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Pim Kinases are Required for Short Lived Plasma Cell Responses by Murine B Cells

John F. Treml
University of Pennsylvania, jtreml@mail.med.upenn.edu

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Abstract
Humoral immunity is characterized by an early primary response of short lived plasma cells (SLPCs) secreting low affinity antibody followed by the formation of germinal center (GC) reactions and subsequent production of high affinity, class switched antibodies by long lived plasma cells (LLPCs). The lineage relationship between these two processes is not well established. We report that the SLPC element of the humoral response is Pim-dependent. Pim-deficient animals have decreased numbers of PreB cells in the bone marrow, but other subsets remain comparable to control animals. In the periphery, only peritoneal B1a B cell numbers are deficient, contributing to low serum Immunoglobulin (Ig) and a lack of natural antibody. Other aspects of the primary B cell pool and splenic architecture reflect normal development. Following immunization, Pim1-/-Pim2-/- animals mount humoral immune responses deficient in the production of SLPCs. T-independent type II responses, comprised entirely of SLPCs, are virtually absent. T-dependent responses are similarly compromised in their production of SLPCs, but do generate germinal center reactions leading to LLPC production. These deficiencies evince two distinct humoral immune responses: the Pim1/2-dependent early production of SLPCs, and the later, Pim1/2-independent, GC / LLPC response. The upstream mediators of these pathways correlate with the expression of TACI on SLPCs and BR3 on GCs / LLPCs, two receptors for the BLyS family of survival factors, known to be vital to B cell survival.

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PIM KINASES ARE REQUIRED FOR SHORT LIVED PLASMA CELL RESPONSES BY MURINE B CELLS

John F. Treml

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Supervisor of Dissertation

Michael P. Cancro, PhD

Graduate Group Chairperson

Steven Reiner, MD

Dissertation Committee

David Allman, PhD

Avinash Bhandoola, MBBS,PhD

Terri Laufer, MD

Michael May, PhD

Dedication

I dedicate this work to my wife, Laura, and son, Harry, who are the foundation of my life.

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Humoral immunity is characterized by an early primary response of short lived plasma cells (SLPCs) secreting low affinity antibody followed by the formation of germinal center (GC) reactions and subsequent production of high affinity, class switched antibodies by long lived plasma cells (LLPCs). The lineage relationship between these two processes is not well established. We report that the SLPC element of the humoral response is Pim-dependent. Pim-deficient animals have decreased numbers of PreB cells in the bone marrow, but other subsets remain comparable to control animals. In the periphery, only peritoneal B1a B cell numbers are deficient, contributing to low serum Immunoglobulin (Ig) and a lack of natural antibody. Other aspects of the primary B cell pool and splenic architecture reflect normal development. Following immunization, $Pim1^{-/-}Pim2^{-/-}$ animals mount humoral immune responses deficient in the production of SLPCs. T-independent type II responses, comprised entirely of SLPCs, are virtually absent. T-dependent responses are similarly compromised in their production of SLPCs, but do generate germinal center reactions leading to LLPC production. These deficiencies evince two distinct humoral immune responses: the Pim1/2-dependent early production of SLPCs, and the later, Pim1/2-independent, GC / LLPC response. The upstream mediators of these pathways correlate with the expression of TACI on SLPCs and BR3 on GCs / LLPCs, two receptors for the BLyS family of survival factors, known to be vital to B cell survival.

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I. Introduction

A. Overview

B lymphocytes are the effectors of humoral immunity, providing defense from pathogens through antibody production. The induction of a humoral immune response involves the selective activation of antigen-specific B cells from previously established pre-immune pool. Each cell in this quiescent population expresses a B cell antigen receptor (BCR) with a unique specificity. When antigens cross-link surface-expressed BCR molecules, intracellular cross-phosphorylation signals are generated that lead to activation and differentiation, culminating in the formation of antibody-forming plasma cells and memory B cells. These antigen-experienced populations maintain protective antibody levels and contribute to anamnestic responses upon subsequent antigen challenges. Accordingly, an effective humoral immune system relies on the maintenance of pre-immune pools that are large enough to contain a broad array of BCR specificities and provide adequate surveillance given the organism's size; as well as upon the generation and accumulation of memory and plasma cell compartments to sustain protective immunity.

Our understanding of how the sizes, compositions, and behaviors of these related but independent pools are regulated has advanced substantially in the last decade, as appreciation for the molecular systems involved has grown. A central feature of current thought is that the establishment and maintenance of B cell pools relies on integrated signals from both the (BCR) and members of the B Lymphocyte Stimulator (BLyS, also termed BAFF) family of cytokines and receptors (1-3). In these processes, the BCR provides specificity-based cues that determine the likelihood of successful maturation, activation, differentiation, and triage into various functional subsets (4). Proper assembly, expression and signaling of the BCR are central to B cell development

and survival. Any mutation that inhibits these functions results in a developmental block or shortened lifespan of the resulting cells (5, 6). In contrast, signals via BLyS family molecules directly mediate survival, determining the proportion of newly formed B cells that survive to enter mature pre-immune pools, the lifespan of these mature primary B cells, and the actions of antigen-experienced B cell subsets (**Figure 1**). Like the pre-immune pool, activated B cell subsets are also thought to have their survival regulated by BLyS family cytokines. In order to distinguish activated B cell pools from the pre-immune pool, a model has emerged where receptor expression, cellular localization and micro-environmental cytokine concentrations effectively manage multiple subsets of cells using only two cytokines and three receptors(7).

The BLyS family is a recent addition to the tumor necrosis factor (TNF) superfamily of biomolecules. It includes at least two ligands: BLyS itself and a "sister" cytokine named A Proliferation Inducing Ligand (APRIL); as well as three receptors, BLyS receptor 3 (BR3, also termed BAFF-R), Transmembrane Activator and Calcium-signaling modulating and cyclophilin ligand (CAML) Interactor (TACI), and B Cell Maturation Antigen (BCMA). As with most TNF superfamily members, receptor ligation initiates interactions with one or more of the TNF receptor associated factors (TRAFs) (8, 9), leading to downstream signaling cascades that can modulate survival and differentiation (10). BLyS can interact with all three receptors; whereas APRIL binds BCMA and TACI, but not BR3. The distinct TRAF interactions of the three receptors, coupled with their dissimilar ligand binding capacities and differential expression patterns, yields a broad range of combinations and thus affords independent regulation of various B cell subsets. For example, antigen-experienced B cells, such as memory B cells and long-lived bone marrow (BM) plasma cells, express different BLyS receptors than pre-immune B cells. Moreover, BLyS ligands may oligomerize or localize within specific anatomic compartments, fostering unique interactions or limiting trophic support to particular anatomic niches. Herein is a discussion of the nature, structure, and expression of BLyS family cytokines and receptors; followed by an in-depth discussion

their actions and signaling characteristics in the context of B lymphocyte development, survival, and activation. Once activated, BLyS receptor expression changes as cells are sorted into new niches. Once these fate decisions are made, survival may be mediated by new cytokine / receptor interactions.

Amongst the downstream mediators of BLyS signals are the Pim kinases. The Pim family of protein kinases are signaling molecules associated with B cell survival and proliferation. These kinases were originally described as proto-oncogenes activated by retroviral insertion resulting in aberrant overexpression. Molecular evidence has emerged linking Pims to specific phosphorylation events associated with cell cycle checkpoint events and expression of anti-apoptotic proteins. Despite these associations, animals lacking Pim kinases have been reported to have minimal immune phenotypes.

Contrary to the previously published work suggesting a minimal role for Pim kinases in B lymphocyte biology, we have found a critical role for Pim kinases in several aspects of B cell biology. First, animals lacking Pim kinases (referred to as PimDKO or $Pim1^{-/-}Pim2^{-/-}$) have significantly reduced Ig titers. To identify the source of this reduction, we have undertaken a complete analysis of the B cell phenotype. At steady state, most B cell subsets were normal, however we did find a reduction in the frequency and number of Pre B cells, particularly the large, cycling Pre B cells. While the B1 B cell defect may partially contribute to the reduced Ig titers in Pim-deficient animals, it does not explain the extent of isotypes affected.

Amongst mature populations, only B1a B cells in the peritoneal cavity are missing. In accord with this phenotype, Pim-deficient animals lack the so-called" natural antibodies produced by these cells. All other mature populations (FO, MZ, splenic B1 and peritoneal B1b) appear in frequencies and absolute numbers comparable to control animals.

Perhaps most significantly, when these animals are challenged with TI-1, TI-2 or TD immunogens, SLPC responses are drastically impaired. Despite this, GC and LLPC responses are reduced, but largely normal. These data indicate that Pim kinases are specifically required for the generation, maintenance or survival of SLPC cells, but not the adaptive elements of a humoral response. Because all SLPCs are affected by Pim deficiency independently of the stimulatory signals, we hypothesize that this phenotype is not mechanistically proximal to antigen-initiated signals, but results from some generalized feature of AFC generation, function or survival. Specifically, we propose a model where survival of SLPCs is dependent upon Pim expression induced by capture of BLyS or APRIL by TACI.

This dissertation explores these deficiencies and what they might reveal about B cell biology. Below is an introduction to humoral immunity as well as the generation of a pre-immune pool of B cells and how these pre-immune pools are activated by antigenic stimulation. Following that are discussions of the nature of the BCR and BLyS receptors and the roles they play in both pre-immune and antigen experienced B cells. Lastly, the role of Pim kinases in cell cycle and survival are introduced. In the results section, we present new findings linking the generation, function and survival of SLPCs made shortly after immunization to Pim kinases; and ultimately to the expression of the BLyS receptor, TACI.

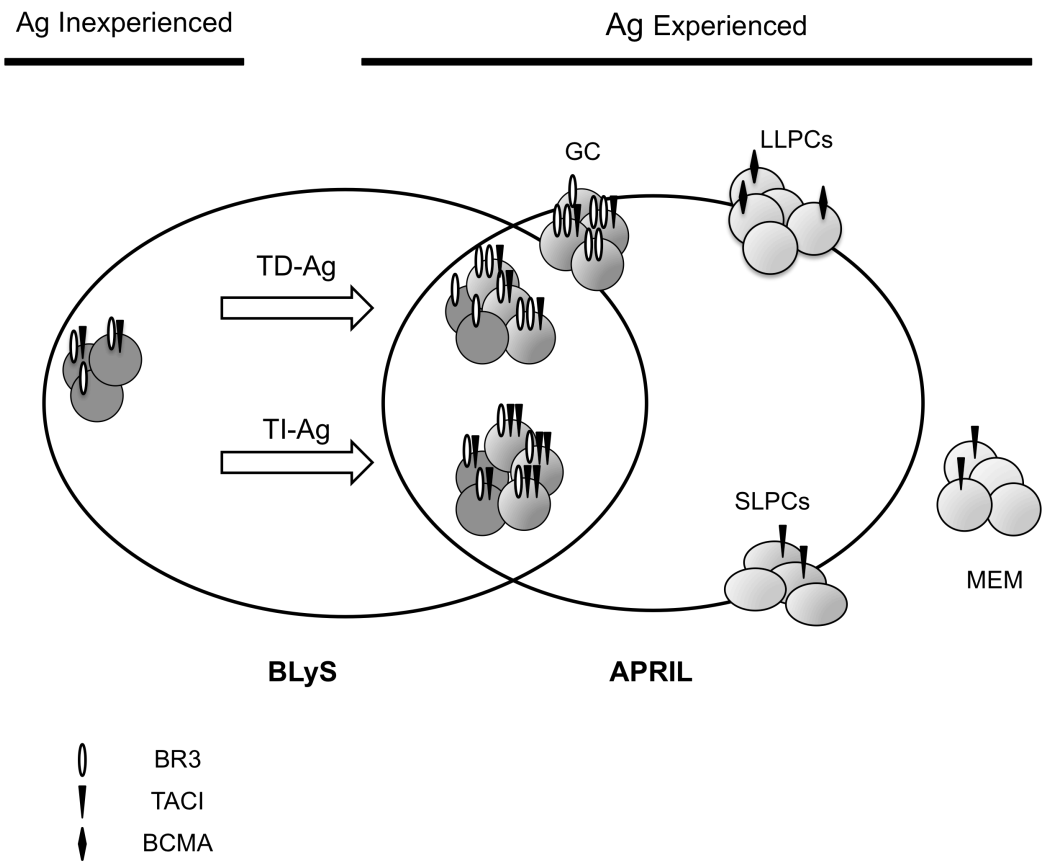

Figure 1: Receptor Expression and Cytokine Dependence. Naïve, mature B cells express both BR3 and TACI, however only BLyS is required for normal development. Stimuli result in changes in receptor expression with TD stimuli favoring BR3 expression and leading to germinal center reactions while TI stimuli favors TACI expression on short lived antigen producing cells. Dependence on BLyS or APRIL specifically has not been fully demonstrated for recently stimulated cells. LLPCs express BCMA and require APRIL for normal development / survival. Memory cells are known to express TACI, however they appear independent of either BLyS or APRIL.

B. B cells and Humoral Immunity

The humoral immune response is characterized by the production of antibodies with specific binding to an epitope present on the immunogen. Antibodies are soluble BCR proteins produced by plasma cells selected from a pre-immune pool of FO B cells that bore antigen receptors specific for the immunogen in question. The antigen-binding portion of the BCR / antibody is generated by random recombination and modulation of pre-existing germline elements in order to bind to a vast array of unknown (non-self) epitopes. The generation of this repertoire of preimmune cells is described below in section II. Antibody binding to these cognate epitopes serves several purposes including coating and sterically interfering with a pathogen or toxin's function, facilitating phagocytosis or mediating cellular cytosis by complement.

The activation of B cells to develop into antibody-secreting plasma cells can result from a variety of stimuli. Humoral immune responses can occur in cooperation with CD4+ T helper cells, a T-dependent response, or without T cell help, a T- independent response. In general, both responses begin in the first week after antigen exposure with the rapid proliferation of extrafollicular plasmablasts and the production of low affinity IgM antibodies. In T-dependent responses, this low affinity response is followed in the second week by the production of higher affinity antibody by plasma cells that have undergone expansion and selection during germinal center reactions (**Figure 2**.) Both of these responses are discussed in greater detail below, including their cytokine and survival requirements in sections IIIb and IIIc.

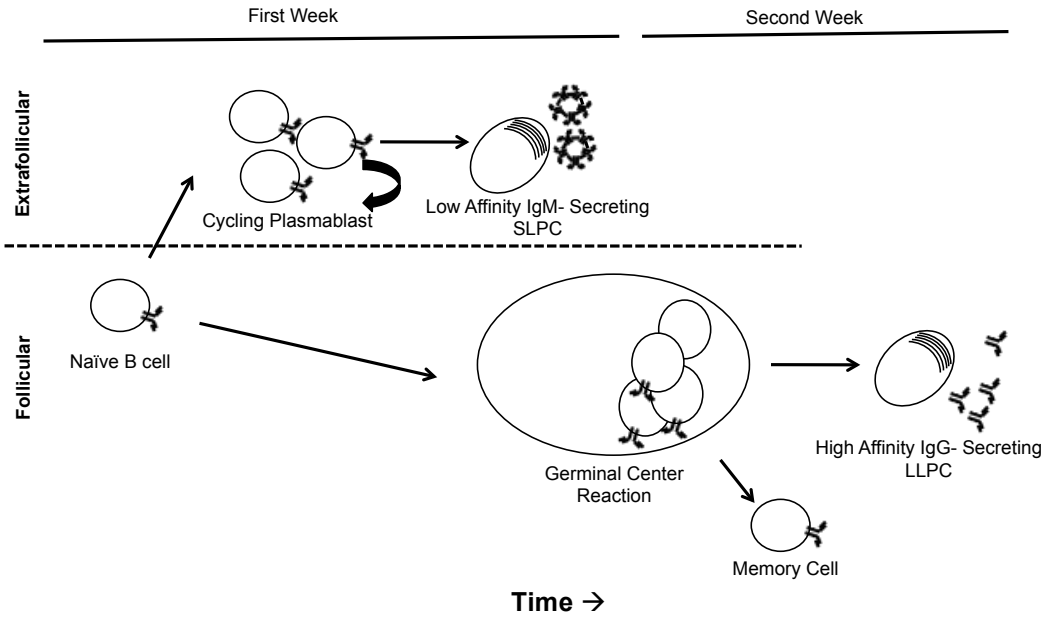

Figure 2: The humoral immune response. Short-lived plasma cells producing low affinity IgM are generated early in the immune response. Later, long-lived plasma cells producing high affinity, class-switched antibody and memory B cells are generated following germinal center reactions.

II. Materials and Methods

Mice C57BL/6 mice were purchased from The Jackson Laboratory and B6x129 mice purchased from Taconic Labs were used as controls for the $Pim1^{-/-}Pim2^{-/-}$ mice. Animals were immunized with preparations of NP-CGG in Alum (50ug in 200ul PBS) or NP-Ficoll in Alum (50ug in 100ul or 200ul PBS), or NP-Ficoll with Fitc-LP910-50ug in 200ul PBS) injected ip. $TACI^{-/-}$ animals were provided by Dr. Richard Bram (Mayo Clinic, Rochester, MN.) $Pim1^{-/-}Pim2^{-/-}$ mice were provided by Dr. Casey Fox (The Jackson Laboratory, Bar Harbor, ME.) All animal husbandry and procedures were carried out in accordance with the Animal Welfare Act.

Flow cytometry Spleen, bone marrow and peritoneal cells were surface stained with Abs that recognize B220 (clone RA3-6B2 BD Pharmingen,San Jose, CA), CD19 (clone 1D3, BD Pharmingen,San Jose, CA), Igκ (clone 187.1 Southern Biotech, Birmingham, AL), Igλ (clone JC5-1 Southern Biotech, Birmingham, AL), Igδ (clone 11-26c.2a BD Pharmingen, San Jose, CA) CD4 (clone H129.19 BD Pharmingen, San Jose, CA), CD8 (clone 53-6.7 eBiosciences, San Diego, CA), F4/80 (clone BM8 eBiosciences, San Diego, CA), GR1 (clone RB6-8C5 eBiosciences, San Diego, CA) and CD23 (clone B3B4 BD Pharmingen, San Jose, CA), GL7 (clone 8C5 BD Pharmingen,San Jose, CA) CD95 (clone Jo2 70 BD Pharmingen, San Jose, CA), CD11b (M1/70 BD Pharmingen, San Jose, CA), CD93 (clone AA4.1 eBioSciences, San Diego, CA), CD5 (clone 53-7.3 BD Pharmingen, San Jose, CA) and CD21 (clone 7G6, BD Pharmingen, San Jose, CA). Cells were analyzed using LSR II (BD Biosciences, San Jose, CA) and Flowjo software (Treestar, Ashland, OR.)

B cell enrichment and culture assays B cells were enriched by positive selection using CD23+ MACS on Miltenyi columns as previously described (11). Resulting cell suspensions were 90%+ follicular B cells as verified by CD23 and IgM FACS. Cells were

enumerated after lysis of red blood cells and cultured in B cell media containing DMEM or RPMI 10% Characterized FBS (Thermo Scientific HyClone, Waltham, MA) Non-essential Amino Acids, L-Gluatamine, 2-Mercaptoethanol, OPI and gentamycin. Cells were cultured for indicated times in the presence of BLyS (Human Genome Sciences, Rockville, MD) or APRIL (Peprotech, Rocky Hill, NJ.) For qPCR, cells were harvested for mRNA by pelleting and extraction of mRNA using Qiashredder and RNeasy kits (Qiagen, Valencia, CA.)

Quantitation of serum antibody and antibody secreting cells Serum antibody specific for NP was assayed by ELISA incubating serial dilutions of sera or control antibody on NP-coated plates and detecting with IgM, lambda or IgG specific reagents, extravidin-HRP and tetramethylbenzidine substrate kit (BD PharMingen, San Jose, CA.) Concentrations of NP-Specific antibody were established by reference to a standard curve. The method for quantitating antibody secreting cells by ELISPOT has been described (12). Briefly, cells were recovered from spleen or bone marrow and enumerated. Serial dilutions of cells were plated on multiscreen HTS plates (Millipore, Billerica, MA) coated with NP, NP3 or NP33 coupled to the carrier BSA and incubated for 4 hrs at 37°C. Plates were washed 5x before adding biotinylated detection antibody (anti-lambda, anti IgG or anti-IgM). Plates were incubated 1hr at RT and washed. SA-AP was added, incubated 1hr at RT, plates were washed and then given BCIP/NBT substrate (Sigma Aldrich, St. Louis, MO) and allowed to develop. Plates were dried and spots were enumerated using an ELISpot reader (CTL, Cleveland, OH) and analyzed with Immunospot software version 3.2 (CTL, Cleveland, OH).

Quantitative PCR Message RNA levels were established by qPCR using commercially inventoried primers in an ABI 7500 analyzer (Applied Biosystems, Foster City, CA.) Relative expression was determined by ddCT method according to manufacturer's methods.

Immunohistochemistry Splenic architecture was observed by microscopy on Zeiss LSM 510 Meta-confocal and Nikon E800 instruments. Spleen sections were harvested and frozen down in OCT under 2-methyl butane. Sections were cut at 5-8 micron thickness, fixed with acetone and stained with antibodies or PNA as indicated.

III. Development and homeostasis of pre-immune B cell pools

A. Primary B cell development

B cells are generated throughout life from stem cells in fetal liver, neonatal spleen, or adult bone marrow (BM). Multi-potent cells arise from these progenitors and, upon initiation of lymphoid gene expression, yield B lineage precursors. Excellent, highly detailed reviews of early B cell development are available elsewhere (13-17). Briefly, immunoglobulin heavy and light chain gene rearrangements occur as cells transit the BM pro- and pre-B stages respectively. These genetic events culminate in the production of an immunoglobulin heavy/light chain pair that is assembled and, in conjunction with the Ig-alpha and Ig-beta signaling molecules, form a functional BCR that is transported to the cell surface. BCR surface expression marks the progress into the Immature (IMM) BM subset. These newly formed B cells continue their maturation after they migrate to the periphery, passing through several so-called transitional (TR) developmental stages before entrance into primary B cell pools. Cells completing these differentiative processes enter one of the major pre-immune subsets: the follicular (FO) and marginal zone (MZ) populations.

B. Primary B cell selection and homeostasis are coupled processes

While details of primary B cell differentiation are extensively reviewed elsewhere (18, 19), a feature critical to the concepts discussed here is that stringent selection, based on BCR specificity, occurs within the IMM and TR pools. This selection transpires at both the IMM and TR stages. Indeed, only about 10% of the IMM cells generated live to exit the BM, and fewer than half of the resulting TR cells survive to finally join mature pre-immune B cell pools (20, 21). Both negative and positive selection events contribute to these cell losses.

Negative selection – the elimination of potentially autoreactive B cell clones – occurs at both the IMM and TR stages. Current thought holds that elimination at the IMM stage is engendered by high avidity BCR engagement, whereas cells undergoing less avid but sustained BCR interactions persist into the TR stages, where they then die before maturation (22). Substantial evidence from transgenic mouse systems confirms that autoreactive clones are eliminated at both of these stages (23-25). More recently this has been confirmed in humans by studies that revealed similar selection against autoreactive BCR specificities during TR maturation (26). Moreover, these cell losses failed to occur in autoimmune patients (27).

Positive selection – the preferential survival of clones whose receptors meet minimum so-called "tonic" BCR signaling requirements - occurs at these stages as well, particularly among TR cells (28-31). Moreover, mature primary B cells continue to require continuous sub-threshold BCR signals for survival, since conditional ablation of either the BCR itself or proximal BCR signaling components leads to the rapid death of most mature B cells (6). The requirement for persistent, low-level BCR signaling is consistent with the observation that BCR engagement has a clear positive effect in cellular survival at the BM / periphery interface and beyond (32). The consequence of this effect is best illustrated in mixed bone marrow chimeras, where the mature B cell pool is derived from differing mixtures of BCR transgenic or other genetically manipulated B lineage

progenitors (33-36). These experiments show a distinct connection between BCR specificity and selection in conditions of interclonal competition.

An overarching conclusion arising from such experiments is that in the TR subsets and beyond, BCR specificity determines a cell's relative fitness to capture or utilize limited trophic resources for survival (34, 35). This in turn suggests that while a range of tonic BCR signaling can afford survival, those cells with optimal tonic BCR signaling levels will be more likely to survive to maturity and will persist longer in per-immune pools. Considered together, these concepts unify specificity-based selective processes with homeostatic control, because B cell production rate and lifespan are the determinants of the primary pool's size. Further, the prediction that limiting trophic factor(s) would be the basis for homeostatic control foreshadowed the discovery of BLyS as the biological metric for "space" in primary B cell compartments.

IV. The BCR and BLyS Receptors

As discussed above, B cell development is controlled by signaling from two key surface receptors, the BCR and the BLyS family receptors. Accordingly, an overview of these receptors and their ligands follows. **TABLE 2** lists pre-immune B cell subsets and their surface phenotypes, their BCR and BLyS receptor expression and BLyS ligand dependence

A. The B Cell antigen receptor

B cell development is defined by the expression and functionality of membrane bound BCR molecules. The BCR is comprised of a pair of light chain molecules associated with a pair of either μ or δ membrane-spanning heavy chains, non-covalently linked to Igα and Igβ elements that bear the intracellular signaling elements. In a pre B cell, the light chains of the preBCR are comprised of lambda5 and VpreB. In the mature

BCR, light chains may be either kappa or lambda. In the mouse only 5% of peripheral CD19+ B cells express lambda.

Prior to the Pre B cell stage, no BCR is expressed, however genomic rearragement of the immunoglobulin genes are active as early as the common lymphoid progenitor stage (37). At the Pre B cell stage cells express a PreBCR comprised of a successfully recombined heavy chain paired with the surrogate light chain elements lambda5 and VPreB. One explanation of this pairing is proposed to be a check on the structural stability of the recently formed heavy chain and an opportunity for successful heavy chains to proliferate as large Pre B cells prior to additional immunoglobulin rearrangement and pairing with light chains (38). In this way these successful heavy chains can be paired with a variety of light chains thus hedging against the loss of cells due to unsuccessful light chain rearrangements.

Once a light chain is made that can productively pair with the heavy chain, mature BCR proteins are expressed on the surface of immature B cells in the bone marrow. Immature B cells then leave the bone marrow to complete their development in the periphery as transitional B cells. B cell development is discussed in greater detail below in section III.

How signaling from the PreBCR and BCR occurs has been a topic of great interest. Since each BCR bears a receptor of unique specificity, the notion of ligand mediated signaling becomes difficult to fathom. However, without a ligand, there is nothing to crosslink BCRs together to allow cross-phosphorylation of signaling elements. The concept of ‘tonic signaling’ has emerged following experiments that demonstrated singaling from BCRs lacking some, or all, extracellular elements (39-42).

B. The BLyS family of cytokines and receptors

Most members of the BLyS family were discovered through genomic homology searches (43-45). As some family members were discovered simultaneously by several

laboratories, they possess multiple names and acronyms. Throughout this dissertation, the two ligands are refered to as APRIL and BLyS; and use the terms BCMA, TACI, and BR3 for the three receptors. The following two sections discuss the biochemical characteristics of BLyS subfamily ligands and receptors respectively.

i. BLyS family ligands

There are two ligands in the BLyS family: BLyS and APRIL. Both cytokines are TNF family receptors expressed by a variety of cell types, particularly among leukocytes. Both are initially synthesized as membrane-spanning monomers, but associate as trimers and are cleaved to soluble form by furin. Although they share homology with other TNF ligands, BLyS and APRIL are smaller than most - consisting of only transmembrane domains, a Furin-cleavage site and a TNF homology domain. In addition, receptor binding face structure and amino acid composition also distinguish these ligands from other superfamily members (46).

a. APRIL

APRIL was isolated based on mRNA expression in cell lines, and so-named because it could provoke proliferation in transfected fibroblasts (47). APRIL can interact with BCMA and TACI, but not with BR3. APRIL also bears an N-terminal region that interacts with surface proteoglycans through a short sequence of basic residues (48-51). The exact biological role(s) of this feature is not yet clear, but may be to facilitate binding of APRIL to TACI, BCMA or other receptors by generating higher order complexes (49).

Because APRIL knockouts lack a profound phenotype, the biological functions of APRIL are only now being explored in detail. Given their overlapping receptor binding specificities, many functions of APRIL may prove redundant with those of BLyS, at least to the extent that unique attributes may be difficult to discern unless studied in the absence of BLyS (52-55). Nevertheless, APRIL clearly plays a modulatory role in certain

aspects of B cell activation and isotype switching (56, 57); and likely interacts with bone marrow plasma cells via BCMA (58, 59). Indeed, while expressed in a variety of tissues, APRIL message is seen at very high levels in both human (60) and mouse osteoclasts. The expression of this cytokine in bone may thus be vital to the support of long–lived plasma cells (LLPCs), as LLPCs reside in the bone marrow and are reduced in mice lacking BCMA (61). Dendritic cells were recently reported to secrete APRIL constitutively and this production is increased following stimulation of these cells with TLR ligands (62). Finally, APRIL fosters the viability or progression of some cancers (63-66).

b. BLyS

Sequence searches also contributed to the discovery of BLyS, and the features of this cytokine were reported simultaneously by several groups (43-45, 67). BLyS can interact with Magnesium, and has an exceptionally deep and acidic cleft that in part explains its unique affinity for the comparatively basic BR3 receptor, as well as its lower affinity for TACI and BCMA when compared to APRIL.

BLyS is produced by a wide variety of cell types, including neutrophils, dendritic cells, monocytes, macrophages, and others (68). BLyS message and furin-mediated release is up-regulated by some cytokines, including interferon gamma and IL10 (69). The furin-cleaved, soluble trimeric form is the primary effector of in vivo function, but BLyS can also exist as a 60-mers whose spectrum of receptor binding activities are expanded, especially with TACI (70). Finally, heterotrimers of BLyS and APRIL exist, although their significance remains poorly explored (71).

ii. BLyS family receptors

There are three receptors for BLyS and/or APRIL: BCMA (72), TACI (73), and BR3, the product of the previously defined *bcmd* locus (Bcmd/BR3) (1, 74-78). The basic features of these receptors are summarized here. More detailed considerations of the

ligand binding motifs, as well as descriptions of key contact residues and structural organization of each receptor, have been reviewed (51).

a. BCMA

BCMA is a 20 kDa Type III transmembrane protein that was originally identified in a human T cell lymphoma (72, 79). BCMA can bind both BLyS and APRIL, but the considerably higher affinity for APRIL suggests this may be the most relevant ligand in vivo. Structurally, BCMA has the least homology to other TNF receptor family members. Like BR3, BCMA contains a single characteristic CRD motif. Indeed, among the TNF family of receptors, only BCMA and BR3 exhibit this feature. BCMA can interact with TRAFs-1, 2 and 3; and downstream effects include activation of NF-kB transcriptional mediators and JNK kinases (80).

The role played by BCMA in B cell biology remains somewhat enigmatic. BCMA is expressed in neither developing nor primary B cells, and BCMA knockout mice show no phenotypic abnormalities within the pre-immune B cell compartments. Further, the abnormalities seen in the primary B cell pools of other BLyS receptor knockouts are BCMA independent. More recently, BCMA expression has been confirmed on LLPC populations, and detailed studies of BCMA knockouts support a role for the generation and maintenance of these cells.

b. TACI

TACI is a 293 amino acid Type III transmembrane protein that was initially identified based on its ability to interact with CAML (80). Similar to BCMA, TACI can bind both BLyS and APRIL but, unlike BCMA, TACI binds each with similar affinities (81). Structurally, TACI bears two CRDs, and has cytoplasmic domains capable of interacting with TRAF 2, 5, and 6. Receptor ligation activates a variety of downstream transcriptional regulatory pathways, including NFAT, AP-1 and the classical NF-kB pathway, presumably through a CAML-independent mechanism (73). Signaling through TACI has been shown

in numerous experimental and transformed cell types to result in the classical NF-kB mediated upregulation of the X-linked Inhibitor of Apoptosis Protein (XIAP), but not bcl2 family members (82).

TACI expression is restricted to B cells and a subset of activated T cells. Although TACI can bind both BLyS and APRIL with comparatively strong affinity, the biological effects of TACI ligation remain unclear and somewhat controversial. Early results from TACI knockout mice suggested a negative role for TACI (83), because these animals exhibited B cell hyperplasia and humoral autoimmune symptoms including glomerulonephritis, proteinuria and anti-dsDNA antibodies. More recently, there has been accumulating evidence that TACI is a positive regulator of B cell survival. For example, APRIL stimulation of primary B cells ex vivo, which should engage only TACI, improves survival (84). Further evidence for positive regulatory roles in survival or differentiation stems from findings that show TACI facilitates isotype switch recombination, particularly to gut-associated IgA isoforms (85, 86). It is unclear whether this dichotomy reflects opposing roles for TACI in different cellular and anatomic contexts, or instead indicates that the B cell hyperplastic phenotype in knockouts is a secondary phenomenon.

c. BR3/BAFFr

BR3 is the most recent addition to the BLyS family receptors. Its discovery resulted from results suggesting that neither TACI nor BCMA dramatically influenced B cell activities, implying the existence of an additional BLyS-binding receptor. Similar to the discovery of BLyS, BR3 was reported simultaneously by several groups (80). Importantly, it proved to be the gene product of a locus that had previously been shown to govern late primary B cell differentiation and mature B cell survival (76). BR3-BLyS interactions have proven critical to the maintenance of primary B cells, as shown clearly in knockout and mutant mice (77, 87). Structurally, BR3 contains one extracellular CRD that interacts with ligand and a single intracellular recognition site for TRAF3. Recent evidence suggests that BR3-mediated interactions with TRAF3 initiate and sustaining downstream signaling

(8). These signaling targets include non-classical NF-kB pathway activation and subsequent upregulation of multiple Bcl-2 family members (detailed below). This strategy contrasts the survival genes regulated by TACI, in that Bcl-2 family members inhibit apoptosis upstream of the mitochondria.

C. BLyS receptor expression and physiology in developing and primary B cells

Extensive evidence links signals via BLyS receptors, particularly BR3, with the survival of TR, FO and MZ B cells. Indeed, BR3 and TACI are first expressed among IMM B cells in the bone marrow, and their expression continues to increase as cells transit through TR stages. Within pre-immune FO and MZ pools, BR3 and TACI reach relatively high and constant levels (88). MZ B cells are highly sensitive to conditions when BLS is limiting, likely related the uniformly high levels of TACI and BR3 expressed (78, 89).

BLyS signaling through BR3 is crucial for the survival of all pre-immune B cell subsets from the TR stage onward. This was initially evidenced by the profound mature B cell deficiency in the A/WySnJ mouse (74-77, 90, 91), a feature that segregated as a single autosomal trait reflecting a 400bp insertion in the intracellular tail of BR3 (78). This and subsequent studies have led to the notion that BLyS signaling via BR3 controls the size and composition of pre-immune B cell pools (reviewed in (92)). For example, either ectopic BLyS expression or exogenous BLyS administration yields B cell hyperplasia and humoral autoimmune manifestations (44, 88, 93). Conversely, both BLyS and BR3 knockout mice display primary B cell deficiencies reminiscent of the A/WySnJ (77, 87).

Thus, the BLyS/BR3 axis is central to the homeostatic control of primary B cell numbers, with current thought favoring the notion that available BLyS levels determine both the proportion of TR cells that survive to enter the mature pools and the lifespan of mature B cells themselves (see (94) for reviews). By controlling the entry rate and lifespan of mature B cells, a set-point for steady-state numbers is thus imposed, whereby

BLyS availability constitutes the “limiting resource” for which cells in these primary pools compete. This prompts a conceptual model whereby TR, FO, and MZ B cells continuously compete for BLyS consumption (binding), such that when consumption and availability are balanced, the set-point for steady state numbers is achieved (92).

The ability to vary the survival of TR B cells has obvious implications regarding negative selection, since it implies that the thresholds for negative and positive selection at the TR stage may vary, based on available BLyS and the tonic BCR signaling capabilities within the competing cohort of emerging cells. Recent studies in several transgenic systems verify this plasticity in TR selection stringency (95-97), showing that when BLyS is non-limiting, self-reactive clonotypes that normally die at the TR stage instead survive and mature. Importantly, BLyS over expression does not rescue cells deleted at the IMM stages, suggesting that a developmental switch affords the ability for BR3 and BCR to cross-regulate one another’s signaling outcomes. This may reflect the onset of BLyS receptor expression per se, but a growing literature suggest that differentiation-dependent changes in intracellular signaling systems that afford cross-talk between the BCR and BR3 may also play a role (42, 98-101).

An extension of this model of BLyS-mediated homeostasis can be used to understand how the same cytokines and receptors are utilized to maintain the survival of antigen experienced B cell subsets. As described above in section III D, SLPCs, GCs, LLPCs and memory B cells all have subset-specific BLyS receptor expression patterns and many of these cells also have well defined physiological niches (GC cells exist in the PALS/follicle interface, LLPCs often reside in the BM in contact with osteoclasts, SLPCs thrive in the splenic red pulp as extrafollicular foci.) In combination, these characteristics may allow for a shift in survival signals from BLyS:BR3 interactions to other combinations.

V. Antigen experience and B cell activation

A. The generation and identity of antigen experienced B cell subsets

The clonal daughters of naïve B cells recruited into immune responses comprise antigen-experienced subsets. These are summarized in **Table 1** and include the responding B cells at various stages of activation, as well as antibody-forming cells (AFCs) and memory cells. B cell responses are generally categorized as either TD or TI, depending on whether cognate T cell help is involved in initiating the response. TD responses arise from FO B cells and are elicited by protein antigens. Following receptor ligation and internalization, such antigens can be processed to short peptides that are complexed with and presented in the context of MHC class II molecules, facilitating T cell costimulation. A distinguishing feature of TD responses is the germinal center (GC) reaction, in which B cells undergo class switch recombination and somatic hypermutation, as well as the specificity-based selection that underlies affinity maturation. Descendants of cells in the GC reaction include LLPCs that home to and reside in the bone marrow; as well as memory B cells, whose exact homing and localization characteristics remain less clear. In contrast, TI responses do not involve T cell help and do not yield a GC reaction. Accordingly, they lack affinity maturation, do not yield long-lived plasma cells, and produce little if any humoral memory. Two types of TI antigens exist: TI-1 antigens induce proliferation and differentiation through the stimulation of pattern recognition receptors such as Toll like receptors (TLRs); whereas TI-2 antigens bear densely repeating epitopes, and are thought to yield activation via exceptionally strong and sustained BCR cross-linking.

Reflecting these two major modes of activation, the participation and chronologic appearance of antigen-experienced subsets follow two general patterns. Both TD and TI responses yield an initial proliferative burst among the activated primary B cell clones that have localized to extrafollicular foci in the splenic red pulp. These rapidly expanding cells quickly give rise to short-lived plasma cells (SLPCs), which generate the initial low affinity

IgM antibodies observed during early primary responses, and in some cases modest amounts of some IgG subclasses. Following these events, most TI responses dissipate within an 8 to 14 day course.

During TD responses, the early generation of LLPCs is paralleled by the initiation of a GC reaction. Within the GC, two populations emerge: one that is rapidly proliferating and one that is less mitotically active. These are polarized within the GC, and corresponded to the historically named “dark zone” and “light zone” respectively. Class Switch Recombination and Somatic hypermutation (SHM) are initiated in the GC, following by the upregulation of enzymatic machinery associated with these processes. *In toto*, these events result in the generation of B cells that have switched heavy chain Ig isotypes, and that have accumulated point mutations in their BCR combining site. Those daughter clones bearing novel specificities generated by SHM then undergo selection against either low antigen affinity or self-reactivity, and positive selection for higher affinity. The mechanisms underlying this selection remain debated, as do the compartmentalization and trafficking patterns of cells in the GC (102-104). Regardless of exact mechanism, cells surviving GC selection give rise to LLPC that migrate to and reside in the BM, and to memory B cells. While the phenotypic characteristics of these cells remain debated, their respective roles in immunity are well documented (105-107). The LLPCs provide ongoing protection from reinfection through continuous high affinity antibody secretion, and are the source of standing antibody titers associated with protective adaptive immunity. Memory B cells provide a long-lived population of expanded, antigen specific cells that are quickly activated upon subsequent antigen encounters, affording the rapid response time associated with anamnestic responses.

Additionally, antigen activation, including those delivered through BCR, TLRs, or CD40 engagement, engender characteristic BLyS receptor profiles. Because of the different ligand preferences for each BLyS family receptor, as well as their disparate

signaling outcomes, acquiring novel BLyS receptor profiles will specify independent or overlapping homeostatic niches (108, 109).

Table 1: Characteristics of Antigen Experienced Subsets

Differentiation Subset		Surface Phenotype	BLyS Receptors	BLyS Ligand Dependence
T-Independent Responses	Early AFC/SLPC	$B220^{lo}CD19^{+/-}$ $sIg^{+/-}icIg^{hi}$	BR3, TACI	?
T-Dependent Responses	Early AFC/SLPC	$B220^{lo}CD19^{+/-}$ $sIg^{+/-}icIg^{hi}$	BR3, TACI	?
	GC	$B220^{+}CD19^{+}$ $GL7^{+}$	BR3, TACI	GC formation ensues but smaller and less persistent in BLyS or BR3 deficiency
	LLPC	$B220^{lo}sIg^{-}icIg^{+}$	BCMA	BLyS or APRIL
	Memory	$B220^{+}sIG^{+}$ IgD^{-}	TACI?	None?
Natural Antibodies	Peritoneal B1a and B1b	$CD43^{+}CD23^{-}$ $CD5^{+/-}$	TACI?	None described

B. T-Independent B cell activation

Following encounter with TI-1-stimulating TLR agonists, B cells dramatically up-regulate TACI (84). A similar finding occurs following stimulation with TI-2 antigens, where TACI expression increases the number of AFCs generated (110). The role played by TACI in these rapidly dividing but relatively short lived responses remains puzzling, but recent findings have suggested it is involved in controlling entry and exit from cell cycle (110). A potential role for BLyS in cell cycle entry and success has been posited, although the receptor involved was not clear from these studies (111). TACI may be directly promoting survival of short-lived AFCs, particularly when bound by multimeric ligand arrays such as those afforded by oligomerized BLyS or surface bound APRIL (70). SLPCs generated during either TI or TD responses also express high levels of TACI, in contrast to the phenotype of LLPCS (see below) which instead up-regulate BCMA. The difference in which receptor is up-regulated in each response may be key to the difference in lifespan.

Information is sparse regarding the signaling systems and downstream targets of TACI in B cells activated by TI antigens. Nonetheless, these likely involve the activation of classical NF-kB signals; and in contrast to the pre-mitochondrial survival mechanisms afforded by BR3 signals, post-mitochondrial anti-apoptoic mechanisms, including stress response proteins such as XIAP, may be involved (82). Since such mechanisms are effective in preventing apoptosis engendered by cell cycle checkpoint failures or unfolded protein responses, these pro-survival mediators might be expected for B cells undergoing extensive division and differentiation to high levels of Ig protein production (112).

C. T-dependent B cell activation and germinal center reaction

The striking parallels between GC and primary B cell differentiation, in terms of negative and positive selection processes based on BCR specificity, suggest mechanistically similar processes. Substantial evidence suggests that members of the BLyS family may indeed play a role in the establishment and proper evolution of the GC reaction, but the members involved and mechanisms of action remain cloudy. In general, BCR engagement per se, as well as with concomitant CD40 ligation, leads to a BLyS receptor signature in which BR3 is up-regulated (32). Alternatively, Qian et all showed that Act 1 functions as a negative regulator of both BR3 and CD40, suggesting potential cross talk and cross-modulation between different TNF family receptors expressed in the GC (113).TACI is elevated following TD stimulation as well, although not to the extent seen with TI antigens. Interestingly, GC B cells retain this phenotype, suggesting that the BLyS-BR3 interactions may play a role in this aspect of TD responses. However, early studies in the BR3 mutant A/WySnJ mouse strain suggested GCs likely form, since TD memory could be generated - albeit with reduced efficacy and less extensive isotype switching (75). In agreement with this, more recent work from the Manser laboratory showed that BR3 is not required for the generation of GCs (114), but plays a role in the appropriate evolution of the overall GC reaction. This finding was extended in studies that used soluble BCMA-Fc to block both APRIL and BLyS, yielding the same results (114, 115). Thus, while not profoundly affected, GCs generated in the absence of appropriate BLyS or BR3 signaling are small, transient and fewer in number. In addition to possible B cell intrinsic activities for BLyS in the GC reaction, follicular dendritic cells (FDC) networks do not mature in GCs when BLyS and APRIL are blocked (116). This may reflect a loss of FDC supporting signals secondary to the decreased number of B cells in the GC, although the exact basis remains speculative.

The potential roles of BLyS family members in the selective processes that follow SHM within GCs have not been directly interrogated. This in part reflects the complexity

associated with blocking BLyS-mediated signals necessary for primary cell survival, as any effects on GC selection might indirectly result from a reduction in primary B cells that seed the GC. In addition, analysis of negative and positive B cell selection in the GC requires single cell analyses, exacerbating the difficulty of this problem.

D. Antigen experienced cell populations: SLPCs, LLPCs and memory B cells

The BLyS receptor expression profiles and cytokine requirements for LLPC and MEM cells appear to be unique. Recent evidence supports a role for BCMA in maintaining LLPC survival (61). As might be expected, these cells are sensitive to only combined withdrawal of BLyS and APRIL, and some are apparently independent of both cytokines. The ability of at least some LLPCs to use APRIL as a survival factor is intriguing, since osteoclasts generate large amounts of this cytokine, and might thus provide localized high levels of APRIL in specialized bone marrow niches. Further, APRIL exhibits the unique ability to bind proteoglycans (49), which are abundantly expressed on resident BM accessory cells where long-lived PCs co-exist. The downstream effects of BCMA signaling in LLPCS are not yet extensively characterized, as these are rare populations of B cells that are difficult to maintain in vitro. However, B cell and plasma cell lines reveal classical NF-kB activation, the upregulation of a number of proteins associated with antigen presentation and co-stimulation, and increased IL-2 production following BCMA ligation (117).

In contrast to LLPCs, memory B cells express elevated levels of TACI, at least early in their generation (59). Despite this, most evidence points to a lack of reliance on either BLyS or APRIL when both cytokines were neutralized using sufficient soluble TACI-Ig to ablate naïve B cells (59). Further supporting BLyS independence, anti-BLyS antibody failed to eliminate B cell memory in a variety of experiments designed to elicit memory B cell generation (118).

SLPCs are the first plasma cells generated in response to antigenic challenge. These cells rapidly migrate out of the follicle and into the red pulp of the spleen. The migration of these cells from the follicle has been attributed to the downregulation of the chemokine receptor CXCR5 and the upregulation of CXCR4. Cells then migrate towards a CXCL12 gradient emanating from the red pulp (119). Once in the red pulp, cells proliferate in extrafollicular foci and secrete low affinity IgM. In TI-1 responses, SLPCs up-regulate the receptor TACI and in animals lacking TACI, SLPC responses are lost (84, 89, 110). These results suggest that TACI may be involved in the capture of pro-survival signals in these cells, however this has not been directly confirmed. Regardless of their generation, these cells persist only for a few days before their numbers involute (120).

Table 2. Characteristics of Primary B Cell Subsets and their Progenitors

Differentiation Subset		Surface Phenotype	BCR Expression	BLyS Receptors
Progenitor Subsets (Bone Marrow)	Pro-B	$B220^{lo}CD43^{+}$ $AA4.1^{+}$	None	No
	Pre-B	$B220^{lo}CD43^{-}$ $AA4.1^{+}preBcR^{+}$	preBCR (Heavy chain, Surrogate Light Chain)	No
	IMM	$B220^{lo}$, $sIgM^{+}$, $sIgD^{-}$, $CD23^{-}$	BCR	No
	IMM	$CD19^{+}B220^{+}$ $sIgM^{+}$, $sIgD^{-}$, $CD23^{+}$	BCR	Br3
Transitional Subsets (Spleen)	T1	$IgM^{hi}CD23^{-}$ $B220^{int}AA4.1^{+}$	BCR	BR3 TACI
	T2	$IgM^{hi}CD23^{+}$ $B220^{+}A4.1^{+}$	BCR	TACI BR3
	T3	$IgM^{lo}CD23^{+}$ $B220^{+}AA4.1^{+}$	BCR	TACI BR3
Mature Primary Subsets	FO	$IgM^{lo}CD23^{+}$ $B220^{hi}$ $AA4.1^{-}$	BCR	TACI BR3
	MZ/ MZP	$CD9^{+}IgM^{hi}IgD^{lo}CD23^{-}CD21^{+}$	BCR	BR3 $TACI^{hi}$
	B1	$CD43^{+}CD23^{-}CD5^{+/-}$	BCR	No?

VI. The Pim family kinases

Recent work demonstrates a role for Pim kinases in antigen receptor signaling (121, 122). The *Pim-1* gene encodes a serine/threonine kinase that was identified as a frequent target locus for Moloney Murine Leukemia Virus retroviral insertion in T cell lymphomas (123, 124). MMLV insertion downstream of the coding region of *Pim-1* results in a highly stabilized mRNA, aberrant gene activation and subsequent transformation (124, 125). Two additional genes, *Pim-2* and *Pim-3*, were subsequently identified by sequence homology. As with Pim-1, *Pim-2* is also expressed in hematopoietic cells, whereas Pim-3 is expressed in non-hematopoietic cells (124, 126). In addition to promoting lymphomagenesis, Pim kinases are associated with a number of other pathologies, inducing polyploidy or more generalized cell cycle control defects (127-129).

A. Pim kinases and cell cycle regulation

All three Pim kinases are members of a larger group of survival kinases whose constitutive expression counters pro-apoptotic signals (121). Pim kinase activity is known only to be regulated transcriptionally. Once expressed, the Pim kinases are constitutively active, as they have no known regulatory elements (121, 130). Pim-1 is involved in cell cycle regulation: loss of function mutations result in G1 checkpoint arrest, overexpression yields aberrant cycle progression with loss of growth factor requirements (131). Pim-dependent effects on cell cycle progression operate by down-regulating the activity of both p21(WAF1) and p27(Kip1) (131-133). Specifically, lymphocytic turmorogenesis associated with the overexpression of Pim-1 is linked to its dysregulation of p21 and inactivation of the pro-apoptotic factor Bad (122, 134).

Despite these apparent roles in cell cycle regulation, no major phenotypic defects have been reported in animals rendered deficient for Pim1, Pim-2, or both (135, 136). In

T cells, Pim-1 is up-regulated following stimulation or cytokine treatment and Pim kinases are part of one of two redundant signaling pathways required for response to CD3 and CD28 signals in vitro (137).

B. Pim kinase signaling pathway and BLyS-mediated survival

Recently, work by the Thompson lab and others demonstrated a role for Pim2 in T cell survival independent of the AKT/mTOR signaling pathway. In B cells, Pim2 was shown to work in parallel with the AKT/mTOR pathway to promote the survival and resistance to atrophy of resting cells stimulated with the obligate B cell survival factor BLyS (138). BlyS-mediated B cell survival in the presence of rapamycin, an AKT/mTOR inhibitor, was dependent on Pim2. When Pim-deficient B cells were cultured with rapamycin, the addition of BLyS did not improve survival, whereas Pim-sufficient B cells were able to utilize BLyS to improve survival. Signaling events downstream of Pim2 facilitated the production of the anti-apoptotic protein Mcl-1 as a mechanism for enhanced survival. Thus, in both T cells and B cells, Pim kinases work in parallel with AKT/mTOR to protect cells against apoptosis.

The process of antigen activation in vivo renders lymphocytes susceptible to apoptosis while markedly increasing their dependence on sustained metabolic activity. Because of the critical role of the Pim kinases in controlling these activities in resting and activated lymphocytes, we wanted to determine if they played a continuing role in the processes of B cell activation, proliferation and differentiation leading to antibody synthesis. To assess the role of Pims we employed mice with targeted mutations in the *Pim* 1 and 2 genes ($Pim1^{-/-}Pim2^{-/-}$.)

C. Pim Kinases and the SLPC fate decision

B cell biology is dominated by the function of the BCR and BLyS family receptors, however important these receptor molecules may be, they rely on a number of signaling proteins to mediate their function. Here we describe the results of the characterization and challenge of animals lacking two of these molecules, and how these results illustrate the important role of Pim kinases in various aspects of humoral immunity. These data support a connection between the short-lived plasma cell fate decision and BLyS receptor function in survival through the Pim kinase signaling molecules.

VII. Results

Despite earlier reports indicating a lack of severe phenotype for Pim1$^{-/-}$Pim2$^{-/-}$ mice, we find severely limited serum Ig levels, indicating that a B cell phenotype exists in association with antibody forming cells. Pim-deficient animals also lack peritoneal B1a B cells as well as natural antibody. Further, these mice fail to mount SLPC responses to a number of immune challenges. This deficiency was found for responses to T cell dependent and T cell independent (type 1 or type 2) antigens. Despite the lack of an early SLPC response, GC reactions in the spleen following immunization with TD antigens were largely intact and resulted in high affinity, class switched antibody. These data show that Pim kinases are vital to control aspects of a normal humoral immune response.

A. Pim1$^{-/-}$Pim2$^{-/-}$ mice have little serum Immunoglobulin

Prior results show that Pim2 is necessary for rapamycin resistant survival of resting mature B cells downstream of BLyS (138). Pim1 and 2 kinases have also been reported to play a role in rapamycin resistant T cell blastogenesis as well as activation (122). Whether or not Pim kinases are important for the activation and maintenance of B cells producing a humoral response has not been investigated. Previous work demonstrates that animals deficient in hematopoietic Pim kinases show little or no impairment in their immune phenotypes. Recently evidence links these proteins to signaling events downstream of BLyS receptors in B cells. Given the importance of BLyS signaling in B cell biology, we examined the B cell phenotype of these animals more closely. Despite the lack of any previously reported phenotype, we found that immunoglobulin titers in Pim1$^{-/-}$Pim2$^{-/-}$ mice (IgM, IgG and IgA) were significantly reduced (**Figure 3**.)

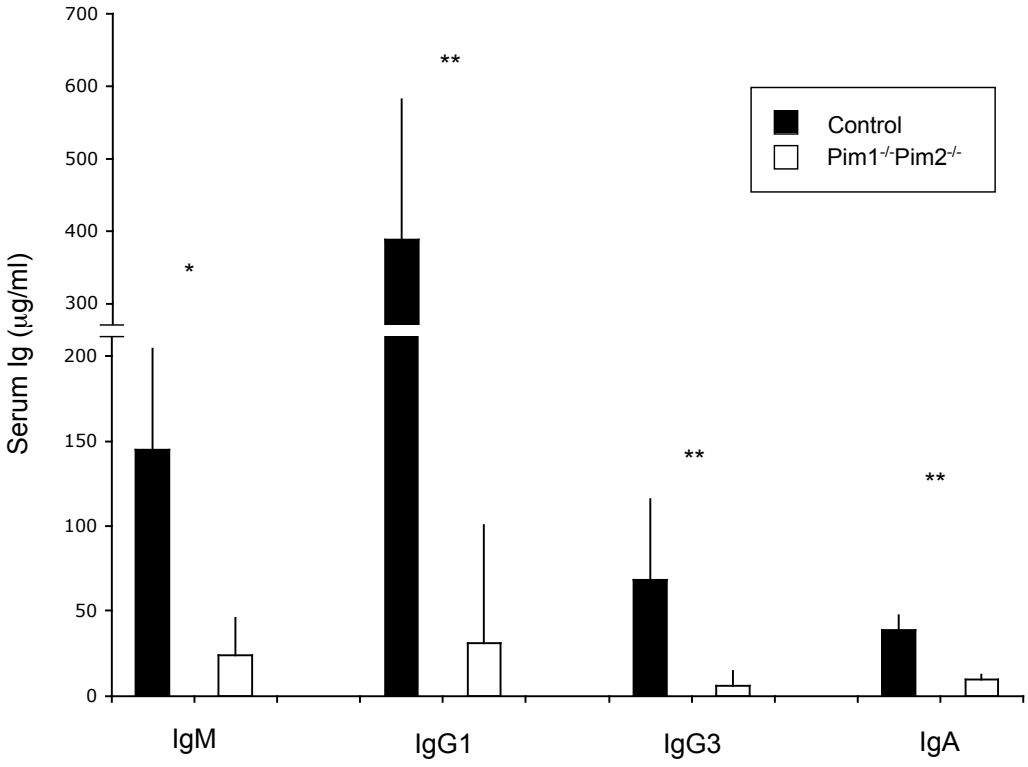

Figure 3: Pim1$^{-/-}$Pim2$^{-/-}$ animals have reduced serum immunoglobulin. Serum Ig levels for IgM,IgG1,IgG3 and IgA were assessed by sandwich ELISA. Plates were coated with goat anti-mouse Ig(light chain), serum was added over a serial dilution and then immunoglobulin were detected using isotype-specific antibody coupled with biotin and extra-avidin-HRP followed by substrate. *p<0.05, **p<0.01

B. Primary B cell pools develop normally in Pim1$^{-/-}$Pim2$^{-/-}$ mice.

To assess the impact of pim-1/2 deficiency on primary B cell pools, the proportions and magnitude of all splenic and bone marrow B cell populations were determined in normal and knockout mice. Little difference in resting populations were found between heterozygous control (Pim1$^{-+/-}$Pim2$^{+/-}$) or Pim1$^{-/-}$Pim2$^{-/-}$ animals. BM precursor populations (ProB, Immature B and recirculating Mature B) were found at normal frequencies, while PreB cells were found to be reduced, similar to reports of Pim1$^{-/-}$Pim2$^{-/-}$Pim3$^{-/-}$ animals (**Figure 4**.) Within the PreB cell compartment, large (cycling) cells can be further distinguished from small (non-cycling) cells by their larger, more granular scatter profiles. Enumeration of cells in each B cell precursor compartment reveals a reduction in absolute number, as well as proportion of small and large PreB cells (**Figure 5**.)

Peripheral follicular (FO), marginal zone (MZ) and transitional populations (as defined by Allman et al 2001(139)) were also present in proportions and absolute numbers similar to control animals (**Figure 6**.) We further examined the spleen and peritoneal cavity for B1 B cells and found that, splenic B1 B cells were present in normal frequency and numbers (**Figure 7**) while peritoneal B1a B cells, but not B1b B cells, were reduced in Pim1$^{-+/-}$Pim2$^{+/-}$ mice (**Figure 8**.) We assessed anti-phosphocholine (PCh) titer and found that serum from Pim1$^{-/-}$Pim2$^{-/-}$ animals contained little of none of this example of natural antibody (**Figure 8**.)

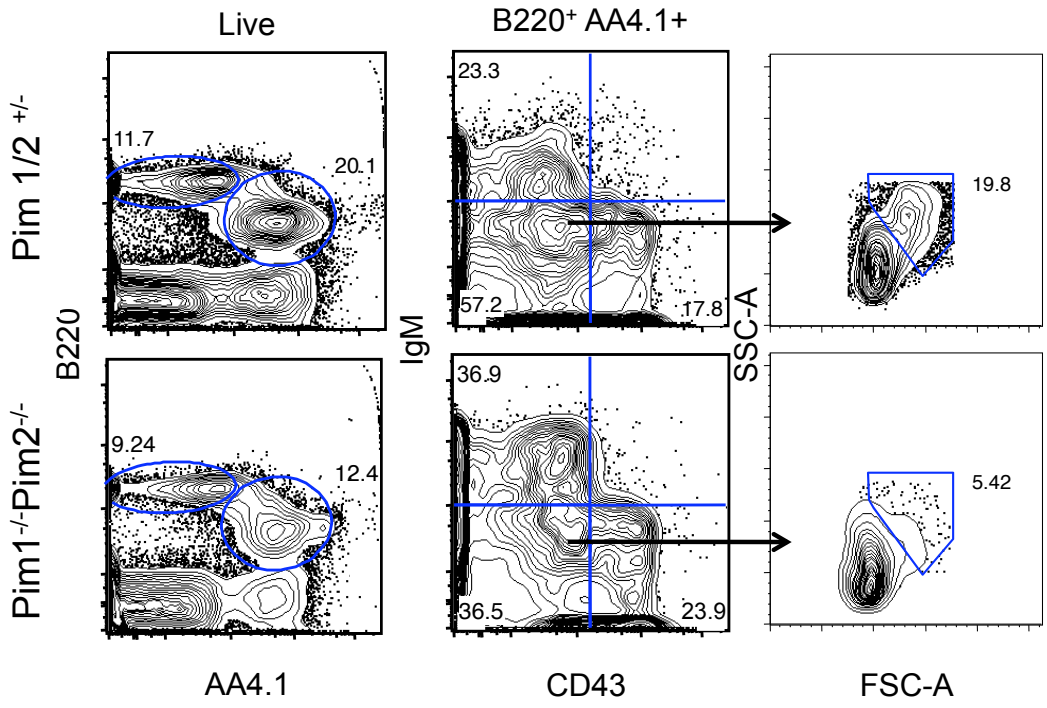

Figure 4: Early B cell development in Pim1$^{-/-}$Pim2$^{-/-}$ animals shows a deficiency in Pre B cells. BM cells were harvested and stained to identify B cell precursor populations by flow cytometry. Representative FACS plots of BM B cell populations ProB (CD43^{+}B220^{int}) PreB(B220^{+}CD43^{-}IgM^{-}IgD^{-}), Immature B(B220^{+}CD43^{-}IgM^{+}IgD^{-}) and Mature B (B220^{+}CD43^{-}IgM^{+}IgD^{+}). Noncycling, small PreB cells are FSC-A^{lo}SSC-A^{lo}, Cycling, Large PreB cells are FSC-A^{mid}SSC-A^{mid}.

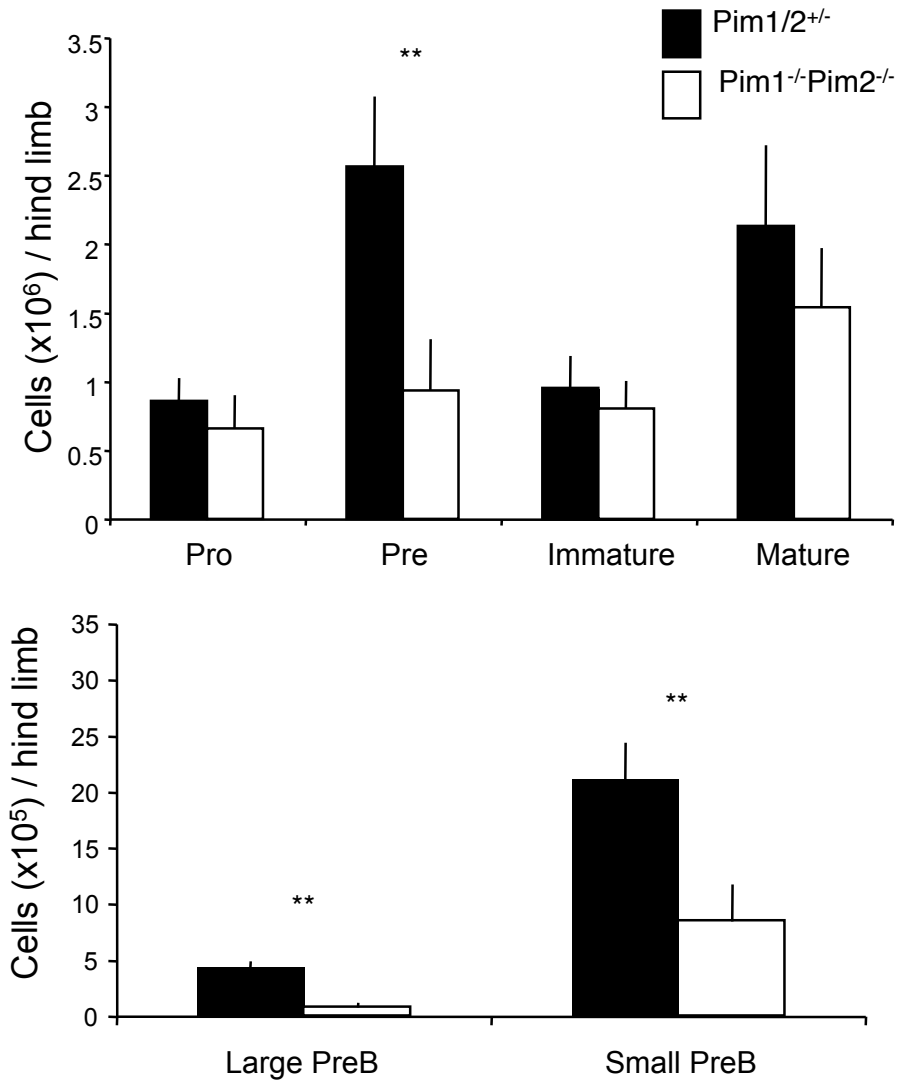

Figure 5: Absolute numbers of B cell progenitor populations in Pim1$^{-/-}$2$^{-/-}$ animals confirm the Pre B cell deficiency. Absolute numbers of BM precursor populations were identified by FACS using counts obtained from a single hind limb of control (Pim1/2$^{+/-}$) or Pim1$^{-/-}$Pim2$^{-/-}$ animals. **P<0.01

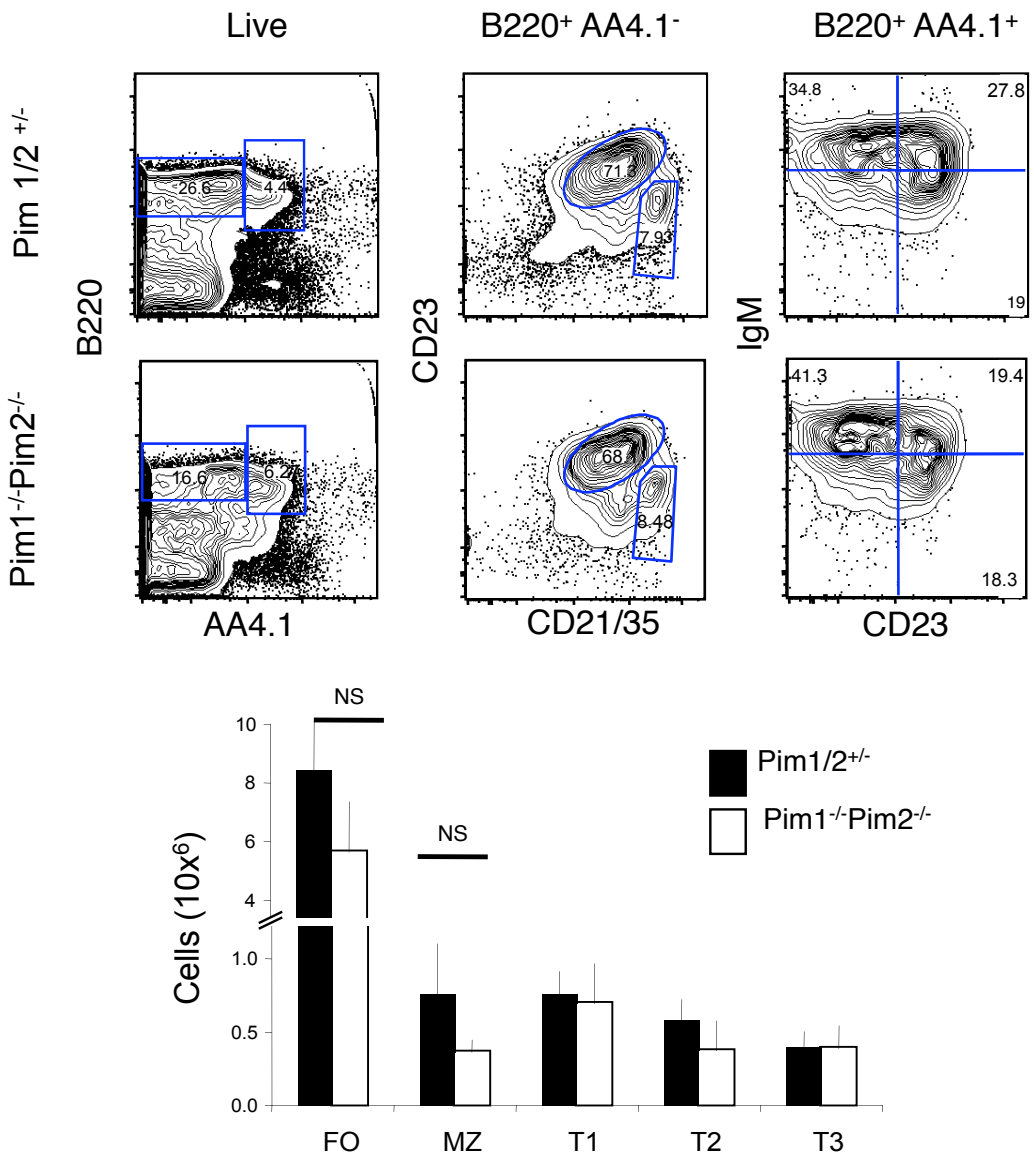

Figure 6: Splenic B cell subsets of Pim1$^{-/-}$Pim2$^{-/-}$ animals show proportions and numbers comparable to control animals. Spleen cells were harvested, RBCs were lysed and remaining cells were stained with antibodies to identify peripheral B cell populations. Representative FACS plots of peripheral mature FO (B220^{+}AA4.1^{-}CD21/35^{int} IgM^{int}) MZ (B220^{+}AA4.1^{-}CD21/35^{hi}IgM^{hi}) and immature T1 (B220^{+}AA4.1^{+}IgM^{+}CD23^{-}) T2 (B220^{+}AA4.1^{+}IgM^{+}CD23^{+}) and T3 (B200^{+}AA4.1^{+}IgM^{mid}.) Total numbers (x10^{6}) of B cell fractions were determined from spleen counts and graphed.

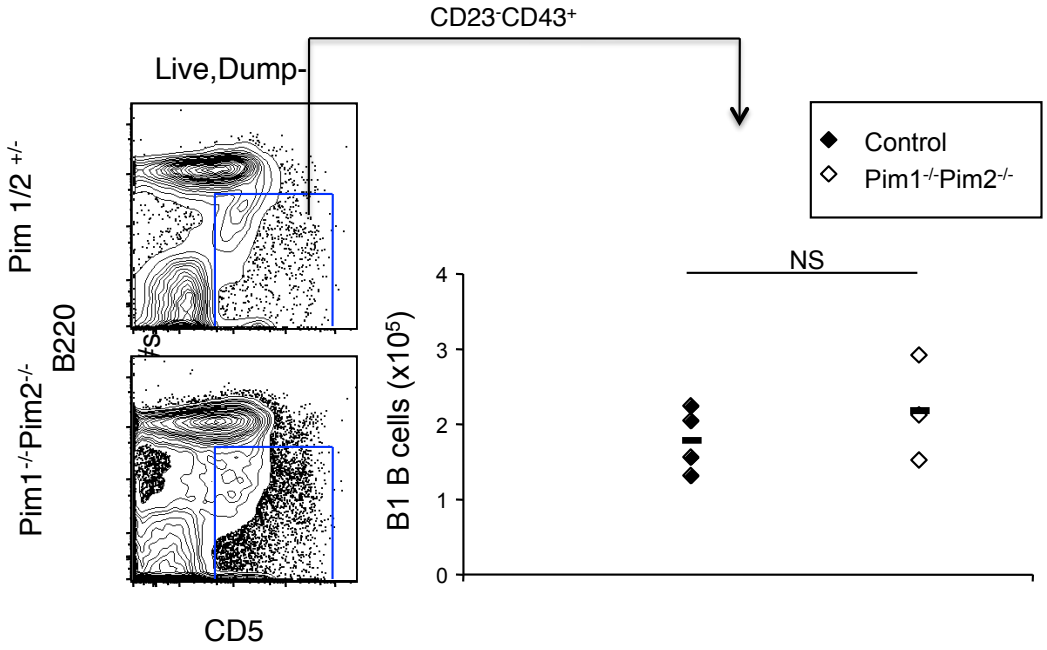

Figure 7: Pim1$^{-/-}$Pim2$^{-/-}$ animals have normal proportions and numbers of splenic B1 B cells. Splenocytes were harvested, RBCs were lysed and remaining cells were stained to identify splenic B1 populations. Representative FACS plots of splenic B1 B cells dump- (CD4-CD8-GR1-F4/80-) CD23-CD43+B220-CD5+ are shown. Total numbers of splenic B1s were determined from spleen counts and graphed.

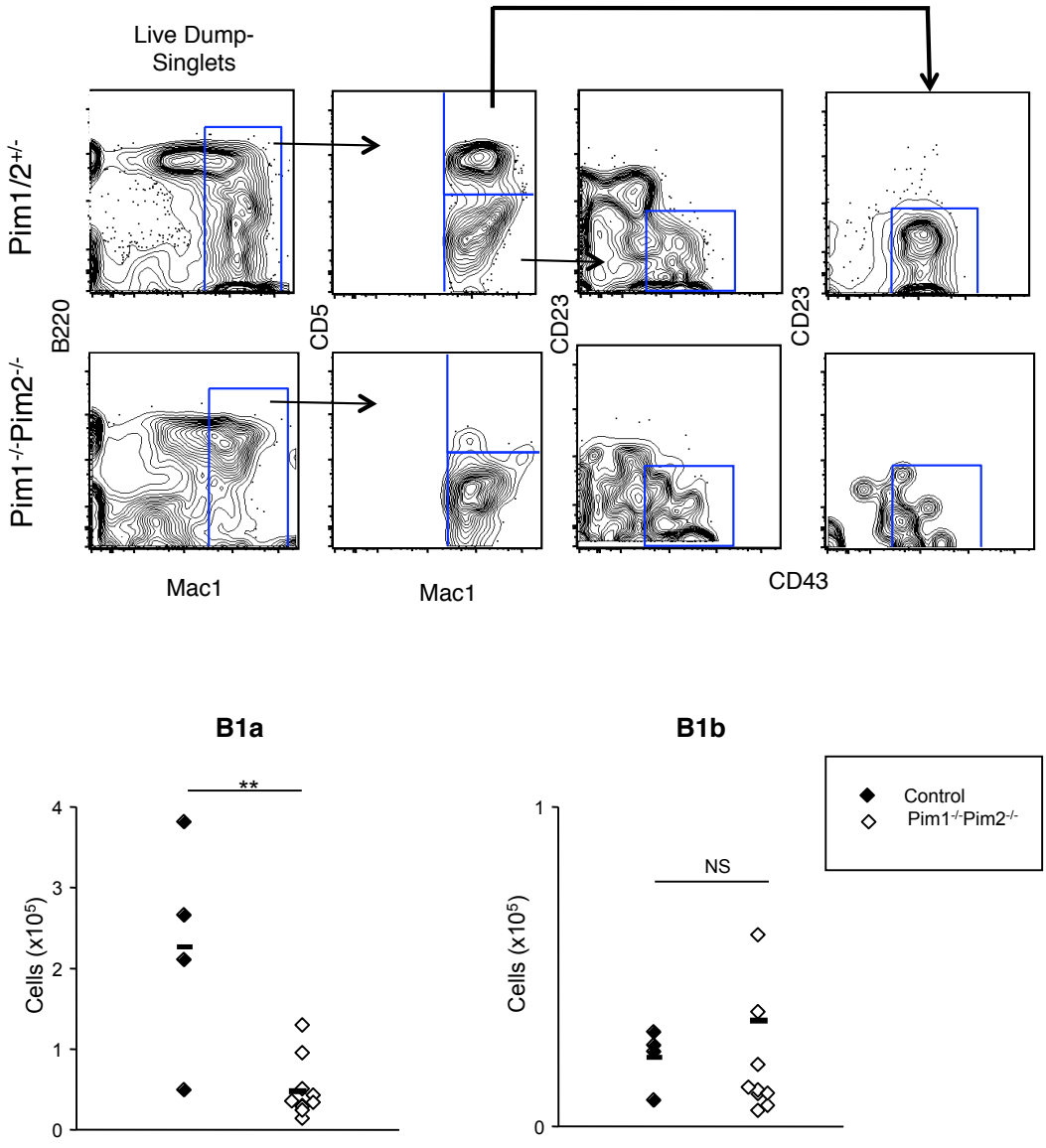

Figure 8: Pim1$^{-/-}$Pim2$^{-/-}$ animals lack peritoneal B1a but not B1b B cells. The peritoneal cavity was lavaged and cells were stained to identify B1 B cell populations. Peritoneal B1 B cells (CD4-CD8-GR1-F4/80-Mac1+) were further parsed into B1a (CD23-CD43+CD5+) and B1b (CD23-CD43+CD5-.) Enumeration of B1a and B1b B cell populations were determined from peritoneal cavity counts. **p<0.01

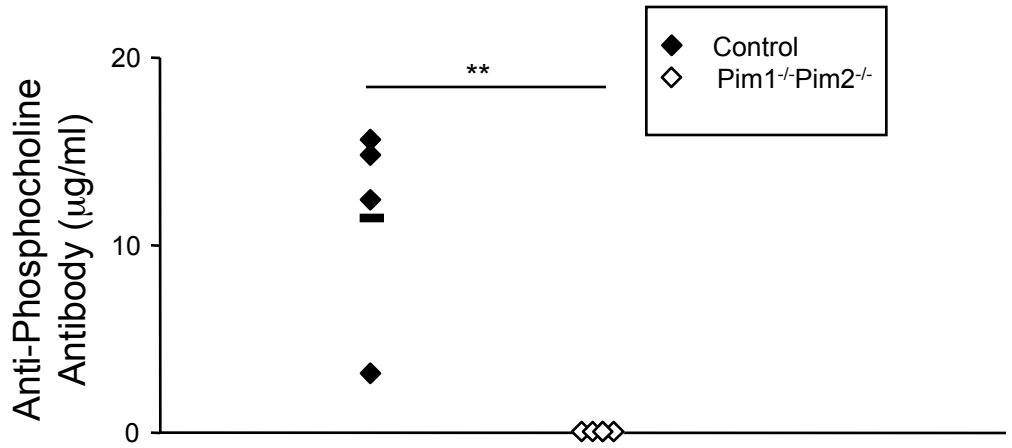

Figure 9: Pim1$^{-/-}$Pim2$^{-/-}$ animals have no detectible natural antibody. Animals were bled and serum was purified by centrifugation. Total anti-phosphocholine antibody was assessed by sandwich ELISA using plates coated with Phosphocholine and detected with anti-Ig (Heavy and Light)-AP + substrate. **p<0.01

C. TI-2 responses require Pim kinases

An integral feature of the humoral immune response is blasting of selected clones following BCR binding of cognate antigen. Because of Pim kinases' involvement in cell cycle, we assessed whether these animals could mount a normal immune response. To examine TI-2 reactions, we immunized animals intraperitoneally with NP-Ficoll and harvested spleens after 5 days. Single knockouts for either Pim1 or Pim2 generated AFCs and displayed normal serum titers in responses to challenge (compared to Pim1$^{+/-}$ Pim2$^{+/-}$), while animals lacking both Pim kinases had significantly attenuated responses, producing minimal NP-specific antibody secreting cells AFCs and little or no NP-specific antibody in the sera (**Figure 10-11**).

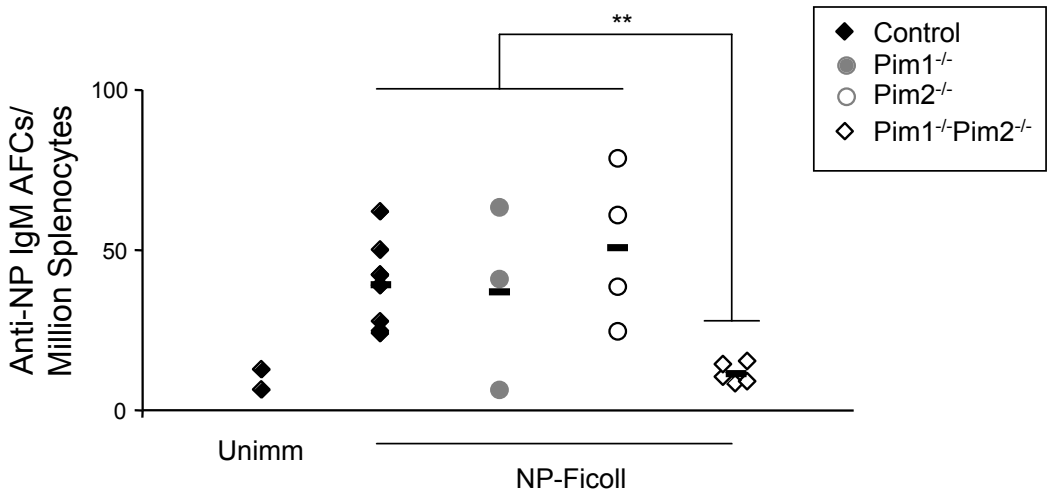

Figure 10: Pim1$^{-/-}$Pim2$^{-/-}$ B cells are deficient in AFC response following TI-2 challenge. ELISpot was performed with isolated splenocytes using plates coated with NP33 as a capture reagent and anti-Igμ-biotin + Extravadin-AP and substrate to detect secreting cells. Unimmunized and immunized controls, Pim1$^{-/-}$, Pim2$^{-/-}$ and Pim1$^{-/-}$Pim2$^{-/-}$ splenic AFCs d5 post NP-Ficoll immunization were analyzed. **p<0.01

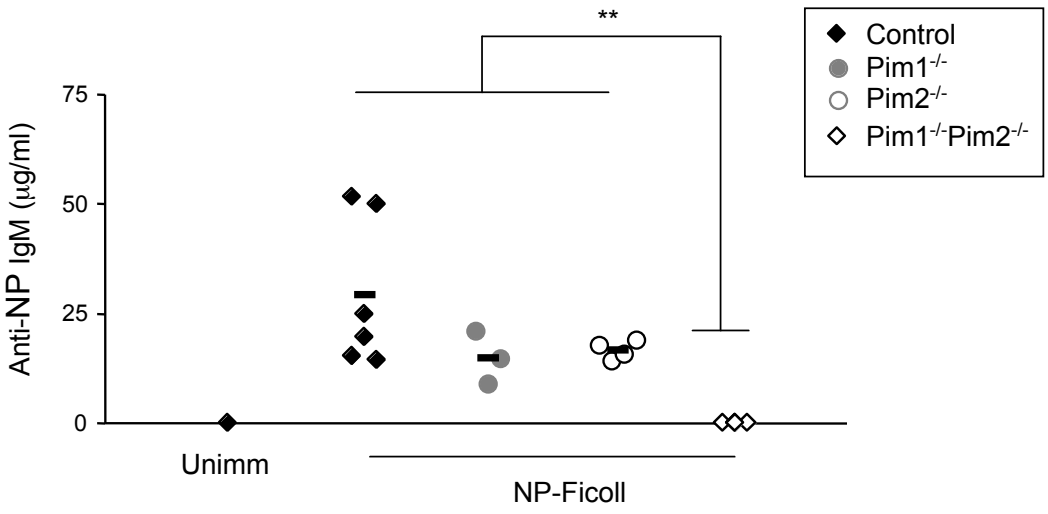

Figure 11: Pim1$^{-/-}$Pim2$^{-/-}$ B cells are deficient in generating antigen-specific antibody following TI-2 challenge. NP-Specific IgM ELISA was performed on serum from Pim1$^{-/-}$ and Pim2$^{-/-}$ single knockouts and Pim1$^{-/-}$Pim2$^{-/-}$ double knowckouts. Splenic AFCs were analyzed d5 post NP-Ficoll immunization. Plates were coated with NP33 as a capture reagent and anti-Igμ-HRP + substrate was used for detection. **p<0.01

In C57BL/6 animals, the NP response is dominated by BCRs expressing lambda light chain. It has been recently shown that the proportion of lambda+ cells are reduced in immature B cells in the bone marrow (BM) of Pim1$^{-/-}$Pim2$^{-/-}$ mice (140). We did not find a similar reduction in lambda+ mature FO B cells (data not shown.) However, to exclude this reduction as the basis of the TI-2 defect we immunized animals with a (T,G)AL peptide ficoll derivative, TG4-Ficoll, that did not favor lambda+ responding cells. We found the TI-2 response to TG4 was similarly diminished in Pim1$^{-/-}$Pim2$^{-/-}$ mice confirming

that the deficiency was not specific to NP or lambda+ B cells (data not shown, produced by K. Willems in the Woodland laboratory, University of Massachusetts).

Peak cellularity of TI-2 responses occurs at d4/d5 (141).To ensure that Pim deficient animals do not display a kinetic difference from wt animals, we assayed for antibody and AFCs at 3, 5, 7, and 10 days post challenge. The lack of response persists throughout this timeframe, indicating no kinetic explanation for the failure of response (Data not shown, produced by K. Willems in the Woodland laboratory, University of Massachusetts).

D. TI-1 responses require Pim kinases

To determine whether the lack of PC generation was specific to BCR signaling, we extended our analysis of Pim requirements for B cell responses to TI-1 antigens. $Pim1^{-/-}Pim2^{-/-}$ and control mice were immunized with FITC-LPS, antibody responses were assessed at d5 by ELISA (Data not shown, produced by K. Willems in the Woodland laboratory, University of Massachusetts.) Similar to the TI-2 defect, antibody responses to FITC were significantly reduced in $Pim1^{-/-}Pim2^{-/-}$ mice when compared to those of control animals. These results demonstrate that Pim-deficient animals fail to generate SLPC responses regardless of activation stimulus.

Altogether, these data support a requirement for Pim kinases in mediating SLPC responses to T independent stimuli and that these kinases have redundant roles in responding cells (at least in TI-2 responses.)

E. TD responses are reduced in animals lacking Pim kinases

To assess the T dependent response of Pim-deficient animals, we immunized $Pim1^{+/-}Pim2^{+/-}$ and $Pim1^{-/-}Pim2^{-/-}$ mice with NP-CGG in alum. In addition to markers to identify cell populations, splenocytes were stained both intracellulary and extracellularly for lambda and NP binding in order to visualize all responding cells. At day 7, control animals had numerous SLPCs secreting NP-specific IgM while $Pim1^{-/-}Pim2^{-/-}$ animals had few AFCs and produced little serum antibody (**Figures 12-13**.) Although similar to the response seen following a TI-2 immunization, the defect was not as severe.

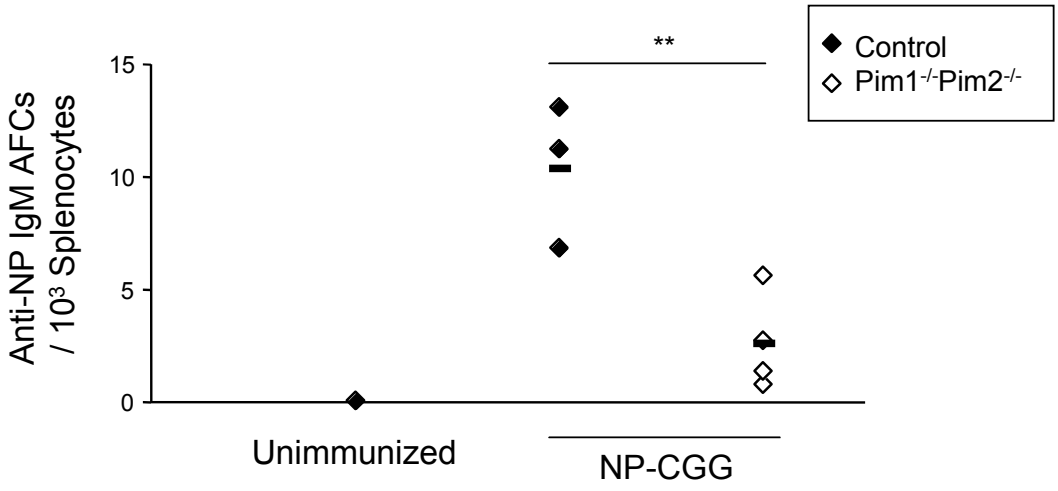

Figure 12. $Pim1^{-/-}Pim2^{-/-}$ animals make few antigen-specific AFCs at d7 following immunization with NP-CGG. ELISpot were performed with isolated splenocytes using plates coated with NP33 as a capture reagent. Anti-Igμ-biotin + Extravadin-AP and substrate were used to detect secreting cells. Unimmunized and immunized controls and $Pim1^{-/-}Pim2^{-/-}$ splenic AFCs were analyzed d7 post NP-CGG immunization. **p<0.01

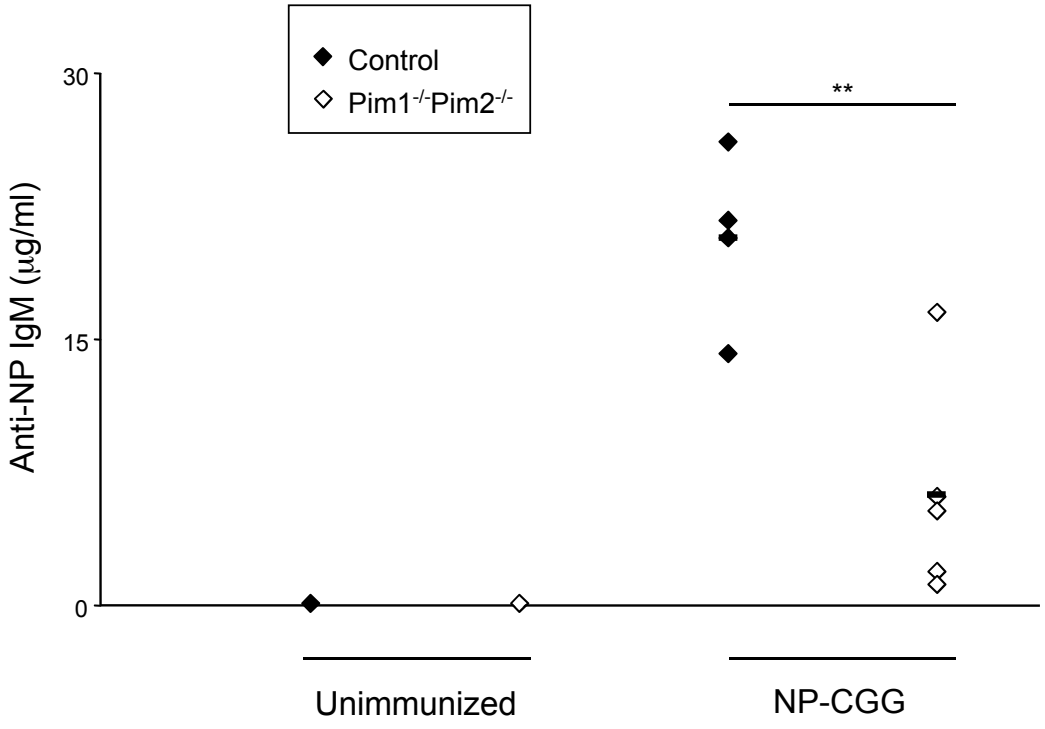

Figure 13. Pim1$^{-/-}$Pim2$^{-/-}$ animals make limited antigen-specific antibody at d7 following immunization with NP-CGG. IgM ELISA was performed with serum from animals at d7 post NP-CGG immunization. Plates were coated with NP33 as a capture reagent and anti-Igμ-HRP + substrate was used for detection. **p<0.01

To specifically address whether GCs were formed in Pim-deficient animals, we examined spleen sections and splenocytes from NP-CGG-immunized animals at d10, during the peak of GC reactions (142). Immunohistochemistry was performed on spleen sections for these animals for the presence of PNA-binding GCs located at the B cell follicle / PALS interface. Bright field microscopy at low magnification shows numerous GCs in the spleens of control and Pim1$^{-/-}$Pim2$^{-/-}$ animals (**Figure 14**). High magnification immunofluorescence demonstrates normal organization of GC cells between CD3-staining T cells and B220-stained B cells (**Figure 15**.) Flow cytometric analysis of Pim1$^{-/-}$ Pim2$^{-/-}$ GC cells (B220^{+}IgD^{-}PNA^{+}Fas^{+}) which are also lambda+ (a surrogate marker for NP-specificity) are not significantly different from wt responders (**Figures 16-17**.)

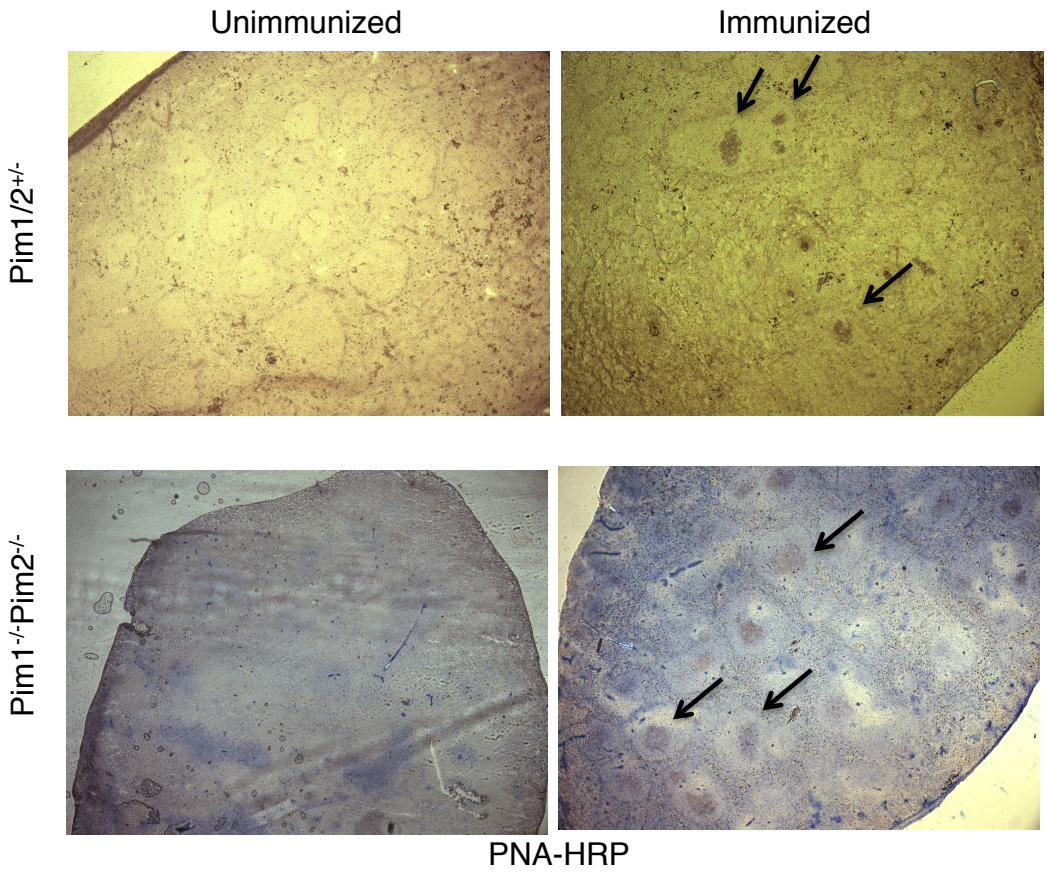

Figure 14: Pim1$^{-/-}$Pim2$^{-/-}$ animals generate germinal centers following TD challenge. Spleens were harvested at d10 following NP-CGG immunization and frozen in OCT solution under 2-methyl butane. Sections were cut at 5-8 micron thickness and fixed with acetone. Sections were then stained with PNA-AP followed by substrate. Bright field immunohistochemistry of spleen sections displays normal germinal center responses indicated by PNA staining regions (arrows.)

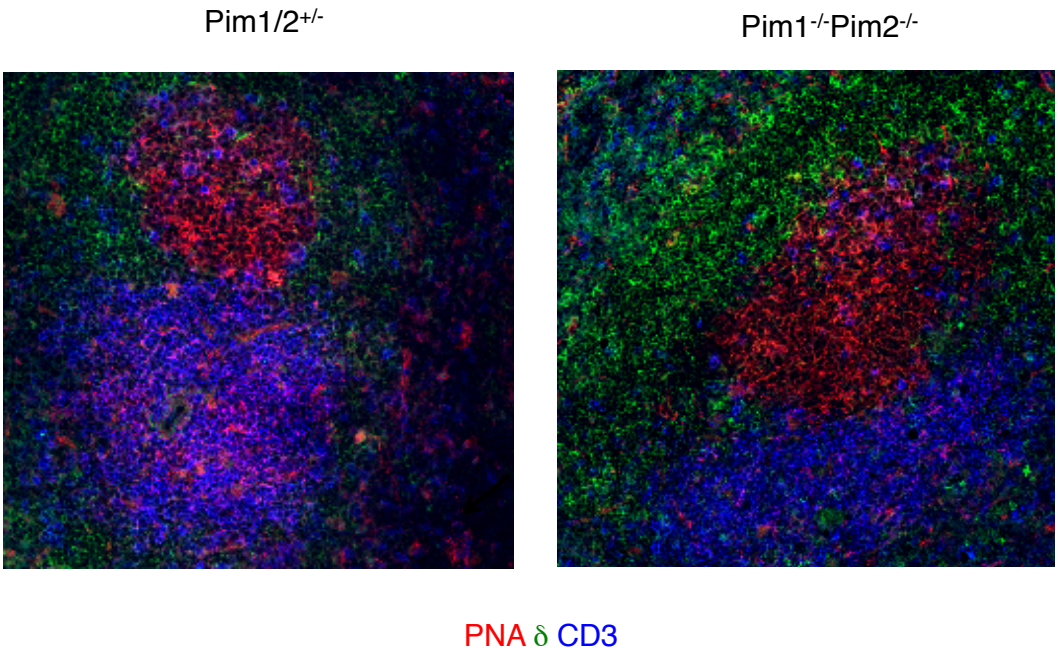

Figure 15: Pim1$^{-/-}$Pim2$^{-/-}$ animals generate germinal centers with normal architecture following NP-CGG immunization. Spleens were harvested at d10 following NP-CGG immunization and frozen in OCT solution under 2-methyl butane. Sections were cut at 5-8 micron thickness and fixed with acetone. Sections were then stained with antibodies (as indicated.) Immunofluorescence of spleen sections at d14 post NP-CGG immunization display normal germinal center architecture. Spleens were stained with antibodies for CD3-Alexa555 (blue), IgD-FITC (green) and PNA-Rhodamine (red.)

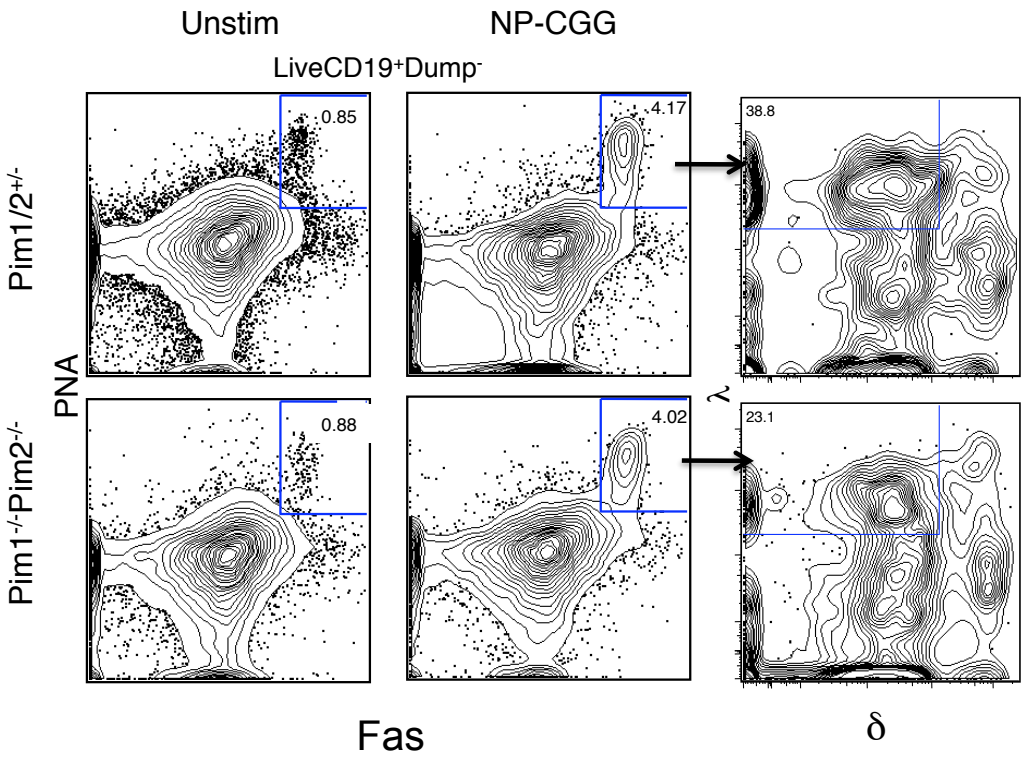

Figure 16: Pim1$^{-/-}$Pim2$^{-/-}$ animals generate germinal centers at d10 post NP-CGG. Spleens were harvested at d10 following NP-CGG immunization. Splenocytes were isolated as previously described and RBCs were lysed. FACS analysis identified germinal center cells as CD19^{+}CD4^{-}CD8^{-}GR1^{-}F4/80^{-}fas^{+}PNA^{+}IgM^{+}. NP-CGG-immunized GCs were further confirmed by IgD^{-} λ^{+} staining.

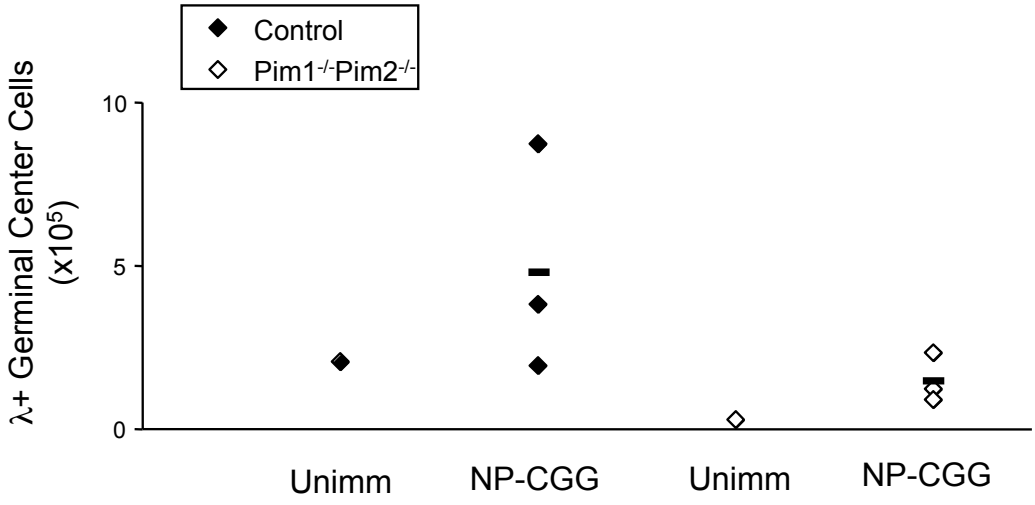

Figure 17: Pim1$^{-/-}$Pim2$^{-/-}$ animals generate germinal centers cells following NP-CGG immunization. Enumeration of total splenic λ+ germinal center cells at d10 post NP-CGG were determined from absolute spleen counts and frequencies identified in figure 16.

At day 14 post immunization, SLPC numbers had reduced in the spleen of control and Pim deficient animals (**Figure 18**) reflecting a defect, rather than delay, in the SLPC response of Pim-deficient animals. Despite the diminished IgM response, high affinity, antigen-specific IgG-secreting AFCs are found in Pim1$^{-/-}$Pim2$^{-/-}$ animals (**Figure 19**.) High affinity antibody is found in the serum, but at lower concentration than control animals' (**Figure 20**.)

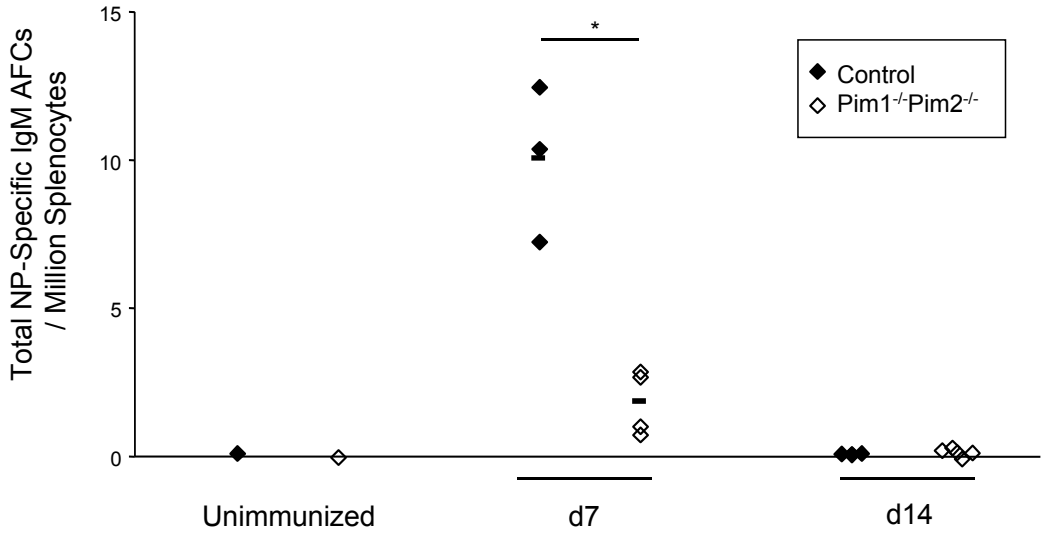

Figure 18: Low affinity SLPC responses in Pim1$^{-/-}$Pim2$^{-/-}$ animals peak at d7. ELISpot was performed for low affinity IgM-secreting AFCs at d7 and d14 post NP-CGG. Splenocytes were isolated and enumerated as previously indicated. Serial dilutions of cells were plated on NP-33-coated HTS plates and allowed to incubate undisturbed for four hours at physiologic temperature. Cells were then washed away and secreted antibody was detected using anti-IgM-biotin followed by SA-AP and substrate. Spots indicating antibody-forming cells were enumerated on an ELISpot reader. *$p<0.05$

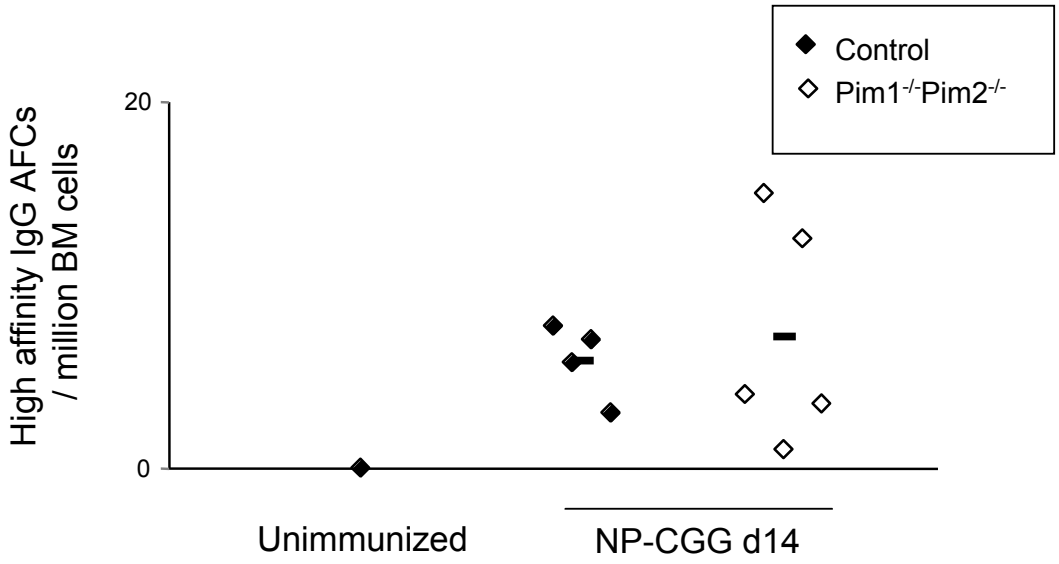

Figure 19: Pim1$^{-/-}$Pim2$^{-/-}$ animals have high affinity AFCs at d14. ELISpots were performed for high affinity NP-specific IgG AFCs d14 post immunization. Splenocytes were isolated and enumerated as previously indicated. Serial dilutions of cells were plated on NP-3-coated HTS plates and allowed to incubate undisturbed for four hours at physiologic temperature. Cells were then washed away and secreted antibody was detected using anti-IgM-biotin followed by SA-AP and substrate. Spots indicating antibody-forming cells were enumerated on an ELISpot reader. No significant difference was seen between control and Pim1$^{-/-}$Pim2$^{-/-}$ sera.

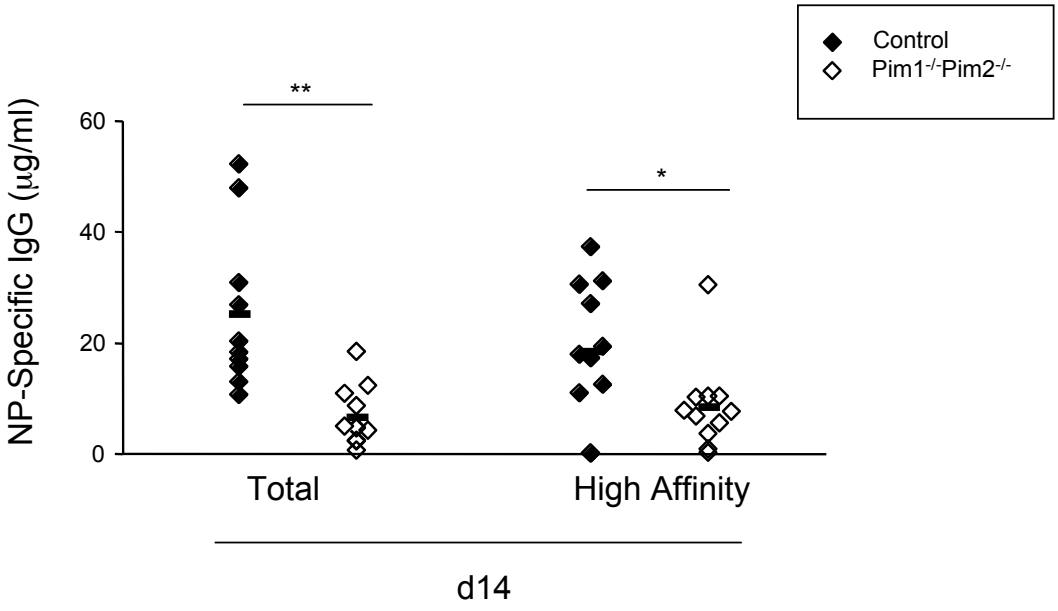

Figure 20: Pim1$^{-/-}$Pim2$^{-/-}$ animals generate high affinity, class switched antibody by d14. ELISA of high affinity NP-specific IgG was performed on serum from NP-CGG immunized aninmals at d14 following NP-CGG immunization**.** Antibody was detected using anti-IgG-biotin + extravidin-HRP and substrate. *p<0.05,**p<0.01

F. TACI is required for Pim kinase regulation by BLyS/APRIL

As discussed above, receptors for BLyS family survival cytokines are modulated following activation of naïve B cells. To assess whether differential expression of these receptors might be upstream of Pim kinase regulation, we assayed for Pim expression following APRIL or BLyS stimulation. At various timepoints, Pim1 and / or Pim2 messages were measured in wt cells following each stimulus.

Naïve FO B cells express BR3 and TACI. Of these two receptors, only TACI binds APRIL. Wt or TACI-/- B cells were co-cultured with APRIL and harvested for analysis of Pim1 message levels at 10, 60 and 90 minute timepoints. Message is only elevated in

response to APRIL in B cells expressing TACI (**Figure 21**) indicating that TACI signaling does positively regulate Pim1.

To determine whether BR3, specifically, is also capable of regulating Pim message we stimulated cultured B cells with BLyS, which can bind either BR3 or TACI. By using both control cells bearing both receptors and cells from a TACI-/- animal that bear only BR3, we could establish whether BR3 can modulate Pim kinase message without TACI signals. Cells were harvested for message analysis after 12 or 18 hrs of culture. We find that only TACI, but not BR3, is capable of regulating Pim message (**Figure 22**.)

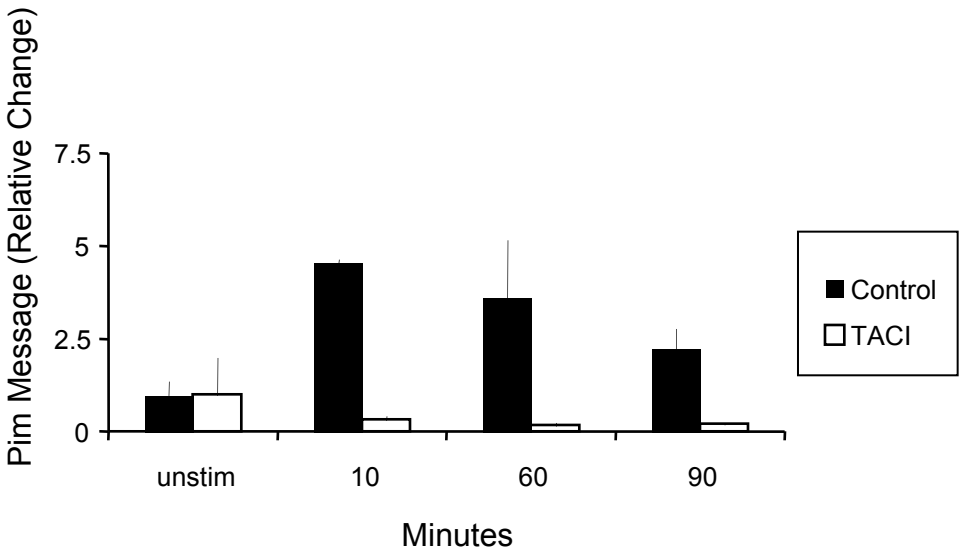

Figure 21: TACI signaling up-regulates Pim1 message in B cells. RT-PCR of Pim1 message was performed at various timepoints following stimulation of B cells with APRIL in vitro**.** Splenocytes were harvested and CD23+ (B cells) were isolated using CD23-biotin and MACS beads. Cells were plated in RPMI and allowed to rest prior to addition of 1μg/ml APRIL. At the indicated time cells were harvested into lysis buffer and RNA was isolated. Pim1 message was assessed by qPCR.

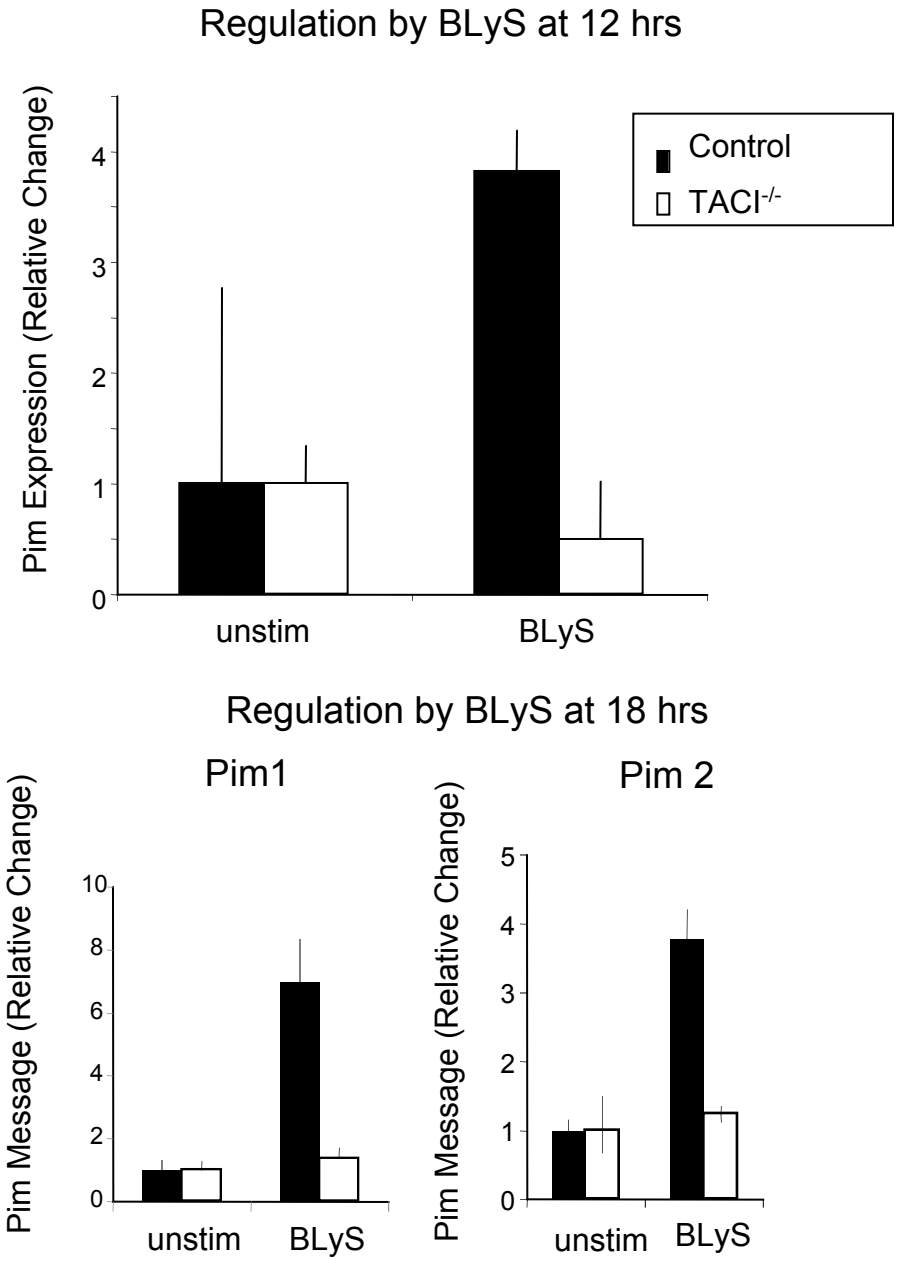

Figure 22: TACI, but not BR3, signaling up-regulates Pim1 message in B cells. RT-PCR of Pim1 message was performed at 12 or 18 hrs following stimulation of B cells with BLyS in vitro**.** Splenocytes were harvested and CD23+ (B cells) were isolated using CD23-biotin and MACS beads. Cells were plated in RPMI and allowed to rest prior to addition of 0.1μg/ml BLyS. At the indicated time cells were harvested into lysis buffer and RNA was isolated. Pim1 and Pim2 messages were assessed by qPCR.

VIII. Discussion

The studies described herein have investigated the roles played by Pim kinases in humoral immune responses. Despite the lack of a major B cell phenotype previously reported in Pim1$^{-/-}$Pim2$^{-/-}$ mice, we found that these animals display drastically reduced Ig titers. To elucidate the source of this defect we pursued two avenues of investigation. First, we completely characterized all developing and mature primary B cell populations in these animals. Second, we assayed the ability of these animals to respond to various types of immunizations. Our results show that despite a reduction in the pre-B cell compartment, the magnitude of most developing and primary B cell subsets is similar to wild-type controls. However, the Pim DKO lack of natural antibody-secreting peritoneal B1a B cells, and fail to generate SLPCs in both TI and TD responses. As a mechanism for this defect, we have intriguing evidence supporting a role for TACI in the regulation of Pim kinases required for SLPC response. Together these observations suggest a critical role for Pim kinases in the differentiation or survival of SLPCs.

In characterizing Pim deficient animals, we found that most of B cell development is comparable to control animals with one exception. In the BM, preB cells cycle following successful heavy chain rearrangement resulting in increased numbers of cells relative to the proB cell compartment. In Pim1$^{-/-}$Pim2$^{-/-}$ animals, this increase in preB cell numbers was not found. These animals have diminished proportions and numbers of small preB cells and have almost no large, cycling preB cells.

Numerous studies have established the role of Pim kinases in cell cycling, often by a generalized mechanism of phosphorylating residues that allows binding of nuclear export machinery and proteosomal degredation. One such mechanism is mediated by the phosphorylation of the CDK inhibitor p27, leading to binding and nuclear export by 14-3-3 and subsequent proteolysis (131, 134). Pim kinases further reinforce p27 inactivating

transcriptional promoters FoxO1a and FoxO3a (131). Together, these functions lead to CDK release and of cell cycle arrest. Pim kinases also regulate p21, which also binds cell cycle promoting proteins. However, when Pim kinases phosphorylate p21, this leads to the stabilization of nuclear p21 complexes also resulting in cell cycle progression (134). A third example occurs when Pim phosphorylation of PAPA-1 similarly leads to its degredation and release of G1 checkpoint arrest (143).

Therefore, the reduction of cycling PreB cells may not be a surprising finding. This reduction may reflect an inability of Pim-deficient cells to proliferate, cell death resulting from failed attempt to proliferate, rapid transit through the preB cells stage or a number of other causes. Given prior evidence of Pim kinase involvement in cell cycle, a failure to proliferate seems reasonable and may be resolved using BrdU incorporation or other cell cycle analysis techniques. Despite this reduction, at steady-state, the defect does not carry forward to later developmental populations. However, this homeostatic compensation is a common effect found in several models of diminished B cell production rates such as aging or induced ablation of developing cells (144, 145). Because the rate of B cell development is independent of the peripheral pool and that peripheral pool size and lifespan is dictated by the availability of BLyS, it is likely that a proliferative defect may reduce the rate that mature cells are generated (146). However, the relative availability of BLyS will promote longevity in this pool resulting in normal steady-state cell numbers. It is conceivable that this proliferative defect may result in diminished diversity of the pre-immune pool, however, this was not investigated in the present study.

What is remarkable is that the proliferative defect in the Pre B cell stage does not translate into an inability of cells to cycle following activation (at least by TD stimuli). This may be compensated by the presence of secondary stimuli such as CD40/CD40L interaction that promotes cell cycle by inducing cyclin dependent kinases and repressed the cell cycle inhibitor $p27^{kip1}$ (147).

B1 B cells have been extensively studied for several decades beginning with their identification by Hayakawa et al as 'Ly-1 B cells,' however much of their biology remains enigmatic (148, 149). They are known to be a distinct population from conventional, B2, B cells, unique in their ontogeny, receptor specificity and lifespan (150). B1 B cells are generated early in life, but are rapidly outnumbered once follicular B cells are produced. It is widely accepted that B1 B cells maintain their numbers by constant, slow cycling and that they constitutively secrete "natural" antibodies favoring proximal arrangements with germline specificities. Their early generation initially suggested that these cells were formed only in the fetal liver, however, more recent work has established that B1 precursors persist in adult animals, but in low numbers (151).

Within the B1 population there are a number of subsets that are primarily distinguished by their variable expression of markers such as CD5 or Mac-1. Additionally, B1 B cells are found both in the spleen and in coelomic cavities (e.g. peritoneum). Cells from each of these loci also exhibit distinctions, including in vitro survival and antibody production. When both cell types are isolated and grown in culture, peritoneal B1s exhibit extraordinary survival compared to both conventional B2 B cells and splenic B1s. Additionally, only B1s isolated from the peritoneal cavity constitutively produced IgM in culture (152). In our own laboratory, we have found that splenic and peritoneal B1 B cells also differ in their requirement for BLyS ligands. Specifically, splenic, but not peritoneal, B1 B cells are lost when animals are treated with BLyS and APRIL-sequestering soluble TACI-fc protein (118). Finally, B1 B cells from the spleen and peritoneal cavities are also distinct in their expression of the B cell differentiation factor, PU.1 (153). These data further show the similarity between splenic B1s and B2 B cells, while peritoneal B1 B cells, which do not express PU.1, may represent a completely unique lineage.

We have found further support for the distinction between B1 subsets in that animals deficient for Pim kinases completely lacked peritoneal B1a B cells, but not splenic or peritoneal B1b B cells. Consistent with the low Ig titers and lack of B1a B cells,

'natural antibody' was completely absent. Altogether, these results suggest that peritoneal B1a B cells are a separate lineage of cells from other B1 B cells, and that these cells alone are responsible for natural antibody production.

We did not investigate whether this lose of peritoneal B1a B cells was due to a developmental or survival defect. This distinction could be made by the assessment of neonate animals for the presence of these cells. At birth, the majority of B cells in the animal are B1 B cells produced in the fetal liver. One may predict that if the requirement for Pims is developmental, B1 B cells will be reduced from birth. Due to observations of reduced fetal liver size in $Pim1^{-/-}Pim2^{-/-}$ animals, we favor a conclusion that B1a's are a separate lineage requiring Pim kinases for their generation (C. Fox, personal communication.)

An alternate explanation would be that Pim kinases are a required signaling molecule in some aspect of the function of AFCs in particular. This could involve the initiation of the plasma cell fate by regulation of transcription factors such as Pax5 or BLIMP-1, which could be assessed more easily with follicular B cells stimulated in vitro. Although, recent publication that B1 B cells do not express BLIMP-1 may require investigation into what transcriptional regulator controls the antibody production program in B1 cells (154). Because B1a B cells constitutively secrete IgM, this could contribute to the Ig deficiency found in Pim-deficient mice, but it does not explain the reduction in other isotypes.

Further investigation of other antibody forming cells revealed a specific loss of SLPC generation / function following a variety of stimuli. TI and early TD responses share characteristics in their rapid production of extrafollilular SLPCs in the first week after immune challenge. Following this early response, follicular B cells engage in GC reactions that produce high affinity, class switched antibody. In the absence of Pim kinases, neither SLPCs nor the resulting low affinity antibody are generated. Because the defect appears downstream of both types of TI response (through the BCR in TI-2; TLRs

in TI-1,) and because these two types of receptors signal in very different manners, the requirement for Pims is unlikely to be due to signals immediately associated with the receptors. Rather, it is more likely to be connected with a more general feature of the plasma cell response or antibody generation.

One possible explanation is that TI responses have been proposed to be functions of MZ and/or B1 B cells. As we have shown in our characterization, MZ B cells are not lacking in $Pim1^{-/-}Pim2^{-/-}$ animals. Further, Tanigaki et al have shown that TI responses may occur even in animals lacking MZ cells (155). Using $CD19^{-/-}$ animals that specifically lack B1a B cells, Haas et al demonstrated by transfer of serum that it is the presence of 'natural' antibody, not an inability to respond to TI-2 antigens, that renders these animals susceptible to *Streptococcus pneumoniae* infection (156). This is not conclusive, however it does suggest that B1a B cells are not exclusively required for a TI-2 response. This could be more accurately assessed by direct immunization of these $CD19^{-/-}$ mice with NP-Ficoll to measure the degree to which these animals generate appropriate AFC responses.

However, the entire TD immune response was not defective. In Pim-deficient animals the SLPC is compromised, but GCs and LLPC generation appear to be largely normal. These data suggest that Pim kinases are specifically required for SLPC responses, but not subsequent AFC generation. These data support our conclusion that the defect is not direct ligand signaling, but a more generic defect occurring after antigen engagement that is specific to SLPC generation or function.

One distinctive feature of the SLPC response is their regulation of BLyS receptors. The BLyS family of receptors and ligands forms a major axis in the homeostatic control of both pre-immune and antigen-experienced B cells. These interactions are most extensively studied in the pre-immune B cell subsets, while an understanding of BLyS family actions in antigen-experienced subsets, including activated, memory, and plasma cells, is only now beginning to emerge. These antigen-

experienced cells display shifts in BLyS family receptor expression, suggesting they display different BLyS family ligand requisites than their pre-immune progenitors.

Prior data from the lab has established that SLPCs specifically up-regulate the BLyS family receptor, TACI, while GC and LLPC cells use other receptors for survival cytokines (84, 109). Further, in PCs induced in vitro, APRIL enhances the survival of Ig secreting, Blimp1+,IRF+,XBP+ cells through TACI (157). In animals lacking either BLyS or BR3, GC reactions are limited (114, 115). Yet, even in a T-dependent stimulation, SLPCs in extrafollicular foci express high levels of TACI, similar to a TI response (data not shown, produced by R. Goenka, University of Pennsylvania.) Together with data from the Woodland laboratory indicating that Pim kinases are regulated by BLyS capture, and evidence of a SLPC defect in $TACI^{-/-}$ animals, we hypothesized that SLPCs use TACI to capture survival factors and that Pims are downstream mediators of this survival. Indeed, we found that TACI does regulate Pim kinases and that BR3 does not.

In addition to survival, recent work by Grundler et al has demonstrated a requirement for Pim1 in proper surface expression of the chemokine receptor CXCR4 (158). Without Pim1, surface expression of CXCR4 was diminished in BM cells resulting in an inability to flux Ca^{++} and migrate towards a CXCL12 gradient. CXCR4 – CXCL12 interactions are involved in both BM retention of HSC as well as chemotaxis of plasmablasts to extrafollicular foci following stimulation (159). These data suggest that the coordinated expression of TACI, Pim kinases and CXCR4 are all required in the generation of localization and survival of extrafollicular foci in response to B cell stimuli. These data suggest a coordinated model where the expression of TACI is induced by antigenic / TLR interaction. Signaling through TACI by BLyS or APRIL then up-regulates CXCR4 in a Pim kinase- dependent manner. All of these players are required for the generation and localization / survival of extrafollicular foci in response to B cell stimuli (**Figure 23**.) Without TACI, B activated B cells receive BLyS signals only through BR3 favoring the generation of abundant GC reactions (R. Goenka, personal communication.)

Without Pims, normal TACI regulation does not lead to the formation of over-abundant GCs, but extrafollicular SLPC responses do not occur. In animals with CXCR4-deficient B cells, TI responses are drastically reduced, while TD responses are comparable to control animals (160).

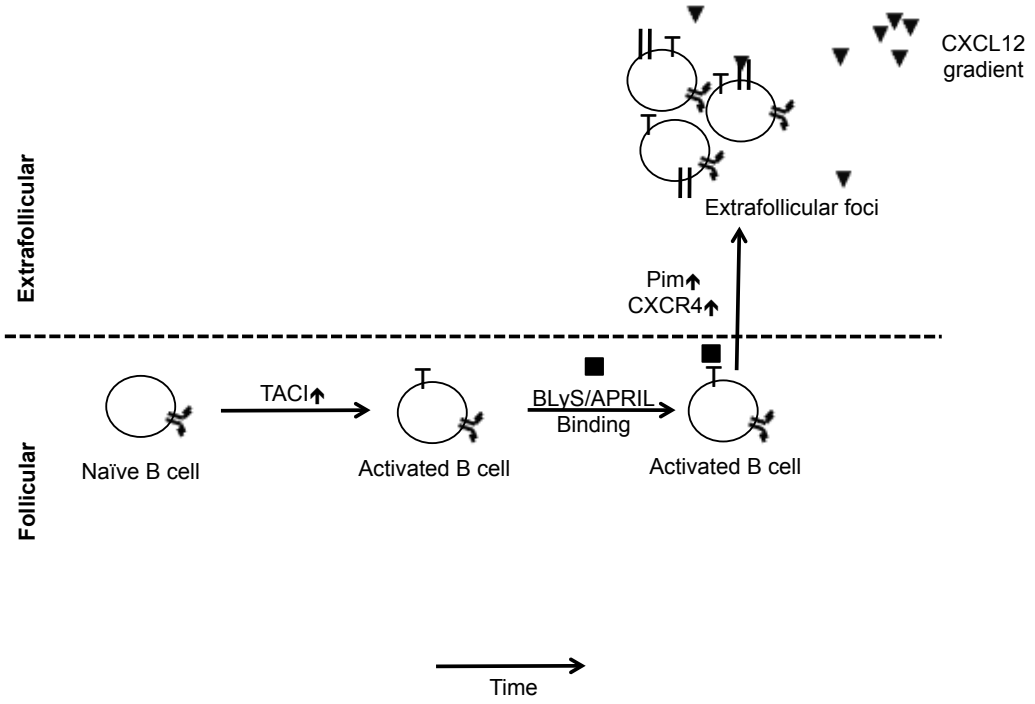

Figure 23: A Model of Short-Lived Plasma Cell Response. Naïve, Follicular B cells are activated by encounter with antigen or TLR stimulus. TACI is up-regulated, allowing cells to bind BLyS or APRIL through this receptor. TACI signaling then up-regulates CXCR4 and down-regulated CXCR5 through a Pim kinase-dependent mechanism. Activated cells can then migrate towards a CXCL12 gradient emanating from the splenic red pulp where extrafollicular foci form.

The similarities between TACI$^{-/-}$ and Pim1$^{-/-}$Pim2$^{-/-}$ animals' response to immunization lends support to the conclusion that these signals are related. This is exemplified by the failure to respond to TI antigens as well as a perceived abundance of GCs in these animals (89, 110). Additionally, there are several reports of cell cycle control in TACI$^{-/-}$ cells that mirror findings in Pim deficient cells. Similar to our model, BLyS addition promotes the transition of BCR-stimulated cells to enter into cell cycle by antagonizing the cyclin dependent kinase inhibitor (CDKI), p18 (111). Although p18 was not one of the CDKIs found to be regulated by Pim kinases, this function is analogous to Pim kinase regulation of other CDKIs, p21 and p27 (131, 134). It remains to be determined whether p18 regulation by BLyS is downstream of BR3 or TACI and Pim.

Future work will be required to address several unanswered aspects of this system. These include a close examination of GC B cells to determine if they show any cycling anomalies, and whether memory B cells are made and can be activated normally. The first question addresses whether Pim kinase involvement in cycling is circumvented in the GC. The second addresses whether there is a qualitative difference between a primary B cell response and a memory response, i.e. are both regulated as SLPC responses? Or have memory B cells bypassed all Pim requirements?

In toto, we have shown that Pim kinases are required for proper cycling or survival of Pre B cells and that the generation and/or survival of activated B cell populations differs between SLPCs, which are Pim-dependent and GCs / LLPCs, that are Pim-independent. The upstream mediators of these pathways correlate with the expression of TACI on SLPCs and BR3 and BCMA on GCs and LLPCs, respectively. Pim kinases also are also required for the generation and/or survival of peritoneal B1a B cells, which may result from either a requirement for Pims in proper cell cycling or may reflect a similarity between SLPC and B1a B cell survival requirements. This combination of AFC abnormalities explains the observed lack of serum Ig in Pim1$^{-/-}$Pim2$^{-/-}$ animals and demonstrates the role of Pim kinase function in humoral immunity.

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