spi-B failing to activate BCR signaling genes, and Spi-C inhibiting those same genes. Reduced Spi-C expression could potentially alleviate defects in antibody production observed in *Spib*$^{-/-}$ mice. It is possible that as *Spic*$^{-/-}$ mice age, these mice could be prone to developing autoimmune or leukemic diseases due to an accumulation of T1 B cells and a failure of peripheral tolerance.

4.10 Concluding Remarks

The long-term goal of this research is to understand how ETS transcription factors regulate B cell development and control immune responses to TI and TD antigens. The studies presented in this thesis demonstrate the importance of the ETS transcription factors PU.1, Spi-B, and Spi-C in directly regulating *Nfkb1* transcription, which is highly involved in humoral immune responses and B cell development. The network of

transcription factors regulating other transcription factors gives appreciable insight to the complexity of gene regulation.

Studies on Spi-C are still at their infancy. Most published studies have attributed Spi-C towards a function in macrophages, but its expression is not limited to one particular cell type. By determining that Spi-C has a negative regulatory role in B cell development and function, our study provides new insight on how PU.1 and Spi-B target genes are regulated. *Nfkb1* is one of many genes likely regulated in opposing fashion between PU.1/Spi-B and Spi-C. Genes activated by PU.1 and Spi-B during antibody responses, drug treatments, and cancer growth could all be influenced by Spi-C's inhibition. Hopefully, the advancement of this work yields new questions and answers in the fields of transcriptional regulation and B cell biology.

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Appendices

Appendix A. Statement of Permission for the Use of Animals for Experimental Research.

All animal experimentation was conducted in compliance with the animal use protocol 2009-10 held by Dr. Rodney DeKoter, principal investigator at the Schulich School of Medicine and Dentistry and the department of Microbiology and Immunology at the University of Western Ontario in London, Ontario, Canada.

Western

AUP Number: 2009-010
PI Name: Dekoter, Rodney
AUP Title: Transcriptional Regulation Of Myeloid And Lymphoid Cell Fates

Approval Date: 04/25/2013

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Transcriptional Regulation Of Myeloid And Lymphoid Cell Fates
" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2009-010::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

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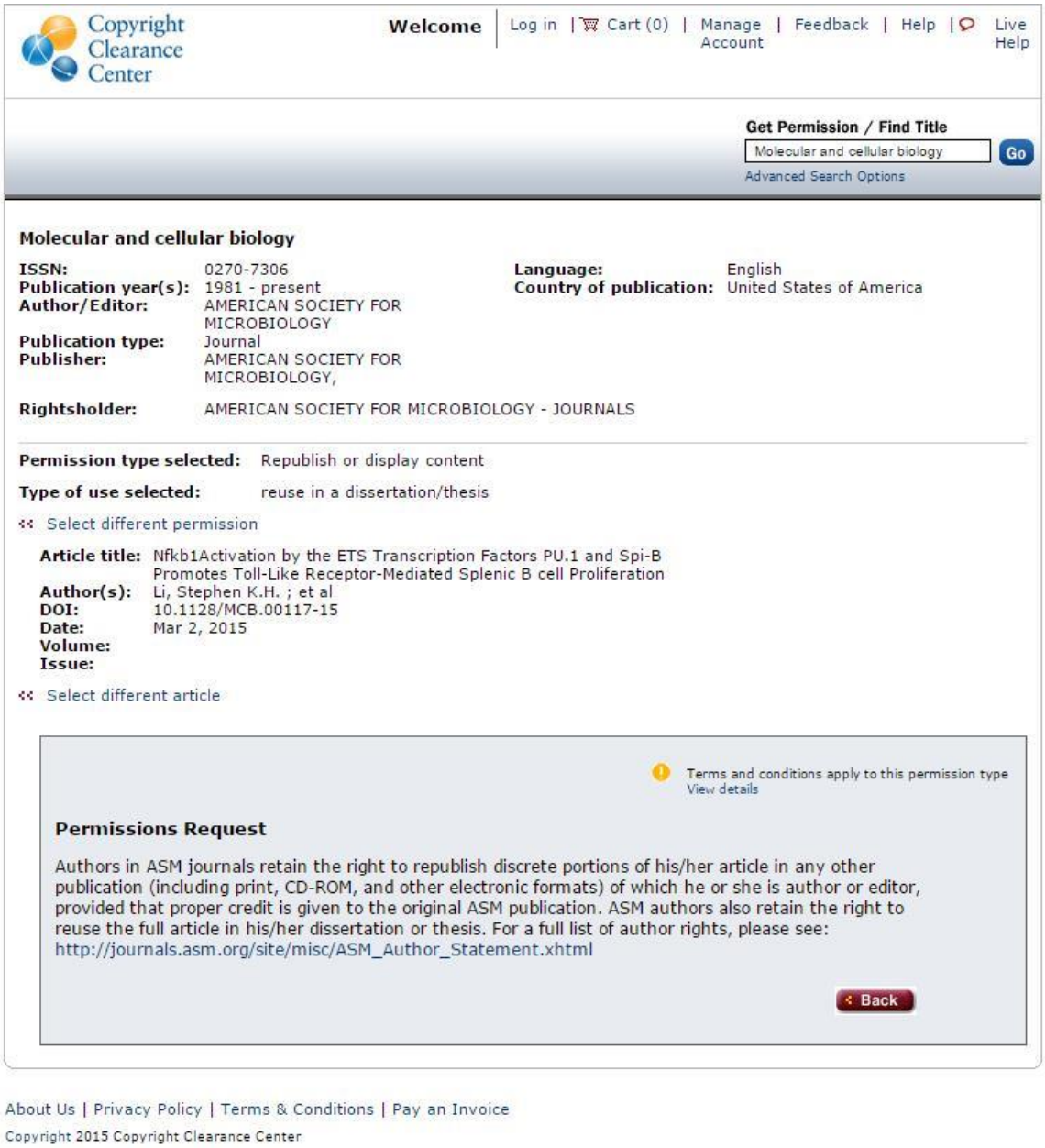

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  Western University ($1000/year)
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PUBLICATIONS

1. **Stephen K. H. Li**, Ali K. Abbas, Lauren A. Solomon, Gaëlle M.N. Groux, and Rodney P. DeKoter. 2015. *Nfkb1* Activation by the ETS Transcription Factors PU.1 and Spi-B Promotes Toll-Like Receptor-Mediated Splenic B cell Proliferation. *Molecular and Cellular Biology*. 35(9):1619-1632.
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3. Lauren A. Solomon, **Stephen K. H. Li**, Jan Piskorz, Li S. Xu and Rodney P. DeKoter. 2014. Genome-wide comparison of PU.1 and Spi-B binding sites in a mouse B lymphoma cell line. *BMC Genomics*. 16:76. doi:10.1186/s12864-015-1303-0.
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CONFERENCE MEETINGS – ORAL PRESENTATIONS

- Li, S. K. H., Solomon, L.A., Fulkerson, P.C., and DeKoter, R. P. 2014. *Identification of a negative regulatory role for Spi-C in the murine B cell lineage*. Keystone Symposia - The Golden Anniversary of B cell Discovery, Banff, Alberta. *(2015)*
- Li, S. K. H., Abbas, A. K., and DeKoter, R. P. *Toll-like Receptor-Mediated Splenic B cell Proliferation Requires Activation of Nfkb1 by the Related ETS Transcription Factors PU.1 and Spi-B.* 5th Annual Developmental Biology Research Day, London, Ontario. *(2013)*
- Li, S. K. H. and DeKoter, R. P. *Regulation of Toll-like Receptor (TLR) Signalling by Transcription Factors PU.1 and Spi-B in Splenic B cells*. 24th Annual Canadian Society for Immunology Conference, Lake Louise, Alberta. *(2011)*

CONFERENCE MEETINGS – POSTER PRESENTATIONS

- Li, S. K. H., Solomon, L.A., Fulkerson, P.C., and DeKoter, R. P. 2014. Identification of a negative regulatory role for Spi-C in the murine B cell lineage. Keystone Symposia - The Golden Anniversary of B cell Discovery, Banff, Alberta. *(2015)*
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- Li, S. K. H. and DeKoter, R. P. *Regulation of Toll-like Receptor Mediated Splenic B cell Proliferation by the Related ETS Transcription Factors PU.1 and Spi-B.* 2012 London Health Research Day, London, Ontario. *(2012)*
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