ROLE OF BRUTON'S TYROSINE KINASE AND INTERLEUKIN-6 IN PLASMA CELL ACCUMULATION AND AUTOANTIBODY PRODUCTION IN LYN-DEFICIENT MICE, A MODEL OF LUPUS

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DEDICATION

To my Mother and my Father, I am eternally grateful for all the love and support.

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Systemic lupus erythematosus (SLE) is characterized by loss of tolerance to nuclear antigens such as DNA and chromatin, resulting in autoantibody production, immune complex deposition, inflammation, and end organ damage such as glomerulonephritis (GN). Currently, only non-specific, immunosuppressive therapies are approved for use in lupus patients. These have undesirable side effects and risks. The development of more targeted therapies is necessary and requires a better understanding of the mechanisms that contribute to the production of autoantibodies.

Mice deficient in Lyn, a gene associated with human lupus, develop several features characteristic of SLE, including peripheral plasma cell accumulation, anti-

dsDNA antibodies, and GN. Lyn is a Src-family tyrosine kinase that, in general, inhibits B cell and myeloid cell activity. Loss of Lyn results in cellular hyperactivity associated with autoantibody production. Bruton's tyrosine kinase (Btk), which is critical to B cell receptor (BCR) signaling, mediates BCR hypersensitivity and autoantibody production in *lyn-/-* mice. B cell hyperresponsiveness is not, however, sufficient for the autoimmune phenotype; additional Btk-dependent events are required. Btk also contributes to myeloid cell function, and generally opposes Lyn action in these cells as in B cells. However, the relative contribution of myeloid hyperactivity to autoantibody production in *lyn-/-* mice is unknown.

Lyn-deficient mice expressing reduced Btk dosage in B cells and no Btk in myeloid cells were utilized to better define how Lyn and Btk regulate and mediate, respectively, the progression from tolerance to autoimmunity. Two major checkpoints regulating autoantibody production were identified and found to be breached in *lyn-/-* mice. The first checkpoint regulates Btk-mediated accumulation of long-lived plasma cells co-incident with polyclonal IgM autoreactivity. This is due in part to impaired migration of *lyn-/-* plasma cells towards SDF-1 and involves a B cell intrinsic effect of Lyn-deficiency. The second checkpoint regulates the class-switching of B cells with lupus-associated autoantigen specificities and the production of pathogenic autoantibodies. This step requires IL-6, which is produced in excess by *lyn-/-* myeloid cells in a Btk-dependent manner.

These results suggest that both B and myeloid defects contribute to autoimmunity in *lyn-/-* mice and identify Btk and IL-6 as potential therapeutic targets for SLE.

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AID – activation-induced cytidine deaminase

ANA – anti-nuclear antibodies

AP-1 – activator protein 1

APC – allophycocyanin

APC – antigen presenting cell

APRIL – a proliferation-inducing ligand

ATF – activating transcription factor

BAFF – B lymphocyte activating factor of the tumor necrosis factor family

BBS – borate-buffered saline

Bcl-2 – B cell CCL/lymphoma 2

BCMA – B cell maturation protein

BCR – B cell antigen receptor

Blimp-1 – B-lymphocyte-induced maturation protein 1

Blk – B lymphoid tyrosine kinase

BLNK – B cell linker protein

BLyS – B lymphocyte stimulator

BMDM – bone marrow-derived macrophages

BrdU – 5-bromo-2-deoxyuridine

BSA – bovine serum albumin

Btk – Bruton's tyrosine kinase

Btk^{lo} – low-dosage Btk

CD – cluster of differentiation

cDMEM – complete Dubelcco's modified Eagle medium

cDNA – complementary DNA

CLC – cardiotrophin-like cytokine

CNTF – ciliary neurotrophic factor

CpG – C—phosphate—G-containing DNA

c-Myc – v-myc myelocytomatosis viral oncogene homolog

CREB – cAMP response element-binding

cRPMI – complete Roswell Park Memorial Institute medium

CSR – class switch recombination

CT-1 – cardiotrophin-1

CTLA4 – cytotoxic T lymphocyte-associated protein 4

CXCL – chemokine (C-X-C motif) ligand

CXCR – chemokine (C-X-C motif) receptor

DAG – diacylglycerol

DC – dendritic cell

dsDNA – double-stranded deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

Elk-1 – Ets like gene 1

ERK – extracellular signal-regulated kinase

FBS – fetal bovine serum

FDC – follicular dendritic cell

FITC – fluorescein isothiocyanate

FO – follicular

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GBM – glomerular basement membrane

GC – germinal center

G-CSF – granulocyte colony-stimulating factor

GM-CSF – granulocyte-macrophage colony-stimulating factor

gp – glycoprotein

Grb2 – growth factor receptor-bound protein 2

GN – glomerulonephritis

GWAS – genome-wide association studies

H – heavy chain

HEL – hen egg lysozyme

ICOS – inducible T cell co-stimulator

IFN – interferon

Ig – immunoglobulin

IKK – I kappa B kinase

IL – interleukin

IRAK – interleukin-1 receptor-associated kinase

ITAM – immunoreceptor tyrosine-based activating motif

ITIM – immunoreceptor tyrosine-based inhibiting motif

JAK – Janus kinase

JNK – Jun N-terminal kinase

κ – kappa light chain

λ – lambda light chain

LIF – leukemia inhibitory factor

lpr – lymphoproliferation gene

LPS – lipopolysaccharide

Mal – MyD88-adaptor-like

MAPK – mitogen-activated protein kinase

MAX – Myc-associated protein X

M-CSF – macrophage colony-stimulating factor

MHCII – major histocompatibility

MRL – Murphy Roths Large

mRNA – messenger RNA

MyD88 – myeloid differentiation factor 88

MZ – marginal zone

NEMO – NF-κB essential modulator

nfi – net fluorescence intensities

NF-AT – nuclear factor of activated T cell

NF-κB – nuclear factor-kappa B

NKT – natural killer T

NMT – N-myristoylationtransferase

NZB/W – New Zealand black x New Zealand white F1

NZM – New Zealand mixed

ODN – oligodeoxynucleotide

OSM – oncostatin M

PAMP – pathogen-associated molecular pattern

PB – plasmablast

PBS – phosphate-buffered saline

PC – plasma cell

PCR – polymerase chain reaction

pDC – plasmacytoid dendritic cell

PDK1 – phosphoinositide-dependent protein kinase 1

PE – phycoerythrin

PH – pleckstrin homology

PI3K – phosphoinositide-3-kinase

PIP2 – phosphatidylinositol-4,5-bisphophate

PIP3 – phosphatidylinositol-3,4,5-triphosphate

PKB – protein kinase B

PKC – protein kinase C

PLCγ2 – phospholipase C-gamma 2

PRR – pattern recognition receptor

PSGL-1 – P-selectin glycoprotein ligand 1

PTEN – phosphatase and tensin homolog

PTK – protein tyrosine kinase

qPCR – quantitative real-time polymerase chain reaction

R – receptor

RA – rheumatoid arthritis

RAG – recombination activating genes

RANK – receptor-activator of NF-kappa B

RANKL – receptor-activator of NF-kappa B ligand

RF – rheumatoid factor

RBCs – red blood cells

RNA – ribonucleic acid

SAPK – stress-activated protein kinase

SCID – severe combined immunodeficiency

SDF-1 – stromal cell-derived factor 1

SFK – Src family kinase

sgp130 – soluble gp130

SH – Src homology

SHIP – SH2-containing inositol-5'-phosphatase

SHM – somatic hypermutation

SHP-1 – SH2-containing phosphatase 1

SLC – surrogate light chain

SLE – systemic lupus erythematosus

SOS – son of sevenless

SPF – specific pathogen free

STAT – signal transducer and activator of transcription

T – transitional

TAB – transforming growth factor-beta-activated kinase 1 binding protein

TAK – transforming growth factor-beta-activated kinase

TACI – transmembrane activator and calcium modulator and cyclophilin ligand interactor

TCR – T cell antigen receptor

Tg – transgene/transgenic

TGFβ – transforming growth factor-beta

Tfh – follicular helper T cell

Th – T helper cell

TI – T-independent

TIR – Toll-interleukin 1 receptor

TLR – Toll-like receptor

TNFα – tumor necrosis factor-alpha

TRAF – tumor necrosis factor receptor-associated factor

Treg – regulatory T cell

TYK – tyrosine kinase

U – units

wt – wild-type

XBP-1 – X-box binding protein 1

xid – x-linked immunodeficieny

XLA – X-linked agammaglobulinemia

Yaa – Y-linked autoimmune accelerator

CHAPTER I

INTRODUCTION

Overview

The primary objective of the immune system is to protect self from non-self. In order to do so, it must first detect the pathogens that would otherwise cause damage to the host. Accordingly, the mammalian adaptive immune system has intricate mechanisms that allow it to recognize the innumerable antigenic variants that we are exposed to daily. Of equal importance are the mechanisms that have developed to maintain immune tolerance to self so that the host's natural defenses will not attack host tissues. Redundant mechanisms protect against occasional inaccuracies, but functional failures are frequently unavoidable. In the case of autoimmune diseases, these immunological breakdowns occur in the regulatory mechanisms that maintain immune tolerance. As a result, the immune system detects and initiates immune responses to self-antigens. The efficacy with which the immune system defends against pathogens is now focused on the host, and the results can be detrimental to the host.

In systemic lupus erythematosus (SLE), tolerance is lost to nuclear antigens, which leads to damage of any host tissues and organs that express these

antigens. The exact etiology of SLE remains unknown, but investigations to distinguish the mechanisms that are critical to disease onset and progression are ongoing. The goal of this work is to characterize defects in Lyn kinase-deficient mice that are associated with autoimmunity and to define how these defects are suppressed by reduced dosage of Bruton's tyrosine kinase. The studies focus on terminal differentiation of B cells, the role of myeloid hyperactivity in autoimmunity, and the net effect of both of these cell types on the production of autoreactive antibodies.

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) affects approximately 1 in 2000 individuals. It is characterized by loss of tolerance to nuclear antigens such as DNA, RNA and chromatin and many others. Anti-nuclear antibodies (ANA) are often present long before the onset of symptoms. This phase is known as benign autoimmunity [Arbuckle *et al*., 2003]. Some ANA-positive individuals never progress to full blown SLE. Others go on to develop pathogenic autoantibodies, including anti-dsDNA, that lead to immune complex deposition, inflammation, and end organ damage such as glomerulonephritis (GN), rash, arthritis, and vasculitis.

A wide range of defects are known to play a role in SLE, and these mechanisms are rarely the same between distinct lupus populations. As such, current standards of therapy involve relatively non-specific immunosuppression with undesirable side effects. The development of novel treatment approaches that target disease-specific mechanisms is therefore of great interest.

SLE is a complex disease with both genetic precursors and environmental cues that affect manifestation and prognosis. It is broadly accepted that the strongest indicator for lupus susceptibility is a genetic predisposition [Deapen *et al*., 1992; Jarvinen *et al*., 1992]. Genome-wide Association Studies (GWAS) have now identified many genes associated with human lupus [Graham *et al*., 2009]. Both naturally occurring polymorphisms and targeted mutations in numerous genes lead to lupus-like autoimmunity in mice. In general, these genes can be characterized into groups depending on the overall mechanism which they affect. For the following review, I will make this distinction into two general categories. One will comprise those genes and mechanisms which directly impact lymphocyte tolerance, with a focus on B cells. This will include mutations that alter antigen receptor signaling or make autoreactive cells resistant to appropriate death signals. The second group will include defects of the innate immune system such as hyperactive antigen-presenting cells, excessive stimulation of TLRs, and over-production of IFNα. In general, the mechanisms in the latter group inevitably lead to loss of tolerance in an indirect manner.

Regardless of the genetic defect(s) that lead to SLE, pathogenic autoantibodies produced by autoreactive B cells are a common feature of the disease. B cells also contribute to lupus pathology through multiple antibody-independent mechanisms including the disruption of T cell tolerance, induction of pathogenic effector T helper 1 (Th1) and T helper 17 (Th17) cells, the attraction and activation of dendritic cells, the inhibition of regulatory T cells, activation of autoreactive memory T cells and the activation and recruitment of follicular B-helper T cells (Tfh) [Manjarrez-Orduno *et al.*, 2009; Townsend *et al.*, 2010]. Thus, the multiple roles that B cells perform in a disease such as SLE are best appreciated with a basic understanding of B cell development and function, which is primarily governed by the expression and function of the B cell antigen receptor (BCR).

B cell development and activation

B cell development and editing

The BCR is not only central to a B cells function in responding to antigen and eliciting an immune response. It is also critical to B cell development, as is exemplified in SCID or RAG-/- mice, which lack the enzymes required for

immunoglobulin gene rearrangement and thus cannot form an intact BCR. These mice are severely immunocompromised due to a lack of lymphocytes [Bosma and Carroll, 1991; Mombaerts *et al*., 1992; Shinkai *et al*., 1992]. B and T lymphocytes share a unique ability to rearrange their immunoglobulin (Ig)/BCR and TCR genes, respectively. This ability allows for the great variation in pattern recognition which is critical to our immunocompetence.

B cell development is defined by stages in which a B progenitor cell rearranges the various gene segments that will ultimately be expressed at the cell's surface as a BCR. The various stages of B lymphopoiesis correlate with the status of Ig gene rearrangements (Table 1.1) [Hardy and Hayakawa, 2001; Pelayo *et al*., 2005, 2006].

Table 1.1. B cell development occurs through several stages.

Stage	Heavy Chain	Light Chain	Ig
Progenitor B cells	germline	germline	-
Early Pro-B cells	D-J rearrangement	germline	-
Late Pro-B cells	V-DJ rearrangement	germline	-
Large Pre-B cells	VDJ rearranged	germline	IgH
Small Pre-B cells	VDJ rearranged	V-J rearrangement	IgH
Immature B cells	VDJ rearranged	VJ rearranged	IgM+
Mature B cells	VDJ rearranged	VJ rearranged	IgM+ IgD+

Formation of the pre-BCR complex is a critical step in B cell development and induces the transition from pro-B to pre-B cell. Immunoglobulin (Ig) heavy (H) chain μ in pre-B cells is associated with λ5 and VpreB, which form a

surrogate light chain (SLC), prior to rearrangement and expression of conventional light chain [Karasuyama *et al*., 1990, 1993; Melchers *et al*., 1993]. This pre-B cell receptor (pre-BCR) assembles with the Ig accessory proteins Igα and Igβ, similar to the B cell antigen receptor (BCR) on mature B cells, and, as with the BCR, plays a critical role in cell differentiation [Karasuyama *et al*., 1996]. At this point in development a proliferative burst occurs, dependent on assembly and signaling of the pre-BCR [Hardy and Hayakawa, 2001]. This serves to expand the pool of B cells that have successfully rearranged an IgH chain. IL-7 also contributes to this proliferative expansion of pre-B cells [Fleming and Paige, 2002].

Next, proliferation ceases and Ig light chain genes rearrange. The intact BCR is expressed in immature B cells, and at this stage the BCR signaling threshold is tuned once again. Productive B cell development requires expression of a light chain that can efficiently pair with the cell's particular heavy chain to form a cell surface BCR. However, the resulting BCR must not signal above some critical threshold (indicative of crosslinking by self antigen) or else RAG expression continues [Lang *et al*., 1996]. Rearrangement will occur at the kappa light chain locus in order to generate a new light chain that will not produce an autoreactive BCR. This light chain editing process can continue on both alleles and eventually proceed to the second light chain locus if unsuccessful at kappa, resulting in lambda light chain expression [Gay *et al*., 1993; Tiegs *et al*., 1993].

Studies in non-Ig transgenic mice have placed estimates of the extent of receptor editing to as high as 25% of the peripheral mature B lymphocyte pool [Casellas *et al*., 2001], indicating this to be a fairly common response to BCR signaling in the bone marrow. In addition to receptor editing, BCR crosslinking at the immature B cell stage can induce two other tolerance mechanisms: i) cells are eliminated by apoptosis (clonal deletion); ii) cells become nonresponsive and likely short lived (anergic).

Peripheral B cell maturation, activation and differentiation

An immature B cell that expresses a non-autoreactive antigen receptor will eventually exit the bone marrow. In the periphery, immature B cells migrate to secondary lymphoid tissues where they mature through several transitional stages of development before becoming either follicular (FO) or marginal zone (MZ) B cells. A B cell's location in either the follicle or marginal zone is indicative of its role and function as a mature cell. A majority of mature, naïve B cells in the periphery are FO B cells. While in the spleen and lymph nodes, FO B cells reside within the B cell zone of secondary lymphoid follicles, which they leave to re-circulate through the periphery many times over until they encounter antigen. Marginal zone B cells do not circulate through the periphery and are found in the marginal zone which encircles a follicle. They have a more innate like response

and serve as a primary defense to antigen encounter through their ability to make antibody quickly and with little co-stimulation from other cells. They are transcriptionally primed to mount rapid responses in the absence of T cell costimulation, forming bursts of Ig-secreting plasmablasts that persist for a limited time after initial antigen exposure [Martin and Kearney, 2000; MacLennan *et al*., 2003; Haas *et al*., 2005; Alugupalli *et al*., 2004]. B-1 B cells, located in the peritoneal cavity, have a similar rapid, innate-like response as MZ B cells [Martin *et al*., 2001]. The rapid differentiation of MZ and B-1 cells provides effective early antibody titers but occurs at the expense of cell proliferation and amplification of the response.

Conversely, a FO B cell that has encountered antigen requires T cell help to enter a germinal center (GC) reaction and make antibody (Figure 1.1). A B cell that is activated through its antigen receptor will internalize the protein and load it onto surface major histocompatibility class 2 (MHCII) molecules in preparation for T cell help. Antigenic stimulation also leads to the up-regulation of co-stimulatory molecules such as CD80 and CD86 [Carreno and Collins, 2002]. To increase its chance of receiving the appropriate help, a B cell will migrate towards the follicular boundary along the T cell zone [Garside *et al*., 1998]. Similarly, naïve Th cells are primed by professional antigen-presenting cells (APCs) such as antigen-experienced mature dendritic cells (DCs) in the T cell zone. Primed Th cells expand, differentiate into effector Th cells, and migrate to the T-B border to

contact B cells presenting MHCII/peptide complexes on their surfaces. B cells thus elicit help from T cells that recognize epitopes contained in the antigen recognized by the B cell. The CD80 and CD86 on B cells provides co-stimulation to the T cell via CD28.

In turn, the T cell provides help to the B cell via the following mechanisms. CD40 ligand (CD40L, also known as CD154 or gp39) is a member of the tumor necrosis factor (TNF) superfamily of transmembrane proteins. The interaction of CD40L on activated T cells with its receptor, CD40 on B cells, promotes B cell proliferation, [Saeland *et al*., 1993; Foy *et al*., 1994; Han *et al*., 1995], GC formation [Renshaw *et al*., 1994; Foy *et al*., 1994; Han *et al*., 1995], and antibody isotype switching [Ledermann *et al*., 1994]. T cell derived cytokines also support these processes as described below. The end result of this interaction is the production of high affinity, long lived antibodies and memory B cells of varying isotypes (and thus effector functions).

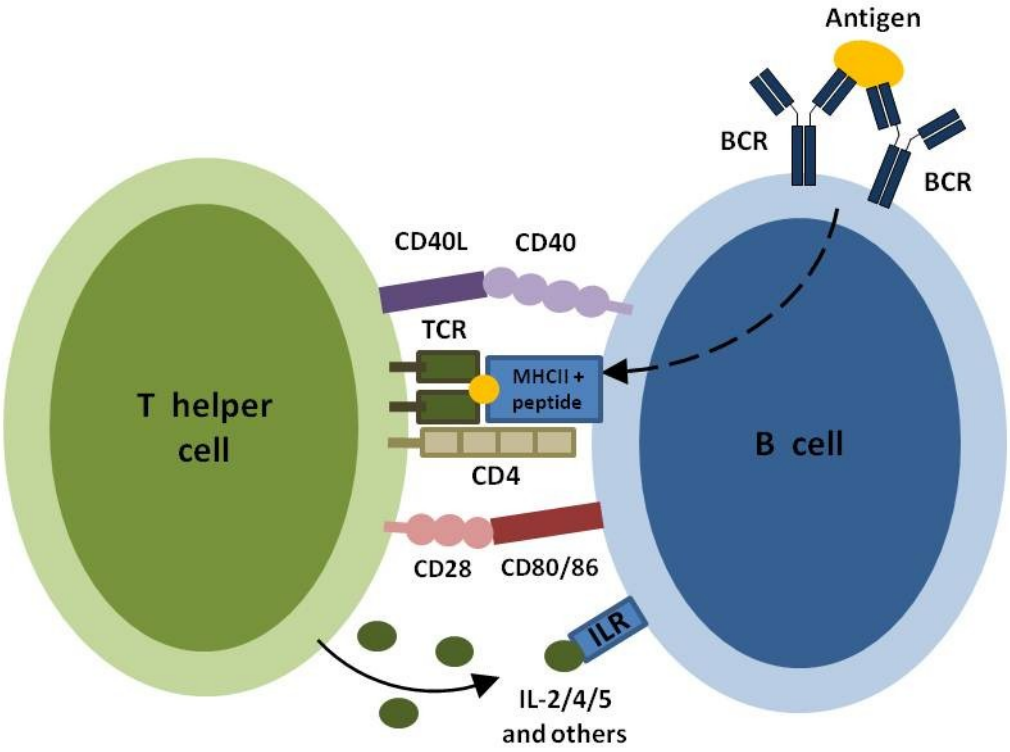

Figure 1.1. Cognate T-B interactions. The two-way interaction between B cells and T cells is critical to the germinal center reaction. B cells must process and present antigen on MHCII molecules to ensure that they interact with T cells that recognize the same antigen. Mutual activation of the interacting B and T cells occurs via the indicated receptor/ligand pairs.

Germinal centers and extrafollicular reactions

The germinal center (GC) provides a microenvironment within which affinity maturation of the humoral immune response takes place [Berek *et al*., 1991; Jacob *et al*., 1991]. During this process, the majority of GC B cells die through apoptosis and only those few that express a receptor with relatively high affinity for the antigen are selected to differentiate into memory and plasma cells [Smith *et al*., 1996]. The initial contact between antigen-experienced B cells and T cells leads to B cell proliferation. These rapidly dividing B cells, called centroblasts, eventually form the GC dark zone. During this period of rapid proliferation, somatic hypermutation (SHM) of the immunoglobulin (Ig) variable (V) region genes is ongoing in Ig-negative centroblasts. These cells subsequently migrate to the light zone as non-dividing centrocytes, which re-express surface immunoglobulin. In the light zone these cells are tested for their ability to bind antigen on follicular dendritic cells (FDCs) [MacLennan *et al*., 1992]. Only B cells with adequate affinity survive, the rest undergo apoptosis. Interacting with antigen on the FDCs is competitive and favors centrocytes with the highest affinity. Those which are positively selected can re-enter the dark zone for successive rounds of mutation and selection. These processes of mutation and selection improve the average affinity of the GC B cell population for presented antigen, affinity maturation [MacLennan, 1994]. In addition to these specialized

FDCs that populate the GC, a specialized subset of follicular helper T cells (Tfh) also play a critical role in GC reactions. Although other T cell subsets such as Th2 cells and γδ T cells [Wen *et al*., 1996] have been known to aid in antibody production, discovery of various molecules that participate in providing T cell help to B cells have helped define this Tfh cells as a specialized group of cells. In addition to CD40L, which stimulates B cell proliferation and, in the presence of appropriate cytokines, isotype switching (see below), Tfh also express specialized/essential molecules including CXCR5, which drives/allows the co-localization of T and B cells in lymphoid follicles [Spolski and Leonard, 2010], inducible co-stimulator (ICOS), which induces the production of helper cytokines [Hutloff *et al*., 1999] and IL-21, which is a potent stimulator of B cell proliferation, isotype switching, and differentiation [Leonard and Spolski, 2005]. At the end of the GC reaction, two types of cells emerge: memory B cells and plasma cells (see below).

Memory B cells persist for long periods of time after antigen exposure and are capable of rapidly responding to antigen re-encounter with secretion of high-affinity antibodies. There are several features of memory B cells that allow them to achieve this including i) increased precursor frequency of antigen-specific memory B cells [McHeyzer-Williams and McHeyzer-Williams, 2005]; ii) strategic location at sites of antigen drainage, such as the splenic marginal zone; [Tangye *et al*., 1998; Dunn-Walters *et al*., 1995]; iii) increased affinity for

specific antigen due to SHM [Rajewsky, 1996]; and iv) constitutive expression of co-stimulatory molecules CD80 and CD86 [Tangye *et al*., 1998, Ellyard *et al*., 2004].

Whether a B cell undergoes a GC or extrafollicular response is most often linked to the antigen eliciting the response and the affinity of the B cell receptor for that antigen [Phan *et al*., 2006]. As described above, GC reactions generate a population of plasma cells that secrete high-affinity antibody of an appropriate class and that persist for a lifetime. The extrafollicular pathway represents an alternate route to differentiation that generally results in the generation of short-lived plasma cells that secrete low-affinity antibody, in part because of limited SHM in these reactions (Figure 1.2).

There is a growing body of work characterizing the cellular interactions that occur outside of the follicle. For example, there is a strong correlation between plasmablast association with $CD11c^{high}$ DCs and their continued survival and differentiation into plasma cells [Vinuesa *et al*., 2010]. There is also a newly described Tfh-like cell that acts as a specialized T helper cell in these extrafollicular regions [Odegard *et al*., 2008]. Accordingly, these cells express a chemokine receptor profile that causes them to migrate to extrafollicular sites, but still exhibit functions typical of classic Tfh cells. They are also unique in their low-level expression of PSGL-1 (P-selectin glycoprotein ligand-1).

Plasma cell numbers are limited in the spleen, but those that persist there seem to be supported regardless of whether they are derived from extra-follicular plasmablasts or GC emigrants [Sze *et al*., 2000]. After an immune response, excess plasma cells are lost during a phase of contraction [Sze *et al*., 2000; Fairfax *et al*., 2008]. This limit to the number of plasma cells sustained within a given area indicates that plasma cells require external factors for survival; these constitute a niche in the microenvironment in which plasma cells are maintained. GC-derived plasma cells migrate to niches in the bone marrow in response to SDF-1/CXCL12 [Hargreaves *et al*., 2001]. There they encounter the factors that support long-term survival. These include IL-6, SDF-1, CD44 ligands, granulocyte- macrophage colony-stimulating factor (GM-CSF), TGFβ and TNFα [Cassese *et al*., 2003; Minges Wols *et al*., 2002]. BAFF, also known as BLyS, and its homologue APRIL also support the survival of at least some subsets of plasma cells through their interactions with TACI and BCMA [Benson *et al*., 2008].

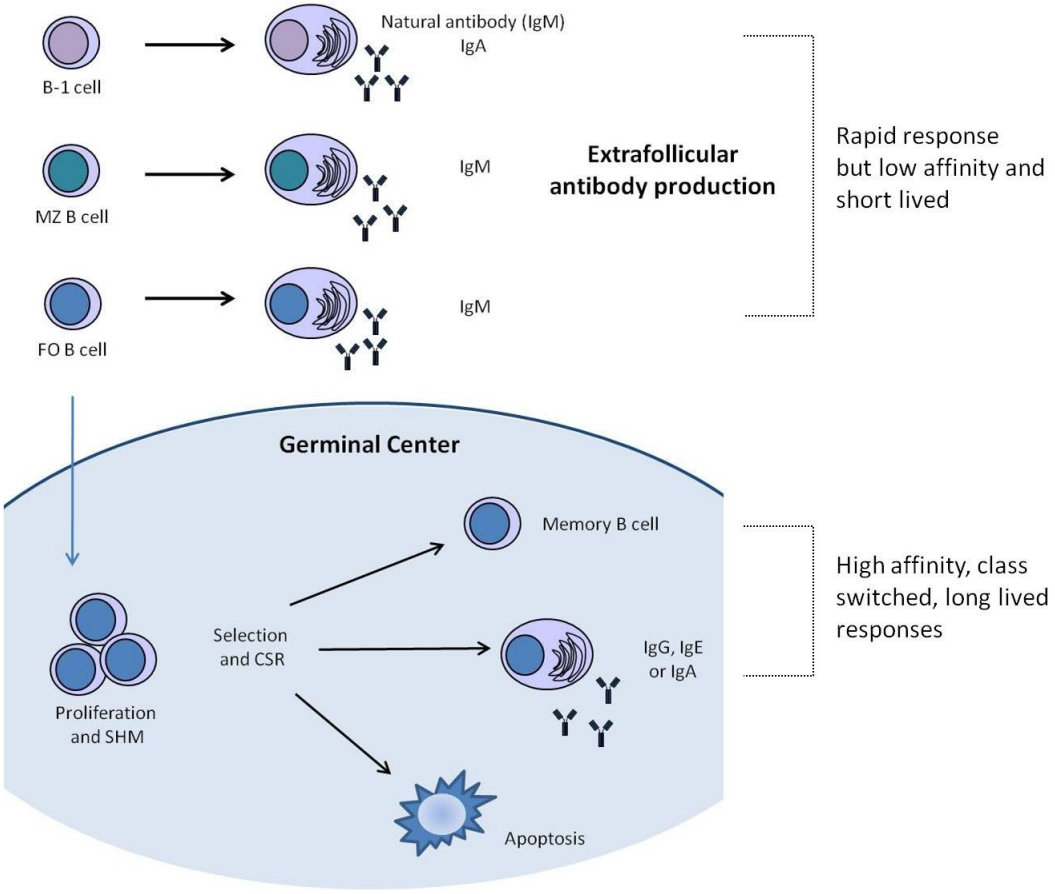

Figure 1.2. Extrafollicular and germinal center responses produce antibodies of different affinity and effector functions. Plasma cell generation in the extrafollicular environment occurs in less time (< 1 week) than responses generated with a germinal center (> 1 week); this allows for early, low-affinity antibody responses that begin working to clear an infection. Plasma cells generated in a germinal center produce high-affinity antibodies that can persist in the body for months after the pathogen has been eliminated. Germinal center reactions are also critical for immunological memory.

Class switching

Class-switching is another process by which a B cell can increase the diversity and effectiveness of the Ig it produces, and like somatic hypermutation, relies on help from a cognate T cell that express CD40 ligand. By substituting the heavy chain C-region of IgM with that of either IgA, IgE or one of four subclasses of IgG, antibodies acquire new effector functions that are crucial for the neutralization of invading pathogens (Table 1.2). Immunoglobulin isotype class switching occurs through class switch recombination (CSR) of DNA and usually requires engagement of CD40 on B cells by CD40 ligand on antigen-activated $CD4^+$ T cells.

T cell-derived cytokines regulate the specificity of a CSR reaction such that they drive the synthesis of particular heavy chain C-regions which allow for the production of either IgG, IgE, or IgA antibodies. For example, IL-4 specifically promotes switching to IgG1 and IgE and IFNγ induces IgG3 and IgG2a [Snapper *et al*, 1997]. Cytokines drive this specificity by activating the appropriate promoter for a given antibody isotype.

Activation-induced cytidine deaminase (AID) is essential for the physiological alterations in immunoglobulin genes that generate antibody memory: class-switch recombination (CSR) and somatic hypermutation [Muramatsu *et al*., 2000; Revy *et al*., 2000].

Table 1.2. Antibody isotypes and functions.

Isotype	Subclasses	Properties	Cytokine Induction[1]
IgA	IgA	Found in mucosal areas, such as the gut, respiratory tract and urogenital tract, and prevents colonization by pathogens. Also found in saliva, tears, and breast milk. Form dimers in mucous secretions but not plasma.[1]	TGFβ IL-5
IgD		Functions mainly as an antigen receptor on B cells that have not been exposed to antigens.[2]	
IgE		Binds to allergens and triggers histamine release from mast cells and basophils, and is involved in allergy. Also protects against parasitic worms.[1]	IL-4 IL-13
IgG	IgG1 IgG2a IgG2b IgG3	Potent activators of complement and activate myeloid cells by binding Fc receptors. The only antibody capable of crossing the placenta to give passive immunity to fetus[1].	IL-4 > IgG1 IFNγ > IgG2a TGFβ > IgG2b IFNγ > IgG3
IgM		Expressed on B cells (monomer) as an antigen receptor and in a secreted form (pentamer). Involved in neutralization and opsonization of pathogens. Also potent activator of complement.[1]	

[1]Janeway, Jr., C. A. (2005). Immunobiology [6th ed.]

[2]Geisberger, R. et al. (2006). Immunology 118(4): 429–437.

There are some instances in which class switching can occur in the absence of T cell help. T cell-independent (TI) antigen, such as viral glycoproteins and bacterial polysaccharides, can elicit CD40-independent CSR and antibody production in extrafollicular MZ and B-1 cells [Balazs *et al*., 2002]. This process requires BAFF/BLyS and APRIL [MacLennan and Vinuesa, 2002; Litinskiy *et al*., 2002; Stein *et al*., 2002], two CD40L-related molecules produced by myeloid cells. TLR signaling can also promote up-regulation of AID expression in B cells [Muramatsu *et al*., 2000; He *et al*., 2004].

CSR must be tightly regulated because certain antibody isotypes can have pathogenic effects. Abnormal IgG and IgA production, for example, may lead to the onset of autoimmunity, while IgE mediates allergic responses.

Mechanisms of autoimmunity

The adaptive and innate immune systems work together in mutually supportive and interdependent roles. Consequently, deficiencies in one branch of the immune system often lead to defects in the other. Lymphocytes that have lost tolerance and become reactive to self-antigen can recruit help from innate immune cells. Conversely, loss of regulation within the innate immune system can lead to a hyper-activated state which can overwhelm the adaptive immune system with production of pro-inflammatory cytokines. In systemic

autoimmunity, and especially in the many models utilized to study the disease (Table 1.3), it is certainly the case that genetic anomalies or environmental triggers that disturb the function of either the adaptive or innate immune system begin a domino effect that presents a challenge to treat. In the following two sections, I will provide examples of the more regularly identified disruptions to both adaptive and innate mechanisms and key checkpoints that are often associated with lupus and lupus-like disease.

Loss of tolerance

Mechanisms of central tolerance are the first line of defense against autoantigen specific lymphocytes. As effector cells of the adaptive immune system, B and T lymphocytes undergo stringent education in the bone marrow or thymus, respectively, to ensure that their antigen receptors will not recognize self-antigen. As described above, antigen receptor signaling is crucial to maintaining central tolerance. Signaling thresholds are carefully calibrated to ensure that non-autoreactive cells survive and autoreactive cells are eliminated. In response to antigen encounter, autoreactive immature B cells undergo either receptor editing, in which a new antigen receptor is rearranged and expressed, or clonal deletion [Tiegs *et al*., 1993; Nemazee *et al*., 1989; Melamed *et al*., 1998]. Autoreactive T

Table 1.3. Genetic and immunological defects in models of systemic autoimmunity.

Spontaneous models of SLE		
	Identified genetic defect(s)	Immunological defect(s)
MRL/*lpr*	Fas receptor	Fatal disease Glomerulonephritis Anti-DNA and anti-rheumatoid factor antibodies Impaired receptor editing Impaired peripheral tolerance Hyperactivated T cells Increase in extrafollicular Tfh-like cells
NZB/W		Fatal disease Glomerulonephritis Anti-nuclear antibodies Impaired B cell deletion, editing and anergy Hyperactivated T/B cells
Strain-derived susceptibility loci/component phenotypes		
	Identified genetic defect(s)	Immunological defect(s)
Sle1†	Ly108 receptor	Non-fatal pathology Anti-chromatin antibodies Impaired B cell deletion, editing and anergy Hyperactivated T/B cells
Sle2†		Non-fatal pathology Polyreactive IgM Hyperactivated B cells
Sle3†		Non-fatal pathology Low incidence of glomerulonephritis Low levels of anti-nuclear antibodies Hyperactived dendritic cells Hyperactivated T cells (extrinsic effect)
Yaa‡	TLR7 duplication	Non-fatal pathology Hyperactivated B/T cells

† NZM2410-derived locus

‡ BXSB-derived locus

cells are deleted by apoptosis at the double positive thymocyte stage [Kappler *et al*., 1987; Kisielow *et al*. 1988].

There are many situations in which disruption of central tolerance mechanisms during lymphocyte education in the primary tissues leads to an increase of autoreactive cells in the periphery. Here I focus on those affecting the B lineage. Over-expression of anti-apoptotic Bcl-2 by the B cell-specific IgH promoter makes B cells resistant to programmed cell death. This allows escape of autoreactive B cells into the periphery and leads to lupus-like GN and early mortality [Strasser *et al*., 1990]. Pre-B clones from NZB/W, but not wild-type mice, can escape central tolerance mechanisms when transferred into SCID recipients. Once in the periphery, NZB/W B cells make anti-DNA antibodies [Reininger, 1992]. Inability to undergo receptor editing leads to autoantibody production in mice of an otherwise non-autoimmune prone genetic background [Vela *et al*., 2008]. Several murine models of lupus, including MRL/*lpr* mice and animals carrying the NZM2410-derived *Sle2* lupus susceptibility locus, display impaired receptor editing [Lamoureux *et al*., 2007; Liu *et al*., 2007]. The *Sle1* lupus susceptibility locus also leads to reduced BCR-induced apoptosis and Rag expression in immature B cells suggesting potential defects in both receptor editing and clonal deletion [Kumar *et al*., 2006]. Allelic inclusion, which occurs in humans as well as in mouse models, can also contribute to the escape of anti-DNA B cells [Giachino *et al*., 1995; Tsao *et al*., 1992, 2008; Iliev *et al*., 1994;

Witsch *et al*., 2008]. During this process, B cells aberrantly co-express two antigen receptors, one that is self-reactive and one that is not. The self-reactive receptor will down-regulate while in the bone marrow but once in the periphery it will become functional and can induce an autoimmune response. Finally, cells newly emerged from the bone marrow in human SLE patients are more likely to be autoreactive than in healthy controls, indicating a defect in central tolerance mechanisms in human lupus [Yurasov *et al*., 2005].

Central tolerance mechanisms are not completely efficient and some autoreactive lymphocytes inevitably make it into the periphery [Limpanasithikul *et al*., 1995; Wardemann *et al*., 2003]. In the case of B cells, it is especially important that additional mechanisms be in place because of the possibility of the *de novo* self-reactivity that can be acquired in the periphery during GC reactions [Diamond *et al*., 1992; Shlomchik *et al*., 1987, 1990]. There are various peripheral tolerance mechanisms that limit the activation of autoreactive cells by passive or activation-induced B and T cell apoptosis, anergy, follicular exclusion and suppression of autoreactivity by regulatory lymphocytes [Cambier *et al*., 2007; Cappione *et al*., 2005; Duan *et al*., 2008; Buckner and Ziegler., 2004]. Failure in any of these mechanisms allows autoimmunity. For example, autoreactive B cells that escape central tolerance are tolerized in the periphery such that they are unresponsive to antigen; these anergic cells typically die prematurely. Loss of the pro-apoptotic Bim, however, results in the survival and

activation of autoreactive B cells [Oliver *et al*., 2006]. In the absence of Bim, autoreactive B cells become desensitized to death so they no longer require survival signals, such as those provided by BAFF, to persist in the environment. Instead, BAFF only affects the differentiation of these cells, resulting in the production of autoantigen-specific antibodies. Likewise, the Fas^{lpr} mutation desensitizes B cells to death in response to *de novo* acquisition of autoreactivity during a GC reaction [Aït-Azzouzene *et al*., 2010]. In SLE patients, multiple studies have identified defects in the suppressive function of regulatory T cells (Tregs) as well [Alvarado-Sanchez *et al*., 2006; Valencia *et al*., 2007].

Hyperactivation of the innate immune system

The lupus interferon (IFN) signature, a peripheral blood gene expression profile consisting of numerous interferon-inducible genes, is compelling evidence of the important role inflammation plays in systemic autoimmunity [Pascual *et al*., 2008]. Type I interferons, IFN-α and IFN-β, are vital to anti-viral immunity and they induce expression of genes associated with inflammation [Takeuchi and Akira, 2009]. Many cell types including lymphocytes, macrophages and NK cells make type I IFN. Dendritic cells (DCs), however, are key mediators of innate immunity and do so effectively through their ability to secrete cytokines. Plasmacytoid DCs (pDCs) are especially proficient at producing substantial

amounts of IFNα [Fitzgerald-Bocarsly *et al*., 2008]. Plasmacytoid DCs are found in reduced numbers in the blood of SLE patients [Blanco *et al*., 2001], but are actively recruited into, and are enriched within, inflamed target tissues such as lupus skin lesions [Blomberg *et al*., 2001; Farkas *et al*., 2001].

In addition to the correlation between the interferon signature and disease activity in SLE patients, IFNα has been implicated in the disease process in murine models. In mice, activation of IFNα in Fas^{lpr} mice resulted in increased severity of renal disease and elevated autoantibody titers, and, conversely, the absence of both Fas and the IFNα-receptor reduce the incidence of kidney disease [Braun *et al*., 2003]. Many of the effects of IFNα on lupus phenotypes involve a positive feedback loop with TLR signaling pathways (see below for more details on TLRs). TLR sensing is critical to the production of IFNα by pDCs [Fitzgerald-Bocarsly *et al*., 2008], and therapies that antagonize TLRs reduce pDC numbers in two mouse models [Ramanujam *et al*., 2006]. Type I interferons, in turn, amplify the response of B cells to TLR7 [Bekeredjian-Ding *et al*., 2005; Green *et al*., 2009] and TLR9 [Giordani *et al*., 2009]. Activation of B cells via TLR7 and TLR9 promotes the production of anti-nuclear antibodies; [Leadbetter *et al*., 2002; Viglianti *et al*., 2003; Marshak-Rothstein *et al*., 2004]. These antibodies then form immune complexes that activate plasmacytoid dendritic cells, via TLRs, to produce additional IFNα [Vallin *et al*., 1999a, 1999b]. Manipulation of TLR7 expression also affects this loop. Significant over-expression of TLR7

leads to a fatal inflammatory syndrome and an expansion of dendritic cells [Deane *et al*., 2007]. A more modest increase in TLR7 levels caused by gene duplication in the *Yaa* (Y-linked autoimmune accelerator) locus exacerbates autoimmunity in models that already exhibited some loss of tolerance [Pisitkun *et al*., 2006; Subramanian *et al*., 2006]. Conversely, TLR7 deficiency abrogates autoimmunity in lupus models [Christensen *et al*., 2006].

Adaptive and innate immunity crosstalk in lupus

The contribution of amplification loops, such as the one described above, to autoimmunity suggests that defects in both the innate and adaptive immune systems are critical to development of full blown lupus. Genetic studies in mouse models have shown that, indeed, the combination of loss of adaptive immune tolerance and innate immune hyperactivity synergize to drive the development of disease. This is clearly illustrated with congenic mice carrying the NZM2410-derived lupus susceptibility loci *Sle1* and *Sle3* on the B6 background. *Sle1* mediates loss of tolerance to chromatin without overtly affecting B or T cell development or function [Mohan *et al*., 1998], but is functionally expressed in B and T cells [Sobel *et al*., 1999; Sobel *et al*., 2002a]. *Sle3* mice display several T cell phenotypes, including spontaneous activation, impaired activation-induced cell death and increased CD4:CD8 ratios [Mohan *et al*., 1999]. *Sle3* is required in

bone marrow derived cells, but not in T or B cells [Sobel *et al*., 2002b], strongly suggesting a myeloid defect. Indeed, these mice demonstrate hyperactive DCs. Neither of these *Sle* loci are sufficient to mediate fatal lupus nephritis [Morel *et al*., 1997]. However, in combination *Sle1* and *Sle3* lead to the development of pathogenic autoantibodies against a wide range of nuclear antigens, severe GN and mortality. Similarly the *Yaa* locus, which contains a duplication of TLR7, synergizes with *Sle1* to drive full blown, severe lupus [Subramanian *et al*., 2006]. The central, initiating event in these models is *Sle1*-mediated loss of tolerance to chromatin. This step is analogous to the benign autoimmunity observed in ANA-positive humans [Arbuckle *et al*., 2003] and is required for the development of fatal lupus. Additional events leading to innate immune system hyperactivity, mediated by *Sle3* or *Yaa*, then promote disease progression.

Lyn-deficient model of lupus

While spontaneous models of lupus and congenic strains derived from these have been a powerful tool to assess the interplay of various susceptibility loci, genetically-engineered models of autoimmunity have been critical to the in depth analysis of specific mechanisms that contribute to autoimmunity. Moreover, with the powerful genetic analysis tools available for the study of

human genes associated with SLE, we have the ability to focus on the most relevant models and mechanisms of action so that what is learned in the mouse might be applied in the clinic.

One of the many lupus models studied in the laboratory is the *lyn-/-* model. Mice lacking the tyrosine kinase Lyn develop an autoimmune disease with several features characteristic of SLE, including anti-dsDNA IgM and IgG, plasma cell accumulation, and glomerulonephritis [Hibbs *et al*., 1995; Nishizumi *et al*., 1995; Chan *et al*., 1997]. Decreased Lyn expression has been observed in SLE patients [Liossis *et al*., 2001; Flores-Borja *et al*., 2005] and polymorphisms in the *lyn* gene are associated with human lupus [Harley *et al*., 2008, Lu *et al*., 2009]. Thus, *lyn-/-* mice are a useful model for understanding SLE.

Lyn structure and function

Lyn is a member of the Src family of nonreceptor protein tyrosine kinases (PTKs), which includes Src, Blk, Fgr, Fyn, Hck, Lck, Yes and Yrk. Each Src family kinase (SFK) shares a highly conserved structure, including multiple Src homology (SH) domains: a short N-terminal SH4 domain that is important for membrane binding and localization, a region that is unique to each kinase; an SH3 domain, which can bind to specific proline-rich sequences; an SH2 domain, which can bind to specific sites of tyrosine phosphorylation; a catalytic tyrosine kinase

domain; and a short C-terminal region containing a conserved tyrosine residue [Boggon and Eck, 2004]. These shared structural elements allude to the similarities in regulation and function amongst SFKs.

The relative activity of Lyn, like all SFKs, is determined by the conformation of the protein. Lyn exists in a dynamic equilibrium between an active "open" conformation and inactive "closed" conformation. Whether the protein is in open or closed conformation is dependent on highly regulated phosphorylation of two key residues that are highly conserved in all nine SFK family members [Liu and Pawson, 1994]. Interaction of the SH2 domain and the C-terminal negative regulatory phosphotyrosine results in a closed conformation with decreased access to Lyn's catalytic site. Dephosphorylation of this site results in adoption of an open conformation. This allows phosphorylation of the tyrosine within the activation loop of the catalytic domain, which is required for full kinase activity. PTKs play a crucial role in mediating the proximal signaling events of many membrane-bound receptors; as such their localization is tightly regulated. An N-terminal myristoylation signal is essential for post-translational modification by N-myristoyltransferase (NMT) [Rajala *et al*., 2000]. In some cases, this is followed by subsequent palmitoylation, which results in constitutive membrane association of all Src subfamily kinases. This allows for rapid activation.

Lyn and B cell receptor signaling

Activation of B cells via BCR engagement results in tyrosine phosphorylation of numerous signaling proteins; phosphatidylinositol turnover; the elevation of intracellular free calcium; and the activation of serine/threonine kinase cascades. The signaling events triggered by the BCR can be modulated by co-receptors such as CD19, FcγRIIb and CD22, which associate with the BCR either constitutively or in a ligand-dependent manner [O'Rourke *et al*., 1997]. These factors, as well as the maturational stage of the B cell, its microenvironment, the nature of the antigen and the availability of T cell help, influence the quality of the BCR response elicited and contribute to determining whether B lymphocytes respond positively to antigen-induced BCR signaling by proliferating or negatively by undergoing receptor editing, anergy or apoptosis. These disparate outcomes are determined through careful tuning and integration of various signaling pathways downstream of BCR crosslinking.

The BCR consists of an antigen-binding subunit, the membrane immunoglobulin (mIg) that is associated with an heterodimeric Igα and Igβ signaling subunit. These signaling subunits contain a single immunoreceptor tyrosine-based activation motif (ITAM) within their cytoplasmic tail that initiates signal transduction following BCR cross-linking [Flaswinkel and Reth, 1994]. BCR crosslinking results in the phosphorylation of the ITAM tyrosine residues on

Igα and Igβ primarily by Src-family PTKs, Lyn, Fyn and Blk [Kurosaki, 1999]. Src-mediated ITAM phosphorylation promotes the subsequent recruitment and activation of additional effector molecules such as tyrosine kinase Syk [Johnson *et al*., 1995; Rowley *et al*., 1995]. Recruitment of Syk to phosphorylated Igα and/or Igβ results in activation of the kinase, which facilitates the initiation of several different signaling pathways (Figure 1.3). Syk is essential to couple the BCR to distal signal transduction elements. One way it does this is via phosphorylation and interaction with the adaptor molecule, B cell linker protein (BLNK, also known as SLP-65) [Fu *et al*., 1998]. BLNK acts as a molecular scaffold for the nucleation of several different signaling pathways (see below).

Lyn and Syk also have a role in PI3K activation. CD19 localizes PI3K to its lipid substrates in the plasma membrane and increases the activity of the p110 catalytic subunit [Gold *et al*., 2000]. Lyn can associate with PI-3K via its SH3 domain and proline-rich regions in the p85 subunit [Pleiman *et al*., 1994]. Thus, not surprisingly Lyn and other Src-family kinases can mediate a transient increase in PI3K activity; but Syk is required for maximal and sustained PIP3 levels [Craxton *et al*., 1999].

PI3K activation by protein tyrosine kinases (PTKs) leads to the phosphorylation and conversion of PIP2 into PIP3, which is essential for recruitment of PH-domain containing effectors, like Btk [Saito *et al*., 2001; Scharenberg *et al*., 1998] or phosphatidylinositol-dependent kinase 1 (PDK1).

PDK1 activates AKT, also known as protein kinase B (PKB), which promotes survival through modulation of transcription factors such as NF-κB [Kane *et al*., 1999]. SHIP and PTEN negatively regulate PI3K signaling by dephosphorylating PIP3, converting it back to PIP2.

BLNK is phosphorylated by Syk following BCR aggregation and serves as the primary docking site for the SH2 domain of PLCγ2 [Fu *et al*., 1998]. BLNK also associates with Btk, which is important for optimal PLCγ2 activation [Hashimoto *et al*., 1999]. Activated PLCγ2 cleaves membrane-associated PIP2 into the second messengers IP3 and DAG. IP3 leads to Ca2+ influx from intra- and extracellular stores. Elevated Ca2+ levels are required for the activation of transcription factors such as NF-κB and N-FAT, which are important for B cell development, proliferation, cytokine production and immunoglobulin class switching [Ruland and Mak, 2003; Gwack *et al*., 2007]. BCR is coupled to NF-AT activation through both the PI3K/Akt and Ca2+/calcineurin pathways [Gold *et al*., 2000; Stankunas *et al*., 1999].

DAG activates protein kinase C (PKC) isoforms which regulate the MAPK family. The guanine exchange factor, Vav, and adaptor complex of Grb2/SOS also associate with phosphorylated BLNK and can activate the Rac- and Ras-dependent MAPK family pathways, respectively. The mitogen-activated protein kinase family, consisting of three members: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK/SAPK) and p38 MAPK

phosphorylate different sets of transcription factors including i) Elk-1 and c-Myc by ERK, ii) c-Jun and ATF-2 by JNK, and iii) ATF-2 and MAX by p38 MAPK [Dong *et al*., 2002; Johnson and Lapadat, 2002].

Lyn is redundant to a significant extent with Fyn and Blk for the activating functions described above as shown by i) the ability of *lyn-/-* B cells to respond to BCR engagement [Wang *et al*., 1996; Chan *et al*., 1997, 1998, Nishizumi 1998] and ii) studies of triple knockouts of these Src kinases [Saijo *et al*., 2003]. However, it plays a unique role in down-regulating B cell activation by inhibitory receptors.

BCR signaling responses are limited by phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on multiple membrane-bound inhibitory receptors, such as FcγRIIb and CD22. Upon phosphorylation by Lyn, these receptors recruit phosphatases like the inositol phosphatase SHIP or the tyrosine phosphatase SHP-1 [Ono *et al*., 1996; D'Ambrosio *et al*., 1996; Chan *et al*., 1998; Nishizumi *et al*., 1998; Smith *et al*., 1998]. This results in inhibition of BCR signaling responses. For example, activation of SHIP leads to dephosphorylation of PIP3, the product of PI3K. Reduced PIP3 levels result in impaired membrane localization and activation of numerous PH domain containing signaling molecules, including Btk (see below). Lyn's role in negative regulation of BCR signaling is unique, as indicated by the hyperproliferative phenotype and the hyperactivation of the Ca++ flux, Akt, ERK,

and JNK pathways in *lyn-/-* B cells [Wang *et al*., 1996, Chan *et al*., 1997, 1998; Nishizumi *et al*., 1998; Smith *et al*., 1998; Li *et al*., 1999]. B cells from mice that are deficient for other proteins involved in BCR signal down-regulation, such as FcγRIIb, CD22, SHP-1, and SHIP, all have exaggerated calcium signaling [O'Keefe *et al*., 1996; Sato *et al*., 1996; Otipoby *et al*., 1996; Nitschke *et al*., 1997; Takai *et al*., 1996; Cyster *et al*., 1997; Liu *et al*., 1998].

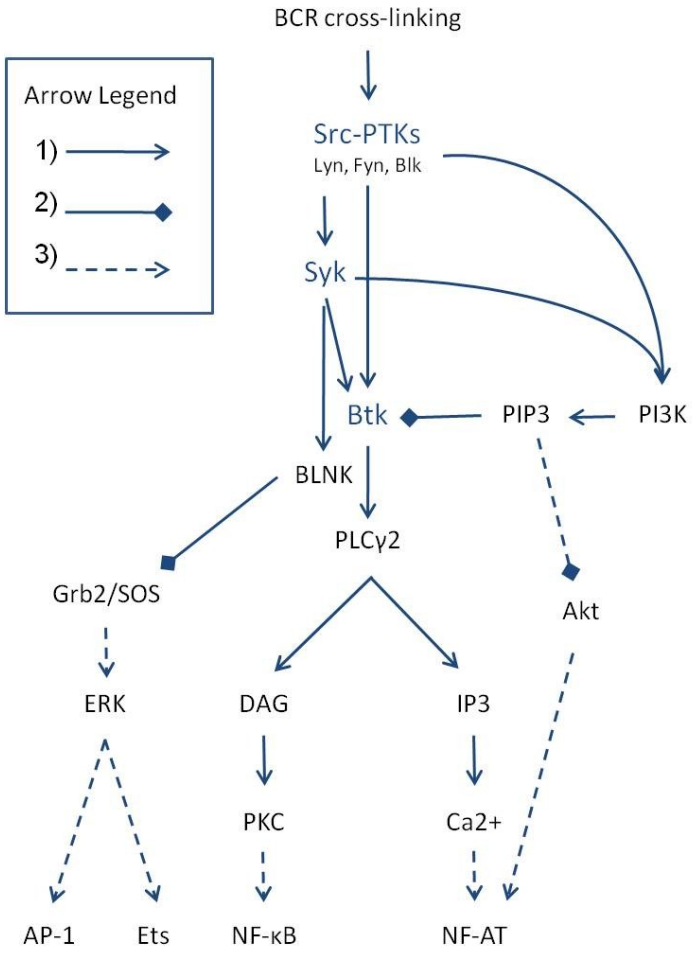

Figure 1.3. Simplified B cell antigen receptor signaling cascade. Activation is dependent on at least three protein tyrosine kinases (PTKs): i) SRC-family PTKs Lyn, Fyn and Blk; ii) Syk; and iii) Btk. The PI3K pathway is activated by both SRC-PTKs and Syk. The generation of PIP3 by PI3K recruits Btk, BLNK and PLCγ2 to the plasma membrane. PLCγ2 generates DAG which activates PKC and leads to NK-κB activation as well as IP3, which leads to opening of calcium channels and activation of the NF-AT. PI3K signaling also activates Akt, which promotes survival via several pathways (some not shown). Additional events mediated by BLNK include activation of the MAP kinase ERK via the Grb2/SOS complex. Arrow legend: 1) Activation event (direct), 2) Recruitment/association, 3) Activation event (indirect)

Defects in lyn-/- mice

There are no appreciable alterations in early B cell development in *lyn-/-* mice. Thus, Lyn's role is minor, or at least redundant, in pro- and pre-B cells [Chan *et al*., 1997; Hibbs *et al*., 1995 Nishizumi *et al*., 1995; Saijo *et al*., 2003]. There is also no defect in the number of immature B cells in the bone marrow; however, there is a significant decrease in the total number of B cells in the peripheral tissues [Chan *et al*., 1997; Hibbs *et al*., 1995, Nishizumi *et al*., 1995]. Overall numbers of follicular and transitional B cells are reduced in the spleen [Allman *et al*., 2001; Meade *et al*., 2002]. Marginal zone B cells are all but absent within the spleen [Seo *et al*., 2001; Whyburn *et al*., 2003]. On average there is little difference in the B-1 B cell population [Hibbs *et al*., 1995; Satterthwaite *et al*., 1998; Whyburn *et al*., 2003], although some studies report either an increase [Chan *et al*., 1997; Nishizumi *et al*., 1995] or decrease [Hasegawa *et al*., 2001] in these cells in *lyn-/-* mice.

B-1 B cells have been considered to be the source of autoreactive antibody in *lyn-/-* mice [Ochi *et al*., 1999]. Increased B-1 B cells have been associated with the production of autoreactive antibody [Murakami *et al*., 1995]. However, B-1 cells do not usually undergo class switching to IgG isotypes that are pathogenic, and autoreactive IgM secreted by these cells is protective [Silverman and Goodyear, 2006]. Furthermore, in mice that carry an immunoglobulin transgene

specific for hen egg lysozyme (HEL), B-1 cells are absent. Despite this, naïve anti-HEL B cells will still secrete antibody concomitant with plasma cell accumulation in the absence of Lyn [Cornall *et al*., 1998]. Thus, B-1 cells are not likely to contribute significantly to either plasma cell accumulation or pathogenic autoimmunity in *lyn-/-* mice.

The role of Lyn in B cell tolerance has been examined in multiple models. In the anti-HEL/HEL transgenic system [Hartley and Goodnow, 1994], negative selection of autoreactive B cells is actually exaggerated in the absence of Lyn. Whereas wild-type anti-HEL B cells are anergized in the presence of soluble HEL antigen, Lyn-deficient anti-HEL B cells were deleted in the bone marrow [Cornall *et al*., 1998]. By measuring expression of RAG, Behrens and colleagues confirmed that receptor editing is intact, and in fact enhanced, in immature *lyn-/-* B cells [Schram *et al*., 2008]. Lastly, in the 3H9 IgH transgene model, B cells express an IgH gene that causes reactivity to DNAs when paired with certain endogenous light chains; these do not secrete anti-DNA antibodies in wild-type mice [Erikson *et al*., 1991]. In this system, 3H9 x *lyn-/-* B cells with anti-DNA specificities are still tolerized in the periphery [Seo *et al*., 2001].

Myeloid defects

Lyn-deficient mice also have several defects in their myeloid cell compartment, indicating an inhibitory role for Lyn in these cells as well. Several such defects have been associated with autoimmunity in other models. Some *lyn-/-* mice display delayed Fc-receptor induced phagocytosis by macrophages [Fitzer-Attas *et al*., 2000]. Reduced clearing of autoantigen from the circulation via uptake by phagocytic cells has also been shown to contribute to autoimmune disease [Hanayama *et al*., 2004; Roszer *et al*., 2011]. Integrin signaling in neutrophils and macrophages is also negatively regulated by Lyn [Pereira and Lowell, 2003]. Activated *lyn-/-* macrophages [Meng and Lowell, 1997], DCs [Silver *et al*., 2007], basophils [Charles *et al*., 2009] and mast cells [Kawakami *et al*., 2000] produce increased levels of pro-inflammatory cytokines such as IL-6, IL-1 and/or TNFα. IL-6 has numerous functions that may contribute to autoimmunity, which are described in detail below. Finally, a characteristic myeloid hyperplasia in the spleens of *lyn-/-* mice has been attributed at least in part to hypersensitivity of myeloid precursors to GM-CSF and M-CSF [Harder *et al*., 2001, 2004; Baran *et al*., 2003]. This may contribute to an overall inflammatory cytokine environment supportive of plasma cell accumulation autoantibody production.

T cells and germinal center reactions in lyn-/- mice

Lyn is not expressed in T cells [Yamanashi *et al*., 1989] but there is evidence that T cells become dysregulated overtime in *lyn-/-* mice. Lyn-deficient mice exhibit Th2 skewing, largely promoted by DCs and basophils [Charles *et al*., 2010]. Also, recent studies indicate that T cells are affected by the general inflammation and immune dysregulation in late-stage, sick animals [Tsantikos *et al*., 2010].

Plasma cell accumulation in *lyn-/-* mice can occur in the absence of antigen [Cornall *et al*., 1998], indicating that B cell antigen presentation to T cells is not required for their differentiation. This suggests that plasma cell accumulation in *lyn-/-* mice may be independent of T cells. However, treatment of Lyn-deficient mice with CTLA4-Ig, a competitive agonist of CD28/B7 interactions, inhibits autoreactive IgG production, suggesting that T cell help is important in the disease process [Oracki *et al*., 2010].

This issue is further complicated by the severe impairment of classic follicular structure observed in the secondary tissues of *lyn-/-* mice. Lyn-deficient mice do not form GCs in response to T-dependent antigens, although somatic hypermutation (SHM) does occur [Hibbs *et al*., 1995; Kato *et al*., 1998]. This would be typical of an extrafollicular response in which low-level SHM and class-switching can occur. In MRL/*lpr* mice, Rheumatoid factor (RF)-specific B cells

are activated in extrafollicular areas, not GCs, and undergo activation, differentiation, and SHM in response to chromatin immune complexes in the absence of T cells [Sweet *et al*., 2010]. As of late, there is more evidence coming to light on the type of cellular interactions that aid B cells during an extrafollicular response, such as interactions with extrafollicular T helper cells or $CD11c^{high}$ DCs [Odegard *et al*., 2008; Vinuesa *et al*., 2010].

Bruton's tyrosine kinase

Btk's role in BCR signaling is critical to B cell development and to diseases related to both immunodeficiencies and autoimmunity. In humans, mutations that disrupt the function of this gene lead to X-linked agammaglobulinemia (XLA), a primary immunodeficiency characterized by lack of mature B cells as well as low levels of immunoglobulins [Tsukada *et al*., 1993]. Animal models of this disease such as *xid* mice, with a naturally occurring point mutation in Btk, and Btk-deficient mice, display a milder XLA phenotype. These animals demonstrate a block in B cell development at the T2 stage, reduced levels of IgM and IgG3, and impaired responses to type II T-independent antigens [Satterthwaite and Witte, 1996]. Btk is also critical to autoimmunity in other models including NZB/W, MRL/*lpr*, and B6.56R strains, the last of which

expresses an anti-DNA Ig heavy chain [Taurog *et al*., 1979; Honigberg *et al*., 2010; Halcomb *et al*., 2008].

Btk structure and role in BCR signaling

Bruton's tyrosine kinase (Btk) belongs to the Tec family of protein tyrosine kinases. Like the Src family kinases, Tec kinases contain the classical SH1, SH2 and SH3 signaling domains. Unlike SFKs, Btk and its family members have a unique and highly conserved N-terminal pleckstrin homology (PH) domain, which is critical to their function. The PH domain facilitates binding to phosphoinositides in the cell membrane [Fukuda *et al*., 1996; Kojima *et al*., 1997; Rameh *et al*., 1997; Salim *et al*., 1996]. PH binding of phosphatidylinositol-3,4,5-triphosphate (PIP3) localizes Btk at the membrane with activating SFKs, including Lyn, which transphosphorylate its catalytic domain and induces kinase activity [Rawlings *et al*., 1996]. Events that block PIP3 binding, such as point mutations within the PH domain or dephosphorylation of PIP3, cause Btk to localize in the cytoplasm instead where it is unlikely to become properly activated [Li *et al*., 1997; Scharenberg *et al*., 1998; Bolland *et al*., 1998; Satterthwaite and Witte, 2000]. Btk activation goes on to potentiate activation of PLCγ2 with concomitant induction of sustained calcium influx and NF-κB activation [Scharenberg and Kinet, 1998; Bajpai *et al*., 2000]. Deficiency of Btk thus leads

to the failure of B cells to proliferate upon antigen stimulation [Khan *et al*., 1995]. Btk also functions as an adaptor molecule in early B cell development [Middendorp *et al*., 2003].

Btk's role in signaling through other receptors

Btk also plays important roles in signaling down-stream of receptors besides the BCR in B cells as well as multiple myeloid cell signaling pathways. Btk mediates signaling for various cytokines and growth factors through its association with the common beta chain of the high-affinity receptors for GM-CSF, M-CSF, IL-3 and IL-5 [Melcher *et al*., 2008; Takatsu *et al*., 2009]. Btk is also activated upon crosslinking of FcεRI in mast cells [Kawakami *et al*., 1994, 1995; Schmidt *et al*., 2009] leading to degranulation and production of inflammatory cytokines [Iwaki *et al*., 2005]. Btk also contributes to RANKL/RANK induced osteoclast differentiation [Lee *et al*., 2008] and collagen-induced platelet activation [Atkinson *et al*., 2003], although it is partially redundant with Tec, a related kinase, for these functions.

Btk in TLR signaling

Btk also has direct involvement in Toll-like receptor (TLR) signaling. TLRs are a family of receptors that are critical to innate immunity and play a role in the priming of antigen-specific adaptive immunity. TLRs are pattern-recognition receptors (PRRs) that have evolved to detect components of foreign pathogens referred to as pathogen-associated molecular patterns (PAMPs) which include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes [Akira *et al*., 2001, 2006; Janeway and Medzhitov, 2002].

TLRs all share similar and often overlapping signaling pathways, and Btk interacts with key proteins from TLR signaling pathways (FIGURE 1.4). Btk interacts directly with myeloid differentiation protein 88 (Myd88), MyD88 adapter-like protein (Mal), and interleukin-1 receptor (IL-1R)-associated kinase-1 (IRAK-1) [Jefferies 2003; Mansell, *et al*., 2006; Doyle *et al*., 2007]. In B cells, Btk has been shown to mediate responses to TLR9, a receptor for hypomethylated CpG DNA. Stimulation of B cells with TLR9 ligand CpG leads to transient phosphorylation of Btk [Lee *et al*, 2008]. In the absence of Btk, TLR9-induced proliferation of B cells can occur, but at reduced levels [Hasan *et al*., 2008, Lee *et al*., 2008, Halcomb *et al*.,2008]. TLR9-induced differentiation of B cells into plasma cells is unaffected in the absence of Btk, however [Halcomb *et al*, 2008].

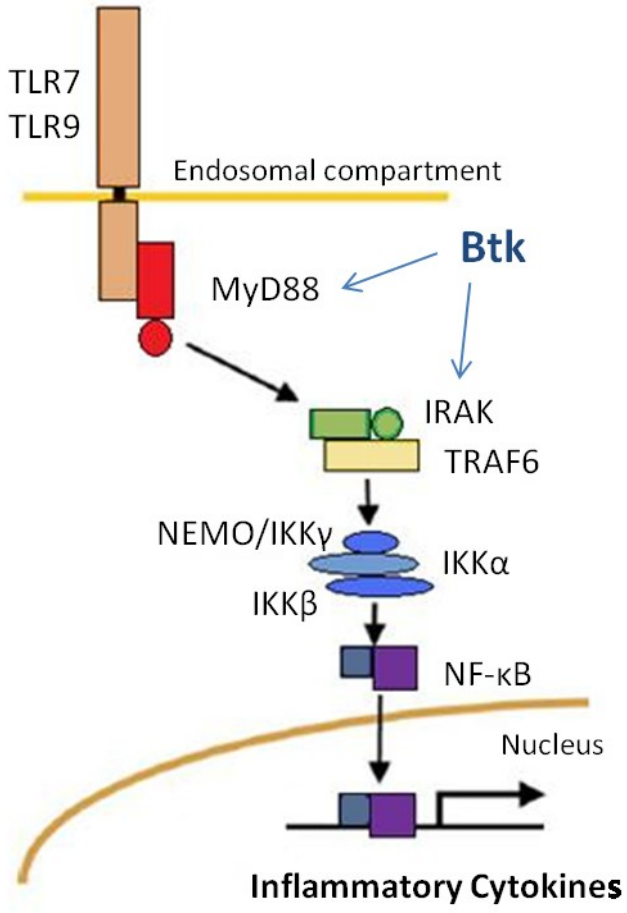

Figure 1.4. TLR signaling cascade. TLRs depend on MyD88 signaling. MyD88 binds to the cytoplasmic portion of TLRs through interaction between individual TIR domains. Upon stimulation, IRAK-4, IRAK-1, and TRAF6 are recruited to the receptor, which induces association of IRAK-1 and MyD88 via the death domains. IRAK-4 then phosphorylates IRAK-1. Phosphorylated IRAK-1, together with TRAF6, dissociates from the receptor and leads to activation of TAK1. TAK1 phosphorylates the IKK complex, consisting of IKKα, IKKβ, and NEMO/IKKγ, which induces the activation of the transcription factor NF-κB. The exact role of Btk in TLR signaling is not known but it is known to associate with MyD88 and IRAK. Loss of Btk affects cytokine production and proliferation in response to TLR stimulation.

Loss of Btk affects B cell cytokine production in response to TLR9 activation; for example, Btk is required for TLR-induced IL-10 production [Schmidt *et al*., 2006; Halcomb *et al*., 2008; Lee *et al*., 2008]. Btk-deficiency also results in increased production of IL-6 and IL-12 in response to TLR stimulation [Lee *et al*., 2008].

Although TLRs are critical for host defense, it has become apparent that loss of negative regulation of TLR signaling, as well as recognition of self molecules by TLRs, are strongly associated with the pathogenesis of inflammatory and autoimmune diseases. The contribution of dysregulated TLR7 signaling to lupus has been described above. Not surprisingly, TLR9 also has implications in autoimmunity. CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10 [He *et al*., 2004]. Loss of Btk in a anti-DNA transgenic model blocks production of class-switched anti-DNA IgG antibodies [Halcomb *et al*., 2008], likely due to reduced up-regulation of the class switching factor AID in response to TLR9.

Functional interaction between Btk and Lyn

Btk is present at similar levels in all stages of B cell development except in plasma cells where expression is down-regulated; Btk is also expressed in myeloid and mast cells but is absent in T cells [Maas *et al*., 1999]. This pattern

of expression is strikingly similar to that of Lyn. Indeed, Lyn has been shown in over-expression studies to phosphorylate and activate Btk [Rawlings *et al*., 1996]. Btk is also a major target of Lyn-dependent inhibition of BCR signaling pathways (Figure 1.5) [Scharenberg *et al*., 1998; Bolland *et al*., 1998; Maeda *et al*., 1999]. For example, SHIP, which requires Lyn for its activation [Nishizumi *et al*., 1998], limits the ability of the Btk PH domain to interact with PIP3 and thus decreases Btk membrane association [Bolland *et al*., 1998; Scharenberg *et al*., 1998] This suggested that increased Btk activity in the absence of Lyn-mediated inhibitory signaling might drive the autoimmune phenotype of *lyn-/-* mice.

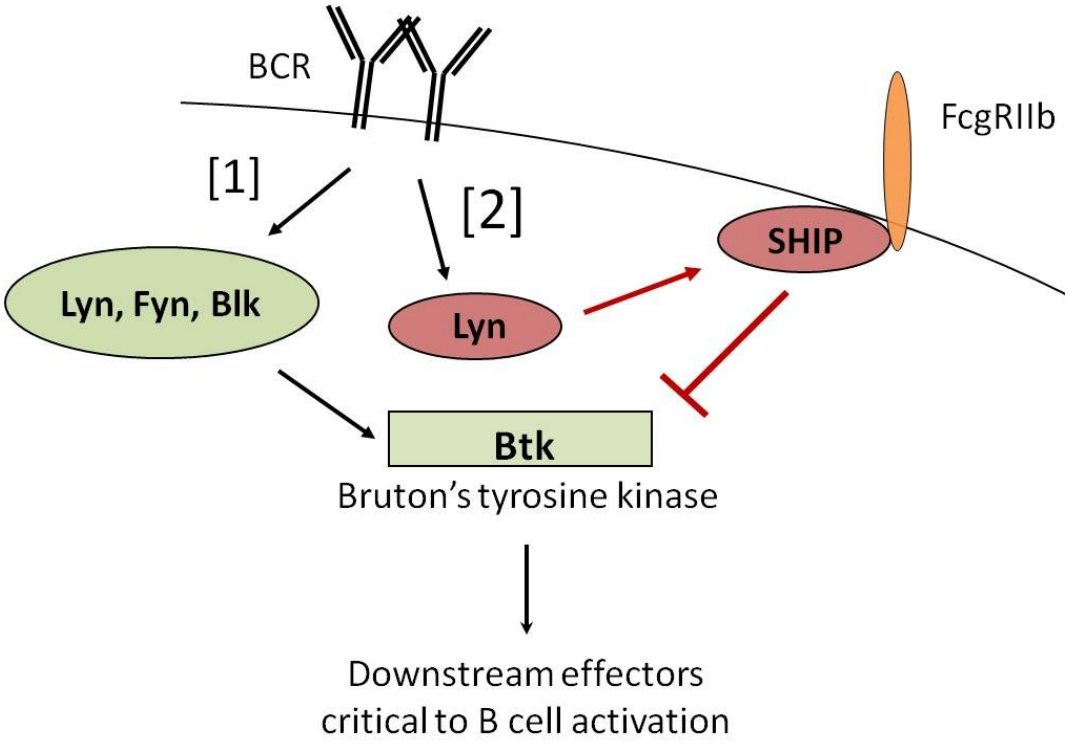

Figure 1.5. A simplified model of Lyn and Btk interaction upon BCR engagement. Btk is activated by both PI3K (not shown) and Src kinases in response to BCR crosslinking [1]. These signals lead to the proliferation and survival of activated B cells. Lyn kinase down-regulates B cell activation in response to BCR crosslinking by phosphorylating inhibitory receptors at the membrane [2]. These receptors are associated with various inositol or tyrosine phosphatases that inhibit the activity of Btk. *lyn-/-* B cells have increased responsiveness to BCR crosslinking.

Effect of reduced Btk dosage in lyn-/- mice

In *lyn-/-btk-/-* and *lyn-/-* x *xid* mice, the hypersensitivity of *lyn-/-* B cells to BCR crosslinking was abrogated and no autoantibodies were observed [Satterthwaite *et al*., 1998, Takeshita *et al*., 1998]. This suggested the role of Lyn as a negative regulator of Btk was more important to the autoimmune phenotype of *lyn-/-* mice. The absence of Lyn, however, exacerbated the effect of Btk-deficiency on B cell development, resulting in a severe reduction in B cell numbers [Takeshita *et al*., 1998; Satterthwaite *et al*., 1998]. So it was unclear if rescue of the autoimmune phenotype in *lyn-/-btk-/-* was due to the loss of Btk-mediated BCR signaling or because of impaired B cell development and diminished B cell numbers.

To address this issue, Whyburn and colleagues [Whyburn *et al*., 2003] took advantage of the Btk^{lo} transgene, which is driven by the B cell-specific Ig heavy chain promoter and expresses 25-percent of endogenous levels of Btk in B cells [Satterthwaite *et al*., 1997]. When crossed onto the *lyn-/-btk-/-* background, this transgene is sufficient to restore B cell numbers to levels similar to those present in *lyn-/-* mice. Like *lyn-/-* B cells, *lyn-/-*Btk^{lo} B cells were hypersensitive to BCR crosslinking [Satterthwaite *et al*., 1998; Whyburn *et al*., 2003]. Intriguingly, the mice did not develop autoantibodies, indicating that BCR hypersensitivity is not sufficient for disease in *lyn-/-* mice.

Myeloid cells in lyn-/-Btk^{lo} mice

The Btk^{lo} transgene is not expressed in myeloid cells as it is driven by the IgH promoter and enhancer [Satterthwaite *et al*., 1997]. Myeloid lineage cells in *lyn-/-Btk^{lo}* mice lack both Lyn and Btk. In general the effects of Btk deficiency on myeloid cell function are subtle, but they often counteract the effects of Lyn deficiency. For example, Lyn and SHIP interact to down-regulate M-CSF signaling [Baran *et al*., 2003], and Btk mediates myeloid hyperproliferation in SHIP-/- mice [Karlsson *et al*., 2003]. Impaired phagocytosis of NZB/W macrophages is normalized in *xid* mice, which have an inactivating point mutation in Btk [Russel and Steinberg, 1983]. Btk deficiency abrogates increased cytokine production from *lyn-/-* mast cells [Kawakami *et al*., 2000; Iwaki *et al*., 2005]. These observations suggest that Btk may contribute to a general pro-inflammatory environment engendered by the hyperactive myeloid compartment in *lyn-/-* mice.

Role of Lyn and Btk in B cell terminal differentiation

Plasma cells are the basis of humoral immunity and antibody-mediated diseases. In SLE, peripheral plasma cell accumulation has been observed in both patients as well as numerous murine models of the disease [Arce *et al*., 2001;

Hoyer *et al*., 2004; Seagal *et al*., 2003; Hang *et al*., 1982; Fukuyama *et al*., 2005; Wang *et al*., 2005, Kersseboom *et al*., 2010]. In some models, researchers have even shown a direct correlation between the presence of atypically elevated plasma cell numbers and the production of pathogenic antibody [Liu *et al*., 2010]. Their role in antibody production makes plasma cells important to antibody-mediated disease such as SLE. Despite this, plasma cells have not successfully been targeted for therapeutic intervention. Until recently, there has not been a clear understanding of the exact nature and regulation of plasma cells that are generated upon B cell terminal differentiation, and currently available B cell depletion therapies fail to deplete long lived plasma cells that secrete pathogenic autoantibodies [Anolik *et al*., 2004]. It is therefore important to understand how these cells are generated and regulated in the periphery.

Disruption of the balance between Lyn and Btk signals results in pathogenic autoimmunity. Studies in *lyn-/-*Btk^{lo} mice indicate that these molecules counteract each other in processes subsequent to or separate from the initial activation of autoreactive B cells by antigen. My work examines how Lyn and Btk regulate B cell terminal differentiation and plasma cell accumulation.

Plasma cells in lupus

Plasma cell accumulation in secondary lymphoid organs is a common feature of human [Arce *et al*., 2001] and murine lupus. NZB/W mice have an increase in short- and long-lived plasma cells in the spleen, both of which contribute to autoantibody production [Hoyer *et al*., 2004]. In these mice, an abnormally favorable plasma cell survival niche in the spleen [Hoyer *et al*., 2004] and impaired plasma cell homing to the bone marrow [Erickson *et al*., 2003] have been implicated in mediating the accumulation. Mice lacking the transcription factor Ets-1 have increased numbers of plasma cells and produce autoantibodies [Wang *et al*., 2005]. In this model, loss of Ets-1 leads to increased activity of the transcription factor Blimp-1, which drives plasma cell differentiation [John *et al*., 2008]. Treatment of NZB/W mice with IFNα induces an increase in short-lived plasmablasts (t ½ less than two weeks) which correlate with increase in anti-DNA antibodies [Liu *et al*., 2010]. In *lyn-/-* mice, plasma cell accumulation is independent of stimulation by autoantigen as it occurs even when the B cell repertoire is restricted to a single specificity (anti-HEL) and the antigen is not present [Cornall *et al*., 1998].

The mechanism for increased plasma cells in *lyn-/-* mice is poorly defined and is a major focus of this work. One likely contributor to this process is IL-6.

Interleukin-6

The family of IL-6-type cytokines comprises IL-6, IL-11, LIF (leukemia inhibitory factor), OSM (oncostatin M), CNTF (ciliary neurotrophic factor), CT-1 (cardiotrophin-1), CLC (cardiotrophin-like cytokine), neuropoetin and IL-27. They activate target genes involved in differentiation, survival, apoptosis and proliferation [Kishimoto *et al*., 1995]. The members of this cytokine family have pro- as well as anti-inflammatory properties and are major players in hematopoiesis as well as acute-phase immune responses (Table 1.4). IL-6 is produced by cells of the innate and adaptive immune system as well as by fibroblasts, synovial and endothelial cells.

Two receptors are required for IL-6 signaling. The α-receptor (IL-6Rα) confers specificity. The signal-transducing receptor chain gp130 (glycoprotein 130) is the common membrane-bound receptor shared by all IL-6 family cytokines. Only when IL-6Rα specifically binds IL-6, can it efficiently recruit/interact with the signaling receptor subunits [Rose-John *et al*., 2006]. Signal transduction involves the activation of JAK tyrosine kinase family members, which lead to the activation of transcription factors of the STAT family. gp130 binds to JAK1, JAK2 and TYK2, but JAK1 is most important to IL-6 signaling [Kishimoto *et al*., 1995]. JAK1 then activates STAT3, and to a minor extent STAT1, leading to transcription of STAT target genes.

Table 1.4. Pleiotropic effects of IL-6[1]

B cells	Ig production Survival of plasma cells Proliferation of myeloma cells Proliferation of EBV-infected B cells
T cells	Proliferation and differentiation Promotes Tfh and T17 cells Inhibits Tregs Differentiation of cytotoxic T cells IL-2R expression and IL-2 production Enhances NK activity
Macrophages	Promotes myelopoiesis Inhibits myeloid leukimic growth by inducing macrophage differentiation
Hepatocytes	Acute-phase protein synthesis
Bone Metabolism	Stimulation of osteoclast formation Induction of bone adsorption
Blood Vessels	Induction of platelet-derived growth factor
Heart Muscle	Negative inotropic effect on heart
Neuronal cells	Differentiation of PC12 cells Induction of adrenocorticotropic hormone synthesis Support of survival of cholinergic neurons

[1]Kishimoto, T. *et al*. (1995). Blood 86(4): 1243-1254.

Although gp130 is ubiquitously expressed, IL-6Rα is tightly regulated [Taga *et al*., 1992]. "Classical" IL-6 signaling occurs in cells expressing both gp130 and membrane-bound IL-6Rα [Rose-John *et al*., 2006]. Soluble forms of IL-6R are also present, formed *in vivo* either by limited proteolysis (shedding) of membrane-bound receptors or by translation from an alternatively spliced mRNA. Once bound by IL-6, sIL-6Rα is able to act agonistically on any cell expressing gp130. In the case of IL-6 signaling in humans, this is more complicated because soluble gp130 (sgp130) is also present in the serum; sIL-6Rα and sgp130 can bind in the serum and act as a buffer against circulating IL-6 [Narazaki *et al*., 1993].

IL-6 is a major inflammatory cytokine that is important for acute-phase responses (Table 1.4). As such, it functions very efficiently at promoting myeloid differentiation from hematopoietic progenitors, and does so at the expense of B lymphopoiesis [Maeda *et al*., 2005]. It can recover emergency granulopoiesis in animals that lack G-CSF and GM-CSF, critical cytokines necessary for myelopoiesis [Zhu *et al*., 2006; Walker *et al*., 2008]. It also induces maturation of megakaryocytes, which can promote plasma cell survival in the bone marrow [Winter *et al*., 2010]. IL-6-mediated myelopoiesis occurs in the context of lupus in B6.*Sle1*.*Yaa* mice as well [Maeda *et al*., 2009].

IL-6 also has several effects on B and T cells that could contribute to autoimmunity (Table 1.4). IL-6 was originally isolated and cloned as a B-cell differentiation factor that induced terminal B cell differentiation and supported the

production of IgG [Yoshizaki *et al*., 1984]. IL-6 transgenic mice eventually die of a fatal plasmacytoma [Suematsu *et al*., 1989], and IL-6 promotes plasma cell survival *in vitro* [Minges Wols *et al*., 2002]. Anti-IL-6 antibodies prevent production of anti-dsDNA antibodies in NZB/W mice [Liang, 2006]. IL-6 can also induce T cell activation. It promotes differentiation of follicular helper T cells (Tfh), and drives Th17 differentiation by inhibiting regulatory T cell (Treg) differentiation in the presence of TGFβ [Nurieva *et al*., 2008; Zhou *et al*., 2007; Kimura and Kishimoto, 2010]. IL-6 has long been associated with disease (activity/severity) in SLE patients and some lupus mouse models [Cross and Benton,1999]. Therefore, IL-6 may contribute to autoimmune disease in *lyn-/-* mice.

In the following chapters I will present work focusing on the mechanisms for and consequences of plasma cell accumulation and IL-6 over-production in *lyn-/-* mice. I will also address the role of Btk in regulating both of these. I identify two major checkpoints regulating autoantibody production in *lyn-/-* mice. The first involves plasmablast and plasma cell accumulation and polyclonal IgM autoreactivity, which depend on Btk and require reduced Lyn dosage in B cells. The second checkpoint regulates the class-switching of B cells with lupus-associated autoantigen specificities and production of pathogenic autoantibodies. This step requires IL-6, which is produced in excess by *lyn-/-* myeloid cells in a Btk-dependent manner.

CHAPTER II

MATERIALS AND METHODS

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Texas Southwestern Medical Center (Dallas, TX). Mice were bred in a specific pathogen free (SPF) colony and housed in a barrier facility. Recipient mice for bone marrow chimera studies were transferred to a sterile husbandry facility approximately two weeks before radiation treatment where they remained until the conclusion of the study. Daily health and husbandry needs for all mice were overseen by the university's Animal Resource Center (ARC) veterinarians and staff. The generation of *lyn-/-* and *Btk-/-* mice has been described previously [Chan *et al*., 1997; Khan *et al*., 1995]. Btk^{lo} mice are *Btk-/-* mice carrying a wild-type Btk transgene driven by the IgH chain promoter and enhancer; generation and characterization of this strain was described previously [Satterthwaite *et al*., 1997]. *lyn-/-*Btk^{lo} mice were generated by crossing *lyn-/-*, *Btk-/-* and Btk^{lo} strains to produce *lyn-/-Btk-/-* mice carrying the Btk transgene; initially, these mice were of mixed C57BL/6 X 129/Sv X Balb/c

genetic background [Satterthwaite *et al*., 1998]. Subsequently, all strains have been backcrossed onto C57BL/6 background for characterization [Whyburn *et al*., 2003; Gutierrez *et al*., 2010]. *IL-6-/-* (B6.129S2-*Il6*^{tm1Kopf}/J) and B6.IgHa (B6.Cg-*Igh*^{a} *Thy1*^{a} *Gpi1*^{a}/J) mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). *lyn-/-* and *IL-6-/-* mice were crossed to generate *lyn-/-IL-6-/-* double knock-out mice. *IL-21-/-* mice (B6.129S-*Il21*^{tm1Lex}/Mmcd) were obtained from the Mutant Mouse Regional Resource Center (MMRRC) and crossed with *lyn-/-* to generate *lyn-/-* and *IL-21-/-* double knock-out mice. These mice are of mixed C57BL/6 x 129 genetic background; Lyn-sufficient littermates were used as wild-type controls in *lyn-/-IL-21-/-* studies. Tail clips were obtained at weaning and used to prepare tail lysates for DNA extraction and PCR genotyping. Mice were used at various ages as indicated in the text and figure legends.

Tissue preparation

Upon collection, tissues were placed in complete RPMI medium (cRPMI) consisting of RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-β-mercaptoethanol. Single-cell suspensions were

made from whole spleen by compressing the spleen between either two frosted glass slides or the plunger of a 3 ml syringe and a tissue culture dish. The latter method was followed by passage through a 23-gauge needle. Using a 25-gauge needle and 10 ml syringe, bone marrow was flushed from femurs and tibias with cRPMI. Single-cell suspensions were depleted of red blood cells (RBCs) with RBC lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na_2EDTA) then washed and passed through a 70-micron nylon mesh cell strainer to remove debris. Cells were resuspended in cRPMI and a small aliquot was counted by Trypan Blue (Lonza/ Biowhittaker, Basel, Switzerland) exclusion using a microscope and hemacytometer.

Flow cytometry

RBC-depleted splenocyte or bone marrow suspensions were Fc-blocked with purified rat IgG2b anti-mouse CD16/CD32 (2.4G2) antibody for 5 minutes at 4 °C prior to primary staining with monoclonal antibodies. The following fluorescein isothiocyanate- (FITC), phycoerythrin- (PE), peridinin-chlorophyll-protein complex- (PerCP) or biotin-conjugated antibodies were used in various combinations to stain for distinct populations: FITC-conjugated antibodies against CD11b (Invitrogen/ Caltag Laboratories, Carlsbad, CA), CD19, CD21, CD23,

Gr-1 (Ly-6G and Ly-6C), IgMb, IgMa, Thy 1.2 (CD 90.2); PE-conjugated antibodies against B220 (CD45R), CD5, CD21, CD23, CD138 (Syndecan-1), Gr-1, IgM, IgMa, Thy1.1 (CD90.1); PerCP-conjugated antibodies against B220, CD4, CD11b, CD86 (Biolegend, San Diego, CA); and biotin-conjugated antibodies against CD11c, CD19, CD23, CD86, CD138, CXCR4, CXCR5, F4/80 (eBioscience, San Diego, CA). Fluorophore- or biotin-conjugated rat IgG2a,κ and IgG1 antibodies were used as irrelevant isotype controls and biotinylated antibodies were detected with strepavidin-allophycocyanin (APC). Incubations for both primary and secondary antibodies were 15 minutes at 4 °C. All antibodies were from BD Biosciences (San Diego, CA) unless otherwise indicated.

When staining for intracellular antigens, cells were fixed and permeabilized according to the manufacturer's instructions for the Cytofix/Cytoperm™ kit (BD Biosciences). Briefly, cells were treated with Cytofix/Cytoperm™ buffer for 10 minutes at 4 °C then incubated with one of the following antibodies for 15 minutes at 4 °C: anti-IgMb-FITC, anti-IgMa-PE, anti-IL-6-PE, Igκ-PE (Southern Biotech, Birmingham, AL) or Igλ-biotin. Prior to intracellular staining for IL-6, cells were incubated with media alone or 10 μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO) for 4 hours in the presence of GolgiPlug™ (brefeldin A) (BD Biosciences), which was used according to the manufacturer's instructions.

Intracellular BrdU staining, was completed according to the manufacturer's instructions for Cytofix/Cytoperm™ Plus kit (BD Biosciences). Briefly, cells were fixed and permeabilized in Cytofix/Cytoperm™ buffer for 20 minutes followed by 10 minutes in Perm Plus buffer and, finally, re-fixed in Cytofix/Cytoperm™ buffer for 5 minutes; all treatments were at 4 °C (BD Biosciences). The samples were treated with DNase (BD Biosciences) for 1 hour at 37 °C prior to intracellular staining with anti-5-bromo-2-deoxyuridine (BrdU)-FITC for 20 minutes at room temperature (approximately 25 °C). All samples were acquired on a FACSCalibur cytometer and analyzed using CellQuest software (both from BD Biosciences).

Serology and ELISAs

Mice were bled by either piercing the saphenous vein (for kinetic and microarray studies) or by cardiac puncture (at terminal sacrifice). Clot-activating microvettes (Sarstedt Company, Nuembrecht, Germany) were used to isolate blood for survival studies. Serum was isolated by centrifugation and stored at -20 °C.

For detection of total IgM and IgG antibodies, flexible 96-well PVC plates (BD Biosciences) were coated with 2 μg/ml goat anti-mouse immunoglobulin (Ig)

(Southern Biotech) and blocked with 1% bovine serum albumin (BSA) in borate-buffered saline (BBS). Serum or Ig standards (mouse IgM and IgG, Sigma-Aldrich) were diluted serially and added to wells in triplicate and incubated for 1 hour at room temperature. For detection of anti-dsDNA autoantibodies, Immulon II plates (Dynatech Laboratories, Chantilly, VA) were pre-coated with 0.1 mg/ml methylated BSA (mBSA) and subsequently coated with 50 μg/ml calf thymus dsDNA (Sigma-Aldrich). After plates were incubated overnight at 4 °C with blocking buffer (PBS, 3% BSA, 0.1% gelatin, 3 mM EDTA), serial dilutions of serum were added in triplicate and incubated for 2 hours at room temperature. For both total Ig and anti-dsDNA ELISAs, bound IgM or IgG was detected by an alkaline phosphatase-conjugated secondary antibody, goat anti-mouse IgM or IgG (Southern Biotech), and developed with an alkaline phosphatase substrate kit (Bio-Rad Laboratories, Hercules, CA). The optical density was read at 405 nm on an absorbance microplate reader (Bio-Tek Instruments, Winooski, VT). Total amounts of IgM and IgG were calculated based on the known concentrations of the Ig standards.

IL-6 was detected using an OptEIA™ anti-mouse IL-6 ELISA kit (BD Biosciences) according to the manufacturer's instructions. Briefly, Immulon II plates (Dynatech Laboratories) were coated with anti-IL-6 antibody overnight at 4 °C. The plates were then blocked and serial dilutions of standards and serum samples or culture supernatants were plated in triplicate for 1-hour incubation at

room temperature. IL-6 was detected with a biotin-conjugated anti-IL-6 antibody and strepavidin-conjugated horseradish pero*xid*ase. Substrate buffer was then added to the plate, stopped after 30 minutes and the optical density was read at 450 nm using an absorbance microplate reader (Bio-Tek Instruments). Total amounts of IL-6 were calculated based on the known concentrations of the recombinant IL-6 standards (BD Biosciences).

Low-density culture and stimulation of bone marrow-derived macrophages (BMDM)

RBC-depleted bone marrow cells from 8- to 12-week old mice were isolated and counted as described above. Cells were resuspended in M-CSF media which consisted of DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 ng/ml M-CSF (R&D Systems Inc., Minneapolis, MN) at a final concentration of $2x10^6$/ml in 25 cm^2-tissue culture flasks, day 0. After overnight incubation at 37 °C, non-adherent cells were transferred into a 75 cm^2-tissue culture flask and fed 0.5X volume M-CSF media. Day 1 transfer of non-adherent myeloid progenitors was necessary to remove adherent stromal cells and mature macrophages from culture. On day 5, old media was aspirated and adherent cells were fed with fresh 1X

volume M-CSF media. On day 7, macrophages were treated with 10 mM EDTA and gently scraped off tissue culture flasks. Cells were washed to eliminate trace EDTA and counted. For 12-hour LPS dose response assay, macrophages were resuspended at a final concentration of $5x10^5$ cells/ml media and plated $1x10^5$ per well in 96-well plate. BMDMs were primed with 20 ng/ml IFNγ (R&D Systems Inc.) and duplicate wells were stimulated with 0.1, 1 or 10 ng/ml LPS. Culture supernatants were collected and stored at -20 °C. IL-6 concentrations were assayed by ELISA as described above.

Autoantigen arrays

Serum was collected as described above from 4-month old and 12- to 14-month old mice. Blind samples were submitted to Dr. Quan-Zhen Li (Department of Immunology, The University of Texas Southwestern Medical Center). Autoantibodies were measured on an autoantigen proteomic array that has been described previously [Li *et al*., 2007]. The array includes seventy autoantigens and four control proteins. 1 µl of each sample was diluted 1:100 and added to the arrays in duplicate. Detection was with Cy3- (indocarbocyanine) labeled anti-mouse IgM and Cy5- (indodicarbocyanine) labeled anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). A Genepix 4000B scanner

(Molecular Devices, Inc., Sunnyvale, CA) with laser wavelengths 532 nm (for Cy3) and 635 nm (for Cy5) was used to generate images for analysis. Images were analyzed using Genepix Pro 6.0 software to generate a GPR file (Molecular Devices, Inc.). Net fluorescence intensities (nfi) were normalized using anti-mouse IgM or IgG spotted onto each array. Values obtained from duplicate spots were averaged. These values were received from Dr. Li and hierarchical clustering analysis of autoantibodies was performed using Cluster and Treeview software (http://rana.lbl.gov/EisenSoftware.htm).

Renal pathology

Mice were euthanized at approximately 10 months of age and sagittal sections of each kidney fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA). Tissues were submitted to Dr. Xin J. Zhou (Department of Pathology, The University of Texas Southwestern Medical Center) for blind examination of the glomeruli, tubules and interstitial areas for evidence of pathology. The kidneys were embedded in paraffin and cut into 3 μm sections then stained with hematoxylin and eosin and with periodic acid Schiff. The glomeruli were examined for crescent formation as well as periglomerular and perivascular lymphocytic infiltration. Glomerulonephritis (GN) severity was

graded on a 0 to 4 scale, where 0 = normal, 1 = mild increase in mesangial cellularity and matrix, 2 = moderate increase in mesangial cellularity and matrix with thickening of glomerular basement membrane (GBM), 3 = focal endocapillary hypercellularity with obliteration of capillary lumina and a substantial increase in the thickness and irregularity of the GBM and 4 = diffuse endocapillary hypercellularity, segmental necrosis, crescents and hyalinized end-stage glomeruli. Similarly, the severity of tubulointerstitial nephritis was graded on a 0 - 4 scale, based on the extent of tubular atrophy, inflammatory infiltrates and interstitial fibrosis, as described previously [Xie *et al*., 2003].

Quantitative real-time PCR

RBC-depleted splenocytes were washed in PBS and resuspended in Trizol® reagent (Invitrogen). Total RNA was prepared using a Qiagen RNeasy Kit (Qiagen, Valencia, CA), and cDNA was generated with a cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturers' instructions. Quantitative real-time PCR was performed in an Applied Biosystems 7300 Real-Time PCR system using TaqMan reagents specific for interleukin (IL)-21, stromal cell-derived factor-1 (SDF-1) and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems).

Data were normalized to GAPDH using the delta comparative threshold cycle (Ct) method.

Mixed bone marrow chimeras

Two-month old B6.IgHa mice were transferred into a sterile husbandry facility with access to a Gammacell 40 irradiator (Nordion/ Atomic Energy of Canada Ltd., Ottawa, Canada). Recipient B6.IgHa mice were exposed to a lethal dose of 1050 cGy cesium-137 gamma radiation in two doses of 525 cGy spaced 3 hours apart. Bone marrow from 3- to 4-month old donor mice was recovered from flushed femurs and tibias and passed through a nylon mesh filter to remove debris. Cells were washed in cRPMI and counted as described above. IgHb-expressing *lyn-/-* (test) or B6.IgHb (wild-type control) bone marrow was mixed at a 2:1 ratio with B6.IgHa bone marrow at a final concentration of 10^8/ml in PBS. Irradiated recipients received 10^7 cells in 100 µl PBS intravenously within 2-3 hours of final exposure to radiation. After the bone marrow transfer, mice were maintained on water supplemented with 2 mg/ml neomycin (Sigma-Aldrich) for two weeks. Fresh neomycin-supplemented water was prepared every 3-4 days. Splenocytes, bone marrow cells and serum were isolated from recipient mice

2-4 months after reconstitution. Analysis of plasma cell accumulation was carried out by flow cytometry as described above.

B cell purification and in vitro polyclonal stimulations

B cells were purified from RBC-depleted splenocytes using MidiMACS™ magnetic beads and columns (Miltenyi Biotec Inc., Auburn, CA), BD IMag™ Cell Separation System magnetic beads and IMagnet™ column-free magnet (BD Biosciences), or a hybrid protocol of both systems. For in vitro LPS and CpG stimulation studies, splenic B cells were positively selected by B220 purification using MidiMACS™ magnetic beads and LS columns according to the manufacturer's instructions (both Miltenyi Biotec Inc.). Purified B cells were >90% $B220^+$ when examined by flow cytometry. B220-positive selection was used rather than negative selection to purify B cells due to complicating factors arising from increased extramedullary hematopoiesis in *lyn-/-* spleens. Traditional negative selection approaches do not allow for isolation of a pure B cell population from *lyn-/-* mice.

Purification of $B220^+CD138^-$ B cells was performed as follows: RBC-depleted splenocytes were washed in PEB buffer (PBS, 0.5% BSA, 2mM EDTA) to remove trace media and incubated with anti-CD11b-FITC/anti-CD43-

FITC/anti-CD138-biotin antibodies (BD Biosciences) according to the manufacturer's instructions. Total splenocytes were indirectly labeled with anti-FITC (Miltenyi Biotec Inc.) and anti-biotin (BD Biosciences) magnetic beads following a merged version of the manufacturers' protocols. $CD11b^{+}CD43^{+}CD138^{+}$ cells were depleted on Miltenyi LD columns and magnets. Following this first depletion step, $CD11b^{-}CD43^{-}CD138^{-}$ cells were incubated in 50 µl of anti-B220 magnetic beads (BD Biosciences) per 10^{7} cells for 30 minutes at 4 °C. After washing, volume was brought 1ml PEB in 5 ml polystyrene tubes and placed on the column-free IMagnet™ (BD Biosciences) for 3 rounds of washing (6 minutes and two 4-minute incubations). The remaining cells were washed and counted. Purified B cells were >90% $B220^{+}CD138^{-}$ when examined by flow cytometry.

Purified $B220^{+}$ B cells from 2- to 4-month old wild-type and *lyn-/-* mice were resuspended at a concentration of $5x10^{5}$/ml and stimulated with 20 µg/ml LPS or 1 µg/ml CpG (ODN 1826) for 72 hours at 37 °C. After 72 hours, cells were harvested and stained with antibodies against B220 and CD138. Culture supernatants were saved for total Ig detection by ELISA.

Transwell migration assays

$3x10^6$ RBC-depleted splenocytes from 2- to 4-month old wild-type, *lyn-/-* and *lyn-/-*Btk^{lo} mice were resuspended in 100 µl of cRPMI were added to the top chambers of a 24-well transwell plate (Corning, Lowell, MA). Recombinant mouse SDF-1 (R&D Systems) was added at a final concentration of 100 ng/ml to the bottom chamber and cells were incubated for 3 hours at 37 °C. Cells remaining in the top chamber as well as those that migrated to the bottom chambers were collected and stained for B220 and CD138. The number of CD138-positive plasma cells for the input sample, upper (non-migrating) and lower (migrating) chambers of the transwell plate were measured by flow cytometry.

In vivo BrdU labeling

Four-month old wild-type, *lyn-/-* and *lyn-/-*Btk^{lo} mice received a 2-mg dose of BrdU (Sigma-Aldrich (Fluka)) on days 1-3, 7-9 and 13. A 10 mg/ml solution of BrdU in PBS was made fresh just prior to injection and 200 µl injected intraperitoneally (IP) with a 25-gauge needle and 1 ml syringe. On day 14, splenocytes and bone marrow cells were isolated, RBC-depleted and stained with

antibodies against B220, CD4, CD138, Gr-1 and BrdU for flow cytometric analysis of intracellular BrdU in B, T, and myeloid cell populations.

Statistical Analysis

Experimental groups were compared by Student's *t*-test as calculated in Microsoft Excel. Values of $p < 0.05$ were taken as significant.

CHAPTER III

BRUTON'S TYROSINE KINASE-DEPENDENT ACCUMULATION OF PLASMABLASTS AND PLASMA CELLS IN LYN-DEFICIENT MICE

A majority of the work presented in this chapter has been published in the *European Journal of Immunology*, volume 40, pages 1897–1905, 2010. Experiments were performed by Maria A. (Toni) Gutierrez unless otherwise noted in the text and figure legends.

Introduction

The mechanisms involved with the onset and progression of autoimmune disease in *lyn-/-* mice are not thoroughly understood. Lyn has both positive and negative regulatory roles in B cells, but early studies characterizing *lyn-/-* mice ruled out any major defects from loss of lyn as a positive regulator of B cell receptor signaling (BCR) [Chan *et al*., 1997, 1998; Cornall *et al*., 1998; Nishizumi *et al*., 1998; Smith *et al*., 1998]. Mutations in a number of molecules

involved with Lyn-dependent inhibitory signaling pathways recapitulate the reduced BCR signaling thresholds and increased autoantibody levels seen in *lyn-/-* mice [O'Keefe *et al*., 1996, 1999; Sato *et al*., 1996; Westhoff *et al*., 1997; Nishimura *et al*., 1998, 1999; Bolland and Ravetch, 2000]. This suggests that hypersensitivity to BCR crosslinking is required for autoimmunity in the model. Despite having hyperresponsive B cells, central [Cornall *et al*., 1998] and peripheral [Seo *et al*., 2001] tolerance mechanisms remain intact in *lyn-/-* mice. In order to understand how loss of Lyn-dependent negative regulation in B cells leads to autoimmunity, these studies consider the role of molecules that mediate activation upon BCR engagement.

Bruton's tyrosine kinase (Btk) is a critical component of BCR signaling pathways [Satterthwaite and Witte, 2000] and is a target of at least two Lyn-dependent inhibitory pathways [Bolland *et al*., 1998; Scharenberg *et al*., 1998; Maeda *et al*., 1999]. Btk is also required for autoimmunity in several other strains of mice with hyperresponsive B cells [Steinberg *et al*., 1982; Smith *et al*., 1983; Seldin *et al*., 1987; Scribner *et al*., 1987]. B cells from mice deficient in both Lyn and Btk fail to respond to BCR crosslinking or produce autoantibodies [Satterthwaite *et al*., 1998; Takeshita *et al*., 1998]. This suggests that Btk mediates autoimmunity at the level of BCR signal transduction. However, further characterization of Btk's role in lyn-mediated autoimmunity is difficult in the *lyn-/-btk-/-* model due to developmental deficiencies in the B cell compartment

[Satterthwaite *et al*., 1998; Takeshita *et al*., 1998]. To bypass these deficiencies, *lyn-/-*Btk^{lo} mice were generated by introducing a transgene that expresses a low level of Btk in B cells into the *lyn-/-btk-/-* strain [Satterthwaite *et al*., 1998]. Reduced Btk dosage in *lyn-/-*Btk^{lo} mice blocks the development of autoantibodies; however, hypersensitivity to BCR crosslinking is not affected [Satterthwaite *et al*., 1998; Whyburn *et al*., 2003]. This indicates that Btk contributes to autoimmunity in *lyn-/-* mice by regulating events subsequent to or separate from the initial hyperactivation of B cells by antigen.

Lyn-deficient mice exhibit aberrant accumulation of antibody secreting cells in the periphery [Hibbs *et al*., 1995; Nishizumi *et al*., 1995; Chan *et al*., 1997]. Plasma cell accumulation is a common feature of human systemic lupus erythematosus (SLE) [Arce *et al*., 2001] as well as other genetically distinct models of murine lupus, including NZB/W mice [Erickson *et al*., 2003; Hoyer *et al*., 2004; Vinuesa *et al*., 2005; Wang *et al*., 2005; Xiang *et al*., 2007]. This phenomenon has been attributed to a variety of mechanisms in different model systems, for example, an unusually favorable splenic plasma cell survival niche and impaired plasma cell homing to the bone marrow in NZB/W and NZM2410 mice, respectively [Hoyer *et al*., 2007;Erickson *et al*., 2003]. However, events that promote increased plasma cells in *lyn-/-* mice are poorly defined.

The goal of the following studies was to determine if Btk had a role mediating plasma cell accumulation in *lyn-/-* mice.

Results

Splenic plasma cell accumulation in lyn-/- mice depends on Btk

Consistent with previous reports that increased antibody secreting cells and total IgM are seen in the absence of Lyn [Hibbs *et al*., 1995; Nishizumi *et al*., 1995; Chan *et al*., 1997; Satterthwaite *et al*., 1998; Takeshita *et al*., 1998; Whyburn *et al*., 2003], these studies reveal an increase in splenic plasmablasts and plasma cells (PB/PCs) in *lyn-/-* mice by flow cytometry (Figure 3.1). Spleens from Lyn-deficient mice had a significantly higher frequency and total number of $CD138^{hi}B220^{+}$ plasmablasts and $CD138^{hi}B220^{lo/-}$ plasma cells than did their wild-type (wt) counterparts (Figures 3.1 B, 3.1 D). A high expression of intracellular Igκ confirmed that gated $CD138^{hi}$ cells were, indeed, plasmablasts and plasma cells (Figure 3.2). For the sake of simplicity, I will refer to $CD138^{hi}$ (intracellular $Ig\kappa^{hi}$) cells as plasma cells. Taking into account the reduced frequency and number of B cells, *lyn-/-* spleens had greater than a 10-fold increase in the number of plasma cells per B cell. The frequency and total number of plasma cells as well as the ratio of plasma cells to B cells were normalized in *lyn-/-*Btk^{lo} mice (Figure 3.1). This was due to reduced Btk levels and not to the integration site of the transgene or unappreciated ectopic expression of Btk, as splenic plasma cells remained elevated in *lyn-/-* mice expressing both the endogenous Btk gene and the

transgene (*lyn*-/-Btk^{hi}) (Figure 3.1). Significant accumulation of plasma cells was seen in mice as young as 6- to 8-weeks old, well before the onset of disease (Figure 3.3 A, D). At 7-9 months of age, the frequency and total number of splenic plasma cells remained elevated in *lyn*-/-, but did not increase in *lyn*-/-Btk^{lo} mice (Figure 3.3 A, C). Thus, reduced Btk dosage does not simply delay the appearance of these cells. Intriguingly, there was a steady rate of increase in the frequency and total number of *lyn*-/- plasma cells starting at 12-13 weeks of age. This expansion did not correlate with an overall increase in B cell numbers (Figure 3.3 B).

Plasma cells do not have the ability to survive without external support such as in a favorable survival niche [Sze *et al*., 2000; Minges Wols *et al*., 2002; Cassese *et al*., 2003]. A vast majority of plasma cells will migrate to the bone marrow where such niches are plentiful. Bone marrow from mice at early (12-13 weeks) and late (32-42 weeks) stages was examined for plasmablast and plasma cell accumulation. No significant difference in the frequency of CD138^{hi}B220$^{lo/-}$ plasma cells was detected in either group (Figure 3.4).

Taken together, the data indicates that *lyn*-/- mice exhibit a Btk-dependent accumulation of plasma cells that is restricted to the spleen. These data also indicate that heightened sensitivity to BCR crosslinking is insufficient for the increase in plasma cells observed in the absence of Lyn.

lyn+/- mice have an intermediate degree of plasma cell accumulation and polyclonal IgM autoreactivity

The increased signaling that occurs in *lyn-/-* mice as a result of impaired inhibitory receptor activity is likely dampened by reduced dosage of Btk. If altering the balance of activating and inhibitory signals in general affects plasmablast and plasma cell accumulation, it was hypothesized that *lyn+/-* mice, which have a partial impairment of lyn-mediated inhibitory signaling [Cornall *et al*., 1998] may exhibit an altered plasma cell phenotype as well. *lyn+/-* mice had plasmablast and plasma cell frequencies between those of wild-type and *lyn-/-* mice (Figure 3.5). This was reflected in an intermediate level of total IgM (Table 3.1). Total IgG levels were not significantly different between *lyn-/-* and *lyn+/-* mice, however.

To assess the effect of altered Lyn and/or Btk dosage on autoantibody profiles, we compared the reactivity of sera from wild-type, *lyn-/-*, *lyn+/-*, and *lyn-/-*Btk^{lo} mice on an autoantigen array containing approximately 70 antigens associated with a range of autoimmune diseases [Li *et al*., 2007]. The antigens include nucleic acids, histones, extracellular matrix components, and various other protein antigens. Sera from 18-week old wild-type, *lyn-/-*, and *lyn+/-* and *lyn-/-*Btk^{lo} mice were hybridized to the array, and reactivity detected with anti-IgM or anti-IgG secondary antibodies. *lyn-/-* mice demonstrated a broad spectrum

of autoreactivity in the IgM compartment (Figure 3.6 A). This result was not due to the increased total IgM levels in *lyn-/-* serum (Table 3.1) as only a subset of the total antigens interrogated were recognized. IgG autoantibodies in *lyn-/-* mice reacted with a more limited set of antigens commonly occurring in lupus, including nucleic acid containing- and kidney antigens (Figure 3.6 B). Intriguingly, several *lyn+/-* mice demonstrated the IgM autoantibody signature characteristic of *lyn-/-* mice, but did not produce autoreactive IgG. These results indicate that reduced lyn dosage is sufficient for increased plasma cells and polyclonal IgM autoreactivity but is insufficient for the production of IgG against lupus related autoantigens. Neither IgM nor IgG autoantibodies were present in *lyn-/-*Btk^{lo} mice (Figure 3.6) [Whyburn *et al*., 2003], consistent with the requirement for Btk in the accumulation of plasma cells.

Discussion

This work shows that *lyn-/-* mice have an increase in splenic plasma cells that is associated with the production of autoreactive IgM against a wide range of self antigens. Both events are observed to some extent even when Lyn dosage is only partially reduced and are dependent on Btk, a target of Lyn-dependent inhibitory pathways. A recent study in which Btk was constitutively activated without manipulating Lyn confirms that excessive signaling through Btk promotes the accumulation of autoreactive plasma cells [Kersseboom *et al*., 2010]. The current studies do not address whether plasma cell accumulation is necessary for autoimmune disease. However, the fact that this is a common feature of human SLE and many distinct murine lupus models suggests that it plays an important role in disease pathogenesis. The microarray results suggest that neither increased plasma cells nor IgM autoreactivity against a wide range of autoantigens are sufficient for the production of pathogenic IgG autoantibodies. Of special interest is the "focusing" of antibody specificities seen in *lyn-/-* mice. In contrast to the wide spread reactivity that is seen from IgM antibodies, IgG autoantibodies in *lyn-/-* mice show a clear pattern of switching that favors antibodies specific for glomerular, DNA- and RNA-containing antigens, all of which are characteristic of SLE.

Possible mechanisms regulating accumulation of plasma cells, polyclonal IgM autoreactivity, and focused switching of B cells specific for lupus-associated self antigens will be addressed in the chapters that follow.

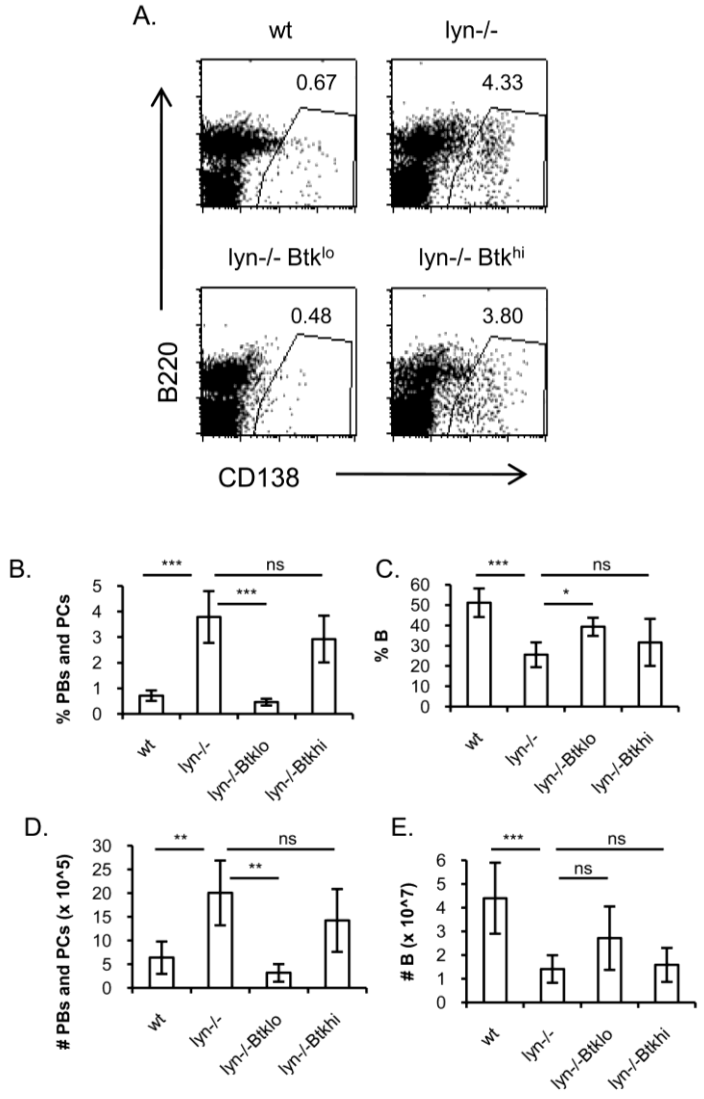

Figure 3.1. *lyn-/-* mice have a Btk-dependent increase in splenic plasma cells. Splenocytes from 15- to 18-week old mice were stained with antibodies against B220 and CD138. A) Representative plots show the frequency of plasma cells ($CD138^{hi}$). B) Frequency of plasma cells ($CD138^{hi}$). C) Frequency of B cells ($B220^{+}CD138^{-/lo}$). D) Total number of plasma cells ($CD138^{hi}$). E) Total number of B cells ($B220^{+}CD138^{-/lo}$). B) - E) Data are mean +/- SD. n = 10 for wt, 8 for *lyn-/-* and 5 for *lyn-/-*Btk^{lo} and *lyn-/-*Btk^{hi}. * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ by Student's *t*-test.

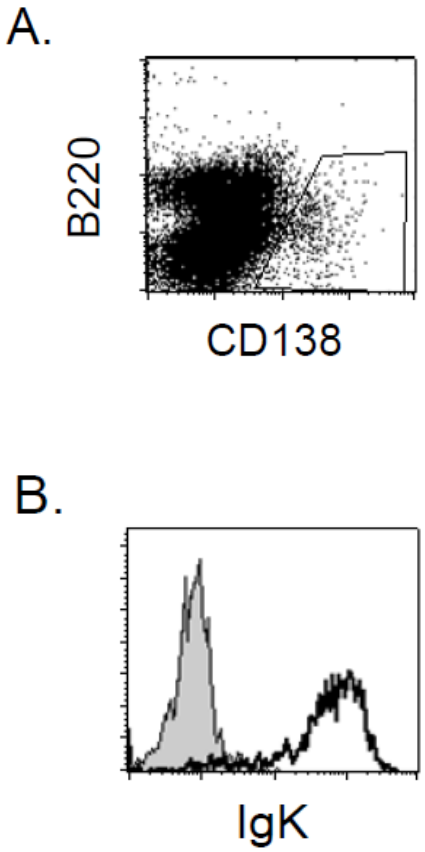

Figure 3.2. CD138^{hi} cells express high levels of intracellular Ig. Splenocytes from a 15- to 18-week old *lyn-/-* mouse were stained with antibodies against B220 and CD138, permeabilized and stained for intracellular Igκ. plasma cells (CD138^{hi}) were gated as shown in A and analyzed for expression of intracellular Igκ (open histogram) compared to isotype control (gray histogram) as shown in B.

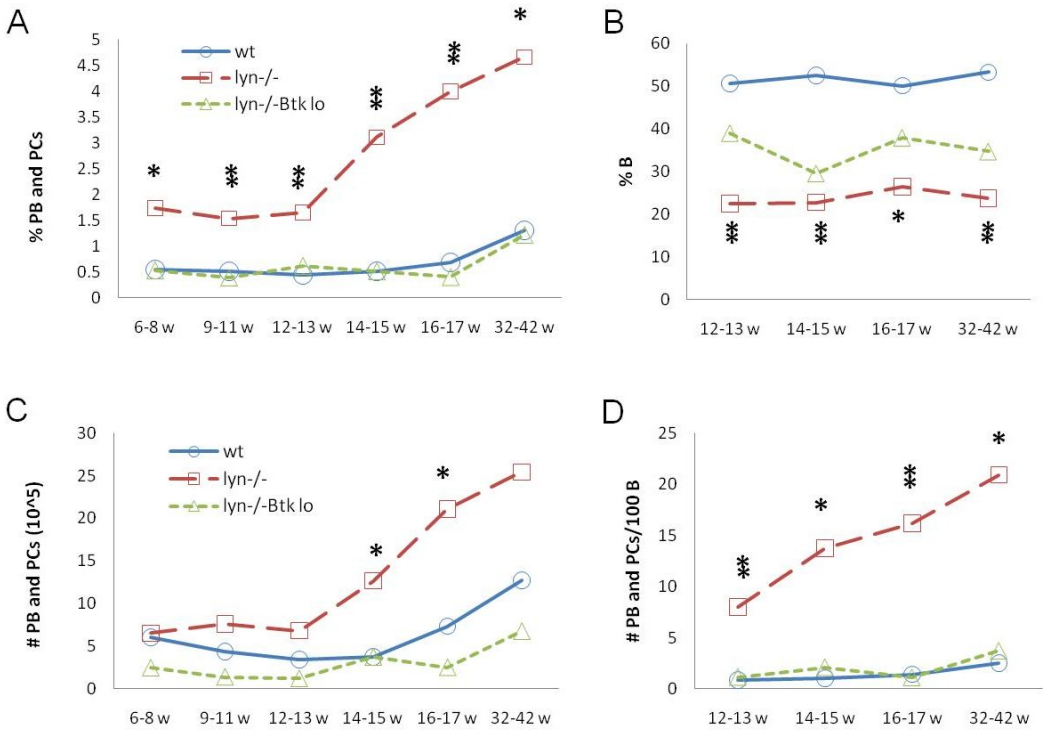

Figure 3.3. Plasmablast and plasma cell accumulation is detectable in *lyn-/-* mice as early as 6-8 weeks and steadily increases over time. Splenocytes were stained with antibodies against B220 and CD138 to identify $CD138^{hi}$ plasma cells and $B220^{+}CD138^{lo/-}$ B cells. A) Frequency of plasma cells ($CD138^{hi}$). B) Frequency of B cells ($B220^{+}CD138^{-/lo}$). C) Total number of plasma cells ($CD138^{hi}$). D) Number of plasma cells per 100 B cells. A) - D) Mean value for wt is represented by open circles, *lyn-/-* by open squares and *lyn-/-*Btklo by open triangles. n = 3-8 for wt, *lyn-/-* and *lyn-/-*Btk^{lo} at every designated interval, weeks (w) with the exception of 9-11 weeks, *lyn-/-*Btk^{lo} (n = 1) and 14-15 weeks, *lyn-/-*Btk^{lo} (n = 2). * $p < 0.05$, ** $p < 0.002$ wt vs. *lyn-/-* by Student's *t*-test.

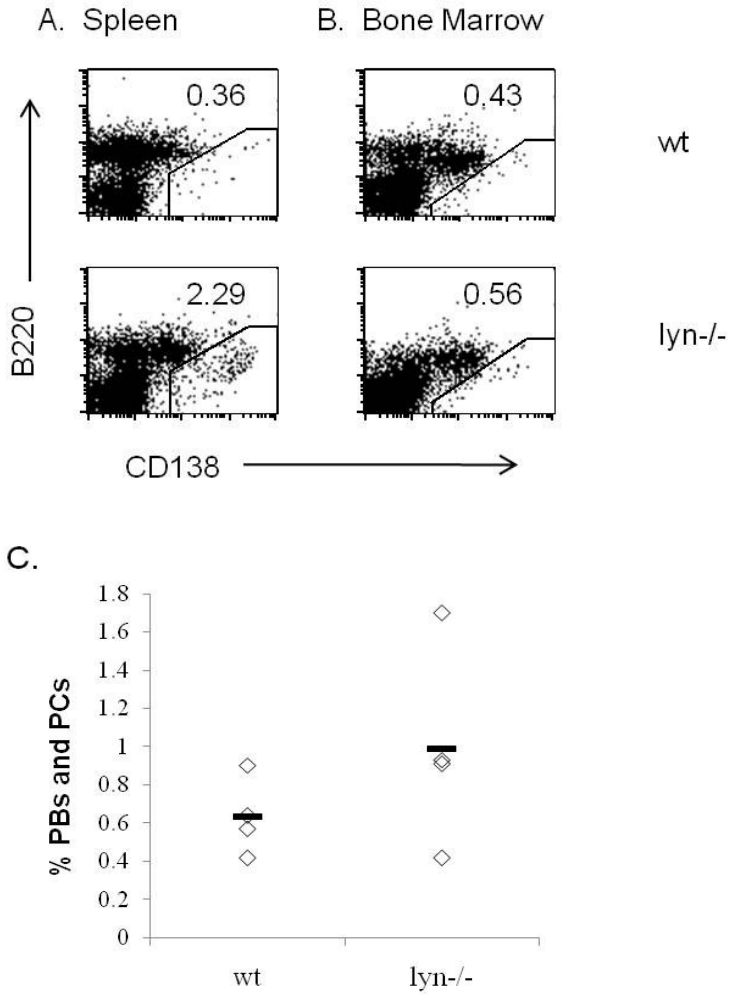

Figure 3.4. Plasma cells do not accumulate in the bone marrow of *lyn-/-* mice. Splenocytes (A) and bone marrow cells (B, C) from mice were stained with antibodies against B220 and CD138. A) - B) Representative plots show the frequency of plasma cells ($CD138^{hi}$) in tissues from 12-13 week old mice. C) Frequency of plasma cells ($CD138^{hi}$) in bone marrow from 32- to 42-week old mice. Each diamond represents an individual mouse, the bar represents the mean. n = 4 for all genotypes. No significance between groups was determined by Student's t-test.

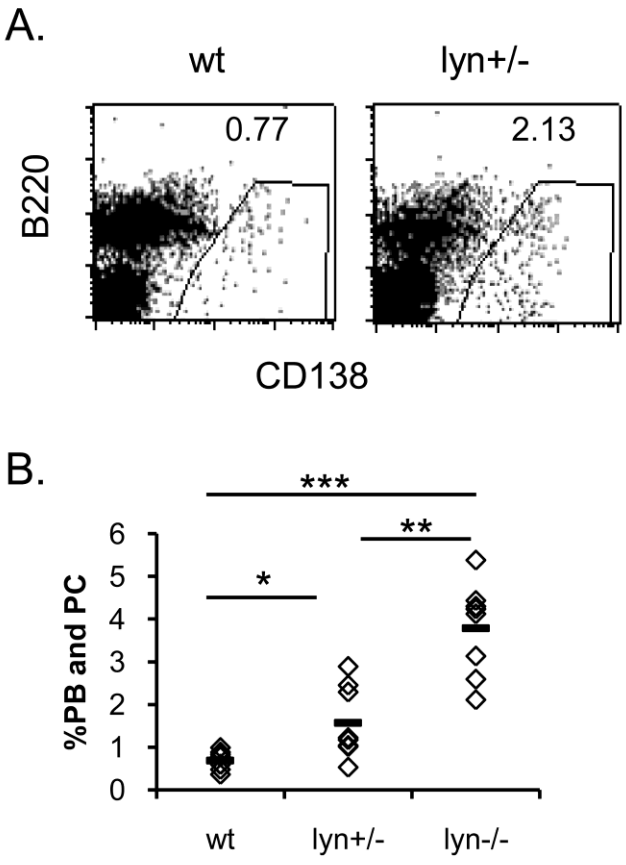

Figure 3.5. *lyn*+/- mice exhibit an intermediate degree of splenic plasmablast and plasma cell accumulation. Splenocytes from 15- to 18-week old mice were stained with antibodies against B220 and CD138. Representative plots showing the frequency of plasma cells ($CD138^{hi}$) are shown in A). B) Frequency of plasma cells ($CD138^{hi}$) is indicated. Each diamond represents an individual mouse. The bar represents the mean. n = 10 for wt, 8 for *lyn*-/-, and 7 for *lyn*+/-. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$ by Student's *t*-test.

	2-4 months		5-7 months	
	IgM	**IgG**	**IgM**	**IgG**
wt	0.18 +/- 0.04	4.6 +/- 0.75	0.64 +/- 0.34	2.24 +/- 0.55
lyn-/-	0.65 +/- 0.22 *	4.3 +/- 0.26	1.95 +/- 0.61 **	2.08 +/- 0.52
lyn+/-	0.41 +/- 0.03 *	4.5 +/-0.64	1.00 +/- 0.15 *	2.2 +/- 0.69

Table 3.1. Total Ig levels in wild-type, *lyn-/-* and *lyn+/-* mice. Total IgM and IgG levels (mg/ml) in the serum of 2- to 4-month or 5- to 7-month old mice were measured by ELISA. Data represent mean +/- SD (n = 3-12). * $p < 0.05$ vs. wt, ** $p < 0.00001$ vs. wt by Student's *t*-test.

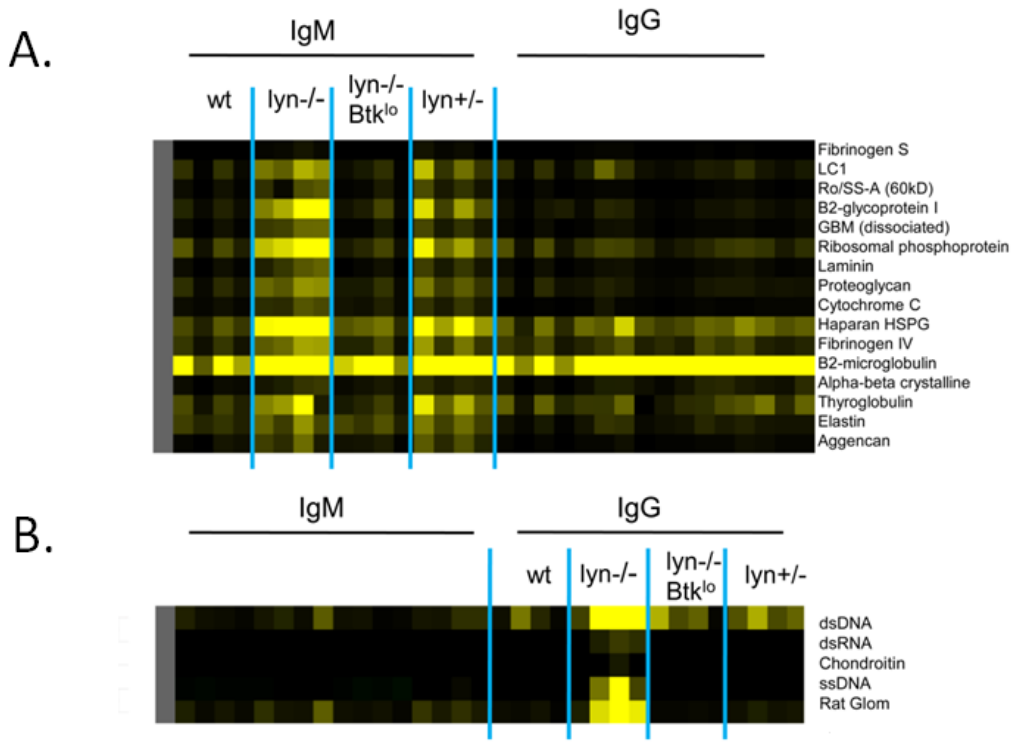

Figure 3.6. Reduced lyn dosage is sufficient for the production of IgM autoreactivity against a wide range of autoantigens in a Btk-dependent manner. Serum from 18-week old mice was hybridized to an autoantigen array and probed with anti-IgM or anti-IgG. Results were clustered hierarchically by antigen. Clusters of IgM (A) and IgG (B) reactivity are shown. The degree of reactivity with the array (fluorescence intensity) is indicated by the intensity of yellow. Black = no reactivity. n = 4 for all genotypes. This work was completed through collaboration with Dr. Quan-Zhen Li.

CHAPTER IV

INTERLUKIN-6 IS REQUIRED FOR PATHOGENIC ANTIBODY, BUT NOT PLASMA CELL ACCUMULATION, IN LYN-DEFICIENT MICE

A majority of the work presented in this chapter has been published in the *European Journal of Immunology*, volume 40, pages 1897–1905, 2010. Experiments were performed by Maria A. (Toni) Gutierrez unless otherwise noted in the text and figure legend.

Introduction

Mounting evidence suggests that myeloid defects also contribute to the development of antibody-mediated diseases such as SLE. This is of interest given that both Lyn and Btk are expressed in myeloid cells. In fact, *lyn-/-* myeloid cells have been show to become increasingly dysregulated with age resulting in increased frequency of myeloid cells and myeloid tumors [Harder *et al*., 2001].

Several myeloid cell functions affected by lyn deficiency have been specifically associated with the generation of autoantibodies in other lupus models. For example, activated *lyn-/-* macrophages produce increased levels of IL-1 and IL-6 [Meng and Lowell, 1997]. The plasma cell survival factor IL-6 has been implicated in the production of anti-DNA antibodies in various murine models of lupus [Suematsu *et al*., 1989; Linker-Israeli *et al*., 1991; Tang *et al*., 1991; Alarcon-Riquelme *et al*., 1993; Finck *et al*., 1994; Minges Wols *et al*., 2002; Cassese *et al*., 2003]. Anti-IL-6 antibodies are sufficient to prevent production of anti-dsDNA antibodies in NZB/W mice [Finck *et al*., 1994]. In lupus patients, elevated serum levels of IL-6 have been observed in several studies; in some, increased IL-6 correlated with disease activity or anti-DNA antibody levels [Linker-Israeli *et al*., 1991; Peterson *et al*., 1996; Grondal *et al*., 2000].

Btk is normally expressed in both the B and myeloid lineage [Smith *et al*., 1994]. However, the Btk^{lo} transgene is not expressed in myeloid cells as it is driven by the IgH promoter and enhancer [Satterthwaite *et al*., 1998]. Myeloid lineage cells in *lyn-/-*Btk^{lo} mice thus lack both Lyn and Btk. Effects of Btk deficiency on myeloid cell function are relatively subtle. However, given their opposing regulatory roles in various cellular functions, it seemed likely that loss of Btk might prevent the effects of Lyn-deficiency and suppress a myeloid contribution to autoimmunity in *lyn-/-*Btk^{lo} mice.

Thus, the following studies were performed to establish whether *lyn-/-* mice expressed increased levels of IL-6, to assess whether IL-6 contributes to autoimmunity in *lyn-/-* mice and to determine if there is any role for Btk in mediating myeloid contribution to pathology in Lyn-deficient mice.

Results

lyn-/- mice exhibit a Btk-dependent increase in IL-6

Lyn-deficient macrophages and dendritic cells secrete increased amounts of IL-6 under certain conditions [Meng and Lowell, 1997; Silver *et al*., 2007]. As proof of principle, the experiments as reported by Lowell and colleagues were replicated. Consistent with previously described results, *lyn-/-* bone marrow-derived macrophages (BMDM) demonstrated greater production of IL-6 in response to IFNγ and LPS than wild-type (Figure 4.1). This increase was not reduced in the absence of Btk. However, serum analysis of IL-6 had shown that there was a trend towards increased serum IL-6 levels in 5- to 7-month old *lyn-/-* mice, but not *lyn-/-*Btk^{lo} mice (Figure 4.2). This was not observed in younger mice (Figure 4.2), although it is possible that local increases in IL-6 occur earlier, becoming systemic with time.

It was considered that the discrepancy seen in the effect of Btk on IL-6 production and serum levels could be due to shortcomings of the *in vitro* system used to test myeloid production of IL-6. *In vitro* generation of BMDM relied on culture in macrophage colony-stimulating factor (M-CSF), and Lyn is a negative regulator of M-CSF receptor signaling [Baran 2003]. In fact, de-regulated M-CSF signaling and the extensive myeloid accumulation which it promotes has

been proposed to contribute to autoimmunity [Lenda *et al*., 2004]. Thus, there was potential for a significant internal bias in the experimental design. It also became evident that *lyn-/-* macrophages were more sensitive to culture conditions (i.e., culture density) than control cells. Despite maintaining similar trends toward increased IL-6 production, the total amount of IL-6 production in *lyn-/-* cultures was quite varied between tests, raising concerns over the reliability of the assays. As reported by Krystal and colleagues, similar studies with macrophages deficient in SHIP, a molecule downstream of Lyn inhibitory signaling pathways, also exhibited this trend [Sly *et al*., 2004].

As such, it was necessary to examine IL-6 production from a more physiologically relevant, and consistent, population of cells. IL-6 production within the splenic microenvironment would likely have the most direct effect on the plasmablast and plasma cell accumulation. Splenocytes were isolated from wild-type (wt), *lyn-/-* and *lyn-/-*Btk^{lo} mice for analysis. The cells were prepared for culture and incubated for 4 hours with brefeldin A with or without LPS. Intracellular IL-6 was subsequently measured by flow cytometry. Minimal IL-6 staining was observed in unstimulated cells (Figure 4.3). However, an increased frequency of IL-6-expressing cells was observed in LPS-stimulated splenocytes from *lyn-/-* mice compared to their wild type or *lyn-/-*Btk^{lo} counterparts (Figure 4.4). Most of these cells were B220-negative and expressed the myeloid cell

marker CD11b. A subset was also positive for the dendritic cell marker CD11c (Figure 4.4).

IL-6 is dispensable for plasma cell accumulation and IgM autoreactivity but mediates the production of lupus-associated IgG autoantibodies

Given the known roles of IL-6 in plasma cell survival and the production of anti-DNA antibodies in other lupus models [Cassese *et al*., 2003; Minges Wols *et al*., 2002; Suematsu *et al*., 1989; Linker-Israeli *et al*., 1991; Tang *et al*., 1991; Alarcon-Riquelme *et al*., 1993; Finck *et al*., 1994] and the requirement for Btk in both the plasma cell accumulation and the increased IL-6 expression observed in the absence of Lyn, it was hypothesized that IL-6 mediates plasma cell accumulation and autoantibody production in *lyn-/-* mice. To address this issue, mice deficient in both Lyn and IL-6 were generated. Surprisingly, there was no difference in the frequency of splenic plasma cells between *lyn-/-* and *lyn-/-IL-6-/-* mice in young or aged mice (Figure 4.5). In fact, the ratio of plasma cells per B cell was similarly elevated in *lyn-/-* and *lyn-/-IL-6-/-* mice. Nor did IL-6 deficiency reduce the frequency of bone marrow plasma cells in either young or aged *lyn-/-* mice (Figure 4.5). Thus, IL-6 is dispensable for both the initial increase in splenic plasma cells in *lyn-/-* mice and the maintenance of this phenotype.

To gain a broad perspective on the role of IL-6 in autoantibody production in *lyn-/-* mice, we employed an autoantigen array containing approximately seventy antigens associated with a range of autoimmune diseases [Li *et al*., 2007]. The antigens include nucleic acids, histones, extracellular matrix components, and various other protein antigens. Sera from 18-week old wild-type, *lyn-/-*, and *lyn-/-IL-6-/-* mice were hybridized to the array, and reactivity detected with anti-IgM or anti-IgG secondary antibodies. As shown in the previous chapter, *lyn-/-* mice demonstrated a broad spectrum of autoreactivity in the IgM compartment, which was also observed in *lyn-/-IL-6-/-* mice (Figure 4.6). This result was not due to the increased total IgM levels in *lyn-/-* and *lyn-/-IL-6-/-* serum (wild-type 0.61 +/- 0.27 mg/ml; *lyn-/-* 4.02 +/- 0.49* mg/ml; *lyn-/-IL-6-/-* 3.68 +/- 1.54* mg/ml; n = 4, * $p < 0.05$ vs. wild-type), as only a subset of the total antigens interrogated were recognized. Thus, IL-6 is also dispensable for IgM autoreactivity.

Quite a different scenario was observed in the IgG compartment, however. Serum from a second set of animals independent from those in Figure 3.6 showed that autoreactive IgG in *lyn-/-* mice was much more focused towards a limited number of antigens which are characteristic of SLE (Figure 4.6). These include the same glomerular, DNA- and RNA- containing antigens that were seen in Figure 3.6. Intriguingly, almost no autoreactive IgG was observed in the absence of IL-6. This was despite the fact that there was no significant difference in total IgG levels between wild-type (2.65 +/- 2.30 mg/ml, n = 4), *lyn-/-* (5.50 +/- 1.58

mg/ml, n = 4) and *lyn-/-IL-6-/-* (4.55 +/- 2.48 mg/ml, n = 4) mice. To determine whether the lack of IL-6 merely delays the appearance of autoreactive IgG, we repeated the array experiments on aged mice. Similar results were obtained with respect to both IgM (data not shown) and IgG autoantibody profiles (Figure 4.7). The absence of anti-DNA IgG in the serum of aged *lyn-/-IL-6-/-* mice was confirmed by ELISA (data not shown).

To confirm that the presence of IgG and not IgM autoantibodies impacted renal pathology, kidneys from 10-month old mice were submitted to an independent pathologist for blind examination. Glomeruli were examined for evidence of pathology such as crescent formation and cellular infiltration, and were scored from 0 – 4 for the severity of glomerulonephritis (GN), 0 being normal and 4 tissue exhibiting the most severe pathology. *lyn-/-* mice showed significant kidney damage as compared to *lyn-/-IL-6-/-* mice as well as *lyn-/-*Btk^{lo} and *lyn*+/- mice (Figure 4.8). This confirms that the presence of IgM antibodies reactive to autoantigens is not sufficient for kidney damage and that it is critical for IL-6 dependent class-switching of autoreactive B cells to occur for the disease to manifest.

IL-6 may affect class-switching via IL-21

There are several possible mechanisms by which IL-6 may control the production of autoreactive IgG. Some of the effects of IL-6 on B cells have recently been shown to be indirect, via $CD4^+$ T cells [Dienz *et al*., 2009]. IL-6 can promote the development of follicular Th cells (Tfh) and Th17 cells [Nurieva *et al*., 2008; Zhou *et al*., 2007], both of which have been implicated in the pathogenesis of lupus [Linterman *et al*., 2009; Hsu *et al*., 2008]. Tfh and Th17 cells are both efficient producers of IL-21 [Chtanova *et al*., 2004; Vinuesa *et al*., 2005; Wei *et al*., 2007], which directly impacts plasma cell differentiation and IgG production [Ozaki *et al*., 2004; Pene *et al*., 2004].

It was therefore hypothesized that the increased IL-6 in *lyn-/-* mice might promote inappropriate expression of IL-21 in the splenic milieu. This could then impact both plasma cell accumulation and class-switching in *lyn-/-* mice. To determine if there was an elevation in IL-21, mRNA was isolated from splenocytes for qPCR analysis of IL-21 expression. There was a trend towards increased IL-21 in *lyn-/-* splenocytes and a significant decrease in IL-21 mRNA expression in *lyn-/-*Btk^{lo} cells (Figure 4.9).

IL-21-deficient mice were then crossed with *lyn-/-* mice to produce double knockouts. As in the *lyn-/-IL-6-/-* mice there was no difference in the frequency of splenic plasma cells between *lyn-/-* and *lyn-/-IL-21-/-* mice (Figure 4.10).

Preliminary results from anti-dsDNA ELISAs indicate that IL-21 is required for IgG autoantibody production (data not shown). Further studies are warranted to determine how IL-21 affects the penetrance, kinetics, or antigen specificity of IgG autoantibodies in *lyn-/-* mice.

Discussion

I have shown in the previous chapter that *lyn-/-* mice have a Btk-dependent increase in splenic plasma cells that is associated with the production of autoreactive IgM against a wide range of self antigens (checkpoint 1, Table 4.1). Despite the observation that *lyn-/-* myeloid cells over-express IL-6 in a Btk-dependent manner, both accumulation and IgM autoreactivity are independent of this plasma cell survival factor. IL-6 is, however, necessary for class-switching of autoreactive B cells (checkpoint 2, Table 4.1). The production of IgG against lupus associated autoantigens is not observed in *lyn-/-IL-6-/-* or *lyn+/-* mice, both of which demonstrate increased splenic plasma cells and widespread autoreactivity in the IgM compartment. This suggests that while many types of autoreactive B cells escape tolerance mechanisms and differentiate into IgM secreting plasma cells in the absence of Lyn, only a subset receive the appropriate signals to induce class switching to pathogenic IgG isotypes. The latter process requires IL-6 and complete deficiency of Lyn.

It has been hypothesized by many that IL-6 effects pathogenic antibody production via alterations in T cell populations and/or their effector function(s). It remains to be determined if, and to what degree, IL-6-deficiency impacts T cell differentiation in *lyn-/-* mice. As of yet, there is only evidence that IL-6-deficiency in *lyn-/-* mice normalizes the expression of T cell activation markers

[Tsantikos *et al*., 2010]. If IL-6 acts through T cells to promote autoantibody production, it may do so by inducing IL-21 expression as indicated by preliminary results from the study of *lyn-/-IL-21-/-* mice. IL-6 may also increase the access to non-IL-21-mediated B cell help by increasing T helper cell numbers. Alternatively, it could also be influencing B cell class-switching and antibody production through the suppression of regulatory T cell differentiation [Bettelli *et al*., 2006] or via alternate non-T cell mechanisms.

The phenotype of *lyn-/-IL-6-/-* mice bears some resemblance to incomplete lupus in humans. These individuals have some autoimmune features but have insufficient criteria for a diagnosis of SLE. Autoantibody array studies similar to those performed here recently revealed that IgM autoreactivity predominates in incomplete lupus patients [Li *et al*., 2007]. SLE patients, in contrast, have a much higher ratio of autoreactive IgG to IgM for most autoantigens. This suggests that the switch to pathogenic IgG isotypes may underlie the transition from incomplete lupus to full blown SLE. It would be intriguing to determine whether IL-6 levels vary between incomplete lupus and SLE patients.

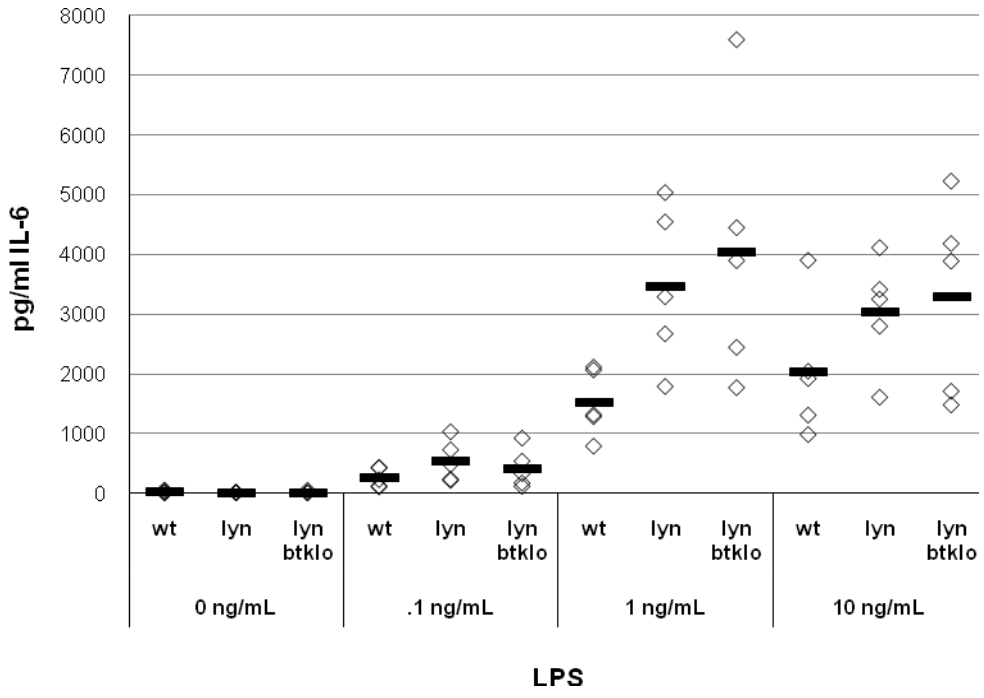

Figure 4.1. Lyn-deficient macrophages secrete increased IL-6 *in vitro*. Bone marrow-derived macrophages (BMDMs) from 2- to 4-month old mice were primed with IFNγ and stimulated with increasing concentrations of LPS. After a 12-hour incubation, the culture supernatant was collected for analysis by ELISA. This data set (n = 5) demonstrates that absence of Lyn correlates with increased secretion of IL-6.

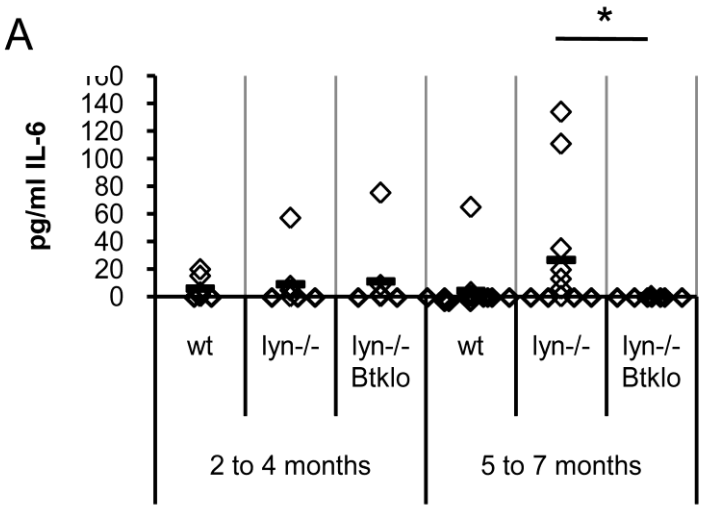

Figure 4.2. Increased level of IL-6 in serum of *lyn-/-* mice depends on Btk. Serum IL-6 levels in 2- to 4- and 5- to 7-month old mice were measured by ELISA. Each diamond represents an individual mouse, the bar represents the mean. 2-4 months: n = 7 for wt, 8 for *lyn-/-* and *lyn-/-*Btk^{lo}. 5-7 months: n = 17 for wt, 12 for *lyn-/-*, and 9 for *lyn-/-*Btk^{lo}. * $p < 0.05$ by Student's *t*-test.

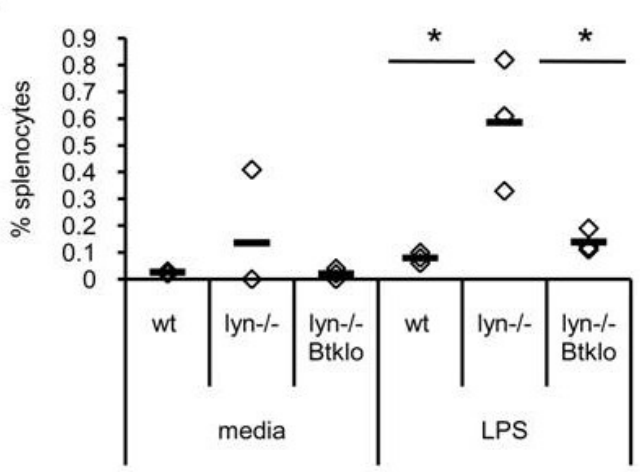

Figure 4.3. Increased expression of IL-6 by splenic myeloid cells is observed in *lyn-/-* mice and depends on Btk. Splenocytes from 5 month old mice were cultured for 4 hours with brefeldin A alone or brefeldin A plus 10 µg/ml LPS. Cells were then stained extracellularly with antibodies against B220, CD11b, and CD11c and intracellularly with isotype control or anti-IL-6. The frequency of $B220^{-}IL\text{-}6^{+}$ cells (with background staining subtracted) is shown for cells incubated with brefeldin A alone (media) or brefeldin A with 10 µg/ml LPS (LPS). Each diamond represents an individual mouse (n = 3), the bar represents the mean. * $p < 0.05$ by Student's *t*-test.

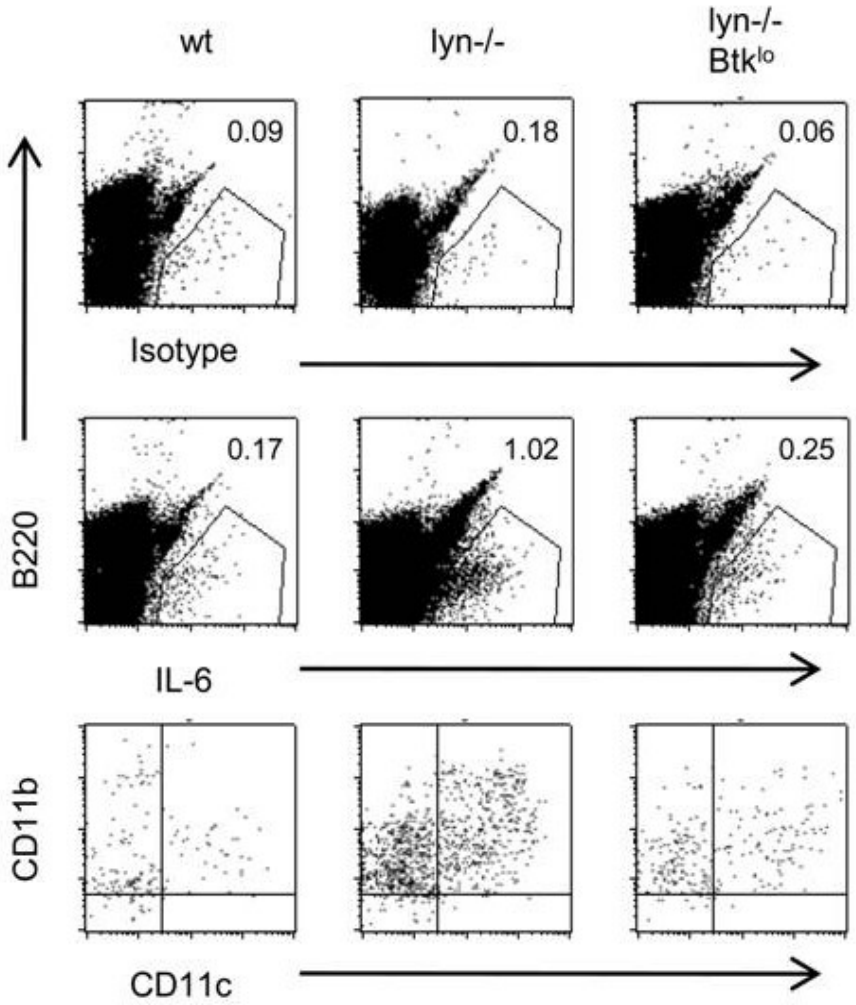

Figure 4.4. Increased expression of IL-6 by splenic myeloid cells is observed in *lyn-/-* mice and depends on Btk. Splenocytes from 5-month old mice were cultured for 4 hours with brefeldin A plus 10 μg/ml LPS. Cells were then stained extracellularly with antibodies against B220, CD11b, and CD11c and intracellularly with isotype control or anti-IL-6. LPS stimulated cells are depicted. The frequency of $B220^-$ cells binding to the isotype control (top panel) or anti-IL-6 (middle panel) is indicated. The bottom panel shows CD11b and CD11c expression on $B220^-IL\text{-}6^+$ cells as gated in the middle panel.

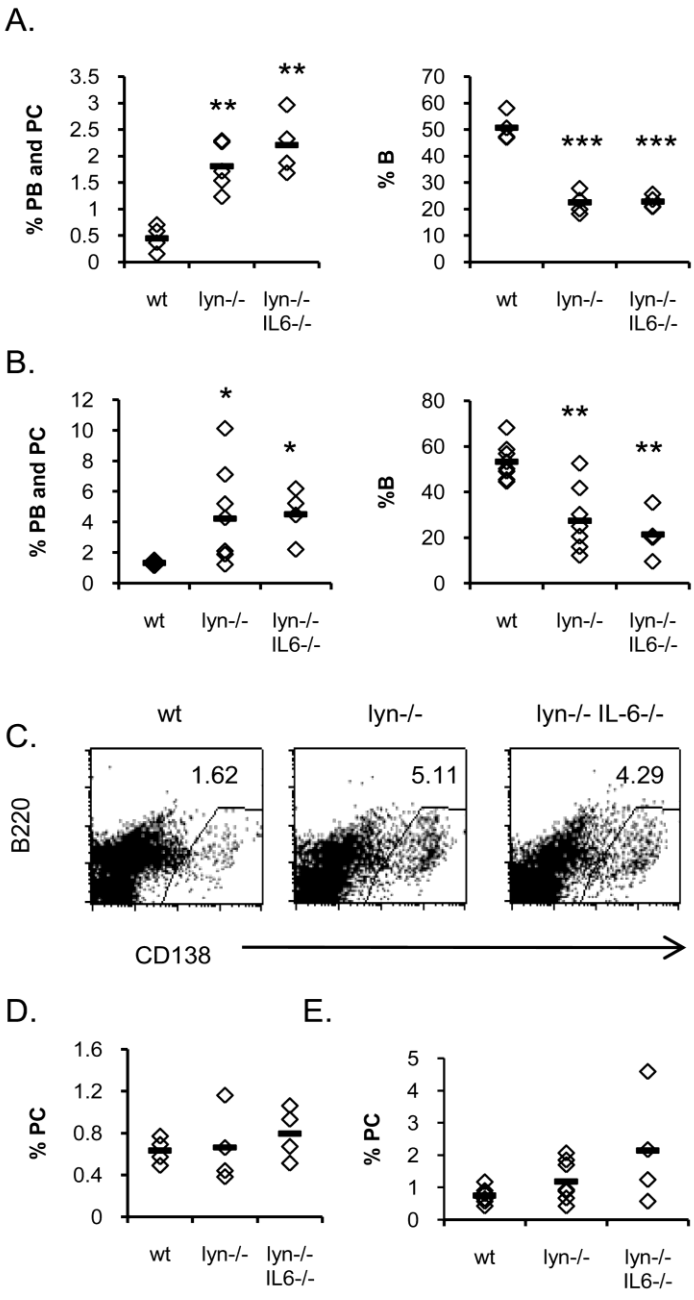

Figure 4.5.
Accumulation of splenic plasma cells in lyn-deficient mice is independent of IL-6. A-C) Splenocytes from A) 12- to 13-week and B, C) 7- to 9-month old mice were stained with antibodies against B220 and CD138 to identify $CD138^{hi}$ plasma cells and $B220^{+}CD138^{lo/-}$ B cells. Results from several mice are compiled in A) and B), while C) shows representative plots indicating the frequency of plasma cells ($CD138^{hi}$). Each diamond represents an individual mouse, the bar represents the mean. A) n = 4 for wt and *lyn-/-IL6-/-*, 5 for *lyn-/-*. B) n = 8 for wt and *lyn-/-*, 4 for *lyn-/-IL6-/-*. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ vs. wt by Student's *t*-test. D, E) Bone marrow from 7- to 9-month old mice was analyzed as in A - C. D) n = 4. E) n = 8 for wt and *lyn-/-*, 4 for *lyn-/-IL6-/-*. There was no significant difference between samples in D and E by Student's *t*-test.

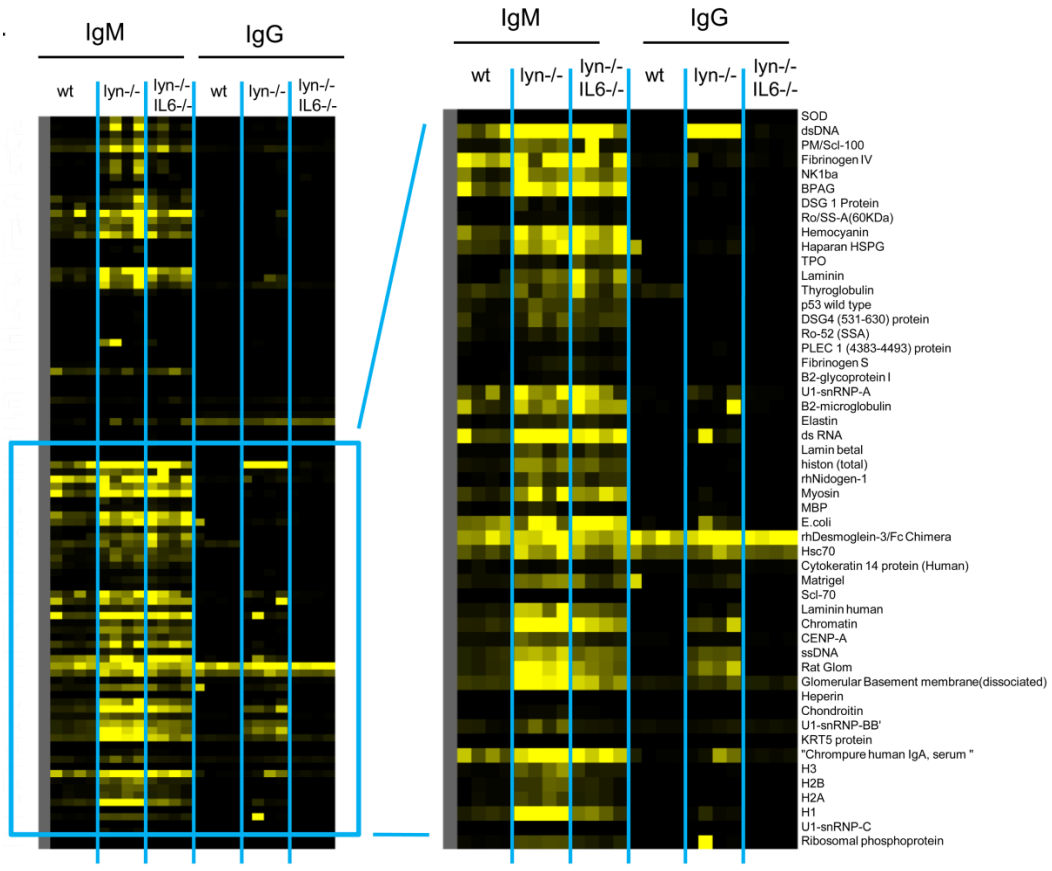

Figure 4.6. *lyn-/-* mice demonstrate IL-6 independent IgM autoreactivity against a wide range of autoantigens and IL-6 dependent, focused IgG autoreactivity against lupus associated autoantigens. Serum from 18-week old mice was hybridized to an autoantigen array and probed with anti-IgM and anti-IgG. n = 4 for all. Results were clustered hierarchically by antigen. Clusters of IgM or IgG reactivity are shown. The degree of reactivity with the array (fluorescence intensity) is indicated by the intensity of yellow. Black = no reactivity.

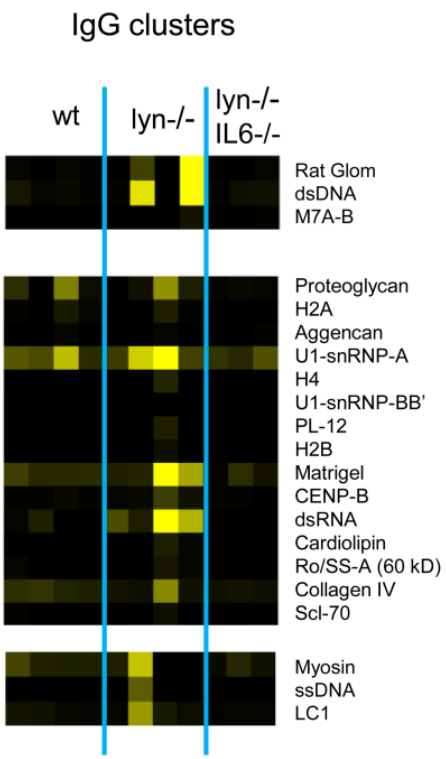

Figure 4.7. IL-6 dependent IgG autoreactivity in *lyn-/-* mice is focused against lupus associated autoantigens. Serum from 10- to 14-month old mice was hybridized to an autoantigen array and probed with anti-IgM and anti-IgG. n = 4 for wt, *lyn-/-* and n = 3 for *lyn-/-IL-6-/-*. Results were clustered hierarchically by antigen. Clusters of IgG reactivity are shown. The degree of reactivity with the array (fluorescence intensity) is indicated by the intensity of yellow. Black = no reactivity.

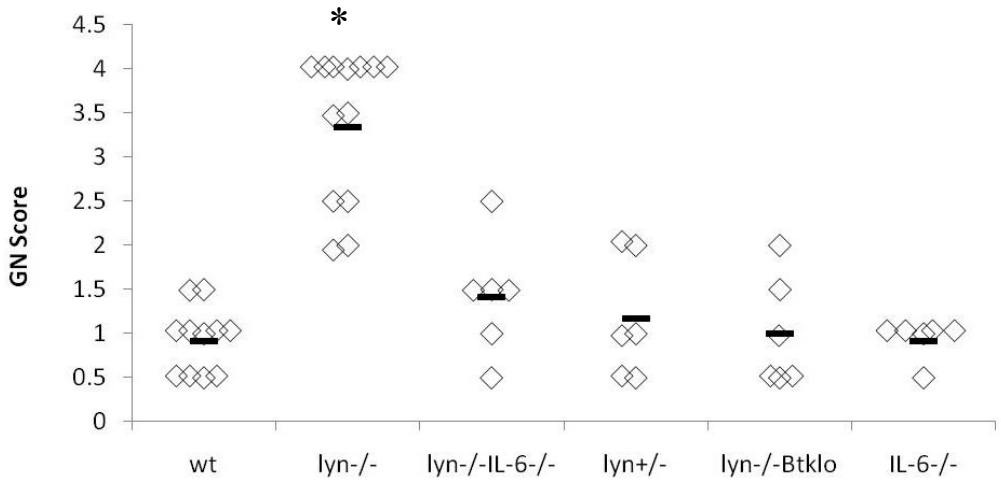

Figure 4.8. Renal pathology correlates with presence of IgG antibodies specific for lupus-associated antigens in *lyn-/-* mice. Sagittal sections of kidneys from 10-month old mice were submitted to Dr. Xin J. Zhou for blind examination. Glomerulonephritis (GN) severity was graded on a 0 to 4 scale, where 0 = normal, 1 = mild increase in mesangial cellularity and matrix, 2 = moderate increase in mesangial cellularity and matrix with thickening of glomerular basement membrane (GBM), 3 = focal endocapillary hypercellularity with obliteration of capillary lumina and a substantial increase in the thickness and irregularity of the GBM and 4 = diffuse endocapillary hypercellularity, segmental necrosis, crescents and hyalinized end-stage glomeruli, as described previously [Xie *et al*., 2003]. n = 11 for wt; n = 12 for *lyn-/-* and n = 6 for *lyn-/-IL-6-/-*, *lyn*+/-, *lyn-/-*Btk^{lo}, *IL-6-/-*. * = $p < 0.00001$ vs. wt as determined by Student's *t*-test**.**

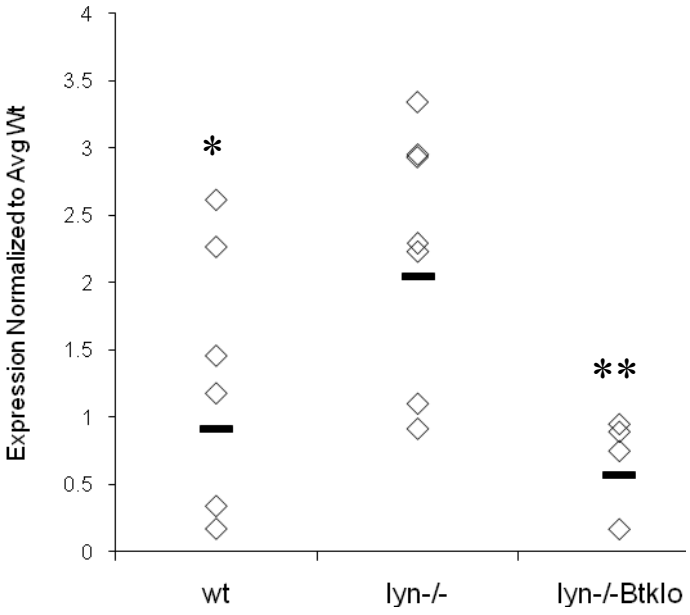

Figure 4.9. Expression of IL-21 mRNA is significantly reduced in the *lyn-/-Btk^{lo}* spleen. Total splenocyte RNAs were isolated from 2- to 4-month old wt, *lyn-/-,* and *lyn-/-Btk^{lo}* mice and used to generate cDNAs. Quantitative real-time PCR was performed using IL-21 Taqman primers. IL-21 expression in each sample was normalized to GADPH and is shown relative to the average expression level in wt samples. * = $p < 0.06$, ** $p < 0.002$ vs. *lyn-/-* as determined by Student's *t*-test**.**

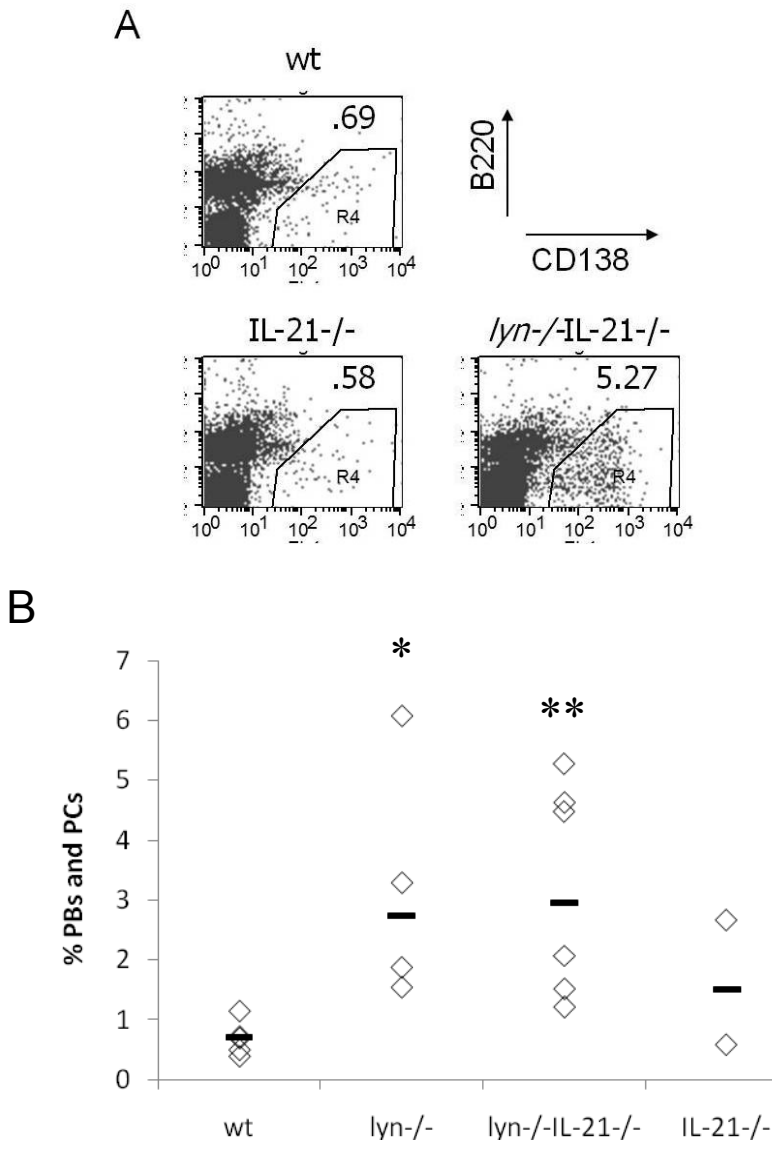

Figure 4.10. Plasma cell accumulation in *lyn-/-* mice does not require IL-21. Splenocytes from 16- to 20-week old mice were stained with antibodies against B220 and CD138 to identify $CD138^{hi}$ plasma cells and $B220^{+}CD138^{lo/-}$ B cells. A) Representative plots indicating the frequency of plasma cells ($CD138^{hi}$). B) Results from several mice are compiled. Each diamond represents an individual mouse, the bar represents the mean. n = 6 for wt; n = 5 for *lyn-/-*; n = 7 for *lyn-/-IL-21-/-*; n = 3 for *IL-21-/-*. * = $p < 0.05$, ** $p = 0.007$ vs. wt as determined by Student's *t*-test**.**

	BCR Signaling	Plasma Cell Accumulation	Widespread IgM AutoAbs	Focused IgG AutoAbs
wild type	+	--	--	--
lyn-/-Btk^{lo}	+++	--	--	--
lyn+/-	++	+	+	--
lyn-/-IL-6-/-	Not tested	++	++	--
lyn-/-	+++	++	++	++

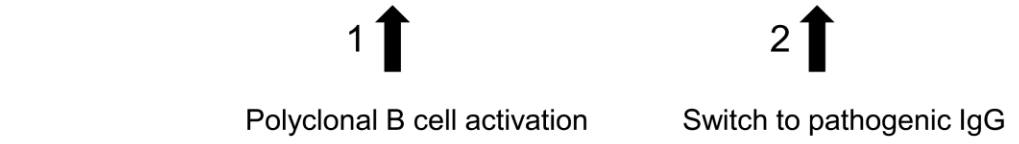

Table 4.1. Multiple checkpoints focus IgG autoreactivity towards lupus-associated antigens in *lyn-/-* mice. The current study identifies two checkpoints that regulate the production of autoantibodies in *lyn-/-* mice. Polyclonal activation and differentiation of B cells results in accumulation of splenic plasma cells and secretion of IgM antibodies specific for a wide range of autoantigens (checkpoint 1). This depends on Btk but not IL-6, requires only a partial reduction in Lyn levels, and involves events other than or in addition to hypersensitivity to BCR crosslinking. An additional checkpoint (checkpoint 2) limits the IgG autoantibody repertoire in *lyn-/-* mice to nucleic acid containing and glomerular antigens. Production of these focused IgG autoantibodies depends on IL-6 and requires complete deficiency of Lyn.

CHAPTER V

CONTRIBUTION OF B CELL-INTRINSIC MECHANISMS TO PLASMA CELL ACCUMULATION IN LYN-DEFICIENT MICE

Introduction

Most plasma cells produced in the spleen either die rapidly due to a limited number of plasma cell niches [Sze *et al*., 2000] or migrate to the bone marrow in response to SDF-1/CXCL12 [Hargreaves *et al*., 2001]. There they encounter a microenvironment supportive of long-term survival. IL-5, IL-6, TNFα, SDF-1, CD44 signaling, and BAFF/BCMA signaling all contribute to this plasma cell survival niche [Minges Wols *et al*., 2002; Cassese *et al*., 2003; O'Connor *et al*., 2004].

Aberrant accumulation of plasma cells in secondary lymphoid organs is a common feature of human and murine lupus. Pediatric SLE patients have increased numbers of peripheral blood plasma cells [Arce *et al*., 2001]. NZB/W mice have an increase in short- and long-lived plasma cells in the spleen, both of which contribute to autoantibody production [Hoyer *et al*., 2004]. This has been

attributed to a favorable plasma cell survival niche in the spleens of these mice [Hoyer *et al*., 2004] as well as impaired plasma cell homing to the bone marrow [Erickson *et al*., 2003]. Increased plasma cell differentiation in response to TLR engagement can also contribute to autoantibody production, as is the case in Ets-1-/- mice [Wang *et al*., 2005]. In *lyn-/-* mice, plasma cell accumulation is independent of stimulation by autoantigen as it occurs even when the B cell repertoire is restricted to a single specificity (anti-HEL) and the antigen is not present [Cornall *et al*., 1998]. Consistent with this observation, reduced Btk dosage uncouples BCR hyperresponsiveness from plasma cell accumulation (Figure 3.1) and autoantibody production [Whyburn *et al*., 2003] in *lyn-/-* mice. Taken together, these observations suggest that control of plasma cell differentiation, survival, and/or homing is a critical regulatory checkpoint that may prevent autoimmunity separate from the activation of autoreactive B cells by antigen.

The observations that reduced Btk dosage i) uncouples B cell hyperresponsiveness from autoantibody production in *lyn-/-* mice [Whyburn *et al*., 2003], and ii) at least partially prevents the increase in IL-6 production by *lyn-/-* macrophages (Chapter 4) indicate that myeloid hyperactivity may contribute to the disease process in this model. In addition to examining B cell function in *lyn-/-* mice, the following studies also sought to reveal whether Lyn-deficiency is required and/or sufficient in either B or myeloid cells for plasma cell

accumulation and autoantibody production. The intention was to illustrate the relative utility of B cell and myeloid cell targets for SLE therapy.

Results

A B cell-intrinsic role of Lyn contributes to splenic plasma cell accumulation

NZM2410-derived congenic strains have allowed for the identification and analysis of distinct lupus-susceptibility loci in the mouse genome [Morel and Wakeland, 2000]. The study of these susceptibility loci has demonstrated that defects in multiple cell lineages can result in autoimmune phenotypes. For instance, mixed bone marrow chimeras have been used to show that *Sle1* must be expressed in B cells to exert its effect on anti-chromatin antibody production while *Sle3* functions in non-lymphoid cells [Wakui *et al*., 2005]. A similar approach was used to determine whether Lyn-deficiency in B cells is required for plasma cell accumulation and autoantibody production. Bone marrow cells were isolated from *lyn-/-* and wild-type control (B6.IgHb) mice, which express the IgHb allotype (Figure 5.1). These cells were then mixed with bone marrow cells from wild-type B6.IgH^{a}Thy-1^{a}Gpi^{a} (B6.IgHa) mice purchased from Jackson Laboratories. Mixtures were then transplanted into lethally irradiated B6.IgHa mice. In this model, the source of both B cells and antibodies produced in the chimeric mice can be identified by flow cytometry or ELISA using detection reagents specific for IgMa (B6.IgHa) and IgMb (B6.IgHb and *lyn-/-*). Similarly, T cells can be identified by expression of either Thy 1.1 (CD90.1) or Thy 1.2

(CD90.2) as having originated from B6.IgHa or B6.IgHb and *lyn-/-* bone marrow, respectively.

Two to four months post-reconstitution, spleen and bone marrow cells were analyzed by flow cytometry. The extent of chimerism was determined by comparing the expression of both IgMa vs. IgMb and Thy 1.1 vs. Thy 1.2. The frequency of B cells derived from *lyn-/-* bone marrow was markedly lower than that of B cells from B6.IgHa (wild-type) bone marrow (Figure 5.2 A). This skewed ratio of *lyn-/-* (Thy 1.2-positive) to B6.IgHa (Thy 1.1-positive) derived cells was not witnessed within the T cell compartment (Figure 5.2 B). Nor did control B cells from B6.IgHb mice exhibit a similar disadvantage against B6.IgHa-derived cells. Cornall and colleagues have also reported this B cell-intrinsic defect in B cell development in the absence of Lyn [Silver *et al*., 2007].

To determine whether the B cells that do develop from *lyn-/-* bone marrow have a greater propensity to become plasma cells than wild type B cells in the same environment, the frequency of plasma cells was examined within the IgMa- and IgMb-positive compartments of splenocytes from chimeric mice. $CD138^{hi}$ plasma cells were detected at a higher frequency among *lyn-/-* cells than among wild type cells (Figure 5.3 A and 5.4). The frequency of B6.IgHa-derived (wild-type) plasma cells was similar whether isolated from B6.IgHb control or *lyn-/-* chimeric mice (Figure 5.3 B and 5.4). This data indicates that plasmablast and plasma cell accumulation is dependent on the loss of Lyn within B cells.

Lyn-deficiency does not sensitize B cells to LPS or CpG in vitro stimulation

Understanding B cell-intrinsic mechanisms for plasmablast and plasma cell accumulation in *lyn-/-* mice may lead to novel therapeutic targets for SLE. To determine if lyn-deficiency resulted in increased differentiation of plasma cells in response to external stimuli, purified B220-positive B cells from wild-type (wt) and *lyn-/-* mice were exposed to the polyclonal activators, LPS or CpG. There was no increase, and in fact a slight decrease, in the terminal differentiation of *lyn-/-* B cells as measured by either CD138 up-regulation (Figure 5.5 A) or IgM production (Figure 5.5 B). This suggested that the increased plasma cells in *lyn-/-* mice resulted from increased differentiation in response to signals other than LPS and CpG, or that plasma cell migration or survival are affected rather than differentiation.

There is a caveat to the manner in which these previous studies were completed, however. It is well-documented that lyn-deficiency causes a significant defect in B cell development, resulting in disparities in the profile of mature B cells populating the periphery [Hibbs *et al*., 1995; Nishizumi *et al*., 1995; Chan *et al*., 1997]. The ratio of mature follicular (FO) B cells (CD21+CD23+) to immature and transitional B cells in the spleens of *lyn-/-* mice is dramatically different than in wild-type mice, and marginal zone (MZ) B cells are virtually absent [Seo *et al*., 2001].

Also of concern, up-regulation of CD138 occurs early in terminal differentiation but CD138-positive pre-plasma cells do not immediately down-regulate B220 [Culton *et al*., 2006]. Thus, there is contamination of $CD138^{+}B220^{+}$ pre-plasma cells when B220-positive purification is utilized. These pre-plasma cells are present at increased frequency in the spleens of *lyn-/-* mice in a Btk-dependent manner (Figure 5.6). Purification methods were adjusted to increase the sensitivity of the *in vitro* assays by removing contaminating pre-plasma cells (data not shown). However, the accuracy of these assays remained unreliable due to the disproportioned B cell profiles (Figure 5.7). In order to determine how the disparate B cell population frequencies within wild-type and *lyn-/-* mice affect the net production of plasma cells *in vitro*, additional work on purification methodology is required.

Diminished migration in response to SDF-1 may contribute to the aberrant accumulation of plasma cells in lyn-/- mice

Impaired plasma cell migration to the bone marrow in response to SDF-1 (CXCL12) has also been suggested as a mechanism for the increase in splenic plasma cells in NZB/W mice. Thus, the ability of wild type, *lyn-/-,* and *lyn-/-*Btk^{lo} splenic plasma cells to migrate in response to SDF-1 was compared as described previously [Erickson *et al*., 2003]. Total splenocytes were plated in the inserts of

transwell plates with media only or varying concentrations of SDF-1 added to the well below. After 3 hours of culture, cells were collected from the upper and lower chambers and the number of plasma cells ($CD138^{+}B220^{lo/-}$) for these samples as well as for the input sample (total splenocytes) was determined by flow cytometry. The total number of plasma cells was then calculated for the upper (non-migrating) and lower (migrating) transwell chambers. By this method, it was clear that *lyn-/-* $CD138^{hi}$ plasma cells had a diminished migratory response to SDF-1 (Figure 5.8). The same does not occur with reduced Btk dosage as *lyn-/-*Btk^{lo} plasma cells were capable of crossing the transwell membrane.

Two approaches were taken to determine what was responsible for the diminished migration response to SDF-1. First, it was possible that there was some inability to detect SDF-1 by its receptor, CXCR4. To determine if there was altered CXCR4 expression, splenocytes were isolated and stained for CXCR4 surface expression on wild-type, *lyn-/-* and *lyn-/-*Btk^{lo} plasma cells. Surprisingly, not only was there no evidence of a decrease in expression, but CXCR4 was found on an increased frequency of $CD138^{hi}$ plasma cells (Figure 5.9 A). There was also a possibility that *lyn-/-* plasma cells were not exposed to the appropriate gradient effect that caused movement across the membrane in the transwell assay as well as out of the splenic microenvironment. SDF-1 expression from *lyn-/-* splenocytes could cause such an effect. Expression of SDF-1 mRNA from total

splenocytes was assessed by qPCR. There was not, however, any change in its expression between wild-type, *lyn-/-* or *lyn-/-*Btk^{lo} splenocytes from 2- to 4-month old mice (Figure 5.9 B). These observations suggest that impaired signaling by CXCR4 in response to SDF-1 underlies the poor migration of *lyn-/-* plasma cells in the transwell assay *in vitro* and from the spleen *in vivo*.

Accumulation of lyn-/- plasma cells is likely dependent on prolonged survival

Plasma cell accumulation has been attributed to abnormally long-lived cells like those seen in NZB/W mice [Hoyer *et al*., 2004]. But autoantibody production has also been associated with rapid production of short-lived plasma cells [Liu *et al*., 2010]. Distinguishing between these two possibilities has clinical relevance, as long-lived plasma cells are spared by B cell depletion therapy [Anolik *et al*., 2004]. Whether plasma cells in *lyn-/-* mice turnover slowly, suggesting increased survival, or are rapidly generated, is unknown. Turnover of plasma cells *in vivo* can be measured by BrdU labeling. In order to ensure that newly generated plasma cells could be visualized by flow cytometry, BrdU exposure was carried out over a two week period. Two milligrams of BrdU were injected intraperitoneally at each treatment. Treatment days were alternated such that BrdU toxicity could be kept at a minimal level. As a positive control for BrdU incorporation and staining, a final BrdU injection was administered at least

12 hours prior to tissue harvest. This final treatment ensured that newly generated cells in the bone marrow could be detected (data not shown).

Splenocytes were stained with antibodies against B220, CD138, CD4 and Gr-1 (Ly-6c). Splenic plasma cells, and B220+CD138- B cells were gated as usual (Figure 5.10 A). CD4+ T cells, which do not express Lyn or Btk, as well as Gr-1+ granulocyte/myeloid cells, which do express both Lyn and Btk, were also analyzed for BrdU incorporation (Figure 5.10 B). To avoid misinterpretation of non-specific or autofluorescent staining, background from the intracellular isotype control was subtracted from the BrdU-positive signal for each sample (Figure 5.10 C). BrdU incorporation was significantly decreased in both *lyn-/-* and *lyn-/-* Btk^{lo} plasma cells indicating a decrease in cellular turnover (Figure 5.11 A). There were no significant differences in BrdU incorporation in B cells (data not shown) or T cells (Figure 5.11 B), indicating that the decrease observed in the plasma cell population was not due to poor labeling in *lyn-/-* and *lyn-/-*Btk^{lo} mice.

Discussion

This work shows that Lyn-deficiency in B cells is critical to the plasma cell accumulation that is seen in *lyn-/-* mice. In mixed bone marrow chimeras, there was no detectable enrichment for B6.IgHa-derived (wild-type) plasma cells in the presence of *lyn-/-* cells at 2 months post-transfer. There is some evidence for B cell-extrinsic effects of *lyn-/-* bone marrow on total IgM production from wild-type cells [Silver *et al*., 2007]. This effect was not reflected in the plasmablast and plasma cell frequencies in our chimeras, however. In *lyn-/-* mice, anti-dsDNA antibodies are detected in the serum after 4 months of age. Unfortunately, only one *lyn-/-*:B6.IgHa chimera of all those generated, including controls, survived to the four month time point. Therefore the effect of non-B cells on autoantibody production could not be assessed in this series of experiments.

Nonetheless, the data suggests that loss of Lyn function in B cells is critical to plasmablast and plasma cell accumulation, independent of its role in BCR hypersensitivity. It was hypothesized that Lyn-deficiency skewed the sensitivity of *lyn-/-* B cells to other activating stimuli. Polyclonal activation with the TLR4 and TLR9 ligands LPS and CpG, respectively, was used to determine if there were any intrinsic differences between wild-type and *lyn-/-* B cell responses to stimulation. No differences were observed in these studies. However, it

became evident that *in vitro* study of B cell terminal differentiation would require more effort to distinguish between splenic B cell sub-populations. There are fundamental differences in how each B cell sub-population in the spleen responds to stimulation. For instance, MZ B cells, which are more prevalent in both wild-type and *lyn-/-*-Btk^{lo} B220-positive cultures, are better suited to respond to TLR stimulation [Gururajan *et al*., 2007; Rubtsov *et al*., 2008]. Purification methods were revised such that more comparable B cell populations could be isolated. Follicular B cells were targeted for isolation because a) they typically comprise the majority of mature B cells in the spleen, b) MZ B cells are virtually absent in *lyn-/-* mice [Seo *et al*., 2001] (Figure 5.7), and c) plasma cell accumulation can occur in *lyn-/-* mice in the absence of B-1 cells [Cornall *et al*., 1998]. Unfortunately, the greatly reduced number of CD21^{+}CD23^{+} FO B cells in *lyn-/-* spleens made the purification of a sufficient number of cells for *in vitro* stimulation impractical.

As an alternate means to determine if *lyn-/-* B cells were sensitized to activation and terminal differentiation, we examined the expression of transcription factors known to regulate B cell fate. Blimp-1 and XBP-1 are critical to B cell terminal differentiation and the generation of antibody-secreting cells [Calame *et al*., 2003]. Increased Blimp activity in Ets-1-deficient B cells is associated with hypersensitivity to TLR9 stimulation and increased frequencies of autoreactive plasma cells [Wang *et al*., 2005; John *et al*., 2008]. Due to

limitations in the cell numbers that could readily be obtained form *lyn-/-* mice, real-time PCR was utilized to detect changes in both Blimp-1 and XBP-1 expression. Results from preliminary experiments did not suggest any change in either transcription factor in FO B cells from *lyn-/-* mice.

Since these initial studies did not reveal an increased propensity for *lyn-/-* B cells to differentiate into plasma cells *in vitro*, other possible mechanisms for the accumulation of plasma cells in these mice were examined. Plasma cells migrate from the spleen because the appropriate plasma cell survival factors are not expressed in the splenic microenvironment [Sze *et al*., 2000; Hargreaves *et al*., 2001]. Plasma cell accumulation could occur if cells are being generated faster than they can be disposed of or if they are being retained longer than they should in the splenic environment. Impaired migration from the spleen was suggested by the observation in Figure 3.4 that plasma cells do not accumulate in the bone marrow of *lyn-/-* mice. Lyn-deficient plasma cells do, in fact, exhibit a diminished capacity to respond to SDF-1. This defect in migration depends on Btk. This establishes a key part of the equation for why accumulation occurs.

The other half of that equation is whether *lyn-/-* mice continuously and rapidly generate plasma cells or whether long-lived plasma cells slowly amass in the spleen. BrdU incorporation was significantly reduced in both *lyn-/-* and *lyn-/-* Btk^{lo} plasma cells indicating that the generation of these cells is actually decreased in the absence of Lyn, regardless of Btk dosage. The *in vivo* BrdU

labeling studies suggest that plasma cell accumulation in lyn-/- mice is due to prolonged survival. These key pieces of information will drive future studies defining the mechanistic contribution of Btk-mediated and Lyn-regulated events.

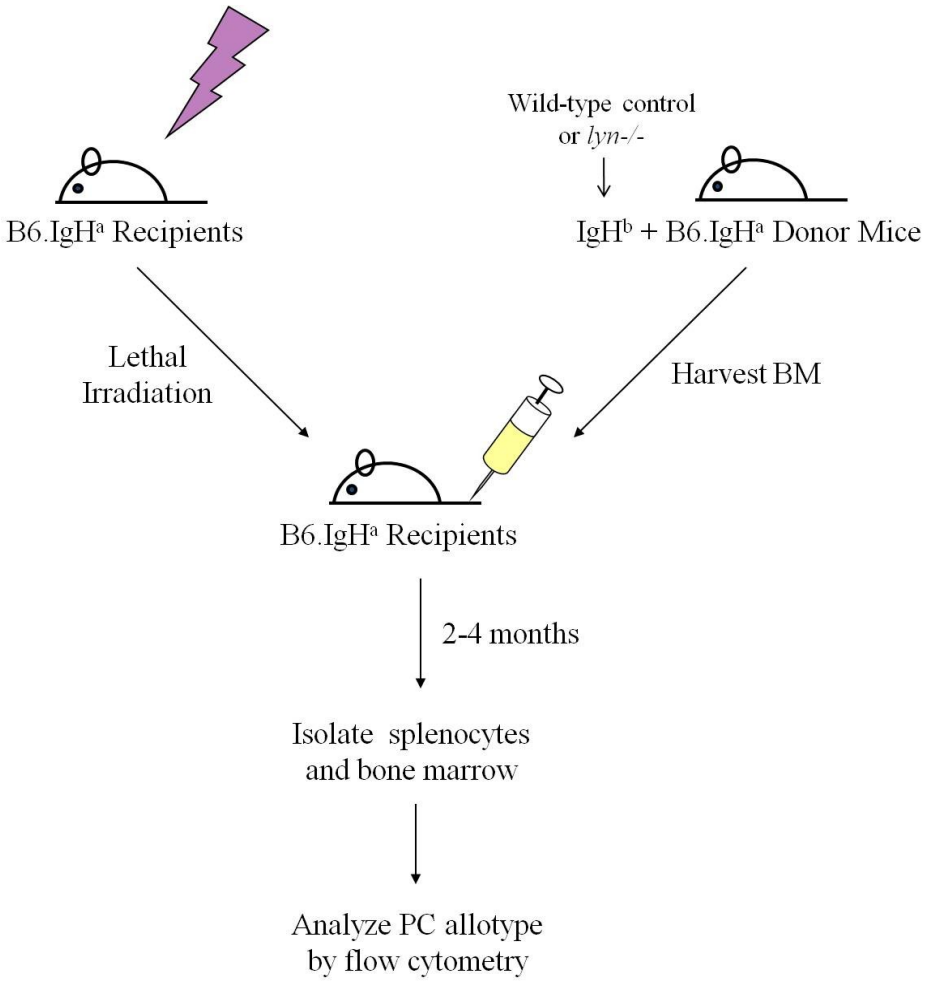

Figure 5.1. Generating mixed bone marrow chimeras to measure B cell-intrinsic vs. –extrinsic effects on plasmablast and plasma cell accumulation in *lyn-/-* mice. Four-month old donor bone marrow from *lyn-/-* or control mice, which express the IgHb allotype, were mixed with bone marrow from B6.IgH^{a}Thy-1^{a}Gpi^{a} (B6.IgH^{a}) mice. Mixtures were then transplanted intravenously into lethally irradiated B6.IgH^{a} recipient mice (2- to 3-months old). At a designated time after transplantation, mice were euthanized and tissues analyzed by flow cytometry. B220^{+}CD138^{-} B cells and CD138^{hi} plasma cells were analyzed for expression of either IgMb (IgHb) or IgMa (IgHa).

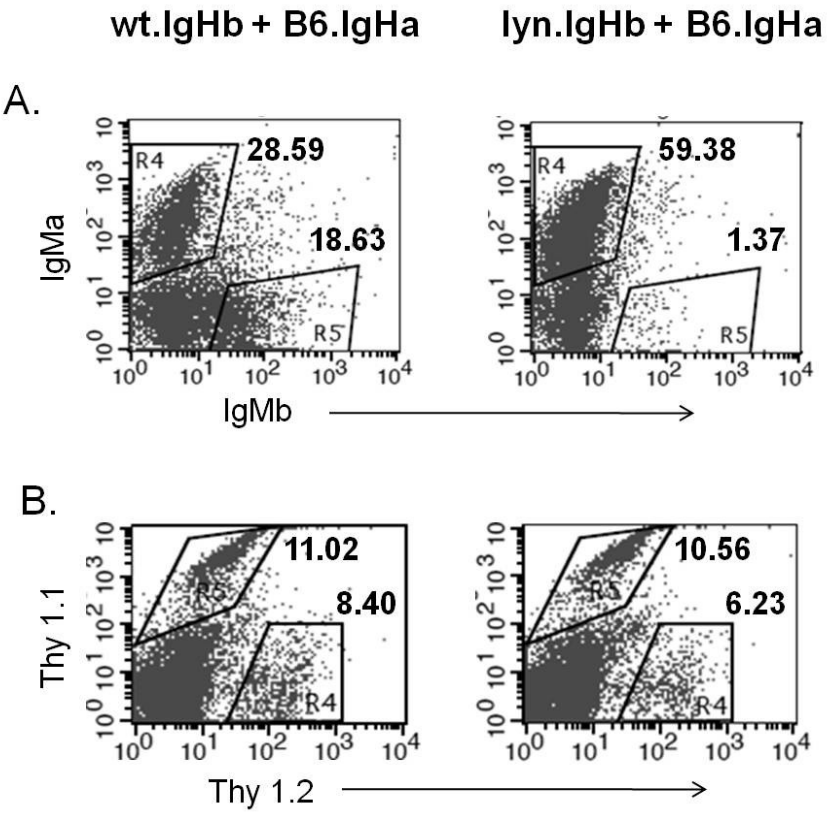

Figure 5.2. IgMb$^+$ cells from Lyn-deficient donor bone marrow exhibit reduced capacity to reconstitute recipient tissues. Splenocytes were isolated from recipient mice 2-4 months post-transfer and stained with antibodies against IgMb and IgMa in (A) or Thy1.2 (IgHb$^+$ mice) and Thy1.1 (B6.IgHa) in (B). A) Representative plots of IgMb$^+$ or IgMa$^+$ splenocytes in control (left panel) and *lyn-/-* (right panel) chimeras. B) Representative plots of Thy1.2 vs. Thy1.1 expression in control (left panel) and *lyn-/-* (right panel) chimeras. Numbers indicate percentage of cells in adjacent gate/region. n = 2 for wt; n = 3 for *lyn-/-*.

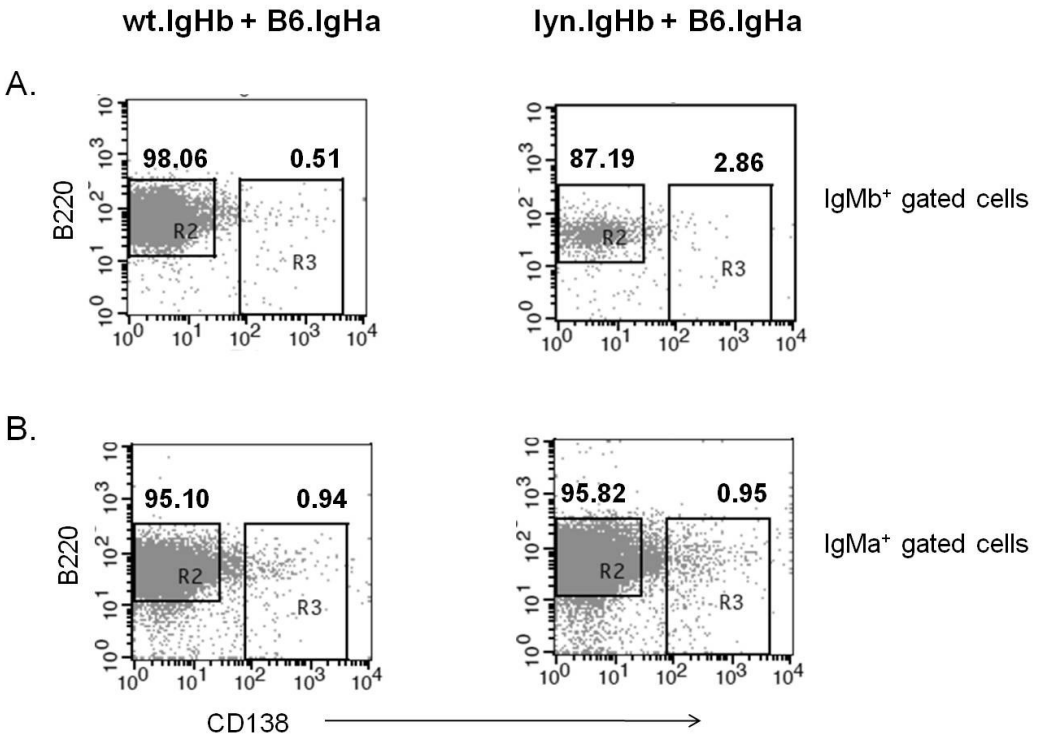

Figure 5.3. IgMb^{+} *lyn-/-* donor cells are more likely to be CD138^{hi} plasma cells. Splenocytes were isolated from recipient mice 2-4 months post-transfer and stained with antibodies against B220, CD138, IgMb and IgMa. IgMb (A) or IgMa (B) single positive cells were gated. A) Representative plots show the percentage of CD138^{hi} plasma cells derived from IgMb^{+} B6.IgHb wt-control (left panel) or *lyn-/-* (right panel) bone marrow. B) Representative plots show the percentage of CD138^{hi} plasma cells derived from B6.IgHa bone marrow mixed with wild-type (left panel) or *lyn-/-* (right panel) bone marrow. Plots are representative of n = 2 for wild-type and n = 3 for *lyn-/-*.

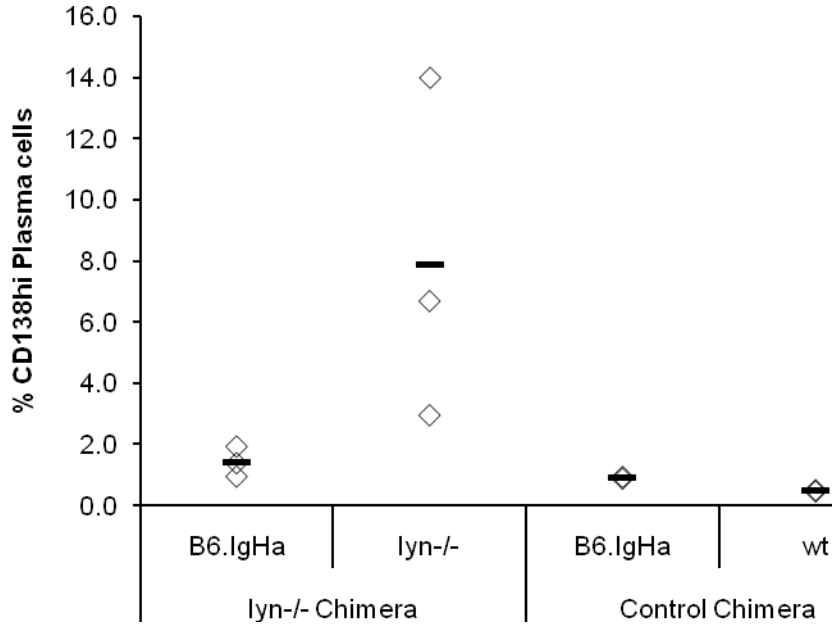

Figure 5.4. B cell intrinsic effect of lyn-deficiency on plasmablast and plasma cell accumulation. Splenocytes (2-4 months post-transfer) from recipient mice were stained with antibodies against B220, CD138, IgMa and IgMb. $IgMa^{+}$ (B6.IgHa) or $IgMb^{+}$ (*lyn-/-* or wt-control) –single positive cells were gated. Graph indicates the percentage of $CD138^{hi}$ plasma cells derived from B6.IgHa bone marrow mixed with *lyn-/-* or B6.IgHb wt-control marrow. n = 3 for *lyn-/-* chimeras and n = 2 for B6.IgHb wt-control chimeras.

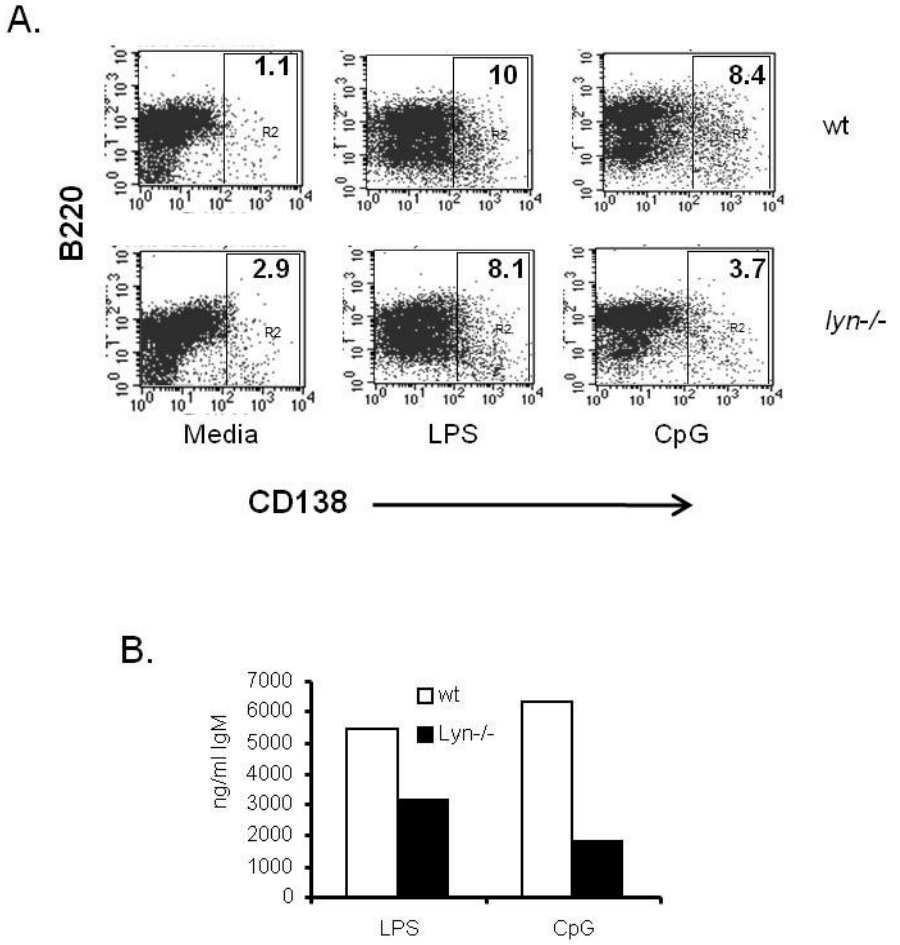

Figure 5.5. Lyn-deficient B cells do not demonstrate increased propensity to become plasma cells in response to LPS or CpG DNA. Purified $B220^{+}$ cells from 2- to 4-month old wt and *lyn-/-* mice were stimulated with 20 μg/ml LPS or 1 μg/ml ODN 1826 (CpG) for 72 hours. A) Cells were stained with antibodies against B220 and CD138. The left-most plots (media) represent unstimulated cells at time 0. The frequency of $CD138^{hi}$ cells is indicated. B) Levels of IgM in the culture supernatant were measure by ELISA. Data are representative of n = 3. These experiments were performed by Kristina Halcomb.

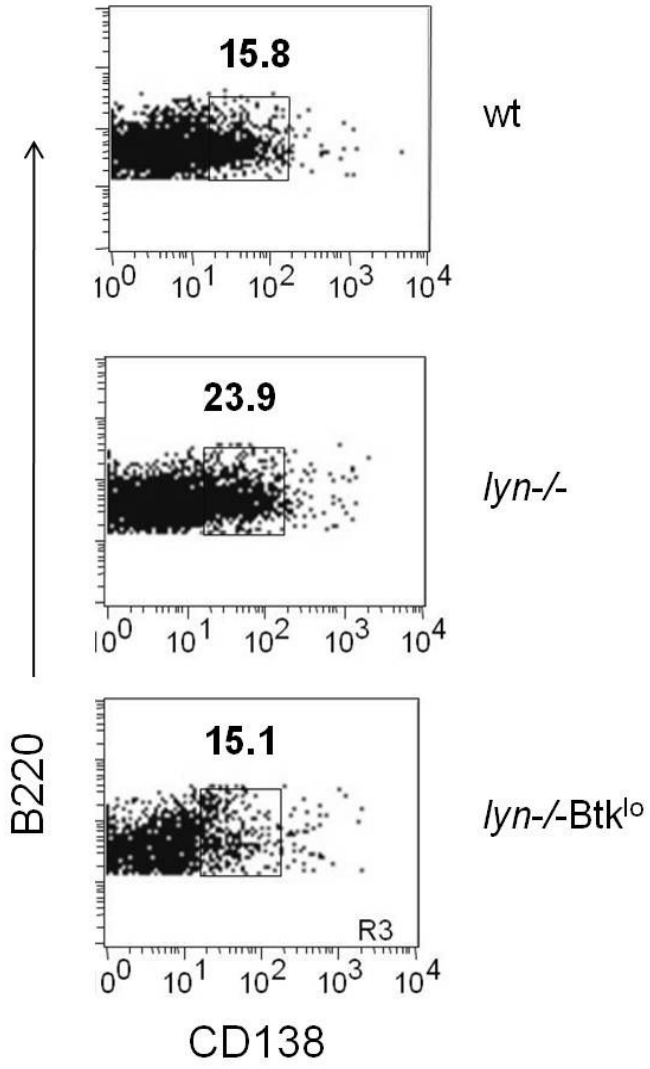

Figure 5.6. Increased frequency of B220+CD138+ pre-plasma cells in *lyn-/-* mice. Total splenocytes from 3- to 4-month old mice were stained with antibodies against B220 and CD138. Representative plots showing the percentage of B220+CD138+ cells among gated B220+ cells are shown to illustrate the increased frequency of pre-plasma cells in *lyn-/-* mice.

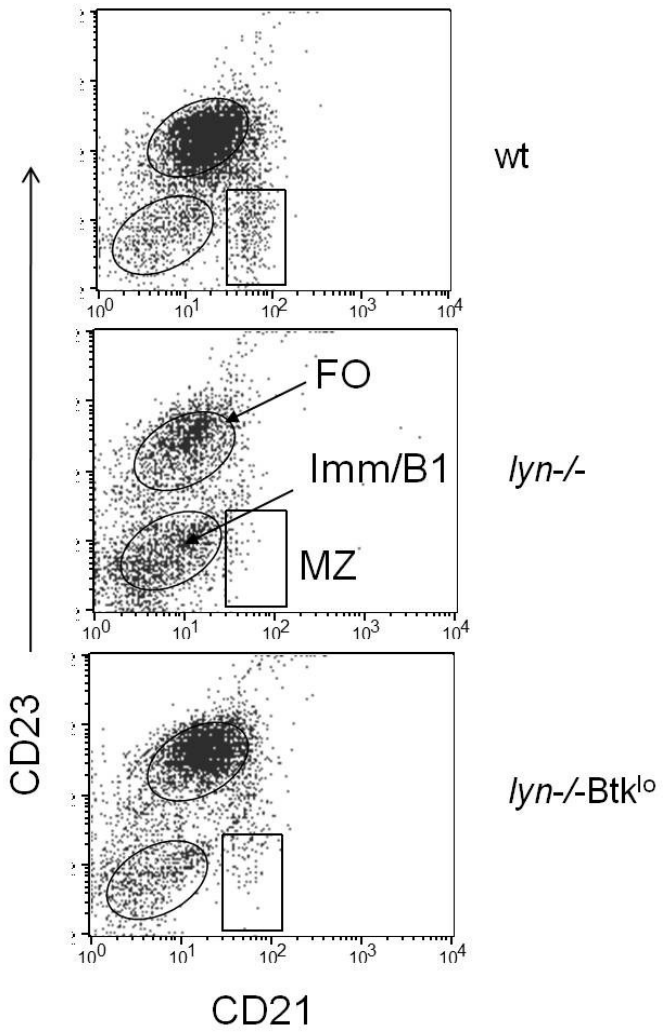

Figure 5.7. Altered B cell populations in *lyn-/-* mice could bias results of *in vitro* studies. $B220^{+}CD138^{-}$ B cells purified from 3- to 4-month old mice were stained with antibodies against CD21 and CD23. Representative plots are shown to illustrate the disparities in splenic B cell subpopulations in wt, *lyn-/-* and *lyn-/-* Btk^{lo} mice.

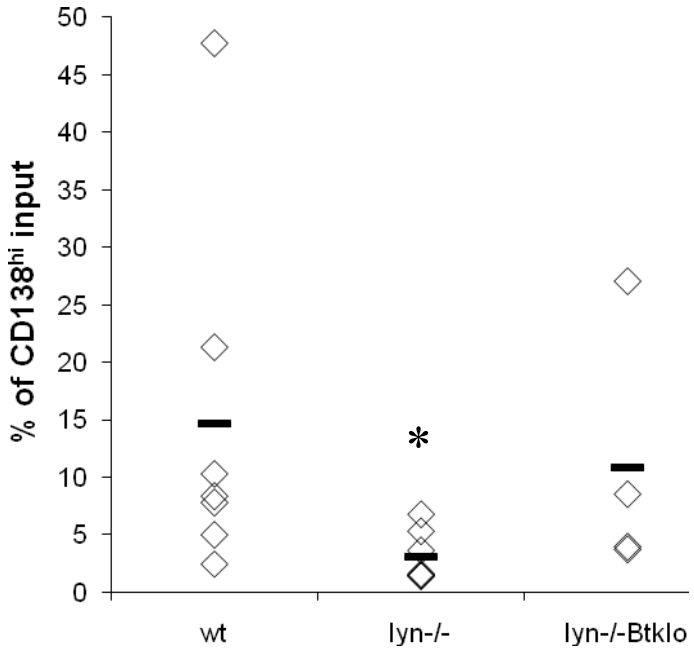

Figure 5.8. Lyn-deficient CD138^{hi} plasma cells show diminished migration in response to SDF-1. Total splenocytes were suspended in media in the upper chamber of a transwell plate and allowed to migrate towards an SDF-1 gradient (100 ng/ml) in the bottom chamber during 3-hour incubation. The percentage of input plasma cells (B220^{lo}CD138^{hi}) that migrated to the lower chamber is shown. * $p < 0.04$ vs. wt by Student's *t*-test. These experiments were performed in collaboration with Whitney Nichols and Dr. Anne Satterthwaite.

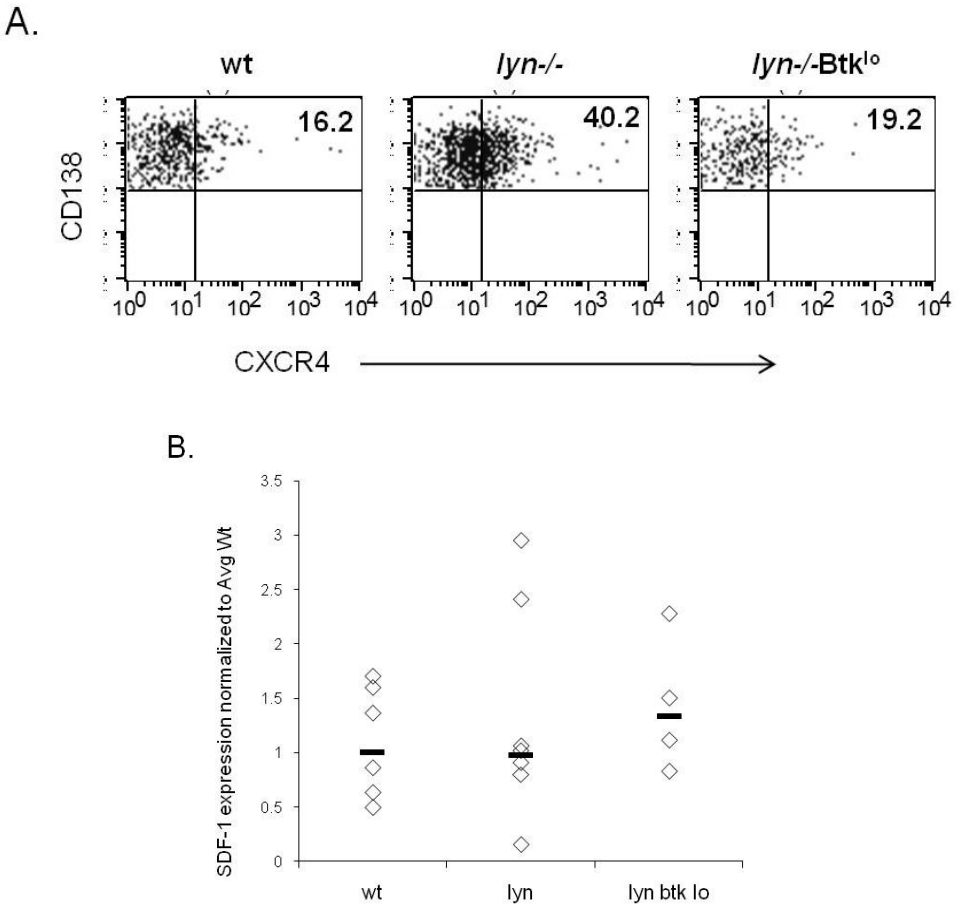

Figure 5.9. Neither decreased CXCR4 (SDF-1 receptor) expression on plasma cells nor increased SDF-1 mRNA expression in splenocytes is responsible for diminished migration of plasma cells in *lyn-/-* mice. A) Splenocytes from 2- to 4-month old mice were stained with antibodies against B220 and CD138 and cells within the $CD138^{hi}B220^{lo/-}$ gate were analyzed for surface CXCR4. Representative plots are shown; number in the upper-right quadrant indicates the percentage of $CXCR4^{+}$ cells for each individual mouse analyzed. n = 4 for all genotypes. p = 0.04 wt vs. *lyn-/-* by Student's *t*-test. B) RNA from total splenocytes were isolated from 2- to 4-month old mice and used to generate cDNAs. Quantitative real-time PCR was performed using SDF-1 Taqman primers. SDF-1 expression in each sample was normalized to GADPH and is shown relative to the average expression level in wt samples. No significance as determined by Student's *t*-test. These experiments were performed in collaboration with Dr. Anne Satterthwaite.

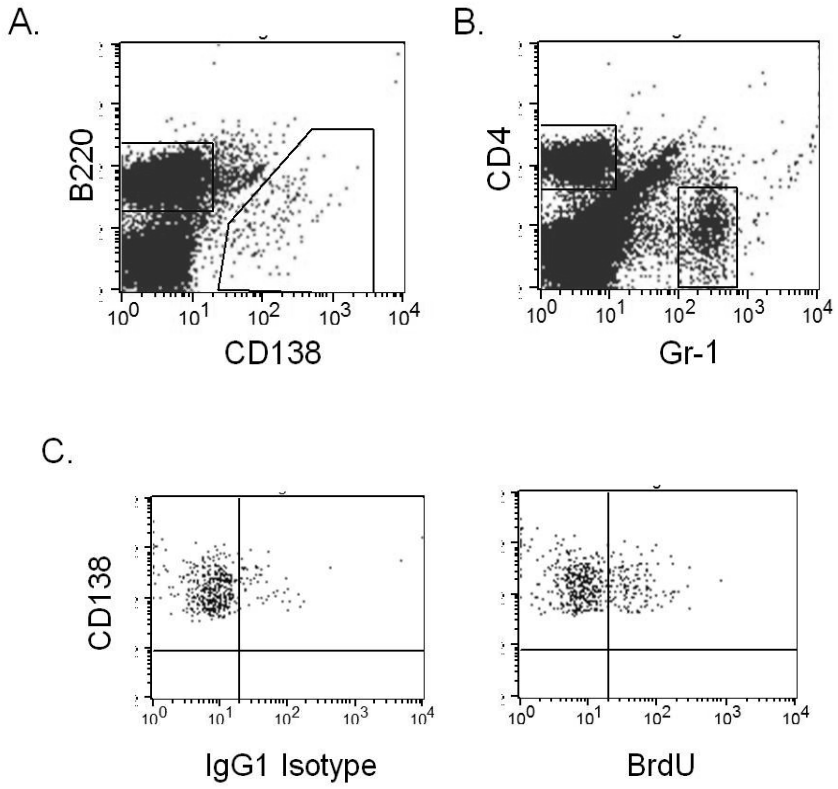

Figure 5.10. Gating strategy for flow cytometric analysis of *in vivo* BrdU incorporation. Four-month old mice were injected intraperitoneally with BrdU over 13 days. Splenocytes were stained with antibodies against B220, CD138, CD4, Gr-1 and BrdU or IgG1 isotype control. Representative plots illustrate gating strategy in A - C. A) B220 and CD138 expression were used to define $B220^{+}CD138^{-}$ B cells and $CD138^{hi}B220^{lo/-}$ plasma cells. B) $CD4^{+}$ T cells and Gr-1^{+} ($Ly6G^{+}$) myeloid cells/granulocytes were gated as shown. C) Gated populations were then analyzed for newly generated $BrdU^{+}$ cells. Representative plots of background (intracellular IgG1 isotype) and BrdU staining in wt $CD138^{hi}B220^{lo/-}$ cells are shown.

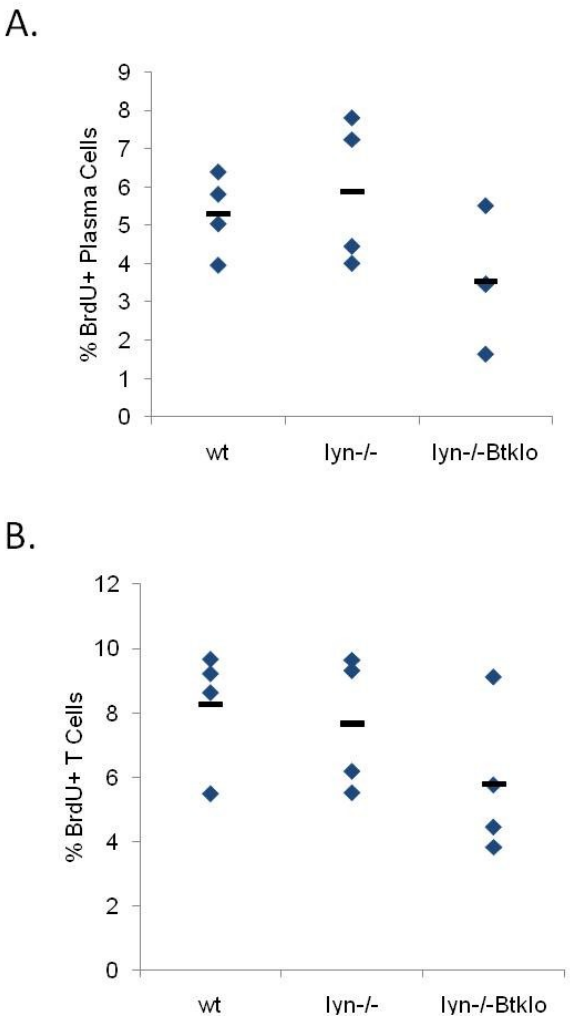

Figure 5.11. Lyn-deficient mice do not exhibit an increase in splenic plasmablast and plasma cell generation/turnover. Splenocytes were harvested from BrdU-injected mice and stained with antibodies against B220, CD138, CD4, Gr-1 and BrdU or IgG1 isotype control. A) The frequency of $CD138^{hi}B220^{lo/-}$ plasma cells that have incorporated BrdU are shown (n = 4). * $p < 0.04$, ** $p < 0.006$ vs. wt by Student's *t*-test. B) The frequency of CD4+ T cells that have incorporated BrdU are shown (n = 4).

CHAPTER VI

DISCUSSION

Novel checkpoints regulate plasma cell accumulation and pathogenic antibody production in *lyn-/-* mice

Currently, only non-specific therapies are approved for use in lupus patients, and these have undesirable side effects and risks. The development of targeted therapies is necessary and requires a better understanding of the mechanisms that contribute to the production of autoreactive antibodies. The presence of antinuclear antibodies (ANAs) is a major element in the diagnosis of SLE, and also a critical factor in the prognosis of disease. There are a handful of ANA-specificities associated with disease in patients, but the presence of some, such as antibodies specific for DNA-related antigens, correlate especially with disease pathology [Li *et al*., 2007]. In the preceding series of studies, I have utilized the Lyn-deficient model of lupus to better understand mechanisms that contribute to the production of anti-DNA antibodies.

The Lyn-deficient model of lupus provides several advantages that aid the overall goals of this research. It was already established that the role of Lyn as a

negative regulator of both B cell and myeloid cell signaling is important to disease in *lyn-/-* mice [Chan *et al*., 1998; Nishizumi *et al*., 1998; Smith *et al*., 1998; Cornall *et al*., 1998; Ho *et al*., 1999; Adachi *et al*., 1998; Okazaki *et al*., 2001]. Understanding of Lyn's regulatory function in BCR signaling resulted in the development of a complementary transgenic model, *lyn-/-*Btk^{lo} mice [Satterthwaite *et al*., 1998], and analyses of this model lead to the discovery that Btk-mediated signaling is critical to autoantibody production in *lyn-/-* mice [Satterthwaite *et al*., 1998; Whyburn *et al*., 2003]. Btk is also an important contributor to myeloid cell function, where it generally opposes Lyn action, as in B cells [Abram *et al*., 2008]. The transgenic model developed to study the impact of reduced Btk dosage in B cells has now been utilized to examine the effect of Btk-deficiency in myeloid cells and the relative contribution of both these cell types on disease. In this way, this work has expanded our understanding of how Lyn and Btk regulate and mediate, respectively, the progression from tolerance to autoimmunity.

Here, I identify two major checkpoints regulating autoantibody production in *lyn-/-* mice. The first involves plasmablast and plasma cell accumulation and polyclonal IgM autoreactivity, which depend on Btk and require reduced Lyn dosage in B cells. The second checkpoint regulates the class-switching of B cells with lupus-associated autoantigen specificities and production of pathogenic

autoantibodies. This step requires IL-6, which is produced in excess by *lyn-/-* myeloid cells in a Btk-dependent manner.

Btk-dependent plasma cell accumulation and polyclonal IgM reactivity in *lyn-/-* mice

Because reduced Btk dosage divorced BCR hypersensitivity from the production of anti-DNA antibodies [Whyburn *et al*., 2003], it was clear that further examination of *lyn-/-* and *lyn-/-*Btk^{lo} mice would be necessary to identify a Btk-dependent phenotype that correlated with autoimmunity. Also, because Lyn and Btk play similarly opposing roles in both B and myeloid activity, the functions of both cell types were evaluated. Not surprisingly, a B cell phenotype was found to correlate with the production of autoantibodies. As described in chapter 3, plasma cell accumulation occurred in the absence of Lyn and depended on the full dosage of Btk expression. Significantly increased frequencies of splenic plasma cells in *lyn-/-* mice were detected in mice at every time-point analyzed from 6 to 42 weeks of age. The ratio of plasma cells to B cells in the spleens of *lyn-/-* mice increased steadily overtime until the last analyses at 32-42 weeks.

Aberrant accumulation of plasma cells in secondary lymphoid organs is a common feature of both human and murine lupus and typically coincides with

significant production of immunoglobulin. Both adult [Dorner *et al*., 2004] and pediatric [Arce *et al*., 2001] SLE patients have increased numbers of peripheral blood plasma cells. Peripheral plasma cell accumulation has also been associated with elevated autoreactive immunoglobulin production in both spontaneous [Hoyer *et al*., 2004; Seagal *et al*., 2003; Hang *et al*., 1982] and engineered [Fukuyama *et al*., 2005; Wang *et al*., 2005, Kersseboom *et al*., 2010] models of lupus. Of note, over-expression of plasma cell differentiation and survival factors, such as BAFF, IL-6 and IL-21 also promote plasma cell accumulation and pathogenic IgG production [Stohl *et al*., 2005; Suematsu *et al*., 1989; Ozaki *et al*., 2004].

Work in *lyn-/-* x anti-HEL Ig mice demonstrates that plasma cell accumulation occurs spontaneously in the absence of antigen [Silver *et al*., 2006]. This is consistent with our results that *lyn-/-*Btk^{lo} mice, which retain their hypersensitivity to BCR crosslinking [Satterthwaite *et al*., 1998; Whyburn *et al*., 2003], do not have increased plasma cells. Antigen-independent plasma cell accumulation might be explained by a B cell-intrinsic defect that affects the activation threshold required to trigger terminal differentiation in Lyn-deficient cells. However, it is also possible that B cells are responding to external cues apart from or subsequent to BCR signaling. As described in chapter 5, mixed bone marrow chimeras suggested that Lyn-deficiency was required in B cells, at least early on, in order for the plasma cell phenotype to be observed. Consistent

with a B cell intrinsic defect mediating plasma cell accumulation, elimination of the B cell extrinsic plasma cell survival and differentiation factors IL-6 and IL-21 did not alter plasma cell frequencies.

The mechanism(s) responsible for plasma cell accumulation in *lyn-/-* mice is not known. There are many possibilities, given the various mechanisms known to affect plasma cell accumulation in other models. These include abnormally long-lived plasma cells [Hoyer *et al*., 2004], increased sensitivity of B cells to TLR engagement [Wang *et al*., 2005], poor migration to the bone marrow plasma cell niche [Erickson *et al*., 2003], or inability to process death signals [Xiang *et al*., 2007]. In the current studies, there was no detectable difference between the differentiation of wild-type and *lyn-/-* B cells into plasma cells in vitro. Lyn-deficient plasma cells did, however, display reduced migration in response to in vitro SDF-1 stimulation and reduced in vivo BrdU incorporation. Taken together, this data suggests that in the absence of Lyn, plasma cells are unable to emigrate from the spleen but gain the ability to survive outside of bone marrow survival niches.

The increase in plasma cells frequency observed in *lyn-/-* spleens was associated with the presence of autoreactive IgM specific for a diverse range of autoantigens. This IgM "bloom" can be seen in 18-week old animals and depends on Btk. Reduced Lyn dosage (*lyn+/-* mice) was sufficient for both the plasma cell accumulation and autoreactive IgM. However, autoreactive IgG was

not observed in lyn+/- mice, nor did they develop kidney disease. This suggests that while many types of autoreactive B cells escape tolerance mechanisms and differentiate into IgM secreting plasma cells in the absence of Lyn, only a subset receive the appropriate signals to induce class switching to pathogenic IgG isotypes.

IL-6 dependent class-switching and pathogenic IgG antibodies

It remains to be seen whether or not BCR hyperresponsiveness is required for autoimmunity in *lyn-/-* mice, however, it is clearly not sufficient [Whyburn *et al*., 2003]. Interest in Btk-mediated B cell-extrinsic signaling and mechanisms has since focused on the role of Btk in myeloid hyperactivity. Work from Lowell and colleagues has illustrated the importance of Lyn and its sister kinases, Fck and Hgr, to myeloid function [Meng and Lowell, 1997]. There is a significant increase in the number of myeloid cells in *lyn-/-* mice, and these become highly dysregulated over time [Harder *et al*., 2001]. In the absence of negative regulation, *lyn-/-* myeloid cells exhibit multiple defects specifically associated with the generation of autoantibodies in other lupus models. Activated *lyn-/-* macrophages produce increased levels of the plasma cell survival and differentiation factor, IL-6 [Meng and Lowell, 1997].

Here, we show that increased IL-6 production by *lyn-/-* splenic myeloid cells depends on Btk. Examination of the role of IL-6 in the autoimmune phenotype of *lyn-/-* mice revealed numerous interesting features of this lupus model. Surprisingly, both plasma cell accumulation and polyclonal IgM autoreactivity are independent of the plasma cell survival factor IL-6. However, IL-6 mediates the production of IgG autoantibodies, which are focused towards a limited number of glomerular, DNA- and RNA- containing antigens, all of which are characteristic of SLE. Consistent with a pathogenic role for these IgG autoantibodies, *lyn-/-IL-6-/-* mice did not develop glomerulonephritis

There are several possible mechanisms by which IL-6 may control the production of autoreactive IgG. Some of the effects of IL-6 on B cells have recently been shown to be indirect, via CD4+ T cells [Dienz *et al*., 2009]. IL-6 promotes the development of both T_{FH} cells and Th17 cells [Nurieva *et al*., 2008; Zhou *et al*., 2007], both of which have been implicated in the pathogenesis of lupus and other autoimmune diseases [Linterman *et al*., 2009; Hsu *et al*., 2008]. Inhibition of Treg function as a result of increased IL-6 expression has also been demonstrated in the *Sle1.Sle2.Sle3* lupus model [Wan *et al*., 2007]. Additional evidence that T cells wplay an important role in disease comes from the observation that treatment with CTLA4-Ig inhibits autoreactive IgG production in *lyn-/-* mice [Oracki *et al*., 2010]. CTLA4-Ig, a competitive antagonist of CD28/B7 interactions prevents lupus by preventing anti-DNA antibody production, somatic

mutations, and class switching [Mihara *et al*., 2000]. It is possible that CTLA4-Ig interferes with an important function of CD28 on plasma cells [Delogu et al., 2006]. However, studies in a model of anti-phospholipid disease indicates that CTLA4-Ig treatment does little to change the progression of disease if administered after autoantibodies are present [Akkerman et al., 2004], indicating that T cell activation through CD28 is critical for disease initiation.

IL-6 has also been shown to promote myelopoiesis in the *Sle1.Yaa* murine lupus model [Maeda *et al*., 2009]. Myelopoiesis [Harder *et al*., 2001] and IL-6 expression are also enhanced in *lyn-/-* mice. This could lead to an increase in T cell activation due to greater numbers of APCs, or promote T-cell independent class switching of anti-DNA B cells in response to myeloid derived factors such as BAFF [Groom *et al*., 2007].

B cells may also be directly affected by IL-6. IL-6 affects the expression of inhibitory receptors and RAG genes in human SLE B cells [Garaud *et al*., 2009; Hillion *et al*., 2007]. In non-autoimmune mice, IL-6 inhibits the differentiation of autoreactive, tolerant B cells [Kilmon *et al*., 2007]. This tolerance mechanism is clearly ineffective in *lyn-/-* mice, as IL-6 has no effect on IgM autoantibodies and promotes IgG autoreactivity in the absence of Lyn**.**

CD11b+ myeloid cells from the spleens of *lyn-/-* mice are likely a major source of increased IL-6 in *lyn-/-* mice. A greater frequency of these cells express IL-6 in response to LPS compared to their wild type counterparts. This was

dependent on Btk, as both serum IL-6 levels and IL-6 expression by CD11b+ cells was normalized in *lyn-/-*-Btk^{lo} mice. The role of Btk in IL-6 production by myeloid cells is controversial based on studies from Btk-/- mice or XLA patients, who have loss of function mutations in Btk. Some studies indicate that Btk has no effect on LPS-induced IL-6 production [Kawakami *et al*., 2006], while others indicate that IL-6 expression is enhanced in the absence of Btk [Schmidt *et al*., 2006]. However, consistent with our results, a Tec kinase inhibitor blocks LPS-induced IL-6 production in human neutrophils [Zemans *et al*., 2009]. Taken together, these observations suggest two possibilities. The requirement for Btk in IL-6 production may differ depending on the context, for example the presence or absence of Lyn. Alternatively, various myeloid cell subsets may have different requirements for Btk in IL-6 production, and a Btk-dependent subset may be preferentially expanded and/or active in *lyn-/-* mice.

The lack of IgG autoantibodies in *lyn-/-*-Btk^{lo} mice may be due to a requirement for Btk in the processes that lead to plasma cell accumulation and polyclonal IgM autoreactivity, which precedes the production of pathogenic IgG. Alternatively, it may be because Btk mediates the over-expression of IL-6, which in turn promotes the switch to pathogenic IgG autoantibodies. Previous studies from our lab have also demonstrated a role for Btk in the production of anti-DNA IgG in the 56R anti-DNA Ig transgenic model [Halcomb *et al*., 2008]. In this system, 56R.Btk^{lo} mice lose tolerance to DNA and produce anti-DNA IgM, but do

not switch to anti-DNA IgG. It will be interesting to determine whether Btk-mediated expression of IL-6 contributes to class switching of DNA-specific B cells in this model.

Focused autoantibody production specific for lupus-associated antigens

Another interesting point that arises from this data is the limited autoantigen specificity of the IgG autoantibodies. This focused autoreactivity could be explained by the observation that B cells specific for DNA or RNA containing antigens can be activated by signals from both the BCR and TLR9 or TLR7, respectively [Leadbetter *et al*., 2002; Viglianti *et al*., 2003; Marshak-Rothstein *et al*., 2004]. This dual signal mode of activation applies to the stimulation of RF B cells by immune complexes containing chromatin, CpG-containing DNA, or RNA as well as the activation of DNA-specific or RNA-specific B cells by CpG-containing DNA or RNA. While antigen is not required for the accumulation of plasma cells in *lyn-/-* mice, it is entirely possible that dual BCR/TLR signals drive class switching of B cells reactive to nucleic acids, contributing to the focusing of autoreactive IgG towards these antigens.

Other groups have shown the importance of TLR signaling to the production of autoantibodies through manipulation of MyD88 activity. MyD88 is an adaptor molecule required for signaling through all TLRs except TLR3

[Takeda and Akira., 2004]. In *lyn-/-* x MyD88 double knockouts, plasma cell accumulation is partially normalized and IgG autoantibody production is abrogated [Silver *et al*., 2006]. Similar results are seen in several other murine models of lupus, including BAFF-transgenic (Tg) mice, FcγRIIb-/- mice, and the extrafollicular activation of RF B cells [Groom *et al*., 2007; Ehlers *et al*., 2006; Herlands *et al*., 2008]. However, MyD88-deficiency normalizes many of the dendritic cell alterations in *lyn-/-* mice, including IL-6, and it has been suggested this contributes to the abrogation of autoimmunity in these mice [Silver *et al*., 2006]. Such a B cell extrinsic mechanism would explain our observations with respect to IL-6 dependency of class switching but would not account for the restricted antigen specificity of the switched cells, however.

There are two major means, not mutually exclusive, by which B cell-intrinsic TLR signaling may induce class switching in B cells. CpG DNA directly induces class switching in vitro [Liu *et al*., 2003; He *et al*., 2004; Ehlers *et al*., 2006]. This is associated with Btk-mediated up-regulation of AID and T-bet, two factors important for the class switching process [Liu *et al*., 2003; Muramatsu *et al*., 2000; He *et al*., 2004; Halcomb *et al*., 2008]. Alternatively, dual BCR/TLR signaling may promote the ability of nucleic acid-specific B cells to recruit T cell help. Such a model would take into the account the effect of CTLA4-Ig on IgG autoantibody production in *lyn-/-* mice [Oracki *et al*., 2010]. Excessive IL-6 production by myeloid cells in response to TLR signaling [Meng and Lowell,

1997; Silver *et al*., 2006] may then further support the ability of T cells to provide help to autoreactive B cells via various mechanisms described above.

The role of global immune dysregulation and inflammatory loops in lupus

A significant increase in the frequency of plasma cells can be detected in *lyn-/-* mice as young as 6- to 8-weeks old (Figure 3.3 A). Although some *lyn-/-* mice present with class-switched ANAs by 11 weeks of age [Tsantikos *et al*., 2010], the frequency of this occurrence is generally rare. The time between the initial observation of plasma cell accumulation and ANA production is quite substantial. Intriguingly, there is a visible increase in the rate of plasma cell accumulation at around 12-13 weeks (Figure 3.3), which correlates with an increased ANA penetrance [Tsantikos *et al*., 2010]. These observations point to some secondary event that culminates in a full break of tolerance and class-switching of B cells specific for lupus-associated antigens.

The occurrence and contribution of a dysregulated immune system and inflammatory environment to disease progression is consistent with the polygenic nature of SLE. The most potent example of this is the IFNα signature that is strongly associated with lupus, despite the various genetically-distinct populations of patients that have been studied throughout the years. It has become clear that

type I interferons play an important role, both early and late, in disease severity [Obermoser and Pascual, 2010].

Recently, there is evidence for various other inflammatory loops that can contribute to lupus pathology. There are now at least two examples of this action occurring in the *lyn-/-* model, and these loops are generally observed later in disease manifestation. One such loop has been described by Rivera and colleagues in their study of Lyn regulation of basophil and mast-cell function [Charles *et al*., 2009, 2010]. In this model, autoreactive plasma cells produce not only IgG, IgM, or IgA autoantibodies, but also IgE autoantibodies, which then activate basophils. The activated basophils then home to secondary lymphoid organs and induce increases in circulating immune complexes that lead to kidney injury and disease in *lyn-/-* mice. Intriguingly, IgE-mediated activation of mast cells does not contribute to this inflammatory loop [Charles *et al*., 2010]. It is not clear yet if this relationship between autoreactive IgE and autoantibody production will be specific to the Lyn-deficient mouse model. Another example arises from the research of Lowell and colleagues who have focused on the over-production of myeloid-derived BAFF/BLyS in Lyn-deficient mice [Scapini *et al*., 2010]. In response to over-stimulation by BAFF, T cells in *lyn-/-* mice produce IFNγ which in turn further enhances myeloid cell hyperactivity. There is also an effect of BAFF over-production on B and plasma cells, of course.

One might imagine how these inflammatory loops fit into our understanding of autoantibody production in *lyn-/-* mice. First, there are early B cell-intrinsic effects that lead to the accumulation of plasma cells. Because there is always some nominal rate of failure for central tolerance mechanisms, it is likely that ANA-specific B cells, plasmablasts or plasma cells may slowly accumulate in the periphery. Simultaneously, there are some cumulative effects in non-B cell populations that lead to general inflammation. The second critical event results in a full break in tolerance that aids class-switching and the production of pathogenic autoantibodies. Similarly, an initial loss of B cell tolerance subsequently amplified by innate immune system defects leads to full blown lupus in several other genetically distinct murine models including *Sle1.Sle3*, *Sle1.Yaa*, and MRL/*lpr* mice (Chapter 1, Table 6.1).

Future directions

The work presented in this thesis, in addition to the work of others, is therefore consistent with a two-step model of immune dysfunction that results in full breach of tolerance and production of pathogenic antibodies in *lyn-/-* mice (Table 6.1). There are, of course, additional details that need to be resolved. It is still not clear if plasma cell accumulation (step one) is required for disease in Lyn-deficient mice. Nevertheless, given that Btk may provide a useful therapeutic

target at multiple checkpoints, it would be useful to determine the precise mechanism(s) by which Btk mediates the accumulation of plasma cells in the spleen.

The data shown in chapter 5 indicate that Btk may play an important role in mediating splenic plasma cell survival and migration. Btk has already been described as an important mediator of signaling by several B and plasma cell survival factors such as IL-5 [Sato *et al*., 1994] and BAFF [Shinners *et al*., 2007]. It would be especially interesting to determine whether BAFF contributes to the enhanced survival of *lyn-/-* plasma cells via Btk, particularly in light of conflicting evidence for whether Btk is required for BAFF-mediated survival [Shinners *et al*., 2007; Sadat, 2009; Hoek *et al*., 2009]. Preliminary attempts to determine if B220+ and/or CD138+ cells from *lyn-/-* and *lyn-/-*Btk^{lo} spleens exhibited altered sensitivity to *in vitro* BAFF exposure were confounded by population disparities (see chapter 5 discussion). Thus, further analyses of comparable B cell and/or plasma cell populations from *lyn-/-* and *lyn-/-*Btk^{lo} mice will be necessary. In addition, inhibitors that target signaling molecules downstream of Btk could be useful in further delineating the precise component(s) of B cell signaling pathways that mediate aberrant survival or migration and could serve as therapeutic agents.

Defining the mechanism(s) that is required for pathogenic autoantibody production (step two) will be of utmost importance. As of yet, it remains to be

seen if a B cell-extrinsic mechanism will be sufficient for disease in *lyn-/-* mice or whether autoreactive antibody production is also dependent on the loss of Lyn expression specifically in B cells. Additional mixed bone marrow chimera experiments will be necessary to obtain sufficiently aged mice so that antibody production can be examined. As mentioned earlier, there are various transgenic animals, such as BAFF-Tg [Stohl *et al*., 2005] or TLR7-Tg [Deane *et al*., 2007] mice that indicate that a pro-inflammatory environment can ultimately lead to IgG autoantibody production. However, preliminary data that was generated in collaboration with Lowell and colleagues indicate that BAFF is not decreased in *lyn-/-*Btk^{lo} mice. If BAFF can still function in the absence of Btk, this observation may provide additional evidence for the importance of an initial B cell-intrinsic defect that may have to occur first before a global inflammatory environment can affect autoantibody production. If, however, BAFF's effects on pathogenic autoantibody production do require Btk, than the normalization of the autoimmune phenotype in *lyn-/-*Btk^{lo} mice may be due at least in part to failure to respond to the excess BAFF in the environment.

Determining how IL-6 exerts its effect on B cell switching will also be necessary. T cells, being the most likely candidates, will need to be analyzed for significant population changes in *lyn-/-*, *lyn-/-*Btk^{lo}, and *lyn-/-IL-6-/-* mice. Flow cytometric analysis of Tfh, Th17, or Treg populations in Lyn-deficient mice could provide further insight into how T cells might be involved in B cell class-

switching. Different T cells provide B cells help in distinctive ways, and this would ultimately lead to a therapeutic approach that could target different aspects of Th cell function. Determining which cells must receive IL-6 signals, and whether "trans" or "classical" IL-6 signaling promotes pathogenic autoantibody production, may also help guide therapeutic approaches targeting IL-6.

Part of understanding what kind of help B cells are receiving will be in further understanding the nature of the pathogenic antibody that is being produced in *lyn-/-* mice. The plasma cells that accumulate in the spleen are clearly polyclonal, as is exhibited by the autoreactive IgM bloom that is observed in the autoantigen arrays (Figures 3.6, 4.6). IgG focusing indicates that only a relatively small number of B cells are actually switching, however. Although *lyn-/-* mice have a severe defect in GC formation [Hibbs *et al*., 1995; Kato *et al*., 1998; Mirnics *et al*., 2004], it is not clear if the switching of autoreactive B cells is occurring in a traditional GC response or if this is occurring through extrafollicular mechanisms. Extrafollicular responses have been characterized fairly well recently and are shown to generate class-switched antibodies [MacLennan *et al*., 2003]. Such responses are responsible for the activation and class switching of RF-specific B cells in MRL/*lpr* mice [Herlands *et al*., 2008]. Recently, Tfh-like cells have been identified, which act like Tfh cells in their ability to aid class-switching, but home to extrafollicular regions [Odegard *et al*., 2008]. These cells are increased in MRL/*lpr* mice. It may be that a non-classical

extrafollicular response is responsible for the production of pathogenic autoantibodies in *lyn-/-* mice.

Clinical applications of the current studies

The results presented here indicate that therapeutic approaches that target the production of IgG autoantibodies (checkpoint 2 in Figure 4.10) may have therapeutic efficacy for SLE even if they do not prevent B cell hyperactivation, plasma cell accumulation, or IgM autoreactivity (checkpoint 1 in Figure 4.10). By targeting mechanisms that control the process of pathogenic IgG production at a late stage, we could avoid the negative side effects of more immunosuppressive therapies such as those currently approved therapies or B-cell depletion.

IL-6 is a particularly promising candidate. To be sure, IL-6 has been a candidate for therapeutics long before there was a clear understanding for the exact mechanism of action. Many groups have shown the correlation of anti-IL-6 treatment and the decreased production of pathogenic antibodies in both lupus patients and experimental models [Tackey *et al*., 2004], and as of late 2010, anti-IL-6-receptor therapy, Tocilizumab, has been approved for use in RA patients. Various phase III trials are ongoing to evaluate the efficacy of anti-IL-6 therapies in SLE patients and results so far are promising [Illei *et al*., 2010]. The

comparison of *lyn-/-* and *lyn+/-* mice may reveal additional potential targets that affect checkpoint 2.

Btk is also a good target because it affects both checkpoints. Its role in checkpoint 1 suggests that blocking Btk function may be more effective at eliminating long lived plasma cells than current B cell depletion therapies. Studies with the Btk^{lo} transgenic system also indicate that only partial inhibition of Btk function need be achieved for successful therapeutic intervention. This is of particular importance since complete deficiency of Btk results in the immunodeficiency disease XLA in humans. Initial studies examining the effect of a Btk inhibitor on autoantibody production and kidney damage in MRL/*lpr* mice have yielded promising results [Honigberg *et al*., 2010].

	Step 1: Initiating event(s)	Step 2: Inflammatory event(s)
	Loss of Tolerance	**Amplification**
MODEL	DEFECT	DEFECT
lyn-/-	Normal central tolerance Accumulation of autoreactive plasmablasts and plasma cells	↑ IL-6 ↑BAFF ↑Basophil activation
Sle1.Sle3	Impaired B cell deletion, editing and anergy Hyperactivated B/T cells	↑ Dendritic cells
Sle1.Yaa	Impaired B cell deletion, editing and anergy Hyperactivated B/T cells	↑ TLR7 expression

Table 6.1. Defects in both adaptive immune tolerance and innate immune responses contribute to autoimmune disease. The occurrence and contribution of a dysregulated immune system and inflammatory environment to disease progression is consistent with the polygenic nature of SLE. Evidence from *lyn-/-* mice as well as other models of systemic autoimmune disease demonstrate the contribution of two separate steps in enhancing disease severity.

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