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# Regulation of Humoral Immunity by Pim Kinases: A Dissertation

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# REGULATION OF HUMORAL IMMUNITY

# BY PIM KINASES

A Dissertation Presented

By

Kristen N. Willems

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## Abstract

Pim (Provirus Integration site for Moloney murine leukemia virus) kinases are a family of three serine/threonine kinases involved in cell cycle, survival and metabolism. These kinases were first identified in malignant cells and are most often associated with their role in cancer. Their role in immunity and lymphocytes is less well known. To date, it has been shown that Pim 1 and/or Pim 2 are important for T lymphocyte survival and activation when the Akt signaling pathway is inhibited by rapamycin. In addition, our laboratory has shown that Pim 2 is critical for BLyS-mediated naive B lymphocyte survival in the presence of rapamycin.

This thesis extends the role(s) for Pim 1 and/or 2 to include functions during B cell activation and the generation of immune responses. We found that during in vitro activation of purified resting splenic B cells from wild type mice with a variety of activators that use multiple signaling pathways, including the BCR, TLR and CD40 receptors, both Pim 1 and 2 kinases were induced by 48 hours post-activation, suggesting that they could play a role in B cell activation and differentiation to antibody secreting or memory B cells. Immunization of Pim $1^{-/-}2^{-/-}$ knockout mice with T cell dependent antigens showed impairment in antibody and antibody secreting cell generation as well as lack of germinal center formation clearly demonstrating an involvement of Pim 1 and/or 2 in the immune response. FACS examination of B cell populations from naive Pim $1^{-/-}2^{-/-}$ knockout mice revealed normal levels of splenic marginal zone and follicular B cells and T cells, however, decreased numbers of all peritoneal B cell

populations and decreased B cells in Peyer's Patches was seen. An examination of serum antibody found in naive Pim $1^{-/-}2^{-/-}$ knockout mice showed decreased levels of natural antibody, which is likely due to loss of the peritoneal B1 cells but does not explain the significantly decreased TD immune response. To determine whether the defect was B cell intrinsic or a more complex interaction between B and T cells, we determined whether Pim $1^{-/-}2^{-/-}$ mice would respond to T cell independent, TI-1 and TI-2, antigens. Antibody production and antibody secreting cell formation were also significantly decreased in these mice supporting our notion of a B cell intrinsic defect. To further examine the B cell response problem, we attempted to establish chimeric mice using either bone marrow derived cells or fetal liver cells from WT or Pim $1^{-/-}2^{-/-}$ donors so that the B cells were derived from Pim $1^{-/-}2^{-/-}$ mice and the T cells would be WT. Unfortunately, we were not able to consistently engraft and develop mature Pim $1^{-/-}2^{-/-}$ B cells, which indicate that there is a stem cell defect in these knockout mice that requires further investigation. Because one of the major failures in activated Pim $1^{-/-}2^{-/-}$ B cells is the generation of antibody secreting cells, an analysis of the expression of transcription factors IRF-4 and BLIMP-1, known to play a role in this process was carried out. Although IRF-4 induction was not affected by the loss of Pim 1 and 2, the number of cells able to increase BLIMP-1 expression was significantly decreased, revealing a partial block in the generation of ASCs. Taken together the data presented in this thesis reveals a new and critical role for Pim 1 and 2 kinases in the humoral immune response.

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# Chapter I

## Introduction

B cell development following gestation begins in the bone marrow when a common lymphoid progenitor commits to the B cell lineage by becoming a pro B cell. Thereupon follows a step-wise developmental and selection process producing sequentially a pre-B cell then an immature B cell that exits the marrow. Immature B cells transit from the bone marrow to the spleen where they mature through the transitional 1, 2, and 3 stages. It is during the transitional 2 stage that B cells become dependent on B Lymphocyte Stimulator (BLyS, also known as B cell Activating Factor (BAFF)), a cytokine in the tumor necrosis factor (TNF) family, for survival. Transitional B cells express two different BLyS receptors, BLyS receptor 3 (BR3) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI). A third receptor, B cell maturation (BCMA) is expressed after B cell activation and is critical for the survival of long-lived antibody secreting cells (1-3). In the final step of B cell development a transitional immature B cell becomes either a Marginal Zone (MZ) or Follicular B cell (Fo); these B cell subsets have different functions discussed below and can be identified by differing expression of cell surface phenotypic markers such as CD21, CD9, CD1d, and CD23 (4).

A number of functional differences, aside from phenotype, distinguish these two naïve mature B cell subsets. The types of immune responses are explained in detail later, but Fo B cells are considered the main B cells involved

in both T cell dependent (TD) and T cell independent type 1 (TI-1) responses. When activated by a specific TD antigen Fo B cells generate extrafollicular short-lived antibody secreting cells and germinal centers (GC) that give rise to long-lived ASCs and memory B cells. Marginal zone B cells respond primarily and rapidly to T cell independent type 2 (TI-2) antigens that traffic into the spleen from the blood and generate short-lived plasma cells, with certain exceptions (5, 6). MZ B cells can also transport antigen to the follicle where GC reactions develop (7). MZ B cells quickly increase BLIMP-1 expression and differentiate into plasma cells after activation, which has been demonstrated in vitro (8). This is likely due to the fact that resting MZ B cells contain more endoplasmic reticulum, express higher levels of BLIMP-1 and lower levels of Bcl-6 than their follicular counterparts (8). These cellular and molecular characteristics are reminiscent of antibody secreting cells (ASC); and suggest that MZ B cells are in a “heightened” state of activation compared to Fo B cells.

Another subset of B-lymphocytes, B1 cells, are found in the peritoneal and pleural cavities as well as the spleen and can migrate to the intestine (5, 9). As with MZ B cells, B1 B cells are poised to rapidly differentiate into ASCs without T cell help (6). B1 B cells have the unique ability to self-renew and have less BCR diversity than splenic B cells and are considered the source of natural antibody (5, 9). B1 B cells can be further divided into B1a and B1b cells, which have been shown to have different but complimentary roles in the humoral immune system. B1a B cells often produce natural antibodies, which provide the first line of defense against infection, while B1b B cells can generate antibodies in response

to infection (10, 11). Natural antibody forms the first layer of protection against invading pathogens. B1b cells can be activated quickly when encountering antigen and have been shown to be the first B cells to produce antibody against the cell wall of an invading bacteria. Therefore, while B1a B cells may spontaneously differentiate into plasma cells and produce natural antibody, which is useful against many pathogens, B1b cells respond quickly in a cognate manor to a specific pathogen to produce antibody against that particular pathogen (5, 9, 11, 12).

All B cell subsets participate in the humoral immune response, in which B cells produce antibodies against an immunization or invading pathogen and stem the spread of disease. There are three types of immune responses resulting in the production of antibody against a pathogen, T cell dependent (TD), T cell independent type I (TI-1), and T cell independent type 2 (TI-2). The type of response elicited depends on the pathogen and the accessory cells participating in the response. A TD antigen will only generate an antibody response if T cells are present to assist in B cell activation. T cells facilitate the differentiation and proliferation of B cells activated by antigen. TD responses induce ASCs via two pathways. An extra-follicular response results in a quick increase in antibody against the antigen. Alternatively, activated B cells enter a splenic or lymph node follicle and form a GC where B cells proliferate rapidly and differentiate into ASCs as well as memory cells (6). Germinal centers are also the site of isotype switching (13) and the selection of high affinity ASC and memory B cells. B cells expressing different isotypes, generated by switching, have varying effector and

trafficking mechanisms associated with the non-antigen binding Fc portion of the immunoglobulin. Affinity maturation ensures that antibody generated later in the immune response will have a higher affinity than early antibody (14, 15).

TI immune responses results in B cell activation without the requirement for cognate T cell help, as the name suggests. In vivo they arise earlier than TD responses, as there is no need for expansion or priming of cognate T cells, and play an important role against many extracellular pathogens. There are two types of antigen, which activate B cells in a T cell independent manner. A TI type 1 (TI-1) antigen contains epitopes recognized by the BCR as well as ligands that can engage pattern recognition receptors. Unlike TD and TI-2 antigens, TI-1 antigens are directly mitogenic, such that at high concentrations of antigen, B cells proliferate and generate ASCs regardless of their BCR specificity by binding of the TI-1 antigen to pattern recognition receptors such as CD14, and TLR4. At low concentrations, a TI-1 antigen activates only those B cells specific for the antigen, resulting in an antigen specific response. TI type 2 (TI-2) antigens have a highly repetitive structure and can only activate B cells specific to that antigen. This response is generally thought to occur extra-follicularly though there is some evidence for a short-lived germinal center response (6, 16).

Humoral immune responses occur not only in the spleen or peritoneal cavity, but also in other secondary lymphoid sites, including the lymph nodes and intestine. The intestine is open to the environment and requires constant monitoring and quick responses to foodborne pathogens. Peyer’s Patches (PP), follicles in the intestine wall that contain lymphocytes, macrophages, and

dendritic cells are the primary sites for lymphocyte activation by environmental stimulation. Germinal centers and isotype switched B cells are found in PP, similar to TD antigen driven responses in the spleen. Unlike the spleen where IgG is the dominant isotype, IgA is the dominant isotype in mucosal defenses including Peyer's Patches (16).

Irrespective of the mode of activation, B cells must differentiate into antibody secreting cells to produce the antibody that is the basis of humoral immunity. This differentiation involves a tightly regulated specific genetic program, which has only been partly elucidated. The most well known critical transcription factor associated with ASC differentiation is B Lymphocyte induced maturation protein 1 (BLIMP-1), the loss of which severely impacts ASC generation (17), whereas over expression can spontaneously induce ASC differentiation (18). Immunization of conditional BLIMP-1 knockout mice with TD and TI-2 antigens resulted in very few ASCs (17). In vitro, BLIMP-$1^{-/-}$ B cells stimulated with LPS proliferated normally but did not generate ASCs. Immunization of mice whose B cells lack BLIMP-1 revealed a 3-fold decrease and 10-fold decrease in ASCs to TD and TI-2 antigens (17). IRF4 is another key transcription factor induced upon B cell commitment to the ASC lineage, which precedes BLIMP-1 induction. There is contradictory evidence of a direct linear relationship between IRF-4 and BLIMP-1 expression as one study found that IRF-$4^{-/-}$ B cells stimulated in vitro with LPS expressed BLIMP-1 normally (19) while another study found that IRF-$4^{-/-}$ B cells were unable to induce BLIMP-1 under the same conditions (20). Consistent with its role in ASC development,

IRF-4$^{-/-}$ B cells generate 95% fewer ASCs than WT B cells when activated polyclonally in vitro, and there are very few CD138$^{+}$ IRF4$^{-/-}$ B cells induced by immunization with TD antigens compared to control B cells (19, 20). A third transcription factor critical to ASC generation is XBP-1 (21). B cells lacking either IRF-4 (19) or BLIMP-1 (17) are unable to induce expression of XBP-1, demonstrating that XBP-1 depends on the expression of both IRF-4 and/or BLIMP-1. Although in vitro experiments revealed that survival and proliferation were unaffected in XBP-1$^{-/-}$ B cells after 4 days with LPS stimulation, XBP-1 deficient mice had 70-fold fewer ASCs, and virtually absent IgM and $IgG_3$ antibody responses to immunization with TI-2 and TD antigens respectively (21).

Pim 1, Pim 2, and Pim 3 kinases comprise a family of proto-oncogenes, which promote cell cycle and protein synthesis while suppressing apoptosis. The Pim kinases have a conserved kinase domain but no regulatory domain and are thought to be constitutively active once expressed (22). Pim-1 (23) and Pim-2 (24) were first identified as frequent proviral insertion sites in murine leukemia virus induced lymphomas. Pim kinases are highly homologous; Pim 2 and Pim 3 share 61% and 71% amino acid identity with Pim 1 respectively (25). Pim 1, 2, and 3, also share a common phosphorylation consensus sequence (26) while Pim 1 and 2 display substrate specificity with a strong preference for basic residues (27-29). All three are serine/threonine kinases (30) that act downstream of cytokine and other receptors affecting several aspects of cell function, including but not limited to, induction of transcription factors NFAT and c-Myb, stabilizing proteins such as SOCS, suppressing apoptosis by inducing Mcl-1

while inhibiting BAD, and promoting cell cycle by inactivating p21 (31-33). All three Pims are transcriptionally regulated and have unique though sometimes overlapping tissue expression patterns (34-36). Pim kinases are found in the cytoplasm, though recent preliminary experiments in our lab have shown that Pim 1 and 2 can localize to the nucleus in B cells stimulated with LPS but not anti-CD40 and IL-4 suggesting that localization as well as Pim targets are dependent on upstream signaling.

Only Pim 1 and Pim 2 are expressed in lymphocytes and both have been shown to play a role in growth and survival in these cells. Pim 1 is important in cell cycle progression (33, 37, 38). Pim 2 is involved in cell growth by increasing glycolysis (39, 40). Pims have been shown to be induced downstream of the Jak/STAT pathway after various stimuli. However, unpublished results from our lab demonstrate that Pim induction downstream of BLyS is not Jak/STAT dependent and therefore other pathways are also capable of inducing Pim. Although Pim induction downstream of BLyS has been linked to NF-kB2 induction (41) this signaling pathway(s) continues to be an active area of investigation. Despite their role in these critical functions, mice with targeted mutations in Pim 1 and Pim 2 (Pim $1^{-/-}2^{-/-}$) display no gross abnormalities. In vitro studies of lymphocyte survival have shown that Pim $1^{-/-}2^{-/-}$ B and T cells behave as WT unless treated with rapamycin, which blocks the Akt kinase pathway downstream at the level of mTOR. WT T cells cultured with IL-4 or IL-7 survive normally with or without rapamycin treatment, whereas, Pim $1^{-/-}2^{-/-}$ and Pim $2^{-/-}$ T cells do not survive when treated with rapamycin even in the presence of survival

cytokines, demonstrating that either Akt or Pim 2 are required for T cell resistance to spontaneous apoptosis. In vitro experiments further showed that Pim $1^{-/-}2^{-/-}$ and WT T cells responded similarly to multiple activators. However, Pim $1^{-/-}2^{-/-}$ T cells were unable to up-regulate cell surface activation markers and blastogenesis was suppressed in the presence of rapamycin demonstrating the involvement of Pim 1 and 2 in T cell survival and activation (42). A similar paradigm was demonstrated for B cells, WT or Pim $1^{-/-}2^{-/-}$ B cells cultured with BLyS resist spontaneous apoptosis, however, when the Akt survival pathway is blocked using rapamycin, Pim $1^{-/-}2^{-/-}$ and Pim $2^{-/-}$ B cells do not survive. These results demonstrate the redundancy of Pim 2 and Akt pathways in BLyS dependent resistance to apoptosis (41). The role of Pim 1 and 2 during B cell activation or differentiation to antibody secreting cells, long-lived plasma cells or memory B cells is unknown. However, it is noteworthy that Pim 1 and 2 are known to play a critical role in cell metabolism, and in protein synthesis processes that are critical to the function of activated B cells.

To investigate a possible role for Pim 1 or 2 in B cells post-activation, we used a genetic approach with Pim 1 and 2 deficient mice to assess the role of these kinases in regulating humoral immunity. Chapter III of this thesis demonstrates that Pim 1 and 2 are induced upon B cell activation by a wide variety of stimuli and documents reduced immunoglobulin found in unimmunized Pim $1^{-/-}2^{-/-}$ mice when compared to wild type controls. To investigate the cause of the immunoglobulin defect, we analyzed the resting B cell populations in a naïve Pim deficient mouse. MZ and Fo splenic B cell subpopulations were not

significantly impaired in number or proportion in Pim $1^{-/-}2^{-/-}$ mice. However, B1 B cells residing in the peritoneal cavity were decreased when either Pim 1 or 2 was lost. B1 B cells are the main contributors to natural $IgG_3$, IgA, and IgM immunoglobulin of unknown specificity, which was lower in Pim deficient mice than WT mice. The loss of $IgG_3$ and IgM natural immunoglobulin is consistent with the diminished peritoneal B1 B cells. However, B1 cells produce IgA not primarily in the peritoneal cavity but at mucosal surfaces such as Peyer's patches found in the intestine. This led us to ask if the Peyer's Patches of Pim $1^{-/-}2^{-/-}$ mice were affected by the loss of Pim 1 and 2. While the number of Peyer's Patches in Pim $1^{-/-}2^{-/-}$ was not significantly different, however, the number of IgA switched B cells found in the Peyer's patches was approximately 2-fold less in Pim $1^{-/-}2^{-/-}$ mice, closely matching the magnitude of the B1 deficiency found in the peritoneal cavity. Whether this B1 deficiency is due to a developmental or migratory defect is not explored in this work. Peyer's Patches also contain GC B cells defined as $B220^{+}GL7^{+}CD95^{+}$, in response to environmental stimuli, but this population was absent in Pim $1^{-/-}2^{-/-}$ mice.

In addition we evaluated Pim induction in B cells activated by various stimuli. Although Pim 1 and 2 are induced with BLyS, anti-CD40, and anti-Ig activation of B cells (41), to the best of our knowledge, this is the first demonstration of Pim induction after activation with LPS, CpG, anti-delta-dextran, or a BCR-specific activator. Pim induction by a BCR-specific antigen is consistent with a critical role for Pim 1 and/or 2 during a B cell response to antigen.

Chapter IV explores the deficiencies found in Pim $1^{-/-}2^{-/-}$ mice immunized with TD antigens. Antigen-specific antibodies as well as ASC numbers were significantly decreased. We also observed a diminished GC response under most circumstances. We found that using a stronger adjuvant or antigen increased the likelihood of Pim $1^{-/-}2^{-/-}$ mice having normal GC numbers. For example, immunization with SRBC resulted in half of the Pim $1^{-/-}2^{-/-}$ mice generating a GC response while the other half did not. This is consistent with the strength of the immunogen playing a key role in the ability of Pim $1^{-/-}2^{-/-}$ to form a GC. We hypothesize that the mechanism behind the decreased GCs is impaired signaling downstream of the BCR and CD40 due to the loss of Pim 1 and 2. As a result, alternative activation signals either downstream of these or other receptors must be stronger to reach a threshold of activation. Consistent with this idea, the affinity of antigen specific antibody induced in Pim $1^{-/-}2^{-/-}$ mice is significantly higher initially than that of WT suggesting that only Pim $1^{-/-}2^{-/-}$ B cells with a high affinity for the antigen are activated. Despite this, we found that the anti-NP antibody in Pim $1^{-/-}2^{-/-}$ mice failed to increase in affinity during a TD response. This could be due to the impaired GC response, as affinity maturation is known to take place in the GC or it could be due to an intrinsic B cell defect. Both affinity maturation and isotype switching are AID dependent processes. In vitro Pim $1^{-/-}2^{-/-}$ B cells were unable to switch to certain isotypes to the same extent as WT B cells, consistent with an intrinsic B cell defect. The defect in both affinity maturation and isotype switching suggested that the loss of Pim 1 and 2 might result in the deregulation of AID. We have shown that at the mRNA level

that AID transcription is unaffected by the loss of Pim 1 and 2, however, this does not rule out a post-translational defect. Our attempts to induce an effective TD response in Pim $1^{-/-}2^{-/-}$ mice by the addition of T helper cells were unsuccessful, demonstrating again an intrinsic B cell defect.

To directly assess the activation of Pim $1^{-/-}2^{-/-}$ B cells in the absence of T cell help, we immunized Pim deficient mice with either TI-1 or TI-2 antigens. After i.p. immunization with a TI-2 antigen, antibody titers in Pim $1^{-/-}$ and Pim $2^{-/-}$ mice were diminished relative to WT mice but not to the extent observed in Pim $1^{-/-}2^{-/-}$ mice. Delivering the TI-2 antigen directly to the intact MZ by i.v. immunization rescued the antibody response in Pim $1^{-/-}$ and Pim $2^{-/-}$ but not Pim $1^{-/-}2^{-/-}$ mice, revealing a defect in the ability of Pim $1^{-/-}2^{-/-}$ MZ B cells to respond to antigen. To determine where this block occurred, we activated isolated splenic B cells from WT and Pim $1^{-/-}2^{-/-}$ mice in culture. Though early activation steps were intact, measured by the appearance of activation markers and proliferation, Pim $1^{-/-}2^{-/-}$ B cells failed to generate normal numbers of ASCs. Investigating the induction of key transcription factors in the ASC developmental program, we found that only a small fraction of Pim $1^{-/-}2^{-/-}$ were able to induce BLIMP-1 upon activation, which therefore impairs the generation of ASCs. Though we have not tested BLIMP-1 expression directly during an in vivo immune response or during the differentiation of B1 B cells into ASCs in a naïve mouse, other groups have shown that BLIMP-1 is critical to ASC generation during responses to TD and TI antigens by Fo, MZ and B1 B cells (17, 43). These results lead to a model in

which impaired ASC generation by the failure to induce BLIMP-1 results in reduced Ig titers in naïve, and TD, and TI immunized mice.

To establish the cellular basis for the TD response deficiency of Pim $1^{-/-}2^{-/-}$ mice, we attempted to generate chimera mice using mixtures of WT and Pim $1^{-/-}2^{-/-}$ bone marrow or fetal liver. We found, described in Chapter VI, a defect in the ability of Pim $1^{-/-}2^{-/-}$ marrow cells to reconstitute lethally irradiated mice but that homing of Pim $1^{-/-}2^{-/-}$ splenocytes was not affected. Our results closely mimic those of another group that failed to rescue lethally irradiated recipients using Pim $1^{-/-}$ bone marrow; which they attributed to a Pim 1 dependent CXCR4 functional defect affecting stem cell trafficking (44). The loss of CXCR4, however, does not account for the failure of Pim $1^{-/-}2^{-/-}$ fetal liver cells to reconstitute lethally irradiated recipients because when $CXCR4^{-/-}$ fetal liver cells are transferred in high numbers reconstitution is not impaired (45). Intriguingly, BLIMP-1 expression results in the up regulation of CXCR4 (46) and the additional defect seen in Pim $1^{-/-}2^{-/-}$ compared to $CXCR4^{-/-}$ fetal liver cells may be due to unknown additional BLIMP-1 targets. Alternatively to a defect in migration, these results may be due to a defect in development, which can be overcome in the intact Pim $1^{-/-}2^{-/-}$ mouse but prevents Pim $1^{-/-}2^{-/-}$ bone marrow or fetal liver cells from competing with WT cells in the same environment.

This dissertation provides an overview of B cell activation as affected by Pim kinases. We have revealed new and critical roles for these enzymes in humoral immunity and suggest additional avenues of investigation.

# Chapter II

# Materials And Methods

## Mice

C57BL/6 and B6.C-Igh$^{a}$ Thy1$^{a}$ Gpil$^{a}$ mice (aged 8-24 weeks) were originally purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6 mice were used as controls for C57BL/6 Pim $1^{-/-}$, $2^{-/-}$, and $1^{-/-}2^{-/-}$ deficient mice originally obtained from Dr. C. Fox (Jackson labs, Bar Harbor, ME) (42) generated from mice given by Paul Rothman (Columbia University, New York, NY) (47). Initially backcrossed to B6 by Dr. C. Fox, backcrossing was continued in our lab by Sarah Kenward. B6x129 F1 and B6x129 F2 mice were purchased from Taconic Labs and used as controls for B6x129 Pim $1^{-/-}2^{-/-}$ received from Dr. C. Fox (Jackson Labs, Bar Harbor, ME). Mice with BCR specificity to the synthetic polypeptide, Poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lysine [(T,G)AL] were generated by electroporating immunoglobulin H and L chains derived from the 15F6 hybridoma into 2x107 E14.1 ES cells. Recombinant ES clones were identified by southern blot and injected into blastocysts and the resulting knock-in mice were backcrossed to a 129/sv background. Aproximaely 80% of B cells generated by these mice express BCRs with both heavy and light chains specific to (T,G)AL. These mice, referred to at GT-GL knock-in mice, were generated and generously provided by Dr. J. Press (Brandeis University, Waltham, MA). C57BL/6 BLIMP-$1^{gfp/+}$ mice were generously provided by Dr. T. Imanishi-Kari (Tufts University, Boston, MA). Construction of BLIMP-$1^{gfp/+}$ mice by Dr. Nutt

(University of Western Sidney, Australia) has been described previously (48). Our lab bred C57BL/6 Pim $1^{-/-}2^{-/-}$ mice with BLIMP-$1^{gfp/+}$ reporter mice to generate BLIMP-$1^{gfp/+}$ Pim $1^{-/-}2^{-/-}$ mice. Spleens from IRF$4^{-/-}$ mice were provided by Dr. R. Ren (Brandeis University, Waltham, MA) and produced as previously described (49). Drs. L. Berg and J. Kang, Univ. of Massachusetts Medical School provided respectively OT II, Rag$2^{-/-}$ mice whose T cells are specific to OVA and allotypically marked C57BL/6-Ly5.2 mice. Dr. R. Gerstein generously provided μMT mice with a mutation in IgM resulting in a loss of the mature B cell compartment originally constructed by Kitamura and colleagues as previously described (50). B6 Rag$2^{-/-}$, and B6-Ly5.2-Cr from Dr. Kang or purchased from NCI (Frederick, MD) were used as recipients in adoptive transfers. In some experiments mice were irradiated using a $^{137}$Cs source (Atomic Energy of Canada Limited, Industrial Products, Toronto, Canada) 2-24 hours before adoptive transfer. All mice were bred and maintained in the animal facilities at the University of Massachusetts Medical School. All animal care and procedures were carried out in accordance to the Animal Welfare act and IACUC rules.

**Immunizations**

In experiments examining thymus dependent responses, animals were immunized with 50 μg NP-CGG (Biosearch technologies Inc, N-5055-5) in 100 μl of endotoxin free PBS mixed for at least 30 minutes 1:1 with Imject Alum (Thermo Scientific, #77161) or 10% v/v Sheep red blood cells (Triple J Farms, BLD300050) injected i.p.. For experiments examining thymus independent type 2 responses, mice were immunized with 50 μg NP-Ficoll (Biosearch, #F-1420) in

200 µl PBS delivered either i.p. or i.v.. In experiments examining thymus independent type 1 responses mice were immunized with 25 µg of Fitc-LPS (055:5B) injected i.p.. Control mice were given phosphate-buffered saline (PBS, Invitrogen) or Imject Alum without antigen.

**B cell isolation**

Intact spleens were injected with Special balanced salt solution (SBSS, 10g glucose, .6g $KH_2PO_4$, 1.84g $Na_2HPO_2$ $H_20$, 1.86g $CaCl_2$ $2H_20$, 4g KCL, 80g NaCl, 2g $MgCl_2$ $6H_2O$, 2g $MgSO_4$ $7H_2O$, 0.1g phenol red in 10 L $H_2O$, pH adjusted to 7.2) supplemented with .3% BSA, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 10 µg/ml gentamicin (SBSS+) before being disrupted by pressing between 2 glass slides. To eliminate red blood cells, the cell pellet was resuspended in 1 ml/spleen of SBSS supplemented with 1% FBS, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 10 µg/ml gentamicin before 3 ml/spleen of Gey's solution (51) was added. After a 3-minute incubation cell suspensions were again washed with SBSS+ solution described above and enumerated by trypan blue and hemocytometer. T cell depletion was accomplished by incubating splenocyte suspensions with anti-Thy1.2 mAb (clone J1J10) on ice for at least thirty minutes followed by a wash with SBSS+. Mouse adsorbed rabbit complement (Pel-Freeze, 31042-2) was added to the suspension at 1:12 with cells at 50 million/ml. Suspensions were left shaking in a $37^0$ water bath for thirty minutes then washed again. In some experiments small resting B cells were isolated after this step by fractionation on a Percoll (Sigma, P1644) step gradient (50, 65, 70, 75%) prepared in Hank's balanced salt solution (HBSS, Sigma,

55021-C). Cultured B cells were kept in RPMI supplemented with 10% FBS (Gibco), 2 mM glutamine, 50 µM 2-mercatoethanol, 10 µg/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin and MEM nonessential amino acids (RPMI CM) then stimulated or not with LPS 055:B5 (10 µg/ml, Sigma, L2880), anti-IgM (10 µg/ml Jackson cat# 115-006-020), anti-CD40 (1 µg/ml eBioscience cat# 160-402-85), CpG (1 µg/ml Invivogen, tlrl-modn-1), anti-delta-dextran (10 ng/ml, Fina Biosolutions LLC), TG4-Ficoll (1 µg/ml) (synthesized in the lab, basic protocol found in (51)), or B Lymphocyte Stimulator (BLyS 100 ng/ml, isolated from a transfected CHO cell line, provided by Dr. R. Noelle, Dartmouth Medical School).

**Peritoneal Cavity Cell Isolation**

Abdominal skin was removed to expose the peritoneal cavity of euthanized mice while not rupturing the cavity itself. Ten mls of SBSS+ was injected using a 21-gauge needle. The body was massaged to move the fluid around the peritoneal cavity and dislodge any cells. Fluid was then removed from the cavity using a syringe and 18-gauge needle. Cell suspensions were spun down and treated with 1x Geys as needed to remove red blood cells (described above) then washed into FACS buffer and enumerated before cell surface staining as described below.

**Flow cytometry**

Cell Surface Staining

Cell suspensions in Hank's Buffered Salt Solution (HBSS) supplemented with 3% FBS and .02% azide (FACS buffer) were incubated on ice for 10 minutes with a mouse Fc receptor block against CD16/32 (clone 2.4G2) then surface stained

by incubating on ice for 30 minutes in the dark with antibodies against B220 (BD Pharmingen cat# 553093), CD19 (ebioscience 17-01091-81), CD93 (ebioscience cat# 175892-82), CD90.1 (ebioscience cat # 12-0900-81), CD90.2 (ebioscience cat# 17-0902), CD4 (Pharmingen cat#01084A or Caltag cat#RM2515), CD8a (Caltag cat# RM2204) and CD23 (ebioscience cat# 12-0232-83), GL7 (BD Bioscience cat# 553666), CD95 (BD Bioscience cat #554256 or ebioscience cat# 12-0951-83), CD11b/Mac-1 (Biolegend cat#101230), CD43 (BDbiosciece cat#560663 or), CD5 (ebioscience cat# 12-0051-82), CD71 (Caltag cat# RM5304), CD69 (Caltag cat# HM4001), CD80 (ebioscience cat #12-0801-81), CD86 (ebioscience cat# 17-0862), CD21 (ebioscience cat# 11-0211), CD45.1 (eBioscience cat#13-0453-81 or 13-0453-82), CD54.2 (Pharmingen cat# 553771 or 553772), $IgD^b$ (BDpharmingen cat #553511), $IgM^b$ (Pharmingen cat# 05102D or 05105B), $IgG_1^b$ (Pharmingen cat# 05172D), $IgG2_a^b$ (Pharmingen cat# 05032D), $IgM^a$ (Pharmingen cat# 05092D or 05094D), $IgG1^a$ (Pharmingen cat# 5553502), $IgG2a^a$ (Pharmingen cat #05002D), $IgD^a$ (Pharmingen cat# 05064D), IgD (ebioscience cat # 11-5993 or southern biotech 1120-09), IgA (Southern Biotech cat # 1040-09). Antibodies used were directly conjugated to APC, PerCP, Fitc, or PE; in cases where the antibody was biotinylated the cells were washed in FACS buffer and incubated 30 minutes on ice with streptavidin-APC (eBioscience, 17-4317-82) or streptavidin-PerCP (BD pharmingen, 554064). After surface staining cells were washed twice with FACS buffer then resuspended in either 275 µl of 2% paraformaldehyde for fixed analysis or 300 µl of FACS buffer for live analysis. Cells were measured using Facs Calibur

instrument by the flow cytometry core facility at UMass Medical School and analyzed by Flowjo software (Treestar, Ashland, OR.). In all experiments 50,00-200,000 events were collected. Dead cells were excluded based on forward and side scatter.

IRF-4 Intracellular Stain

Cultured B cells were collected and washed into 1xPBS + 0.5% BSA (FCM buffer). 2% paraformaldehyde was added 1:1 to create a final 1% solution. Cells were left at room temperature for 20 minutes. Cells were washed with 1xPBS + .5% BSA + .03% saponin (wash buffer) then resuspended in 25 µl of 1xPBS + .5% BSA + .3% saponin (perm/stain buffer) with 5 µl of donkey serum. Primary antibody was added at 1:20 either anti-IRF-4 (Santa Cruz biotechnology cat# sc-6059) or normal goat IgG (Santa Cruz Biotechnology cat# sc-2028) and incubated for 20 minutes on ice. Samples were washed twice with wash buffer, resuspended in 100 µl of perm/stain buffer with 5 µl of normal donkey serum, and donkey anti-goat-fitc (Santa Cruz cat# sc-20204) secondary antibody was added 1:100. Samples were incubated 20 minutes on ice then washed twice with wash buffer. Samples were resuspended in 300 µl of 2% paraformaldehyde then measured using Facs Calibur instrument by the flow cytometry core facility at UMass Medical School and analyzed by Flowjo software (Treestar, Ashland, OR.). In all experiments 200,000 events were collected. Dead cells were excluded based on forward and side scatter.

Cell Sorting

B cells isolated from C57BL/6 BLIMP-$1^{+/gfp}$ or C57BL/6 Pim $1^{-/-}2^{-/-}$ BLIMP-$1^{+/gfp}$ mice were cultured for 4 days with 10 μg/ml LPS with or without 100 ng BLyS at $0.5x10^6$/ml in RPMI supplemented with 10% FBS (Gibco), 2 mM glutamine, 50 μM 2-mercatoethanol, 10 μg/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin and MEM nonessential amino acids. Cells were sorted based on GFP expression using either a Digital Vantage or Aria II instrument by the flow cytometry core facility at UMass Medical School and analyzed by Flowjo software (Treestar, Ashland, OR.).

**Cell labeling**

Splenic B and T or B cell suspensions $25x10^6$ cells/ml were labeled with 10 μM CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester) (Molecular probes) in 1x PBS for 5 minutes at room temperature or 10 minutes shaking in a 37°C water bath. Cells were washed twice with SBSS+BSA+P/S/G (defined above) then cultured in B cell media containing RPMI supplemented with 10% FBS (Gibco), 2mM glutamine, 50 μM 2-mercatoethanol, 10 μg/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin and MEM nonessential amino acids. In migration experiments cells were washed and resuspended in endotoxin free PBS for I.V. injection.

Cells capable of expressing GFP could not be labeled with CFSE as CFSE and GFP use the same FACS detection channel. As an alternative cells were labeled with cell proliferation Dye eFluor 670 (ebioscience). B cell suspensions were washed two times in 1xPBS and stained at $20x10^6$ cells/ml

with 1.25 µM eFluor 670 in PBS for 10 minutes at $37^0$ in the dark. After labeling cells 4-5 volumes of RPMI supplemented with 10% FBS (Gibco), 2 mM glutamine, 50 µM 2-mercatoethanol, 10 µg/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin and MEM nonessential amino acids was added to the cell suspension which were then left on ice for 5 minutes. Cells were washed 3 times with the same RPMI solution and enumerated. Only B cells for culture were stained with eFluor 670 to track proliferation.

**Isotype Switching**

B cells were isolated as described above or using a 75% Ficoll cushion in place of a step percoll gradient. Isolated B cells were cultured in RPMI supplemented with 10% FBS (Gibco), 2 mM glutamine, 50 µM 2-mercatoethanol, 10 µg/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin and MEM nonessential amino acids with one of the following cytokine cocktails to induce switching to the isotype in parentheses; LPS+IL4+BLyS ($IgG_1$), anti-CD40+IL4+BLyS ($IgG_1$), LPS+IL4+IL5+TGFβ+BLyS (IgA), LPS+INFγ+BLyS ($IgG_{2a}$), LPS+TGFβ+BLyS ($IgG_{2b}$), anti-CD40+TGFβ+BLyS ($IgG_{2b}$), LPS+anti-δ-dextran+BLyS ($IgG_3$), or LPS ($IgG_3$). IL4 (Peprotech) and IL5 (Peprotech) were used at 20 ng/ml, TGFβ (R&D systems) at 2 ng/ml and INFγ at 20 ng/ml (Perprotech). Cultures were collected on day 3 and the percent of switched B cells was measured by flow cytometry with cell surface antibodies against specific isotypes (described above).

**Adoptive Transfer**

Cells were washed twice in sterile PBS-BSA (0.3% w/v) then resuspended in endotoxin free PBS at the appropriate concentration. Cells (0.1-0.2 ml) were injected intravenous via the periocular sinus.

**Chimeras**

Recipient mice were either exposed to a single dose of 900 or 1200 rads 24 hours prior to transfer, or a split dose of 1200 rads given as 600 rads 6 and 2 hours prior to transfer, or were not irradiated. Bone marrow was collected from donor mice by flushing the hind leg bones with SBSS supplemented with 0.3% BSA, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 10 µg/ml gentamicin then aspirating with a needle and syringe to break up clumps. Bone marrow cells were enumerated then washed in sterile PBS and resuspended in endotoxin free PBS at the appropriate concentration for transfer. Fetal liver cells were collected on or about day 14 of gestation and pressed between 2 glass slides while submerged in RPMI supplemented with 10% FBS and pipetted to create a cell suspension. The fetal liver cell suspension was counted, washed in sterile PBS then resuspended in endotoxin free PBS at the appropriate concentration for transfer.

**ELISA**

Serum antibody specific for NP was assayed by an Enzyme-linked immunosorbant assay (ELISA); incubating serially diluted sera or control antibody on plates coated with $NP_{7\text{-}9}$-BSA (synthesized in the lab, basic protocol found in (51)). 96 well polyvinyl chloride plates (Falcon) were coated with 10 or 20 µg/ml of NP-BSA in PBS and left at least overnight at $4^{o}$C. Plates were

washed 8 times with PBS and blocked with 1% BSA PBS for at least 30 minutes at room temperature. After dumping the blocking buffer serial dilutions of serum were incubated on the plate for 2 hours at room temperature or 4$^{o}$C. NP specific antibodies were detected with biotinylated anti-mouse IgM (Southern Biotech, 1020-08), anti-mouse λ (Southern Biotech, 1060-08) or anti-mouse $IgG_1$ (Southern Biotech, 1070-08) specific reagents at 1:2000 dilution in 1% BSA PBS left for 2 hrs at room temperature or 4$^{o}$C. To measure Fitc specific antibody in sera from mice immunized with Fitc-LPS, a Fitc-BSA plate coat was used at 20 ug/ml in PBS and antibody was detected using an anti-mouse $F(ab)_2$-biotin conjugate (Jackson ImmunoResearch, 115-066-072). Total $IgG_1$, $IgG_3$, and IgM antibody in naïve mice was measured using and anti-$F(ab)_2$ (Jackson ImmunoResearch, 115-006-006) plate coat at 10 or 20 μg/ml and an isotype specific biotinylated detecting antibody described above or anti-mouse $IgG_3$-biotin (SouthernBiotech, 1100-08). Total IgA antibody in naïve mice was measured using an anti-mouse IgA (SouthernBiotech, 1165-01) plate coat at 5 μg/ml followed by and anti-mouse IgA-biotin (SouthernBiotech, 1040-08) detecting antibody. For all assays washing off the detection antibody was followed by 1:4000 Strepavidin-Alkaline phosphatase (SA-AP) (SouthernBiotech, 7100-04) in blocking buffer left 30-60 minutes at room temperature and washed off. 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma, N4645-5G) dissolved in 2-diethylalomine at 1 mg/ml was incubated on the plates until color had developed. Optical density was determined at 405 nm on a department plate reader (Molecular Devices, Spectramax Plus 384). Relative antibody

concentrations were established by reference to a NP (Dr. T. Imanishi-Kari, Tufts), IgM (SouthernBiotech, 0101-01), IgA (SouthernBiotech, 0106-01), $IgG_3$ (Bionetics, 8402-28), or $IgG_1$ (Bionetics, 8402-03) standard curve. For ELISAs measuring specific antibody after immunization serum from unimmunized mice was used as a background control. ELISAs measuring antibody in naïve sera used either μMT or $rag1^{-/-}$ sera as the background control.

**ELISpot**

To enumerate ASCs from splenocyte or cultured B cell suspensions, Milipore multiscreen HTS plates previously coated with 10 μg/ml of NP-BSA or anti-mouse $F(ab)_2$ (Jackson ImmunoResearch, 115-006-006) and left at least overnight at 4°C were washed eight times with water and blocked with RPMI supplemented with 10% FBS (Gibco), 2 mM glutamine, 50 μM 2-mercatoethanol, 10 μg/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin and MEM nonessential amino acids for at least 30 minutes at 37°C. Cells were recovered from spleen, bone marrow, or in vitro cultures and enumerated as described above. Serial dilutions of cells in RPMI were plated on previously antigen coated and blocked plates and incubated for 4 hours at 37°C. Plates were washed then blocked overnight at 4°C with 1% FBS in PBS before dumping the blocking buffer and adding the detection antibody, either biotinylated anti-mouse IgM (Southern Biotech, 1020-08), anti-mouse λ (Southern Biotech, 1060-08) or anti-mouse $IgG_1$ (Southern Biotech, 1070-08) specific reagents at 1:2000 dilution in 1% FBS PBS. Plates were incubated 3 hours at room temperature then washed with water. SA-AP (Southern Biotech, 7100-04) diluted in 1% FBS PBS at 1:4000 was added to

each well. The plate was incubated 1 hour at room temperature then the fluid was dumped and the plates were washed eight times with water. The substrate solution was made by dissolving one premeasured reaction mix (SIGMA*FAST* BCIP/NBT, Sigma, B565) in 10 mls of Millipore water then vortexing until completely dissolved. The resulting BCIP/NBT suspension was added to the plates and allowed to develop. When spots became visible the BCIP/NBT was removed, the plates were washed eight times with water dried and stored in the dark. Spots were enumerated using an ELISpot reader (CTL, Cleveland, OH) and analyzed with Immunospot software version 3.2 (CTL, Cleveland, OH).

**RNA extraction**

Cells collected from culture or sorted on the basis of GFP expression were spun down, dried by dumping the supernatant and absorbing left over moisture with a sterile q-tip then resuspended in 500 µl of TRI Reagent (Applied Biosystems, AM9738). Samples could be stored at -80$^{o}$C at this step then thawed when RNA was to be isolated. 100 µl of Chloroform (Sigma 472476) was added to thawed tri-reagent samples, samples were shaken then incubated at room temperature for fifteen minutes. Samples were spun at 13,200 rpm at 4$^{o}$C for fifteen minutes. All spins were done in a tabletop microfuge. The aqueous phase from each sample was transferred into a clean microfuge tube and 250 µl of isopropanol added. Samples were vortexed for ten seconds, incubated at room temperature for ten minutes, and then spun at maximum speed for ten minutes at 4$^{o}$C. Supernatant was discarded and 500 µl of 75% ethanol was added to each pellet. Samples were spun at 10,000 rpm at room temperature for five minutes. Ethanol

was removed and samples were air dried briefly. The pellet was resuspended in 30 µl of RNAse free water. The concentration of RNA isolated from each sample was determined by measuring 1.5 µl of sample on a nanodrop spectrophotometer.

**Quantitative Reverse Transcription-Polymerase Chain Reaction**

cDNA was made as follows; one microgram of RNA from each sample was added to a clean tube and brought to a total volume of 15 µl with RNAse free water. Oligo dT primers (Promega) were diluted to a 5 µM working stock and 5 µl of this stock was added to each sample. Samples were incubated at 65$^{o}$C for twenty minutes. A master mix of the following proportions was prepared for each sample: .25 µl of 25 mM dNTPs, 5 µl of 5x Promega M-MLV Reverse transcriptase buffer (50 mM Tris-HCL pH 8.3, 75 mM KCL, 3 mM $MgCL_{2,}$ 10 mM DTT), .4 µl RNAsin (Promega), 1 µl M-MLV-Reverse transcriptase (Promega), and 3.35 µl RNAse free water. The samples were then incubated at 42$^{o}$C for an hour. The resulting cDNA was stored at -20$^{o}$C. The quantitative polymerase chain reaction was performed using Roche Sybr Green master mix (Roche) with primers diluted to 30 µM, 5 µl of cDNA template, and RNAse free water to bring the final volume to 50 µl per reaction. The following primers were purchased from integrated DNA technologies; BLIMP-1 F 5'-GACGGGGGTACTTCTGTTCA-3' R 5'-GGCATTCTTGGGAACTGTGT-3' XBP-1 F 5'-GAGTCCGCAGCAGGTG-3' R 5'-GTGTCAGAGTCCATGGGA-3' Mcl-1 F 5'-TCAAAGATGGCGTAACAAACTGG-3' R 5'-CCCGTTTCGTCCTTACAAGAAC-3'. B-Actin F 5'-CCCTAAGGCCAACCGTGAA-3' R 5'-

CAGCCTGGATGGCTACGTACAAG-3' was generously provided by Dr. R. Gerstein. Relative levels of gene expression were established by qPCR with specific primers using in a Gene Opticon 2 machine generously shared by Brain Ackerly, University of Massachusetts Medical School.

**Immunohistochemistry**

Spleens from immunized, naïve, and alum control mice were harvested and preserved in formalin. Sections were cut, fixed with acetone, and stained with antibodies as indicated, by the DERC core facility at UMass Medical School. Germinal centers were identified under a light microscope at 4x by binding to biotinylated PNA followed by an immunohistochemical reaction using horseradish peroxidase avidin D (vector labs, a-2004) described at http://www.umassmed.edu/morphology/protocols/immunohistochemistry.aspx. PNA+ positive germinal centers over 2 separate spleen sections were enumerated by eye.

# Chapter III

# Unimmunized Pim $1^{-/-}2^{-/-}$ Mice Have Impaired Natural Immunity

## Introduction

B Lymphocytes are the effectors of humoral immunity; each has its own BCR specificity. As a result, a large pool of naïve mature B cells must be maintained in secondary lymphoid organs to ensure that when an antigen is encountered, a subset of B cells will respond to it in a cognate fashion and mount a humoral response. These naive B cells rely on B Lymphocyte stimulator (BLyS) for survival (1-3). Previous work from our lab has shown that BLyS dependent survival of naïve B cells depends upon two signaling pathways, one acting through Akt kinase, and the other through Pim 2 kinase (41). A previous paper documented a delay in B cell development that disappeared by 12 wks in Pim $1^{-/-}2^{-/-}3^{-/-}$ as assessed by spleen cell analysis and normal splenic B cell numbers in Pim $1^{-/-}2^{-/-}$ mice (52). However, our lab has observed diminished splenic B cells numbers in the Fo compartment in Pim $1^{-/-}2^{-/-}$ mice (41). These conflicting reports indicated that a more thorough analysis of B cell populations the Pim $1^{-/-}2^{-/-}$ deficient mice was needed, which is provided in this chapter.

A second pool of mature B cells capable of producing antibody is found in the peritoneal cavity. Splenic B cells are primarily B2 cells with a small population of B1 cells; in contrast, B cells in the peritoneal cavity are mostly B1, with a small cohort of B2 B cells. The development of these two B cell subpopulations

remains the subject of some debate, with some evidence pointing to different origins in either the fetal liver or bone marrow. Chimeric mice produced by the transfer of fetal liver reconstitute the peritoneal B1 cell compartment more efficiently than transferred bone marrow. The reverse is true of splenic mature B2 cell reconstitution. Some success has been reported in generating peritoneal B1 cells from adoptively transferred bone marrow (53) suggesting that a B1 cell precursor is not only found in the fetal liver. B1 and B2 cells are mainly distinguished by differing expression of specific cell surface markers. Whereas B1 and B2 cells have long been know to differ in their phenotypic expression it has only recently been revealed that B2 and B1 cells in the peritoneal cavity differ from their respective counterparts in the spleen (54). Function is another characteristic separating peritoneal and splenic B cells. Peritoneal B cells have a lower activation threshold and are often activated and producing antibody in a non-immune mouse (6). Production of this "natural antibody" is mainly attributed to peritoneal B1a B cells (11, 55, 56).

B1 cells also migrate from the peritoneal cavity to the intestine, which is exposed to the environmental microflora and constantly under immunological surveillance. Protection against environmental pathogens in these areas is referred to as mucosal immunity and lymphocytes are primarily localized in Peyer’s Patches which are organized secondary lymphoid areas on the intestinal wall containing macrophages, dendritic, B and T cells. Germinal centers initiated by environmental stimulation largely contain B cells expressing the IgA isotype, the dominant isotype in mucosal immunity.

The role of Pim 1 and 2 in the maintenance of B cells and humoral immunity in naïve mice is poorly understood. Reports on the numbers of splenic B cells have been contradictory and to date no analysis of the B1 compartment or natural immunity of naïve Pim $1^{-/-}2^{-/-}$ mice has been published. In this chapter we present evidence that Pim 1 and 2 are critical for regulation of natural humoral immunity in naïve mice. While the numbers of splenic B cells are normal in Pim single and double deficient mice, the B1 cell peritoneal compartment relies on both Pims to achieve normal numbers. Whether this is due to impaired generation or maintenance of the B1 cell subset is not explored here. A consequence of the diminished B1 cell pool is diminished natural Ig, which is produced by B1a cells. We also find that mucosal immunity in the Peyer's Patches is compromised. It has been suggested that B1 cells migrate from the peritoneal cavity to the gut and back so a deficiency in the number of B1 cells in the peritoneal cavity of Pim $1^{-/-}2^{-/-}$ is most likely linked to the decreased mucosal immunity in the gut. These data demonstrate the requirement for Pim 1 and 2 for the generation or maintenance of humoral immunity in naïve mice.

## Results

### Mature splenic B cell subsets are not impaired by the loss of Pim 1 and/or 2

To determine if the number and subpopulations of resting mature splenic B cells were intact in Pim $1^{-/-}2^{-/-}$ mice, we examined them using FACS analysis. Spleens were dissociated and red blood cells were lysed, total mature B cells were identified as $B220^{+}$ $AA4.1^{-}$ then further subdivided into marginal zone ($CD23^{lo}$ $CD21^{hi}$) or follicular ($CD23^{hi}$ $CD21^{lo}$) B cells. Our first experiments were performed on B6x129 mixed background mice. When examining the absolute B cell number in these mice, we observed that Pim $1^{-/-}2^{-/-}$ mice have a 60% reduction in the number of total and follicular B cells. The marginal zone B cells were not significantly different (Figure 3.1 A). Our experiments using purchased B6x129 F1 mice as controls, were consistent with published results on this background that used littermate controls (41).

We repeated these analyses with Pim $1^{-/-}2^{-/-}$ mice that were fully backcrossed (more than 10) to the C57/BL6 strain. Unlike the results with the mixed background, the absolute numbers and subpopulations of B cells in the DKO were not different than wild type B6 mice (Figure 3.1 B). Splenic B cell analysis in single knockout B6 backcrossed Pim 1 (Pim $1^{-/-}$) or Pim 2 (Pim $2^{-/-}$) were the same as the B6 Pim $1^{-/-}2^{-/-}$ and no different than wild type (Figure 3.1 B). Though these results were contrary to what we observed in our mixed background mice, collaborators in Dr. Cancro's lab at the University of Pennsylvania confirmed our observation.

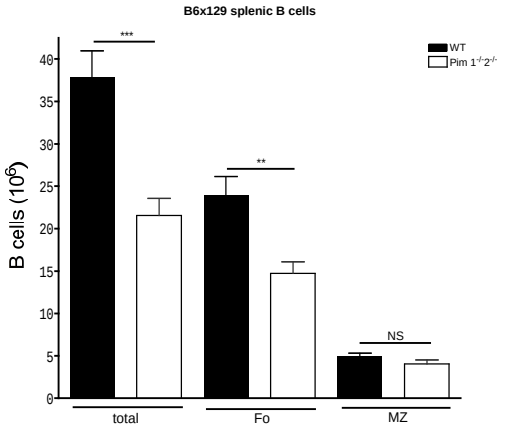
Splenic T cell subpopulations were also examined in control and

Pim $1^{-/-}2^{-/-}$ mice. CD8+ T cells were present but significantly decreased in Pim $1^{-/-}2^{-/-}$ after compiling 2 experiments. CD4+ T cells were not significantly different consistent with T cell help being intact during the response to a TD antigen. Neither Pim single knockout mouse had any impairment.

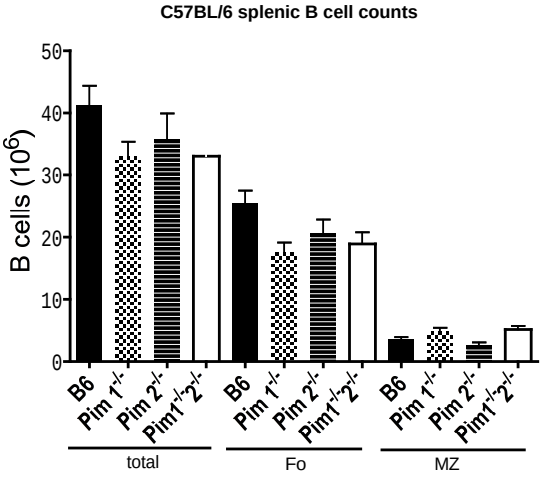
**Figure 3.1. The loss of Pim 1 and 2 does not impair the number of mature splenic B cells.** Splenocytes from B6x129 (A) or C57BL/6 (B) control and Pim $1^{-/-}2^{-/-}$ mice were analyzed for total ($B220^{+}$), Fo ($B220^{+}CD23^{hi}CD21^{lo}$), and MZ ($B220^{+}CD23^{lo}CD21^{hi}$) B cells. (C) Gating strategy for parts A and B. Error bars are shown. WT n=21, Pim $1^{-/-}2^{-/-}$ n=14 in (B); WT n=19 Pim $1^{-/-}$ n=9, Pim $2^{-/-}$ n=11, Pim $1^{-/-}2^{-/-}$ n=23 in (C). Statistics were determined by Student's t test *=p<.05, **=p<.01, ***=p<.001

## The loss of Pim 1 and 2 does not impair the number of mature splenic B cells

**A.**




**B.**




**C.**

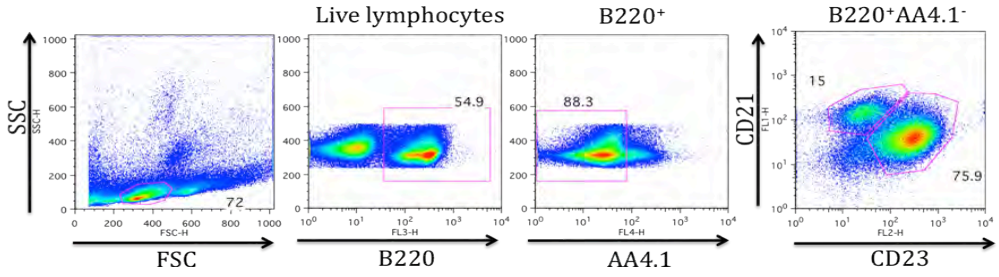
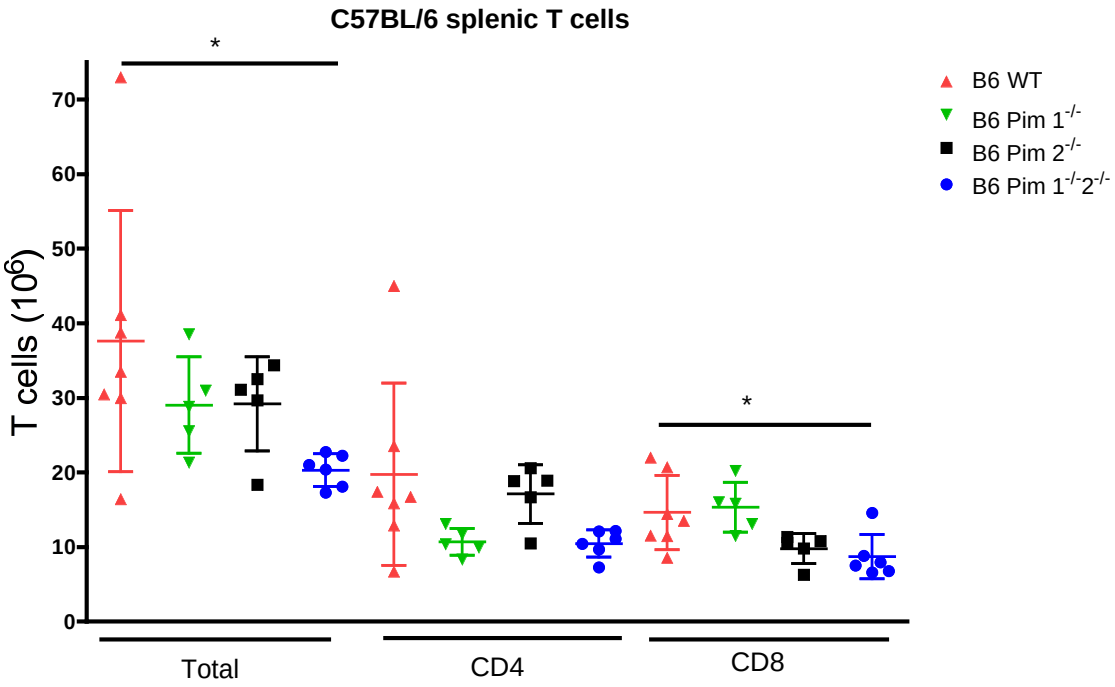


**Figure 3.2 The CD8$^{+}$ T cell subset is diminished in Pim 1$^{-/-}$2$^{-/-}$ mice.**

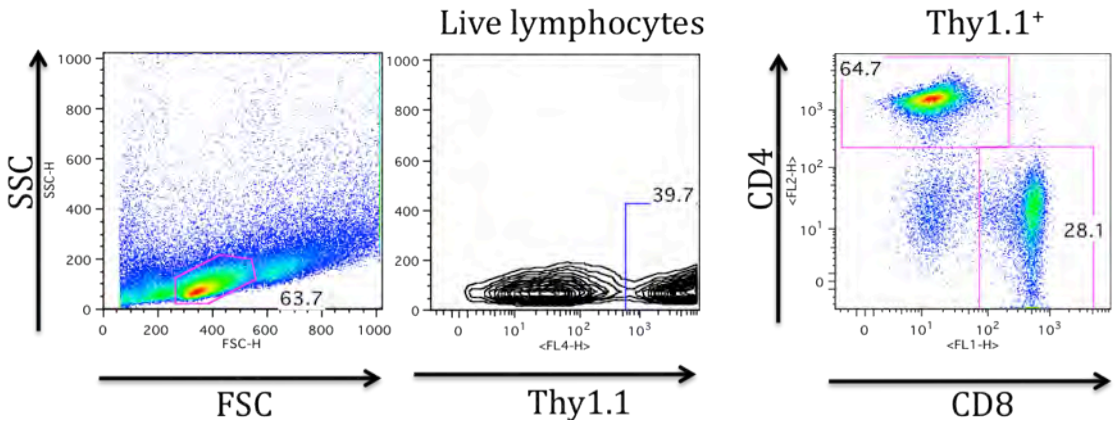
Splenocytes from C57BL/6 control, Pim 1$^{-/-}$, Pim 2$^{-/-}$, and Pim 1$^{-/-}$2$^{-/-}$ mice were analyzed by flow cytometry for total (anti-thy1.2$^{+}$), CD4 (anti-thy1.2$^{+}$CD4$^{+}$) and CD8 (anti-thy1.2$^{+}$CD8$^{+}$) T cell subsets. (A) Numbers of T cells in each subset (B) gating strategy for part A. Bars are the mean and standard deviation for each genotype, each symbols represents an individual mouse. WT n=7, Pim 1$^{-/-}$ n=5, Pim 2$^{-/-}$ n=5, Pim 1$^{-/-}$2$^{-/-}$ n=6. Statistics were determined by Student's t test *=p<.05, **=p<.01, ***=p<.001

## The CD8$^+$ T cell subset is diminished in Pim 1$^{-/-}$2$^{-/-}$ mice

**A.**




**B.**



**Peritoneal Cavity B cells are diminished by the loss of Pim 1 or 2**

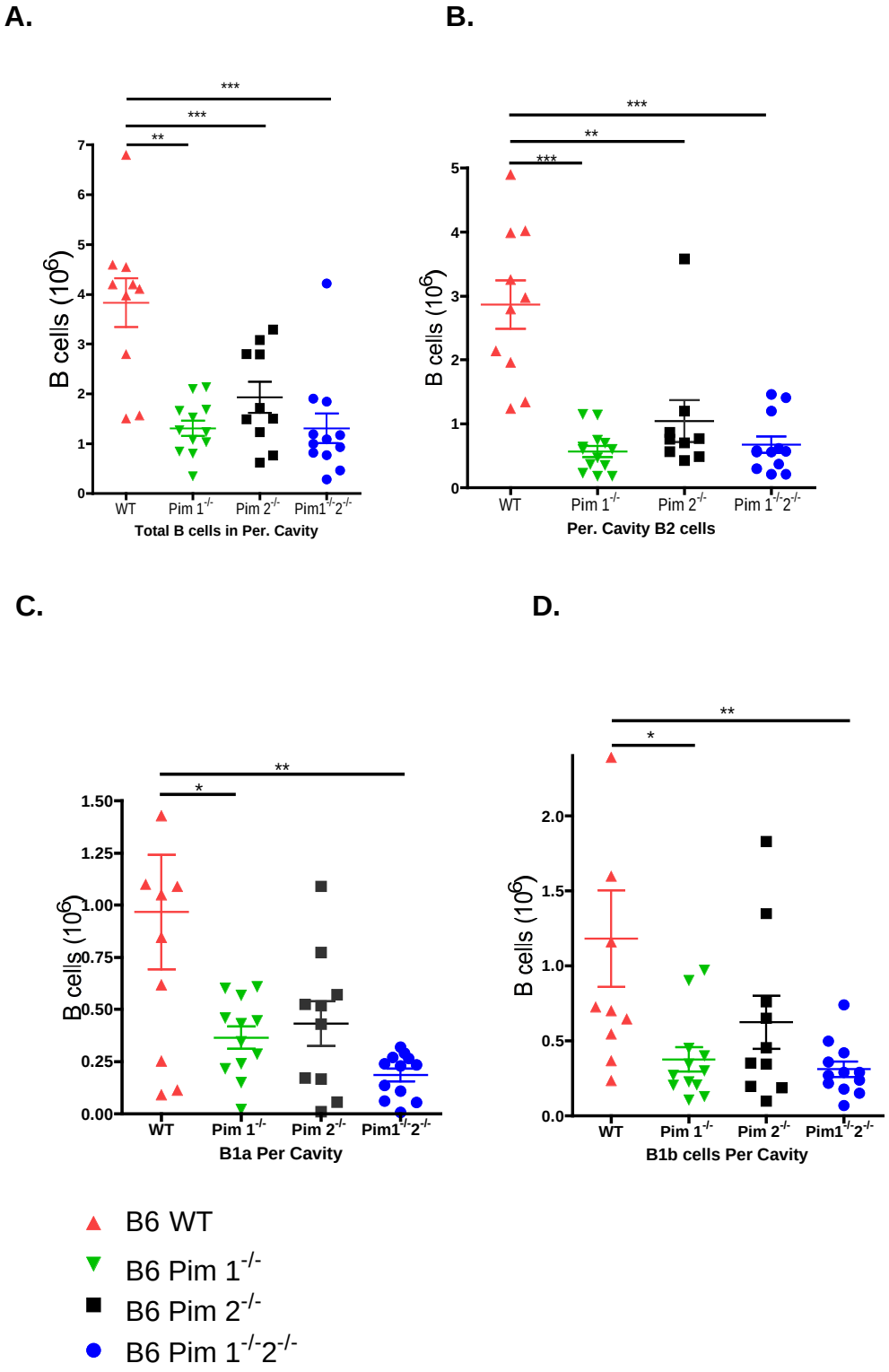
Peritoneal B1 B cells are important mediators of natural and adaptive immunity. To determine if the loss of Pim 1 and/or 2 would impact peritoneal B cell populations, we analyzed the subpopulations of B cells in peritoneal washes from normal and Pim $1^{-/-}2^{-/-}$ mice by flow cytometry. We observed a significant 4-fold reduction in the total number of peritoneal B ($B220^{+}$) cells in $Pim1^{-/-}2^{-/-}$ compared to B6 controls ($1.3x10^{6}$ ± .29 vs. $3.8x10^{6}$ ± .49, p<.001) (Figure 3.3 A). Within this population B1a ($B220^{+}CD43^{+}CD5^{+}$) B cells were also reduced 4-fold relative to B6 controls ($.18x10^{6}$ ± .03 vs. $.97x10^{6}$ ± .27, p<.01) (Figure 3.3 C). A second subpopulation of B1 cells, B1b B cells ($B220^{+}CD43^{+}CD5^{-}$), which can produce antigen specific antibody and B cell memory in response to blood borne antigens (10, 57) were also decreased by 50% when compared to wild type mice ($.31x10^{6}$ ± .05 vs. $1.2x10^{6}$ ± .32, p<.01) (Figure 3.3 D). Therefore, the loss of Pim 1 and 2 results in a defect in two distinct peritoneal B1 cell populations. We noted that the reduction in the B1 subpopulations did not account for the differences in total peritoneal B cells we found between Pim $1^{-/-}2^{-/-}$ and B6 mice. Although the majority of B2 cells reside in the spleen and other secondary lymphoid tissues, a small population recirculates and can be found in the peritoneal cavity. As with B1 cells, B2 ($B220^{+}CD43^{-}$) B cells in the peritoneal cavity were decreased more than 3-fold, with $0.67x10^{6}$ ± .12 B2 cells in Pim $1^{-/-}2^{-/-}$ mice compared to $2.8x10^{6}$ ± .38 B2 cells in B6 controls (p<.001) (Figure 3.3 B).

To determine which Pim kinase is critical for the maturation and/or maintenance of peritoneal B cell populations, we analyzed Pim 1 and Pim 2

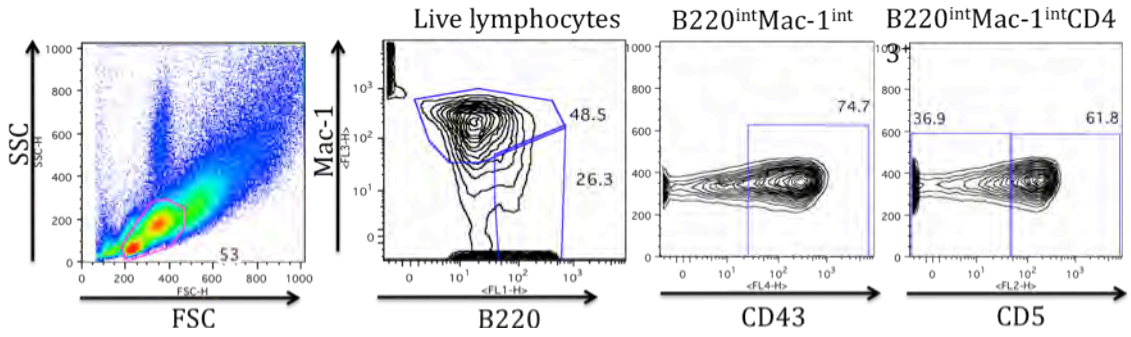
single knockout mice. Total peritoneal B cells were significantly decreased in both Pim $1^{-/-}$ and Pim $2^{-/-}$ mice (Figure 3.3 A). B1a cells in Pim $1^{-/-}$ mice were decreased more than 2-fold with $.36x10^6 \pm .05$ compared to $.97x10^6 \pm .2$ in WT ($p<.05$) whereas Pim $2^{-/-}$ mice were diminished but did not reach significance (Figure 3.3 C). B1b cells in the single knockout mice followed the same pattern with a significant decrease, about 3-fold ($p<.05$), found in the Pim $1^{-/-}$ mice. Although Pim $2^{-/-}$ mice did not have a significant difference in the number of peritoneal B1 cells the mean was very close to Pim $1^{-/-}$ and Pim $1^{-/-}2^{-/-}$ (Figure 3.3 D). Only Pim 1 B1 cell numbers were significantly less than WT but the loss of Pim 2 does not result in normal B1 cell numbers suggesting that both Pims contribute to the generation or homeostasis of peritoneal B1 cells. Peritoneal B2 B cells were significantly decreased in both Pim $1^{-/-}$ ($p<.001$) and Pim $2^{-/-}$ ($p<.01$) mice being reduced to less than a third of that seen in B6 mice (Figure 3.3 B). From these results we conclude that both Pim 1 and Pim 2 play a role in the development and/or maintenance of normal B cell numbers in the peritoneal cavity though the data suggests that Pim 1 may be more important than Pim 2.

**Figure 3.3. Peritoneal B cell subsets are diminished in Pim deficient mice.** Cells were isolated from the peritoneal cavity of control or Pim deficient mice and analyzed by flow cytometry. B cell subsets were identified as total ($B220^{+}$) (A), B2 ($B220^{+}Mac\text{-}1^{-}CD43^{-}$) (B), B1a ($B220^{+}Mac\text{-}1^{int}CD43^{+}CD5^{+}$) (C), or B1b ($B220^{+}Mac\text{-}1^{int}CD43^{+}CD5^{-}$) (D). (E) Gating strategy for A-D. Bars indicate means and SEM; each symbol represents an individual mouse, WT n=10, Pim $1^{-/-}$ n=12, Pim $2^{-/-}$ n=10, Pim $1^{-/-}2^{-/-}$ n=12. Statistics were determined by Student's t test *=p<.05, **=p<.01, ***=p<.001

**Peritoneal B cell subsets are diminished in Pim deficient mice**



**E.**


Live lymphocytes
B220intMac-1int
B220intMac-1intCD4
SSC
FSC
Mac-1
B220
CD43
CD5
48.5
26.3
74.7
36.9
61.8
53

**Natural antibody is impaired by the loss of Pim 1 or 2**

Peritoneal B cells are a major contributor to natural antibodies, found in healthy naive mouse serum that serves as a first line of defense against many environmental pathogens. The source of this serum Ig has been mainly attributed to B1a cells (11, 55, 56), which we have shown are reduced in Pim $1^{-/-}$ $2^{-/-}$ mice. We asked whether this reduction was enough to impair natural Ig in these mice. Serum from B6x129 control F1 and Pim $1^{-/-}2^{-/-}$ mice 10-14 weeks old were measured by ELISA with a light chain binding plate coat and an isotype specific detecting antibody. IgM antibody was nearly 14-fold less in Pim $1^{-/-}2^{-/-}$ animals with 52 ± 15 µg/ml versus 725 ± 173 µg/ml in control mice ($p<.01$). IgG3 antibody revealed a similar deficiency as Pim $1^{-/-}2^{-/-}$ mice had only 88 ± 20 µg/ml while control mice measured 734 ± 227 µg/ml ($p<.05$) (Figure 3.4 A). These studies demonstrate a Pim dependent loss of natural antibody.

In order to confirm the requirement for Pim 1 and 2 for natural antibody production, we repeated the serum Ig analysis with Pim $1^{-/-}2^{-/-}$ and control mice backcrossed onto the B6 background. Serum from naïve 14-week-old C57BL/6 control and Pim $1^{-/-}2^{-/-}$ mice was assayed for antibody as before. We found that the relative antibody in Pim $1^{-/-}2^{-/-}$ vs. wild type mice for IgM (18.54 ± 2 vs. 738 ± 199 µg/ml, $p<.01$), IgG3 (131.2 ± 14 vs. 814.1 ± 101 µg/ml, $p<.001$), and IgA (31.68 ± 4 vs. 87.6 ± 12 µg/ml, $p<.001$) were significantly reduced (Figure 3.4 B). In contrast, the levels of $IgG_1$ and IgE did not differ between Pim $1^{-/-}2^{-/-}$ and control animals. These results were consistent with our previous findings in B6x129 mice.

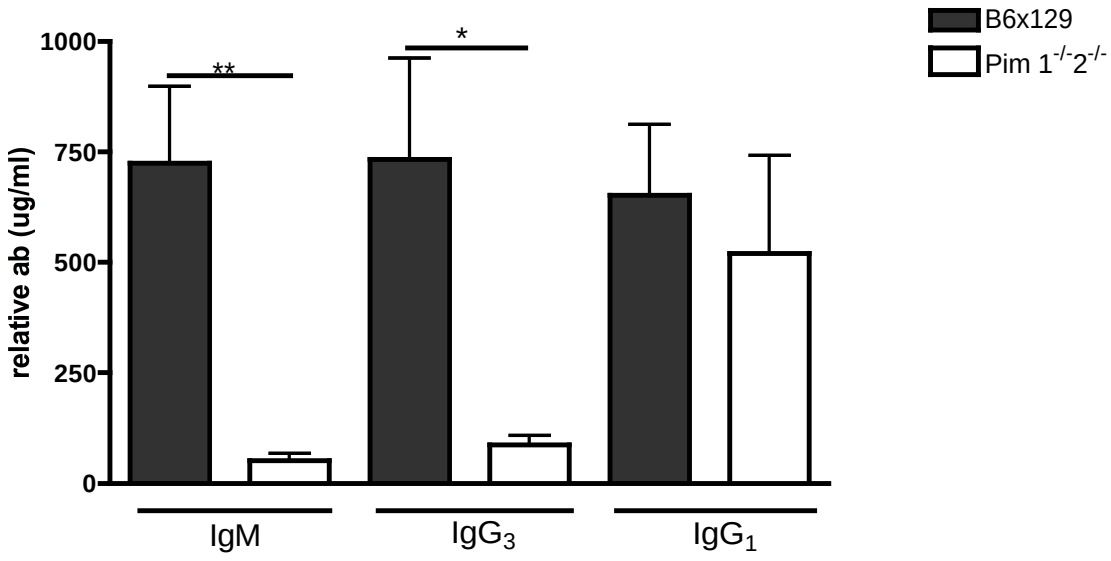
To determine which Pim contributed to this defect, we measured the serum from single knock out non-immune mice. Both Pim $1^{-/-}$ and Pim $2^{-/-}$ had significantly decreased IgM serum levels, 55.75 ± 5 µg/ml ($p<.01$) and 47.27 ± 10 µg/ml ($p<.01$) respectively versus 738 ± 199 µg/ml in control mice. IgA levels were significantly decreased in Pim $1^{-/-}$ mice like the Pim $1^{-/-}2^{-/-}$ mice, 24.68 ± 7 µg/ml versus 87.6 ± 12 µg/ml in control mice ($p<.05$) but were normal in Pim $2^{-/-}$ mice (Figure 3.4 B). From this data we concluded that the loss of either Pim 1 or 2 caused a severe decrease in IgM and the loss of Pim 1 caused the IgA antibody to decrease by half. In contrast, Pim $1^{-/-}2^{-/-}$ mice have normal $IgG_1$ serum levels while Pim $2^{-/-}$ mice had significantly higher levels of $IgG_1$.

The decrease in B1 cells, responsible for production of natural IgM, IgA, and some IgG3 antibody, fits with the profile of antibody levels we observed in Pim deficient mice. Even though our data indicates that Pim $2^{-/-}$ mice do not contain significantly fewer B1 cells in the peritoneal cavity compared to WT mice, there is a large scatter in the data points and the overall average of both B1a and B1b cells are in fact closer to that of Pim $1^{-/-}2^{-/-}$ mice. Keeping this in mind, then it is not very surprising that both Pim $1^{-/-}$ and Pim $2^{-/-}$ mice have significantly impaired IgM levels.

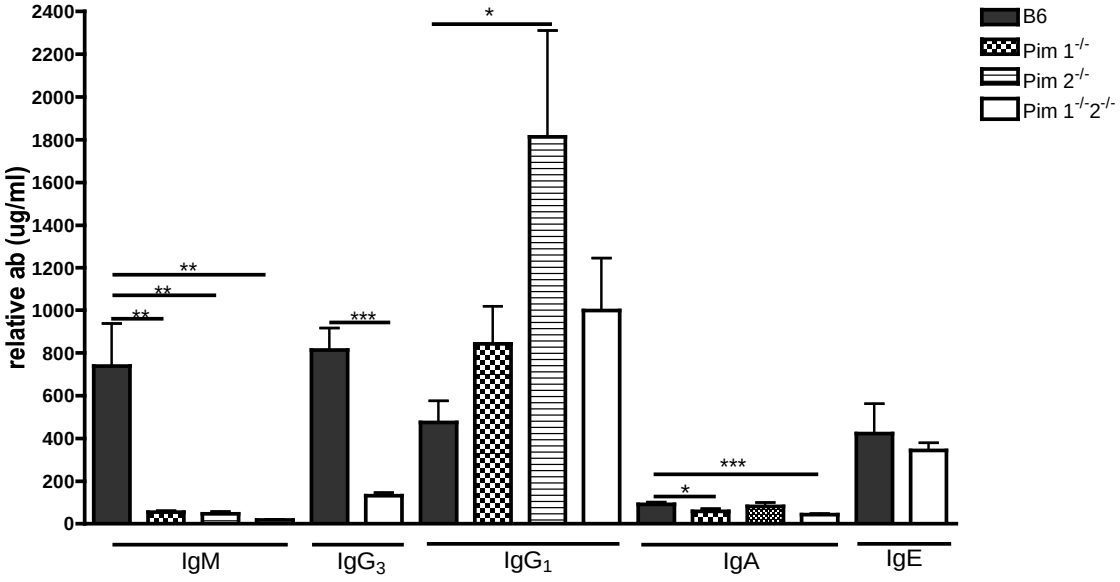
**Figure 3.4. The loss of Pim 1 and/or 2 impairs natural antibody.** Sera collected from naïve Pim $1^{-/-}2^{-/-}$ and control B6x129 (A) or Pim $1^{-/-}$, Pim $2^{-/-}$, Pim $1^{-/-}2^{-/-}$, and control C57BL/6 (B) mice was measured by ELISA for total antibody of a specific isotype indicated on the y-axis. Bars represent SEM, in A for IgM n=7, $IgG_3$ n=6, and $IgG_1$ B6 n=4 Pim $1^{-/-}2^{-/-}$ n=3. In B for each column n=5, except IgA where for B6 n=7, Pim $1^{-/-}$ n=4, and Pim $1^{-/-}2^{-/-}$ n=12. Statistics were determined by student's t test *=p<.05, **=p<.01, ***=p<.001

**The loss of Pim 1 and/or 2 impairs natural antibody**

**A.**




**B.**



**Peyer's Patches have diminished B cell numbers**

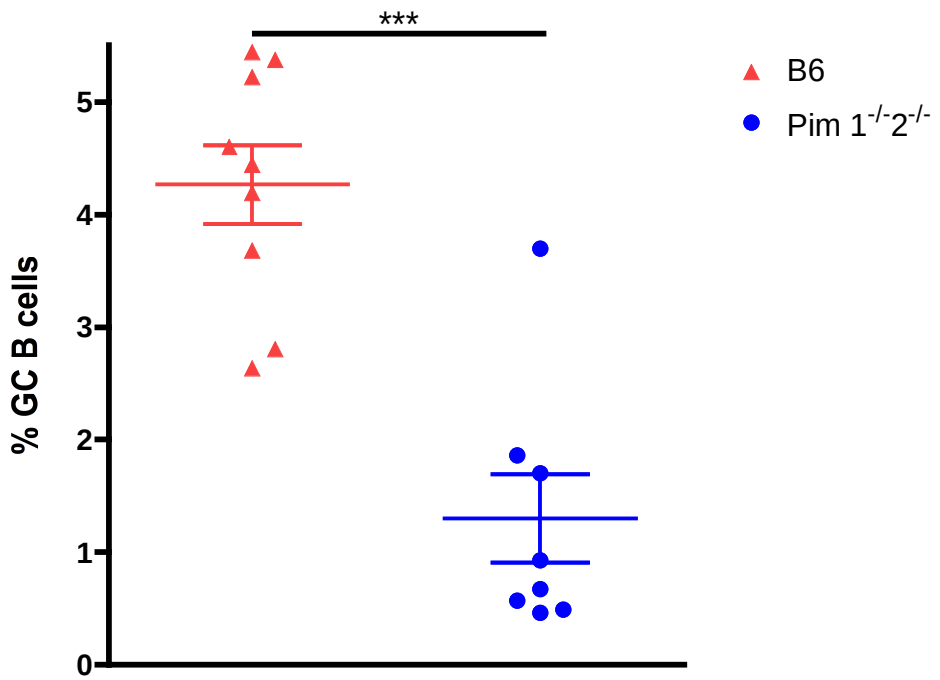
The main source of $IgG_3$ and IgM antibody in naïve mice are peritoneal B1 cells, IgA is generated mainly by B1 cells, which have migrated to sites of mucosal immunity, primarily in the lung and intestine. Along the intestine are Peyer's patches, organized secondary lymphoid tissues containing B, T, and other immune cells that respond to environmental pathogens. Peyer's patches in Pim double mutant mice were visibly smaller but normal in number, however, flow cytometry analysis revealed that a germinal center B cells ($B220^+GL7^+CD95^+$) were rare compared to wild type B6 Peyer's Patches (Figure 3.5).

We also determined the absolute number of B cells in the Peyer's patches of WT and Pim deficient mice and found a decrease in the Pim $1^{-/-}2^{-/-}$ ($1.3x10^6$ ± .2), Pim $1^{-/-}$ ($.44x10^6$ ± .2), and Pim $2^{-/-}$ ($1.6x10^6$ ± .6) mice compared to WT ($3.5x10^6$ ± .4). In addition, consistent with our observation that IgA antibody in the serum of naive Pim $1^{-/-}$ and Pim $1^{-/-}2^{-/-}$ mice was half that of controls, we found that the number of $IgA^+$ B cells in Peyer's patches was decreased with the loss of Pim 1. We also found that the $IgM^+$ B cell population in the Peyer's Patches was diminished whether Pim 1 or 2 was lost (Figure 3.6). The diminished $IgM^+$ and $IgA^+$ B cells in Peyer's Patches of Pim $1^{-/-}2^{-/-}$ mice is consistent with the difference observed in the peritoneal B cell cavity most likely because B cells migrate between these two areas.

**Figure 3.5. Peyer's Patches in Pim $1^{-/-}2^{-/-}$ mice have significantly fewer GC B cells. (A)** Cells isolated from Peyer's patches of control or Pim $1^{-/-}2^{-/-}$ mice were analyzed for the percent of GC B cells ($B220^{+}CD95^{+}GL7^{+}$). (B) Gating strategy for part A. Lines represent the mean and SEM of each group, each symbol represents an individual, WT n=10, Pim $1^{-/-}$ n=2, Pim $2^{-/-}$ n=3, Pim $1^{-/-}2^{-/-}$ n=12. Statistics were determined by student's t test *=p<.05, **=p<.01, ***=p<.001

**Peyer's Patches in Pim $1^{-/-}2^{-/-}$ mice have significantly fewer GC B cells**

**A.**




**B.**

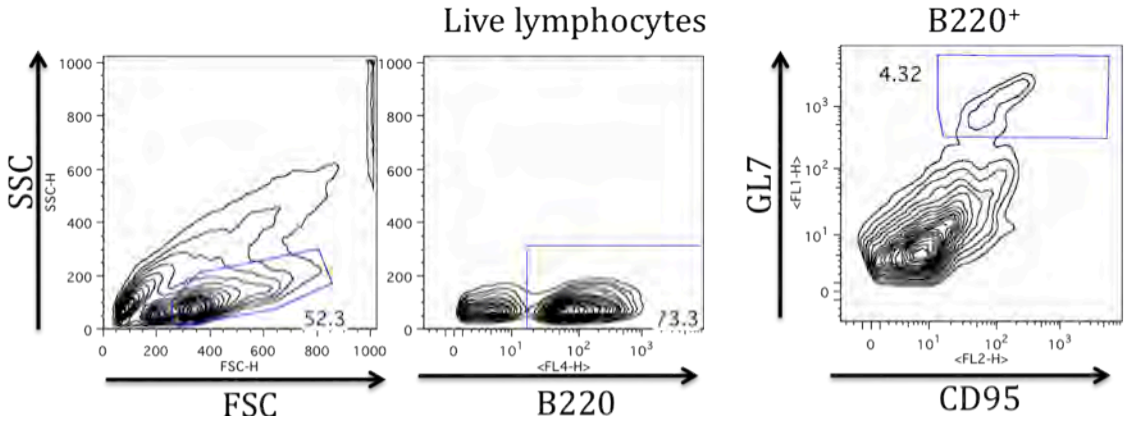
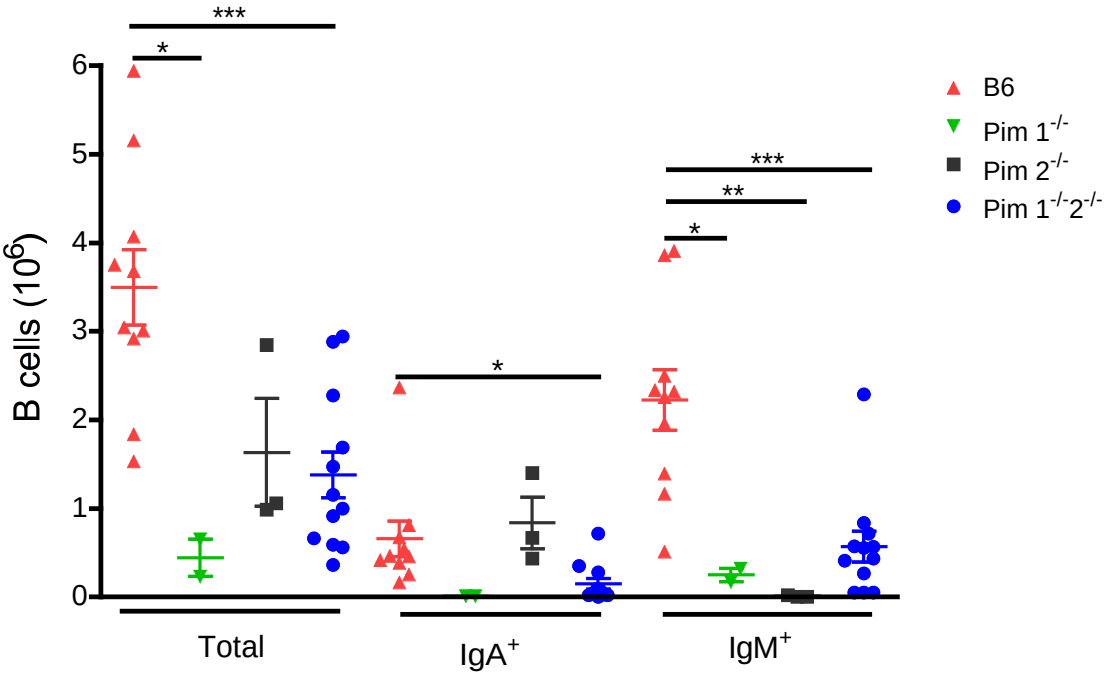


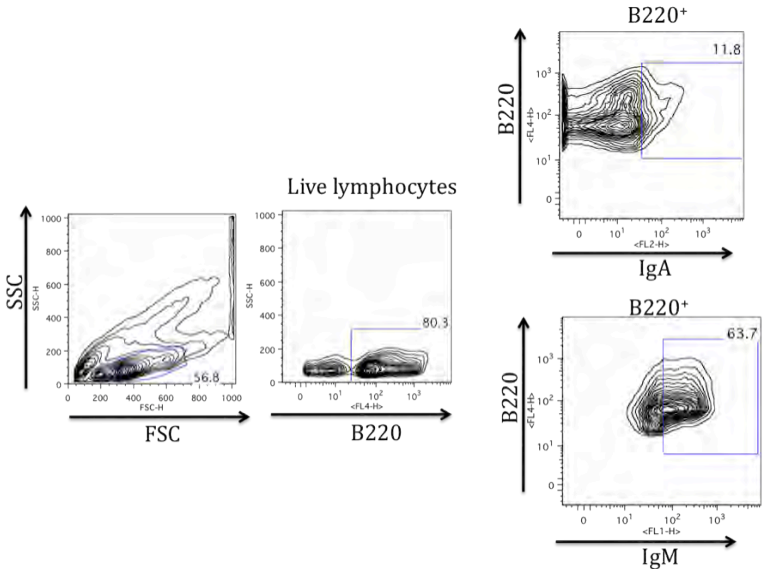
**Figure 3.6. Peyer's Patches from Pim $1^{-/-}2^{-/-}$ mice have diminished B cell populations. (A)** Peyer's Patches were isolated from the intestine of WT, Pim $1^{-/-}$, Pim $2^{-/-}$, and Pim $1^{-/-}2^{-/-}$ mice. B cells were identified as $B220^{+}$ then further categorized as $IgA^{+}$ or $IgM^{+}$. (B) Gating strategy for part A. Bars represent the mean and SEM, each symbol is an individual mouse (n=6). Statistics were determined by student's t test *=p<.05, **=p<.01, ***=p<.001

**Peyer's Patches from Pim $1^{-/-}2^{-/-}$ mice have diminished B cell populations**

A.




B.



**Pim induction after in vitro stimulation of Resting B cells**

The loss of GC and switched B cells in the Peyer's patches as well as the reduction in natural antibody suggests that Pim 1 and 2 kinases are important after B cell activation. To assess the induction of Pim kinases after activation and determine which pathways may involve Pims, we stimulated B cells in vitro with a number of mitogens that engage receptors on the B cell; such as the B cell receptor (anti-IgM), anti-CD40, BLyS receptors (BR3, TACI), or toll like receptors (LPS, CpG).

Percoll purified small resting B cells were obtained from GT-GL donor mice produced by Dr. Joan Press (Brandeis University) that express a immunoglobulin H and L chains specific for the synthetic polypeptide, Poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lysine [(T,G)AL] .Using B cells from this double knock-in mouse allow us to assess Pim induction when the BCR engaged a cognate peptide antigen presenting the dominant (TG)AL epitope, TG4 (TyrGluGluGluGluTyrTyrGluGluGluGluTyr) conjugated to Ficoll, as well as BLyS, anti-CD40, anti-delta-dextran, anti-IgM, CpG and LPS stimulators. The results clearly show that stimulation through the BCR, CD40, BR3, TLR4 and TLR9 all induced both Pim 1 and Pim 2 with the expected kinetics previously established (42, 58) (Figure 3.7). These results demonstrate that the Pim kinases are regulated by common B cell stimuli, including activation by a TI-2 cognate antigen (TG4).

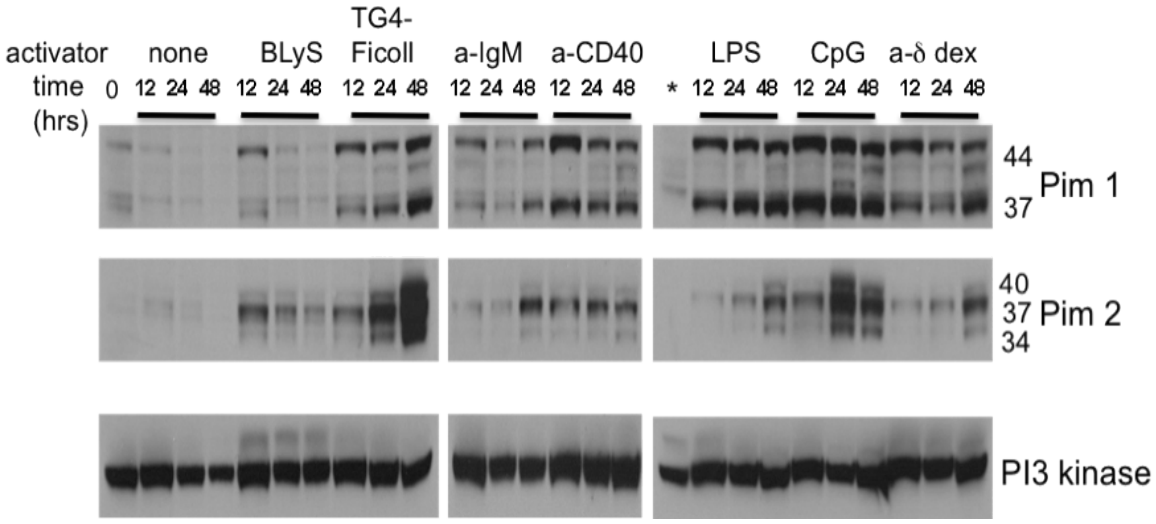
This diverse group of stimuli mimic, as best we can in vitro, activation pathways used by TI and TD antigens during B cell activation in vivo. The most

direct correlation is stimulation with TG4, a TI-2 antigen, which cross-link BCRs to activate the B cell. Using this same pathway anti-IgM and ant-delta-dextran also mimic TI-2 activation. However, signaling through the BCR is also a key component to TD B cell activation. In vivo the BCR signal requires an additional signal through the CD40 receptor, which we have examined alone using anti-CD40. Finally, we attempted to copy signaling through the TLRs, which occurs during a TI-1 response, by activating the B cells in vitro with CpG and LPS. Although each of these pathways can activate NF-kB signaling they also contain non-overlapping downstream targets. Whether these divergent pathways result in divergent roles for Pim 1 and 2 is discussed in chapter 7.

In this chapter we have described several impairments in natural and mucosal immunity found in naïve mice due to the loss of Pims. Though splenic B cell populations are intact, peritoneal B cells are diminished, consistent with the decreased sera Ig in these mice. B cells in the Peyer's Patches were also lower in number and had significantly fewer GC B cells suggesting impairment in GC formation in Pim $1^{-/-}2^{-/-}$ mice. These observations are consistent with a role for Pim 1 and 2 in activated B cells. Finally, we demonstrated that Pim 1 and 2 are induced in vitro under several activation conditions. To our knowledge this is the first demonstration of Pim induction after BCR-specific activation, which is consistent with a role for Pim 1 and 2 in humoral immunity. Considering these results together, we decided to assess whether Pim 1 and 2 kinases are required for normal B cell function during an in vivo humoral immune response to antigen.

**Figure 3.7. Pim kinases are induced upon B cell activation.** B cells isolated from GT+ V(D)J knock-in mice were cultured for 12, 24, or 48 hours with mitogens indicated. At each time point protein extracts were made form each cell sample and analyzed for induction of Pim 1 or 2 by western blot. Each Pim isoform is labeled, PI3 kinase was used as a loading control. This blot represents 3 separate experiments.

**Pim kinases are induced upon B cell activation**



# Chapter IV

# Thymus Dependent Responses Are Impaired By The Loss Of Pim 1 And 2

## Introduction

In a T cell dependent (TD) immune response, B cells are activated through T cell help, which facilitates the differentiation and proliferation of antigen specific B cells. These cognate activated B cells then proliferate and can develop into antibody secreting cells (ASCs) either extrafollicularly, which is generally limited to the beginning phase of the response or within a follicle, which is the pathway that dominates the response to TD antigens (6, 59). B cells within a follicle initiate a germinal center reaction where they can also undergo further diversification through isotype switching and affinity maturation (13-15). Isotype switching involves the replacement of the Cμ region of the antibody with the heavy chain region of another isotype. This does not change the affinity but modifies the effector activity of the antibody. The affinity of an antibody can be altered by somatic mutations in the variable region of the heavy and light chain, this combined with selection for the highest affinity BCR, is a process known as affinity maturation. Both of these processes rely on activation-induced cytidine deaminase (AID) an enzyme expressed exclusively in B cells about 40 hours after activation (16).

B cells eventually exit the germinal center either as an ASC or a memory B cell. How a B cell decides between these two different paths is not completely

understood but is likely influenced by the affinity of the BCR and the signal strength of other co-stimulatory cascades (59).

We have demonstrated in the previous chapter that Pim 1 and 2 are induced upon B cell activation via the BCR and CD40. Both of these receptors play are critical to a B cell's ability to respond to a TD antigen (59) suggesting that Pim 1 and 2 play a role in that response. To measure the affect of the loss of Pim 1 and/or 2 on these events, we measured the immune response of Pim deficient mice to a TD antigen using several different methods including ELISA, ELISpot, immunohistochemistry, and flow cytometry.

We find that Pim $1^{-/-}2^{-/-}$ mice display impaired TD antibody to multiple TD antigens. This is accompanied by reduced ASCs in the spleen early after immunization and in the bone marrow months after immunization, indicating a failure to generate and/or sustain long-lived ASCs. Antibody titers in Pim single knockout mice were significantly depressed compared to WT but less so than Pim $1^{-/-}2^{-/-}$. Germinal centers, a hallmark of the response to a TD antigen, were also impaired in Pim $1^{-/-}2^{-/-}$ mice with isotype switching and affinity maturation. We further show that adoptively transferred WT antigen specific T cell help cannot rescue the response to a TD antigen in Pim $1^{-/-}2^{-/-}$ mice, demonstrating at a minimum a B cell defect. These data point to a critical role for both Pim 1 and 2 in the response to a TD antigen.

## Results

### Antigen Specific antibody

In vitro B cell activation by various mitogens resulted in the induction of Pim 1 and 2. If these kinases are important in activated B cells, then it is reasonable to think that the humoral immune response in an immunized animal may also be affected. In vivo antigen stimulation results in a pool of ASCs producing antigen specific antibody, which can be measured in the blood. We immunized mice with NP-CGG in alum, a TD antigen, and then examined the NP specific antibody response early (day 7 or 10) and late (day 20).

Our first experiments were performed on B6x129 control and Pim $1^{-/-}2^{-/-}$ mice, which were available from Dr. C. Fox at Jackson Labs. Lambda positive antibody specific to NP was 7-fold less in Pim $1^{-/-}2^{-/-}$ mice with an average of 131.2 ± 44.9 μg/ml in control mice compared and 18.6 ± 6.4 μg/ml ($p<.05$) in Pim $1^{-/-}2^{-/-}$ mice ten days after immunization. This defect was not due to a delay in antibody production, as antibody at 20 days after immunization had not increased in Pim $1^{-/-}2^{-/-}$ mice and continued to be significantly ($p<.01$) less than controls (7 ± .9 vs. 198.1 ± 29 μg/ml) (Figure 4.1 A). These data indicated that Pim 1 and/or 2 are critical to a specific humoral response against a TD antigen. We originally measured anti-NP $\lambda^+$ antibody, which does not distinguish between isotypes so we next asked if this defect was seen in all isotypes or only, unswitched (IgM) or switched ($IgG_1$, $IgG_3$) antibody. We found that anti-NP $IgM^+$ antibody in Pim $1^{-/-}2^{-/-}$ was 26.8 ± 9.4 μg/ml compared to 71.9 ± 15.7 μg/ml in control mice, an almost 3-fold drop ($p<.05$) ten days after immunization. Twenty days after

immunization anti-NP $IgM^+$ antibody in control mice dropped to an average of 40.7 ± 17 μg/ml, however, the gap widened as Pim $1^{-/-}2^{-/-}$ mice produced 8-fold less with an average of only 4.7 ± 1 μg/ml ($p<.05$) (Figure 4.1 B). These same serum samples were analyzed for anti-NP specific $IgG_1^+$ antibody revealing an almost 6.5-fold difference between control and Pim $1^{-/-}2^{-/-}$ mice at day ten post-immunization (126.9 ± 38.7 vs. 19.7 ± 8.4 μg/ml, $p<.01$) as well as a significant difference at 20 days post-immunization (245 ± 82 vs. 93 ± 29 μg/ml, $p<.05$) (Figure 4.1 C). Finally, anti-NP $IgG_3^+$ antibody was measured 10 days post-immunization and although titers were low, Pim $1^{-/-}2^{-/-}$ mice had significantly less than controls at day 10 post-immunization (3.8 ± .6 vs. 1.1 ± .2 μg/ml, $p<.001$) (Figure 4.1 D). The low concentration of $IgG_3$ antibody was not unexpected as it is not a dominant isotype in TD responses. We concluded that switched and IgM antigen specific antibody was impaired during the response to a TD antigen.

The response to NP is almost entirely dominated by lambda antibodies. Studies on the bone marrow of Pim $2^{-/-}$ mice have demonstrated a diminution of lambda antibodies (60). To ensure that this failure was not specific to NP or due to a limited number of lambda positive peripheral B cells, we also immunized B6x129 Pim $1^{-/-}2^{-/-}$ mice with a synthetic polypeptide, Poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lysine (T,G)AL, another TD antigen whose response is dominated by kappa antibodies. Pim $1^{-/-}2^{-/-}$ mice still could not mount a TD response to (T,G)AL (Figure 4.2). These results demonstrate a critical role for Pim 1 and/or 2 in the generation of antigen specific antibody, independent of antigen or light chain.

To pursue this defect in TD antibody further we took advantage of single and double Pim knockout mice, which became available backcrossed onto C57BL/6 and were again generously provided by Dr. C. Fox. We repeated our TD immunization experiments with C57BL/6 control and Pim $1^{-/-}2^{-/-}$ mice and measured antibody at multiple days after immunization. A significant defect in antibody was again observed in Pim $1^{-/-}2^{-/-}$ mice 7 days (243.4 ± 94 vs. 53.36 ± 22 µg/ml, $p<.05$), 10 days (394.7 ± 65 vs. 72.52 ± 18 µg/ml, $p<.001$), and 14 days (692.5 ± 36 vs. 87.18 ± 27.8 µg/ml, $p<.001$) after immunization. By 20 days after immunization antibody in control mice decreased but was still more than 3-fold higher than Pim $1^{-/-}2^{-/-}$ antibody (248.1 ± 66.8 vs. 64 ± 32.5 µg/ml, $p<.05$). This difference continued to thirty days after immunization (302.4 ± 57.9 vs. 104.7 ± 51.3 µg/ml, $p<.05$) demonstrating a defect and not delay in the ability of Pim $1^{-/-}2^{-/-}$ mice to produce antigen specific antibody (Figure 4.2 A).

To determine if only Pim 1 or 2 were critical to the humoral immune response we immunized C57BL/6 Pim $1^{-/-}$ and Pim $2^{-/-}$ single knock out mice with 50 µg of NP-CGG in Imject alum i.p., these mice were bled every few days to monitor antibody to NP. Pim $1^{-/-}$ mice produced less than half the antibody of control mice 7 days after immunization (243.4 ± 94 vs. 108.8 ± 22.9 µg/ml) (Figure 4.2 B) while Pim $2^{-/-}$ mice produce 4-fold less (243.3 ± 94 vs. 49.39 ± 18.9 µg/ml, $p<.05$) (Figure 4.2 C). By 14 days after immunization both single knockout mice lagged behind at less than one-third of control mice with Pim $1^{-/-}$ mice averaging 219.6 ± 77 µg/ml ($p<.001$) and Pim $2^{-/-}$ at 211.5 ± 77.4 µg/ml ($p<.001$). Twenty days after immunization all samples began to decline and single

knockout mice were half of controls, both with just over 100 µg/ml while C57BL/6 mice produce over 200 µg/ml of antibody on average. These numbers stayed approximately the same at thirty days after immunization. Though antibody titers in the single knock out mice were a little higher than Pim double knockouts they were not significantly different from Pim $1^{-/-}2^{-/-}$ antibody titers at most time points. The exceptions being 10 days after immunization for Pim $2^{-/-}$ and 14 days after immunization for Pim $1^{-/-}$ mice (Figure 4.2 B&C). These experiments demonstrated that the loss of either Pim 1 or 2 results in a defect in antigen specific antibody production but the loss of both compounds the defect.

We continued our analysis of the C57BL/6 serum by assaying for NP specific antibody of different isotypes, as we did with B6x129 mice. Impairment in isotype switching would result in a diminution of $IgG_1$ but not IgM antibody. Alternatively, a general defect in activation or the production of ASCs would result in all isotypes including IgM antibody to be diminished after immunization of C57BL/6 Pim $1^{-/-}2^{-/-}$ mice, as we observed in B6x129 Pim $1^{-/-}2^{-/-}$ mice.

Serum from C57BL/6 Pim $1^{-/-}2^{-/-}$ mice immunized with NP-CGG was measured again, this time for NP specific antibody of certain isotypes. We found that in Pim $1^{-/-}2^{-/-}$ and Pim $2^{-/-}$ but not Pim $1^{-/-}$ mice, IgM was significantly impaired. On day 10, C57BL/6 control mice averaged 654.8 ± 76.5 µg/ml while Pim $2^{-/-}$ and Pim $1^{-/-}2^{-/-}$ mice were significantly lower with 135.4 ± 24.6 ($p<.001$) and 116.3 ± 54.3 µg/ml ($p<.01$) respectively. On day 14 after NP-CGG immunization, C57BL/6 control mice averaged 395.7 ± 69.8 µg/ml while Pim $2^{-/-}$ and Pim $1^{-/-}2^{-/-}$ mice averaged 79.8 ± 26.6 ($p<.001$) and 52.22 ± 15 ($p<.01$) µg/ml

respectively. This depression in antibody continued to 20 days post immunization when control mice averaged 237.2 ± 48.1 µg/ml compared to 55 ± 12.8 µg/ml ($p<.01$) in Pim $2^{-/-}$ mice and 25.6 ± 11.7 µg/ml ($p<.01$) in Pim $1^{-/-}2^{-/-}$ mice. Pim $1^{-/-}$ mice were not significantly different from wild type controls (Figure 4.3 A). From this data we conclude that Pim 2 and not Pim 1 is required for normal IgM antibody during a TD humoral immune response.

Now that we know that Pim $1^{-/-}$ mice have normal, while Pim $2^{-/-}$ mice have diminished IgM antibody titer, we wanted to compare $IgG_1$ antibody (a common switched isotype) in the same mice. Samples were measured for $IgG_1$ NP specific antibody at 7 and 14 days after immunization. C57BL/6 controls averaged 108.3 ± 41.7 µg/ml 7 days after immunization, which was significantly more than Pim $1^{-/-}$, Pim $2^{-/-}$, and Pim $1^{-/-}2^{-/-}$ averaging 36.9 ± 9.3 ($p<.05$), 12 ± 3.7 ($p<.05$), and 28.3 ± 12 ($p<.05$) µg/ml respectively. One week later, 14 days after immunization, the amount of $IgG_1$ antibody in the WT response had increased as expected to an average of 681.1 ± 132 µg/ml. The increase in Pim $1^{-/-}$ and Pim $2^{-/-}$ mice was far less averaging 139.8 ± 37.3 ($p<.01$) and 26.15 ± 7.5 ($p<.01$) µg/ml on day 14 respectively while $IgG_1$ in the Pim $1^{-/-}2^{-/-}$ samples decreased to an average of 7.1 ± 1.6 ($p<.01$) µg/ml (Figure 4.3 B). This data extends our previous observations by demonstrating that isotype switched as well as IgM antibody is impaired in the TD response of Pim $1^{-/-}2^{-/-}$ and Pim $2^{-/-}$ mice while only $IgG_1$ antibody was found to be deficient in Pim $1^{-/-}$ mice. The loss of $IgG_1$ antibody could be caused by a defect in isotype switching. Whereas, the loss of IgM

antibody in Pim $1^{-/-}2^{-/-}$ mice suggests that a generalized defect in B cell differentiation or survival also exists.

**Figure 4.1. Impaired TD responses in B6x129 Pim $1^{-/-}2^{-/-}$ mice.** B6x129 WT or Pim $1^{-/-}2^{-/-}$ mice were immunized i.p. with 50 µg of NP-CGG in Imject Alum. Sera was collected at 10 and 20 days after immunization and analyzed for lambda (total) (A), IgM (B), $IgG_1$ (C), and $IgG_3$ (D), NP specific antibody. Graphs include 3 experiments, lines indicate mean and SEM of each group, each symbol represents an individual mouse. Red triangles are WT, blue circles are Pim $1^{-/-}2^{-/-}$. Significance was determined by Student's t test *p<.05, ** p<.01, ***p<.001. For WT: unimmunized n=1, Imject Alum only n=3 ($IgG_3$ and IgM) or n=2 (λ and $IgG_1$), day 10 n=9 ($IgG_3$ and $IgG_1$), n=8 (IgM), or n=12 (λ), day 20 n=6 ($IgG_3$ and IgM), n=5 ($IgG_1$), n=3 (λ); Pim $1^{-/-}2^{-/-}$ Imject Alum only n=1, day 10 n=9 (IgM, $IgG_1$, and $IgG_3$), n=12 (λ), day 20 n=6 ($IgG_1$, IgM, and $IgG_3$) n=3 (λ).

## Impaired TD responses in B6x129 Pim $1^{-/-}2^{-/-}$ mice

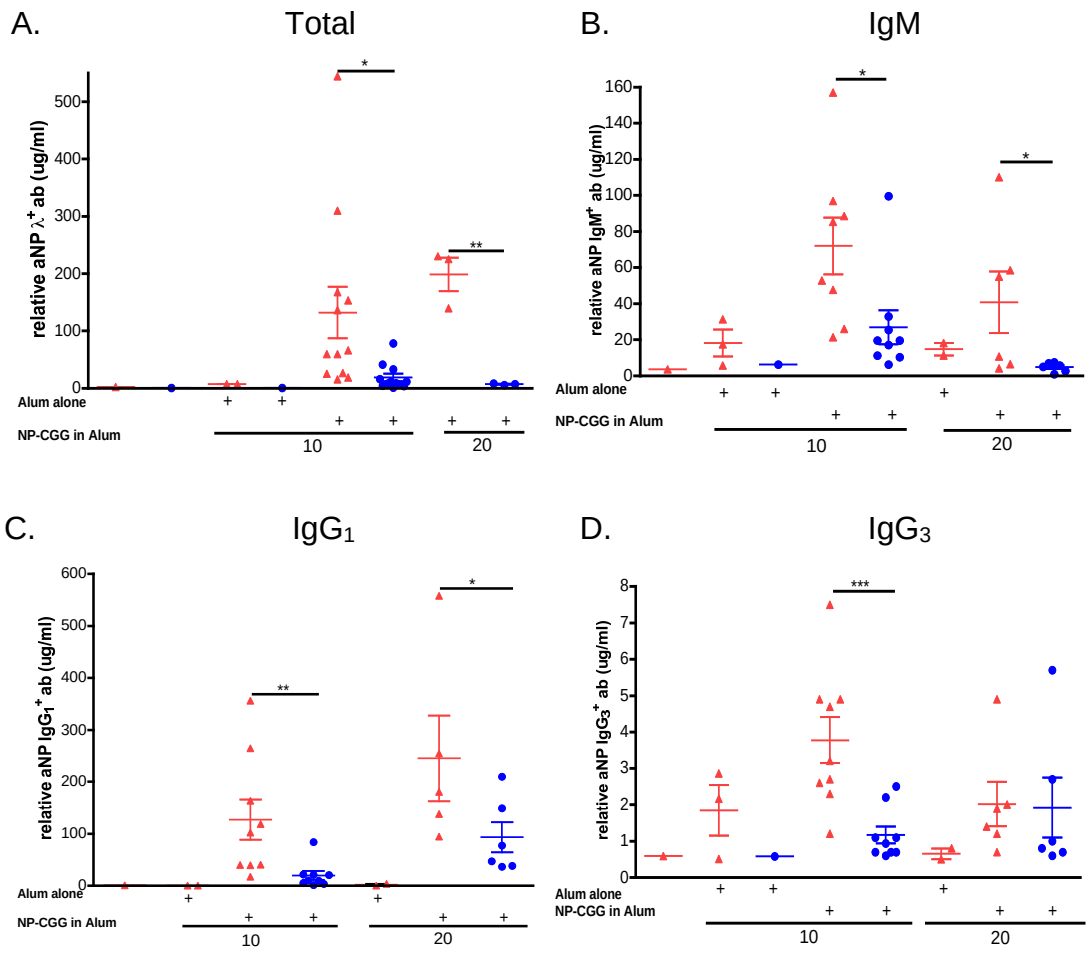


**Figure 4.2. The Pim $1^{-/-}2^{-/-}$ antibody deficiency is not specific to one antigen**. B6x129 control and Pim $1^{-/-}2^{-/-}$ mice were immunized i.p. with 50 µg of (T,G)AL, sera was collected at 7, 14, 21 days after immunization (X-axis) and measured for TG4 specific antibody (y-axis). WT n=3 (day 7 and 21) or n= 2 (day 14); Pim $1^{-/-}2^{-/-}$ n=1 (day 7) or n=2 (day 14 and 21). Lines indicate mean and SEM of each group, each symbol represents an individual mouse. Red triangles are WT, blue circles are Pim $1^{-/-}2^{-/-}$. Significance was determined by Student's t test *p<.05, ** p<.01, ***p<.001.

**The Pim $1^{-/-}2^{-/-}$ antibody deficiency is not specific to one antigen**

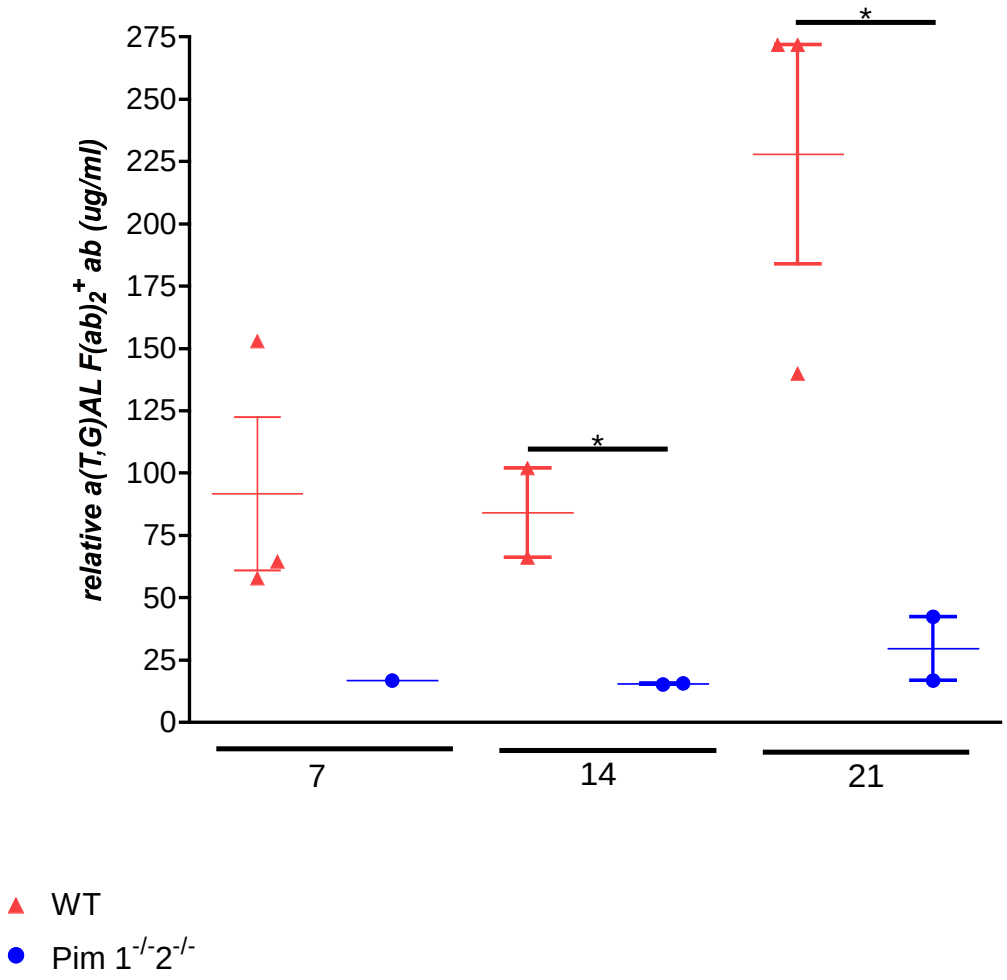
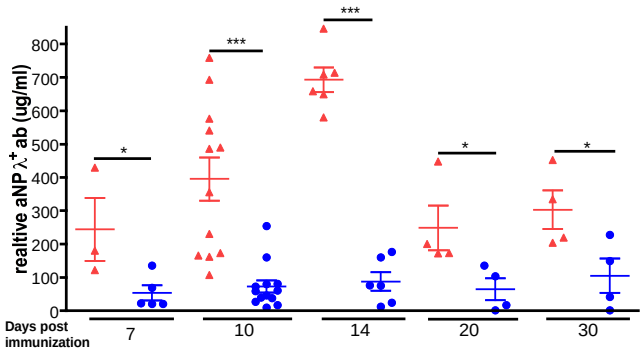


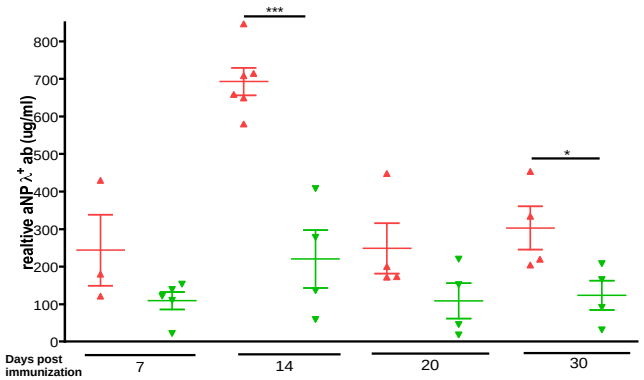
**Figure 4.3. Impaired TD responses in C57BL/6 Pim $1^{-/-}$, Pim $2^{-/-}$, and Pim $1^{-/-}2^{-/-}$ mice.** C57BL/6 WT and Pim $1^{-/-}2^{-/-}$ (A), Pim $1^{-/-}$ (B), and Pim $2^{-/-}$ (C) mice were immunized i.p. with 50 µg of NP-CGG in Imject Alum, sera was collected at days indicated and measured for anti-NP $\lambda^{+}$ antibody. WT and Pim $1^{-/-}2^{-/-}$ data includes 4 experiments, Pim $1^{-/-}$ data is 1 experiment, Pim $2^{-/-}$ data includes 3 experiments. WT day 7 n=3, day 10 n=12, day 14 n=6, day 20 and 30 n=4, Pim $1^{-/-}$ day 7 n=5, day 14 n=6, day 20 and 30 n=4, Pim $2^{-/-}$ day 7 n=4, day 10 n=11, day 14 n=5, day 20 and 30 n=5, Pim $1^{-/-}2^{-/-}$ day 7 n=5, day 10 n=13, day 14 n=6, day 20 and 30 n=4. Lines indicate the mean and SEM, each symbol represents an individual mouse, red triangles are WT, blue circles are Pim $1^{-/-}2^{-/-}$, green inverted triangles are Pim $1^{-/-}$, Black boxes are Pim $2^{-/-}$, statistical significance was determined by student's t test *p<.05, ** p<.01, ***p<.001.

## Impaired TD responses in C57BL/6 Pim $1^{-/-}$, Pim $2^{-/-}$, and Pim $1^{-/-}2^{-/-}$ mice.

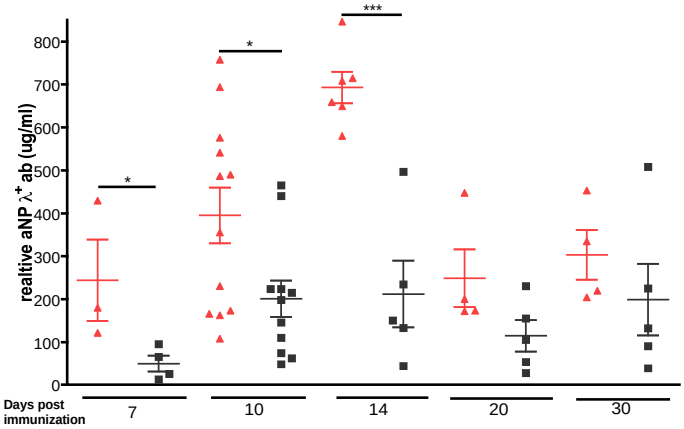
**A.** Pim $1^{-/-}2^{-/-}$




**B.** Pim $1^{-/-}$




**C.** Pim $2^{-/-}$

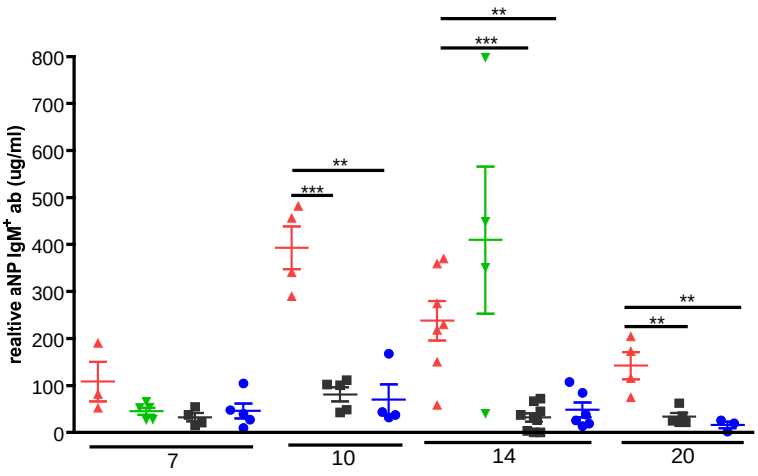



- B6 WT
- B6 Pim $1^{-/-}$
- B6 Pim $2^{-/-}$
- B6 Pim $1^{-/-}2^{-/-}$

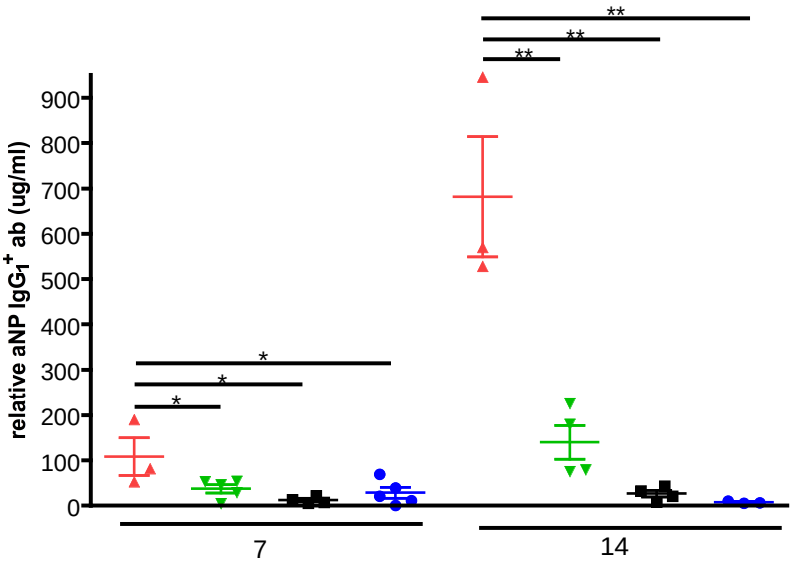
**Figure 4.4. Pim $1^{-/-}$ mice are deficient in $IgG_1$ but not IgM.** C57BL/6 control and Pim deficient mice were immunized i.p. with 50 µg of NP-CGG in Imject Alum, sera were collected at days indicated and measured for IgM (A) and $IgG_1$ (B) antibody. A includes 2 experiments, WT day 7 n=3, day 10 and 20 n=4, day 14 n=7, Pim $1^{-/-}$ day 7 n=5, day 14 n=4, Pim $2^{-/-}$ day 7 n=4, day 10 and 20 n=5, day 14 n=9, Pim $1^{-/-}2^{-/-}$ day 7 n=5, day 10 n=4, day 14 n=6, day 20 n=3. B includes 1 experiment, WT n=3, Pim $1^{-/-}$ day 7 n=5, day 14 n=4, Pim $2^{-/-}$ n=4, Pim $1^{-/-}2^{-/-}$ day 7 n=5, day 14 n=3. Bars indicate the mean and SEM of each group, each symbol represents an individual mouse, red triangles are WT, blue circles are Pim $1^{-/-}2^{-/-}$, green inverted triangles are Pim $1^{-/-}$, Black boxes are Pim $2^{-/-}$, statistical significance was determined by student's t test *p<.05, ** p<.01, ***p<.001.

## Pim $1^{-/-}$ mice are deficient in $IgG_1$ but not IgM

**A.** **IgM**




**B.** **$IgG_1$**




▲ B6 WT

▼ B6 Pim $1^{-/-}$

■ B6 Pim $2^{-/-}$

● B6 Pim $1^{-/-}2^{-/-}$

**Antigen specific ASC numbers are decreased in Pim $1^{-/-}2^{-/-}$**

Whether through the germinal center or the extra-follicular response exposure to a TD antigen results in the generation of ASCs. These terminally differentiated B cells cease to proliferate and exist to produce antigen specific antibody and will do nothing else until they die. A reduced population of antibody secreting cells (ASCs) would be consistent with the decrease in antigen specific antibody of multiple isotypes we have observed in Pim $1^{-/-}2^{-/-}$ mice and a role for Pim 1 and/or 2 in the generation or maintenance of this population. To enumerate the number of ASCs in WT and Pim $1^{-/-}2^{-/-}$ mice we used an antigen specific ELISpot.

Initial experiments done in B6x129 Pim $1^{-/-}2^{-/-}$ mice immunized with NP-CGG in Imject Alum revealed that ASCs specific to NP in Pim $1^{-/-}2^{-/-}$ mice were reduced 2-fold on day 7, 4-fold on day 10, 3-fold on day 14 and 20-fold on day 20 post immunization compared to WT (Figure 4.4 A).

C57BL/6 WT, Pim $1^{-/-}2^{-/-}$, and Pim $2^{-/-}$ mice immunized with NP-CGG were sacrificed 10 days after immunization and ASCs were enumerated by ELISpot. WT mice averaged 28 ± 7 ASCs/$10^6$ splenocytes (n=10), significantly higher than Pim $1^{-/-}2^{-/-}$ mice, which averaged 12 ± 1 ASCs/ $10^6$ splenocytes (n=11). Pim $2^{-/-}$ mice did not produce many more ASCs than Pim $1^{-/-}2^{-/-}$ at 14 ± 6 ASCs/$10^6$ splenocytes but were not significantly different than WT most likely due to the scatter and small number of individuals (n=3) (Figure 4.4 B). These data show that consistent with the lower antibody Pim $1^{-/-}2^{-/-}$ mice have fewer ASCs and suggest that Pim 2, at least, is important for normal ASC development after immunization with a TD antigen. This decrease in ASCs could be due to a defect

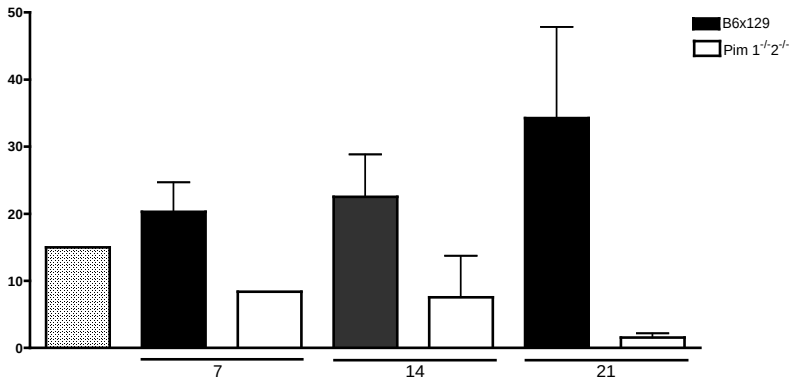
in the generation or survival of these cells. This question will be addressed in Chapter V.

**Figure 4.5. ASC numbers are significantly reduced in Pim $1^{-/-}2^{-/-}$ mice.**

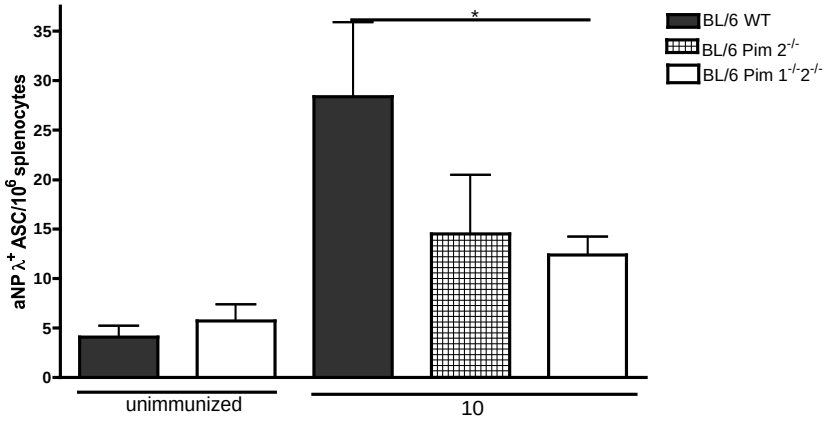
B6x129 control and Pim $1^{-/-}2^{-/-}$ mice immunized with 50 µg (T,G)AL absorbed to Imject Alum (A) or C57BL/6 control and Pim $1^{-/-}2^{-/-}$ mice immunized with 50 µg NP-CGG absorbed to Imject Alum (B) were sacrificed at days indicated, splenocytes were isolated and ASCs were enumerated by ELISpot. (A) Is 1 experiment, unimmunized and Pim $1^{-/-}2^{-/-}$ day 7 n=1, WT n=3, Pim $1^{-/-}2^{-/-}$ day 14 and 21 n=2 (B) Includes 3 experiments, unimmunized n=5, WT n=11, Pim $1^{-/-}2^{-/-}$ n=10, Pim $2^{-/-}$ n=3. Bars represent SD, significance was determined by student's t test *p<.05, ** p<.01, ***p<.001.

## ASC numbers are significantly reduced in Pim $1^{-/-}2^{-/-}$ mice

**A.**




**B.**



**Pim 1 and/or 2 are not absolutely required for germinal center formation**

Antibody secreting cells are generated in a TD response either extrafollicularly and/or in a germinal center. Though the number of ASCs in Pim $1^{-/-}2^{-/-}$ was significantly decreased, we do not know if this is due to impairment in one or both pathways. The presence of germinal centers in splenic follicles is a classic hallmark of a TD response. Phenotypic markers can identify B cells participating in a germinal center reaction as $B220^{+}CD95^{+}GL7^{+}$. Flow cytometry analysis of splenocytes from B6x129 controls and Pim $1^{-/-}2^{-/-}$ mice at 7, 10, 14, and 21 days post-immunization with NP-CGG or (T,G)AL revealed that in Pim deficient individuals, a GC population could not be identified. The number of splenocytes counted for each mouse was multiplied by the percent of $B220^{+}GL7^{+}CD95^{+}$ cells found by flow cytometry to determine the number of GC B cells per mouse. B6x129 control animals averaged $2x10^{6}$ ± .5 GC B cells per mouse (n=5) at the peak of the response (10-14 days) while GC B cell populations in Pim deficient animals remained at background level with .14 ± .07 GC B cells per mouse (n=4, $p<.01$). We also observed that naïve Pim $1^{-/-}2^{-/-}$ mice had fewer GC B cells than WT controls. This observation was consistent with less Ig in the sera of naïve animals, discussed in the previous chapter. Late in the response (3 weeks), B6x129 GC B cell numbers declined as expected but remained significantly higher than Pim $1^{-/-}2^{-/-}$ mice, which also had background levels of GC B cells (Figure 4.5 A1).

Backcrossed C57BL/6 Pim $1^{-/-}2^{-/-}$ and control mice were immunized with NP-CGG in alum and the same analysis was performed. Again, Pim $1^{-/-}2^{-/-}$

mice had significantly fewer GC B cells than controls. Ten days after immunization, WT mice averaged $0.8x10^6$ ± .1 GC B cells per mouse (n=7), 2.8 times higher than Pim $1^{-/-}2^{-/-}$ at $0.28x10^6$ ± .06 GC B cells per mouse (n=9). At 15 days after immunization that gap increased to 3.7 times as WT controls averaged $.93x10^6$ ± .9 GC B cells per mouse (n=4) and Pim $1^{-/-}2^{-/-}$ mice averaged $.25x10^6$ ± .03 GC B cells per mouse (n=5). Pim $2^{-/-}$ mice did not differ from WT controls, suggesting that Pim 1 is responsible for the defect in germinal center B cell numbers (Figure 4.5 A2). This can be tested directly in the future by analyzing GCs in Pim $1^{-/-}$ mice immunized with NP-CGG. We found that Pim $1^{-/-}2^{-/-}$ mice on both the B6x129 and C57BL/6 background generate significantly reduced GC B cell populations following a TD immunization.

We also looked for germinal center reactions in the spleen by immunohistochemistry. Spleen sections from WT or Pim $1^{-/-}2^{-/-}$ as well as unimmunized control mice were fixed and stained with biotinylated peanut agglutinin (PNA-biotin), a lectin that identifies germinal centers by binding to a certain carbohydrate sequence and can be detected by an enzymatic reaction following SA-HRP binding. Counting the number of germinal centers visible in spleen sections revealed that in both the B6x129 mixed and C57BL/6 background Pim $1^{-/-}2^{-/-}$ had significantly fewer germinal centers visible in the spleen (Figure 4.5 B1). This was consistent with our flow cytometry data on germinal center B cells for the same mice. In B6x129 experiments only 2 Pim $1^{-/-}2^{-/-}$ mice had germinal center numbers above background. Several Pim $1^{-/-}2^{-/-}$ mice had no PNA positive follicles consistent with the lack of GC B cells

observed by flow cytometry. Experiments using C57BL/6 Pim $1^{-/-}2^{-/-}$ mice and controls portrayed the same phenomena though each experiment had an outlier, which created more scatter when 4 experiments were compiled than we had seen in the B6x129 experiments or in the flow cytometry data. Despite the scatter, there was a significant difference in the number of $PNA^+$ germinal centers at 10 and 15 days after immunization. Ten days after immunization WT controls averaged approximately 16 ± 3 $PNA^+$ GCs per spleen section (n=11) while Pim $1^{-/-}2^{-/-}$ mice averaged half as many with just below 8 ± 2 $PNA^+$ GCs per spleen section (n=15). Five days later Pim $1^{-/-}2^{-/-}$ mice averaged .6 ± .4 $PNA^+$ germinal center per spleen section (n=5), well below the background determined by WT naïve mice (1.4 ± .9, n=5). WT controls also decreased but by a much smaller margin, falling from 16 ± 3 to 12 ± 2 $PNA^+$ germinal centers per spleen section (n=4), remaining significantly higher than the Pim $1^{-/-}2^{-/-}$ mice ($p<.01$) (Figure 4.5 B2). From this data in both backgrounds, we concluded that the germinal center reaction is impaired in Pim $1^{-/-}2^{-/-}$ mice. Where this impairment is we cannot determine from these experiments but the number of mice with zero or background $PNA^+$ numbers suggests that these mice are unable to initiate germinal centers during a TD response.

Un-published reports from the Cancro lab at the University of Pennsylvania who were conducting similar experiments with C57BL/6 Pim $1^{-/-}2^{-/-}$ mice from the same source contradicted our results described above. In that they found the germinal center reaction to be intact in Pim $1^{-/-}2^{-/-}$ mice after NP-CGG immunization. In comparing experimental protocols we found that though we both

used NP-CGG from Biosearch, the antigen-alum mixture used for each of our experiments was prepared differently. Our lab absorbed antigen onto pre-mixed Imject Alum according to manufacturer's instructions while the Cancro lab performed a precipitation of antigen as described in (61). Briefly, instead of a sterile pre-mixed solution as with our procedure, the protein solution is mixed with sodium bicarbonate and aluminum potassium. The precipitate is spun off and the protein resuspended. To determine if the different antigen-adjuvant preparations influenced the results, I performed 2 experiments using NP-CGG in alum precipitated by the Cancro lab to immunize our mice. Mice given 50 µg of NP-CGG in precipitated alum were sacrificed 15 days after immunization. In both experiments, the number of germinal center cells in the spleen was determined by flow cytometry as described above. These results were similar to our previous observations with a significant difference ($p<.05$) between control and Pim $1^{-/-}2^{-/-}$ germinal center B cells (Figure 4.5 C1). However, we noted that 2 out of the 5 Pim $1^{-/-}2^{-/-}$ mice in one experiment and 3 out of the 5 in the other experiment had a germinal center population detectable by flow cytometry similar in magnitude to the WT controls. This was something we had not observed with our previous immunizations. $PNA^{+}$ germinal centers were measured in one experiment and the same 2 Pim $1^{-/-}2^{-/-}$ mice whose flow cytometry profiles revealed a germinal center B cell population also had WT numbers of $PNA^{+}$ germinal centers by immunohistochemistry (Figure 4.5 C2). These results were particularly striking because of the separation between these two mice and the three who did not

have a germinal center population which suggested that the germinal center response in Pim $1^{-/-}2^{-/-}$ mice was either on or off with nothing in between.

We hypothesized that the loss of Pim 1 and/or 2 impaired signaling downstream of the BCR and CD40 receptor, and as a result Pim $1^{-/-}2^{-/-}$ mice could only initiate a germinal center response if signaling through Pim independent pathways were increased. Increased signaling may be achieved by a more antigenic stimulus, such as the precipitated alum instead of the pre-mixed Imject Alum. Others have proposed that total signal strength through the BCR, CD40, and possibly other environmental cues upon encounter with a TD antigen determines a B cell's fate (59). In addition, it has been shown that although a low affinity BCR does not exclude WT cells from participating in a germinal center reaction, those with a higher affinity have a competitive advantage, accumulate in the GC and survive better (62, 63).

We demonstrated in the previous chapter that both BCR and CD40 activation induces Pim 1 and 2 in vitro. Both of these receptors are activated in the TD response. Our data demonstrating a significant decrease in the number of germinal center B cells and $PNA^{+}$ germinal centers in Pim $1^{-/-}2^{-/-}$ mice over several experiments is consistent with a role for Pim downstream of both of these receptors. It is important to note that as the strength of the adjuvant or antigen increased so did the number of Pim $1^{-/-}2^{-/-}$ mice producing germinal center populations by PNA staining and flow cytometry. So the loss of Pim 1 and 2 does not make it impossible for a germinal center response to proceed as normal, but clearly impacts the likelihood of its occurrence. In the immunohistochemistry

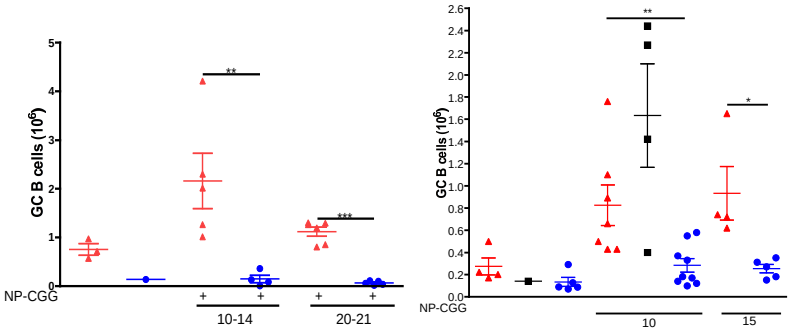
data, Pim $1^{-/-}2^{-/-}$ mice seem to fall into 2 groups, those at background level, and those with normal numbers of $PNA^{+}$ germinal centers, with nothing in between. This suggested to us that there was a threshold which must be reached to switch the germinal center program on. We hypothesized then that Pim 1 or 2 lowers the threshold of activation in B cells downstream of CD40 and the BCR. This idea is also consistent with our finding that NP specific antibody in Pim $1^{-/-}2^{-/-}$ mice has a higher affinity than B6 early in the response, which we will discuss in a later section.

In addition, Pim $2^{-/-}$ mice were not impaired in the number of germinal center B cells or $PNA^{+}$ germinal centers, suggesting that Pim 1 alone may be responsible for this defect. Limitations in our mouse colony prevented us from pursuing this further to date, but testing Pim $1^{-/-}$ mice in the future would determine if Pim 1 is responsible for the germinal center defect or both Pim 1 and 2 must be lost for the germinal center to be impaired.

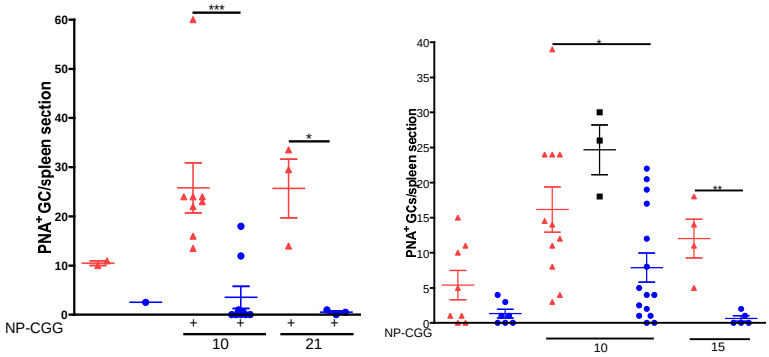
**Figure 4.6. Pim 1 and/or 2 are not absolutely required for the germinal center response.** A-B. Mice were immunized with NP-CGG absorbed to Imject Alum A) B6x129 (1) (WT unim n=3, Pim $1^{-/-}2^{-/-}$ unim n=1, day 7 WT n=2, Pim $1^{-/-}2^{-/-}$ n=1, day 10-14 WT n=5, Pim $1^{-/-}2^{-/-}$ n=4, days 20-21 WT n=6, Pim $1^{-/-}2^{-/-}$ n=5) and C57BL/6 (2) (Unimmunized: WT n=4, Pim $2^{-/-}$ n=1, Pim $1^{-/-}2^{-/-}$ n=5, day 10 WT n=7, Pim $2^{-/-}$ n=4, Pim $1^{-/-}2^{-/-}$ n=9, day 15 WT n=4, Pim $1^{-/-}2^{-/-}$ n=5) germinal center B cells were identified as $CD95^{+}GL7^{+}B220^{+}$ by flow cytometry and enumerated B) B6x129 (1) (Unimmunized WT n=2, Pim $1^{-/-}2^{-/-}$ n=1, day 10 WT n=8, Pim $1^{-/-}2^{-/-}$ n=9, day 20 WT n=3, Pim $1^{-/-}2^{-/-}$ n=3) and C57BL/6 (2) (Unimmunized WT n=5, Pim $1^{-/-}2^{-/-}$ n=7, day 10 WT n=11, Pim $1^{-/-}2^{-/-}$ n=15, Pim $2^{-/-}$ n=3) day 14/15 WT n=4, Pim $1^{-/-}2^{-/-}$ n=5) spleen sections were fixed and stained with PNA to identify germinal centers. C. Mice were immunized with NP-CGG precipitated in alum 1) germinal center B cells were identified by facs and enumerated as in A (Unimmunized n=4, day 15 n=10) 2) $PNA^{+}$ germinal centers were identified as in B (Unimmunized n=2, day 15 n=5). D. Gating strategy for identifying splenic GC B cells by flow cytometry. Bars indicate mean and SEM, each symbol represents 1 mouse, red triangle are WT, black squares are Pim $2^{-/-}$, blue circle are Pim $1^{-/-}2^{-/-}$. Statistical significance was determined by student's t test *p<.05, ** p<.01, ***p<.001

**Pim 1 and/or 2 are not absolutely required for the germinal center response**

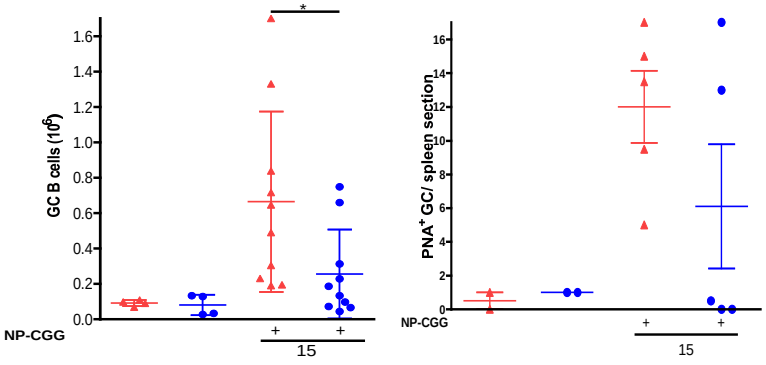
**A. 1) B6x129 2) C57BL/6**




**B. 1) B6x129 2) C57BL/6**



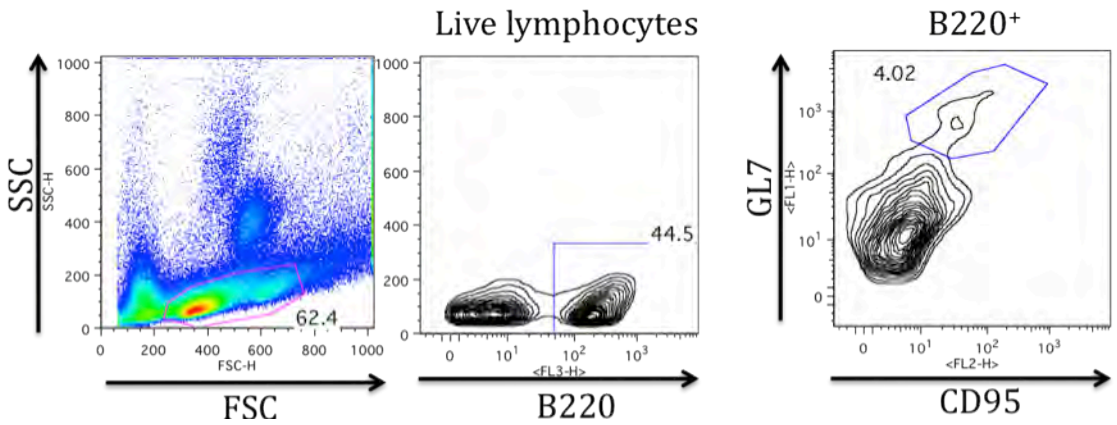

**C. 1) GC B cells 2) PNA+ GCs**




▲ WT

● Pim $1^{-/-}2^{-/-}$

**D.**


Live lymphocytes
B220+
SSC
SSC-H
FSC-H
62.4
FSC
44.5
<FL3-H>
B220
GL7
<FL1-H>
4.02
<FL2-H>
CD95

**Pim $1^{-/-}2^{-/-}$ B cells have impaired isotype switching**

An activated B cell in the germinal center is often induced by its microenvironment to switch isotype, so the effector function of the expressed antibody best fits the type of pathogen that elicits the response. Cultured B cells can be induced to switch to specific isotypes by engaging different ligands and using different cytokine cocktails. After observing a significant decrease in NP specific $IgG_1$ antibody in Pim $1^{-/-}2^{-/-}$ mice, we asked if Pim 1 and 2 also played a role in isotype switching in B cells. B cells isolated from WT or Pim $1^{-/-}2^{-/-}$ mice were cultured with different cytokine cocktails (as described in materials and methods and the figure legend), to induce switching to a specific isotype. The percent of switched isotype B cells was determined by flow cytometry. An example of this data can be seen in figure 4.7 where CFSE is measured on the x-axis and switching to either IgG1 or IgG2a is measured on the y-axis. Proliferation was also examined and determined to be normal. Due to the variability found in individuals as well as in separate experiments, the data is analyzed as percent of switched B cells in Pim deficient animals divided by the percent of switched B cells in WT cultures of the same experiment, resulting in the "percent of WT" charted for each culture condition in figure 4.8. Experiments using Pim $1^{-/-}2^{-/-}$ B6x129 mice revealed that the loss of Pim 1 and 2 impaired switching to every isotype tested (Figure 4.8 A). This was consistent with our in vivo observations that anti-NP $IgG_1$ and $IgG_3$ antibody was diminished in Pim $1^{-/-}2^{-/-}$ mice. We also observed that switching to $IgG_1$ was more severely impaired in the anti-CD40 cultures than the LPS cultures (Figure 4.8 A). Encouraged by

these results, we repeated the experiments using Pim $1^{-/-}2^{-/-}$ C57BL/6 mice. Again, switching by Pim $1^{-/-}2^{-/-}$ B cells to each isotype was impaired. Though scatter was larger in the B6 Pim $1^{-/-}2^{-/-}$ samples induced to switch to IgA, preventing a significant difference, the mean was only 50% of WT indicating an impairment as was observed in B6x129 Pim $1^{-/-}2^{-/-}$ mice. The same phenomenon was observed with regard to $IgG_1$, as B cells cultured with anti-CD40 showed a more severe impairment than those cultured with LPS suggesting varying dependency on Pims downstream of these receptors. In these experiments, we used additional conditions for switching to $IgG_{2a}$ and $IgG_3$. The impairment in switching to $IgG_{2a}$ was more severe in cultures given anti-CD40 rather than LPS, consistent with results for $IgG_1$. With $IgG_3$, however, both cultures conditions (anti-delta-dextran or LPS) resulted a similar level of deficiency (Figure 4.8 B). These data indicate that Pim 1 and/or 2 are required for efficient induction of isotype switching in a B cell intrinsic mechanism.

To investigate whether Pim 1 or 2 is required for effective isotype switching, we examined Pim single knockout mice. Pim 1 showed a similar pattern and magnitude of impairment for all isotypes tested, consistent with this being the key kinase (Figure 4.8 C). The results are too limited to draw a significant conclusion, however, there was very limited impairment seen in the Pim $2^{-/-}$ (Figure 4.8 D). The only severe impairment seen in the Pim $2^{-/-}$ mouse was switching to $IgG_1$ under conditions with anti-CD40, not LPS. This suggests that switching in the Pim $1^{-/-}2^{-/-}$ B cells under this condition may be worse than switching to $IgG_1$ when stimulated with LPS because both Pims are important

downstream of anti-CD40 but not LPS for isotype switching. More experiments are needed to pursue this hypothesis.

In order for isotype switching to occur, the constant (C) region of the targeted heavy chain segment must be accessible. It is thought that which C region is accessible, and therefore which isotype the B cell will switch to, is controlled by signal cascades downstream of receptors activated by cytokines in the B cell's microenvironment (64). One explanation for the impaired isotype switching we have observed is that the C region is not accessible. Accessibility is indicated by the presence of sterile transcripts, therefore if the C region is not accessible, we would expect RNA sterile transcripts to be decreased. Dr. Madelyn Schmidt in our lab isolated RNA from cultures of B cells undergoing switching with anti-CD40+IL4 for $IgG_1$ and LPS + anti-delta-dextran then measured the sterile transcripts representing one of the most impaired switching condition in the C57BL/6 background, $IgG_{2a}$ with CD40 by semi-quantitative PCR (Figure 4.9). $IgG_{2a}$ sterile transcripts were unaffected in Pim $1^{-/-}2^{-/-}$ samples so neither Pim 1 or 2 are involved in the accessibility of the C region. Isotype switching also relies on the induction of AID. To test whether AID mRNA levels were affected by the loss of Pim 1 and 2, Lyn again used the RNA samples from B cells induced to switch to IgG2a in culture and measured AID mRNA by semi-quantitative PCR. Pim $1^{-/-}2^{-/-}$ and control mice expressed comparable levels of AID mRNA (Figure 4.9). So although the loss of Pim 1 and 2 impairs isotype switching it is not by limiting accessibility to the C region or decreased expression

of AID mRNA. This does not exclude the possibility that the quantity AID protein is abnormal, or that Pim 1 or 2 affects the function of AID post-translationally.

In fact, a number of AID co-factors have been implicated in CSR though biochemical evidence is scarce (65). The aberration of any one of these components due to the loss of Pim 1 and 2 may be responsible for the decrease in isotype switching we have observed. However, because AID is unquestionably required for both isotype switching and affinity maturation, which is also impaired in Pim $1^{-/-}2^{-/-}$ mice (described below), we have focused our attention there. There are several steps AID at which could be impaired. Although AID expression in Pim $1^{-/-}2^{-/-}$ B cells appears at WT levels we have yet to determine where it is localized. AID is normally transported to the nucleus after BCR stimulation (which also induces Pim 1 and 2) but the mechanisms regulating this transport are not fully elucidated and so could involve Pim 1 and/or 2 (65). In addition to transcriptional and localization control, post-translational control is critical to AID regulation. Phosphorylation at serine and threonine residues regulates AID activity and mutations at these residues have been shown to interfere with isotype switching and somatic hypermutation, critical to affinity maturation (66). Given the requirement for serine/threonine phosphorylation of AID for normal isotype switching and somatic hypermutation and the established role for Pim 1 and 2 as serine/threonine kinases we hypothesized that Pim 1 or 2 kinases modify AID protein thereby affecting the level of isotype switching and affinity maturation. We are currently pursuing this idea.

**Figure 4.7. Flow cytometry analysis of isotype switching and proliferation in Pim $1^{-/-}2^{-/-}$ B cell cultures.** B cells isolated from Pim $1^{-/-}2^{-/-}$ or control mice were stained with CFSE and cultured for 3 days with LPS+IL4 or LPS+anti-delta dextran+interferon gamma to induce isotype switching to $IgG_1$ or $IgG_{2a}$ respectively. Cultures were measured by flow cytometry for expression of these switched isotypes, proliferation shown by CFSE dilution on the x-axis, $IgG_1$ or $IgG_{2a}$ is shown on the y-axis. Numbers in the top right indicate the percent of switched B cells within each gate. These plots represent 4 separate experiments; switching efficiency for each condition tested is given in figure 4.9.

**Flow cytometry analysis of isotype switching and proliferation in Pim $1^{-/-}2^{-/-}$ B cell cultures**

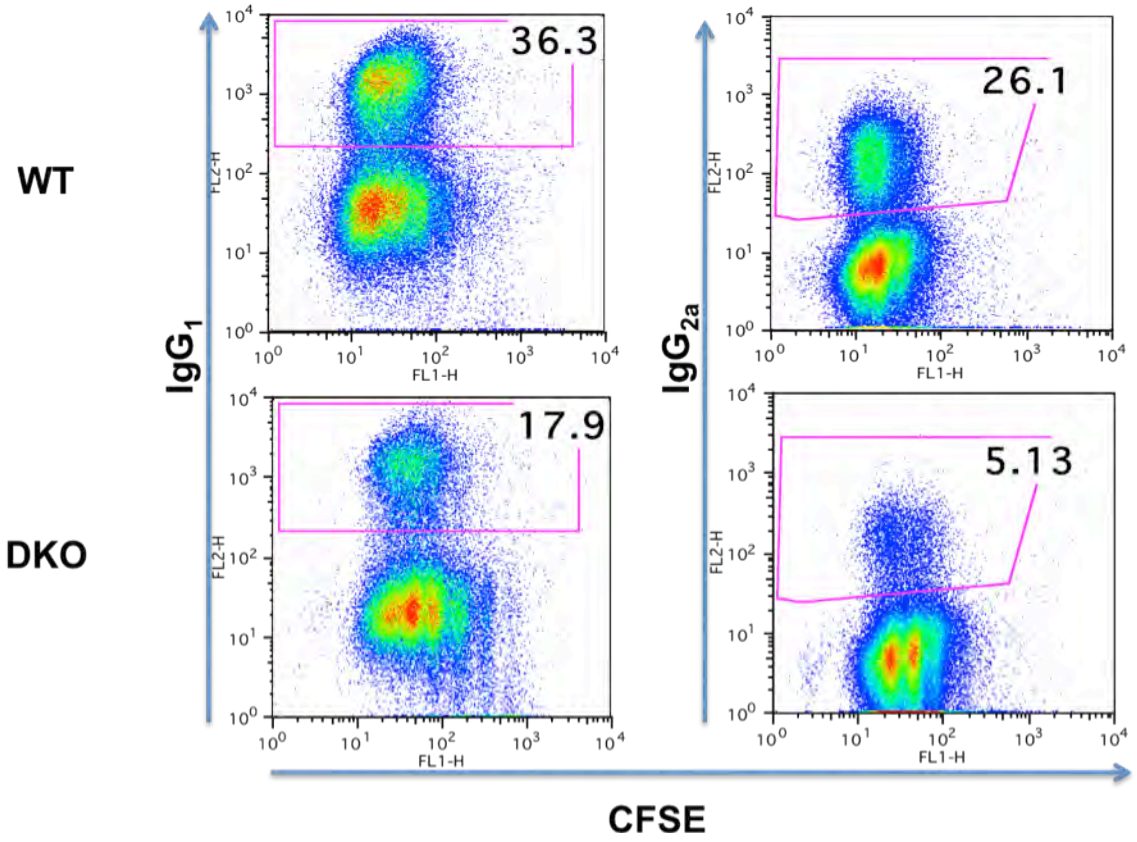
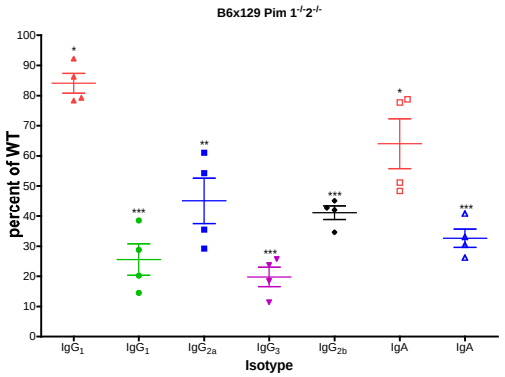


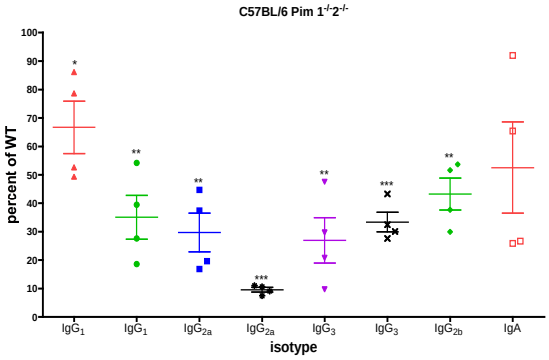
**Figure 4.8. Pim $1^{-/-}2^{-/-}$ B cells have impaired isotype switching.** B cells isolated from B6x129 (n=4)(A) or C57BL/6 (n=4) (B) WT and Pim $1^{-/-}2^{-/-}$ mice, Pim $1^{-/-}$ (n=2) (C), or Pim $2^{-/-}$ (n=1) (D) were cultured with on of the following cocktails; LPS+IL4+BLyS ($IgG_1$, red triangles), anti-CD40+IL4+BLyS ($IgG_1$, green circles), LPS+IL4+IL5+TGFβ+BLyS (IgA, open red squares), anti-CD40+IL4+IL5+TGFβ (IgA, open blue triangles), LPS+INFγ+BLyS ($IgG_{2a}$, blue squares), anti-CD40+INFγ+BLyS ($IgG_{2a}$, black asterisks), LPS+TGFβ+BLyS ($IgG_{2b}$, green diamonds), LPS+anti-δ-dextran+BLyS ($IgG_3$, inverted purple triangles), or LPS ($IgG_3$, black X's) to switch to isotypes indicated in parenthesis and on the Y-axis. A-C includes 2 experiments, D is 1 experiment, lines indicate mean and SEM, each symbol above one isotype represents cells isolated from an individual mouse.

## Pim $1^{-/-}2^{-/-}$ B cells have impaired isotype switching

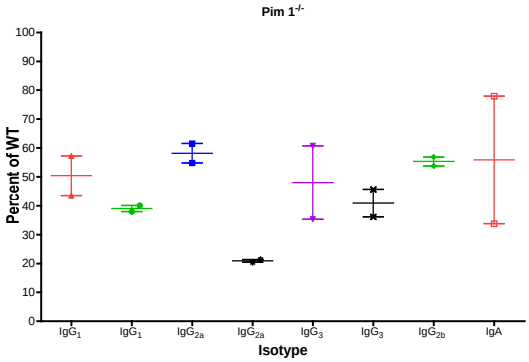
A.




B.




C.




D.

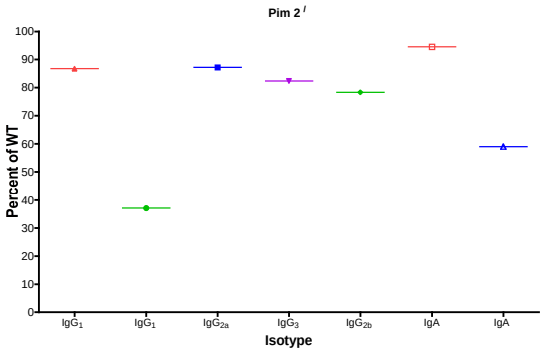
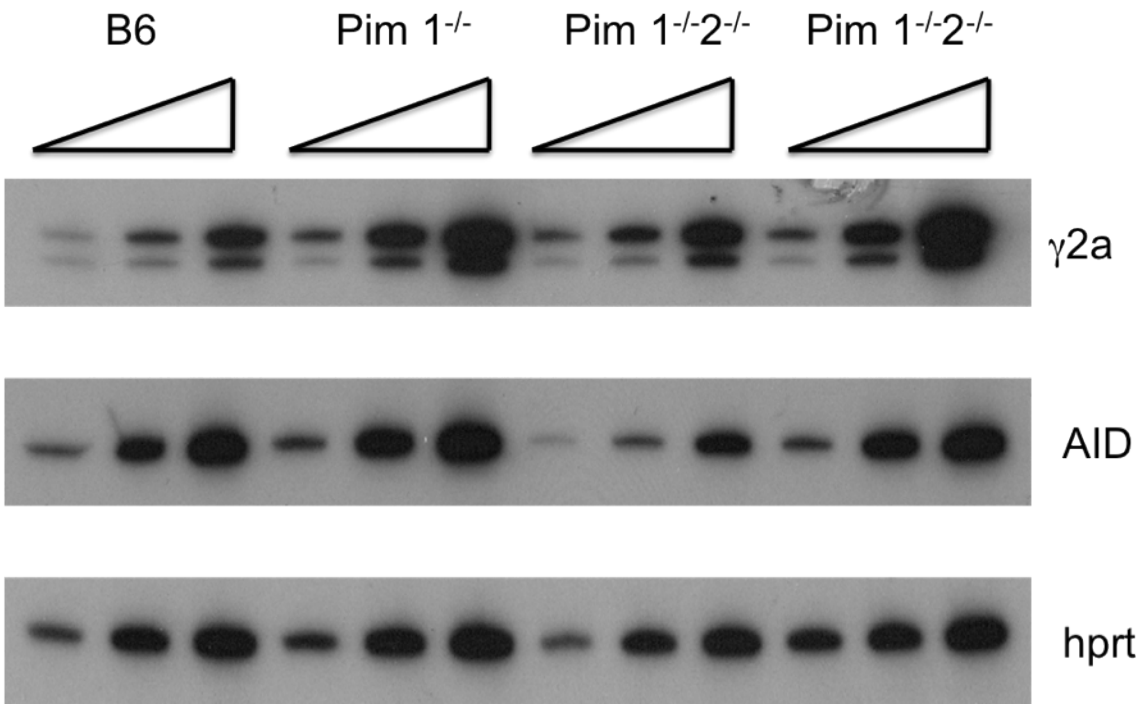


**Figure 4.9. Sterile transcripts and AID induction are unaffected in Pim $1^{-/-}2^{-/-}$ B cells.** cDNA was made from 1 µg of RNA isolated from B cells of indicated genotypes. These B cells had been cultured under conditions conducive to switching specifically to IgG2a. Semi-quantitative PCR was used to measure $IgG_{2a}$ sterile transcripts (top row) and AID expression (center row). Hprt was used as a loading control. Blot is representative of 2 separate experiments.

**Sterile transcripts and AID induction are unaffected in Pim $1^{-/-}2^{-/-}$ B cells**



**Affinity maturation is impaired in Pim $1^{-/-}2^{-/-}$ mice**

In addition to isotype switching, the germinal center is the location of affinity maturation. Affinity maturation results in higher affinity antibody late in the immune response and in a memory response and occurs through two mechanisms. The first is somatic hyper mutation; a high rate of random mutations in the variable region alters the binding affinity of the BCR to the antigen. Second, antigen selection means that B cells compete with each other in binding antigen, such that those that bind with the greatest affinity continue to proliferate and therefore out compete those B cells with a lower affinity BCRs. Rounds of mutation and selection result in higher affinity antibody to the antigen. Affinity of the BCR has been linked to whether the B cell eventually becomes a long-lived plasma cell or a memory cell (59), ensuring that a secondary response to the antigen has a higher affinity than the initial response. To investigate the ability of Pim $1^{-/-}2^{-/-}$ mice to undergo affinity maturation, we employed a NP specific ELISA using 2 NP-BSA plate coats that had different substitution ratios of hapten. $NP_4$-BSA will bind only the high affinity antibody whereas $NP_{14}$ will bind both high and low affinity NP antibody. Each sample was run on both plates and the ratio of high affinity to total antibody detected. The closer this ratio is to 1 the higher the antibody affinity in that sample.

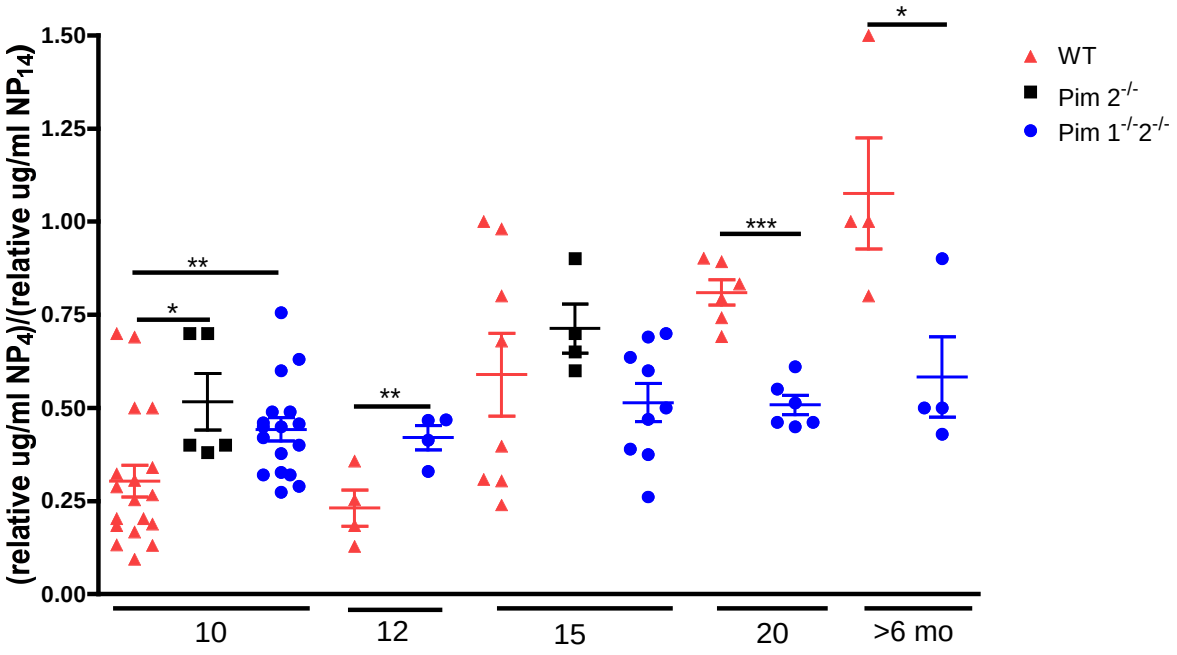
Sera from previous experiments immunizing WT and Pim $1^{-/-}2^{-/-}$ mice with NP-CGG in alum were assayed again on each plate coat to determine the antibody affinity at various days after immunization. Early in the response WT samples averaged a ratio of 0.3 ± .04 indicating low affinity antibody, which is expected.

Pim $1^{-/-}2^{-/-}$ samples, however, were significantly higher than controls averaging over .44 ± .03. Pim $2^{-/-}$ mice were also measured during one experiment and were very similar to Pim $1^{-/-}2^{-/-}$, averaging .5 ± .07, significantly higher than controls 10 days after immunization. The same results for WT and Pim $1^{-/-}2^{-/-}$ mice were obtained 12 days after immunization. As expected, the affinity of antibody from control mice increased throughout the duration of the response. This is first apparent at 15 days after immunization when the mean affinity of control samples was .58 ± .1 and the difference between control and Pim $2^{-/-}$ or Pim $1^{-/-}2^{-/-}$ samples disappeared. Twenty days after immunization a significant difference between control and Pim $1^{-/-}2^{-/-}$ samples reappeared, this time because the affinity of control samples was higher than that of Pim $1^{-/-}2^{-/-}$ samples, .8 ± .03 versus .5 ± .05 respectively. Finally more than 6 months after immunization mean affinity continued to climb in control mice while Pim $1^{-/-}2^{-/-}$ mice averaged 0.5 at 15 days, 20 days, and 6 months post immunization (Figure 4.10). These experiments led to 2 interesting observations. The first is that Pim $1^{-/-}2^{-/-}$ affinity was initially higher than controls. The most likely explanation for this is that only the high affinity B cells are becoming activated in Pim $1^{-/-}2^{-/-}$ mice. This also fits with our germinal center data described above, where the initiation of a germinal center becomes more likely as the immunogen gets stronger. If a Pim deficient B cell has a higher activation threshold than its WT counterparts, a higher affinity BCR could deliver the increased signal strength needed to push it past the threshold. This sub-population of B cells activated in the Pim $1^{-/-}2^{-/-}$ mice would produce higher affinity antibody than controls early in the response.

The second observation was that the affinity of Pim $1^{-/-}2^{-/-}$ antibody did not improve over the course of the response so Pim 1 and/or 2 may be involved in affinity maturation. This could be an intrinsic B cell defect as we have shown with isotype switching. Both processes rely on AID, so a necessary post-translational modification dependent on Pim could be responsible for both defects. The other possibility is that we are not seeing affinity maturation in the Pim $1^{-/-}2^{-/-}$ mice because the germinal center reaction is impaired. During a TD response affinity maturation is thought to occur in the germinal center and may be impaired simply due to the decrease in germinal centers in Pim $1^{-/-}2^{-/-}$ mice.

**Figure 4.10. Affinity maturation is impaired in Pim $1^{-/-}2^{-/-}$ mice.** WT, Pim $2^{-/-}$ and Pim $1^{-/-}2^{-/-}$ mice were immunized with 50 μg NP-CGG absorbed to Imject Alum, sera was collected at days indicated and affinity was determined by measuring the ratio of anti-NP $\lambda^{+}$ antibody in each sample binding to a NP-BSA plate coat with a low ($NP_4$) and high ($NP_{14}$) substitution ratio. Graph includes 5 experiments, lines indicate the mean and SEM of each group, each symbol is an individual mouse. Day 10, WT n=18, Pim $2^{-/-}$ n=5, Pim $1^{-/-}2^{-/-}$ n=17, day 12 n=4, day 15 WT n=8, Pim $2^{-/-}$ n=4, Pim $1^{-/-}2^{-/-}$ n=9, day 20 n=6, 6 months n=4.

**Affinity maturation is impaired in Pim $1^{-/-}2^{-/-}$ mice**



**Pim $1^{-/-}2^{-/-}$ B cells do not respond to secondary challenge**

We have shown that Pim $1^{-/-}2^{-/-}$ mice immunized with NP-CGG generate impaired TD responses. The germinal center reaction can occur but is less frequent in Pim deficient mice, possibly due to the ability of an immunogen to sufficiently activate Pim $1^{-/-}2^{-/-}$ B cells. Memory B cells are generated in the germinal center and whether an activated B cell becomes a memory B cell is thought to be closely linked to its affinity. In normal mice, high affinity memory B cells develop from the germinal center and migrate to the bone marrow along with antigen specific long lived ASCs. To determine if Pim $1^{-/-}2^{-/-}$ mice are able to respond to a secondary challenge, we immunized Pim deficient and control mice with 50 µg NP-CGG absorbed to Imject Alum then transferred B cells from these mice mixed with T cells from KLH primed B6 mice into irradiated B6 recipients. WT and Pim $1^{-/-}2^{-/-}$ B cell donors were immunized with 50 µg of NP-CGG in alum more than seven months prior to the transfer of memory B cells, T cell donors were primed with KLH in alum more than 8 weeks prior to transfer.

WT B6 mice were exposed to 600 rads the day before the transfer. Recipient mice were given 5 or 7 million T cells (varied by experiment) isolated from KLH primed WT B6 mice and 2 or 10 million B cells from either WT B6 or Pim $1^{-/-}2^{-/-}$ NP primed mice. 24 hours after transfer recipient mice were challenged with 50ug of NP-KLH and bled 10 days later. An NP specific ELISA was used to measure antibody generated in recipient mice. Mice receiving 2 million B cells from NP-CGG immunized WT or Pim $1^{-/-}2^{-/-}$ animals did not mount a strong antibody response to NP-KLH. Mice receiving 10 million B cells from WT

animals immunized previously with NP-CGG generated an antibody response averaging 76.63 ± 15.7 µg/ml (n=7) compared to 3.75 ± 1.9 µg/ml (n=9) by mice receiving 10 million Pim $1^{-/-}2^{-/-}$ B cells (Figure 4.11 B). Mice receiving only WT B cells, only T cells, or no antigen were used as controls to determine background NP antibody.

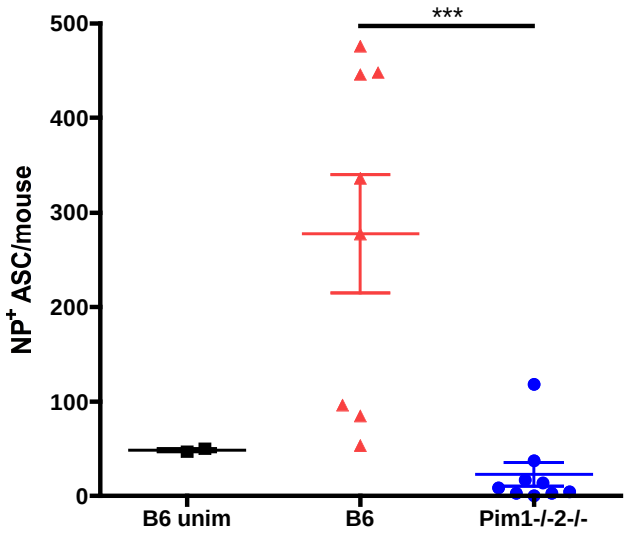
Bone marrow was also taken from the B cell donors and examined by ELISpot for NP specific long-lived ASCs. This revealed that Pim $1^{-/-}2^{-/-}$ mice had significantly fewer long lived ASCs in their bone marrow with an average 23 ± 12 $NP^{+}$ ASC/mouse compared to 277 $NP^{+}$ ± 62 ASC/mouse in WT mice (Figure 4.11 A).

Our results show a significant defect in the ability of Pim $1^{-/-}2^{-/-}$ B cells to respond to a secondary challenge. This could reflect the inability of Pim $1^{-/-}2^{-/-}$ B cells to generate memory cells particularly because long-lived ASCs and memory B cell are products of the germinal center response, which was impaired in Pim 1-/-2-/- mice. Alternatively our results could reflect a defect in the ability of an intact memory B cell pool to differentiate into antibody secreting cells.

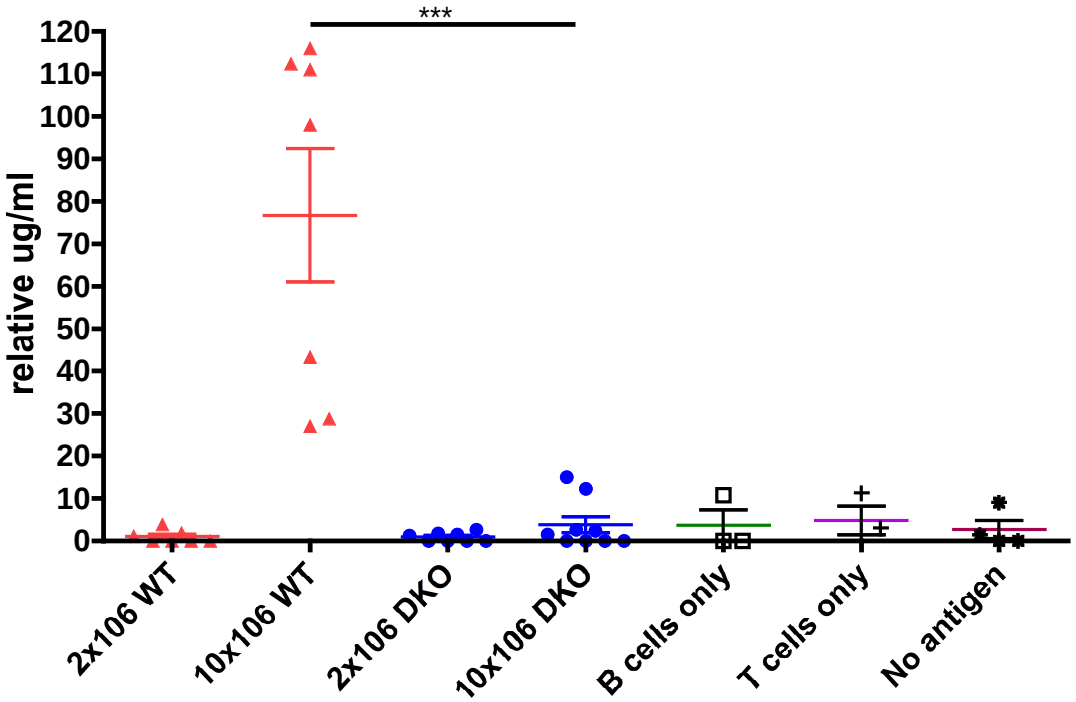
**Figure 4.11. Pim $1^{-/-}2^{-/-}$ mice do not respond to a secondary challenge.** Mice immunized more than 6 months prior with 50 μg NP-CGG absorbed to Imject Alum were sacrificed. (A) anti-NP $\lambda^+$ ASCs in the bone marrow were enumerated by ELISpot; Unimmunized n=2, WT n=8, Pim $1^{-/-}2^{-/-}$ n=9 (B) 2 or 10 million B cells were transferred along with KLH primed T cells into irradiated recipient mice who were immunized with NP-KLH 24 hours after transfer. Sera was collected at day 10 and measured for anti-NP $\lambda^+$ antibody. WT n=7, Pim $1^{-/-}2^{-/-}$ n=8 ($2x10^6$) or 9 ($10x10^6$), B or T cells only n=3, antigen only n=4. Graphs include 2 experiments. B or T cells were transferred alone as a control, “no antigen” refers to mice receiving $10x10^6$ WT B cells with KLH primed T cells who were not immunized with NP-KLH after transfer. Each symbol represents an individual mouse, lines represent the mean and SEM.

**Pim $1^{-/-}2^{-/-}$ mice do not respond to a secondary challenge.**

**A.**




**B.**

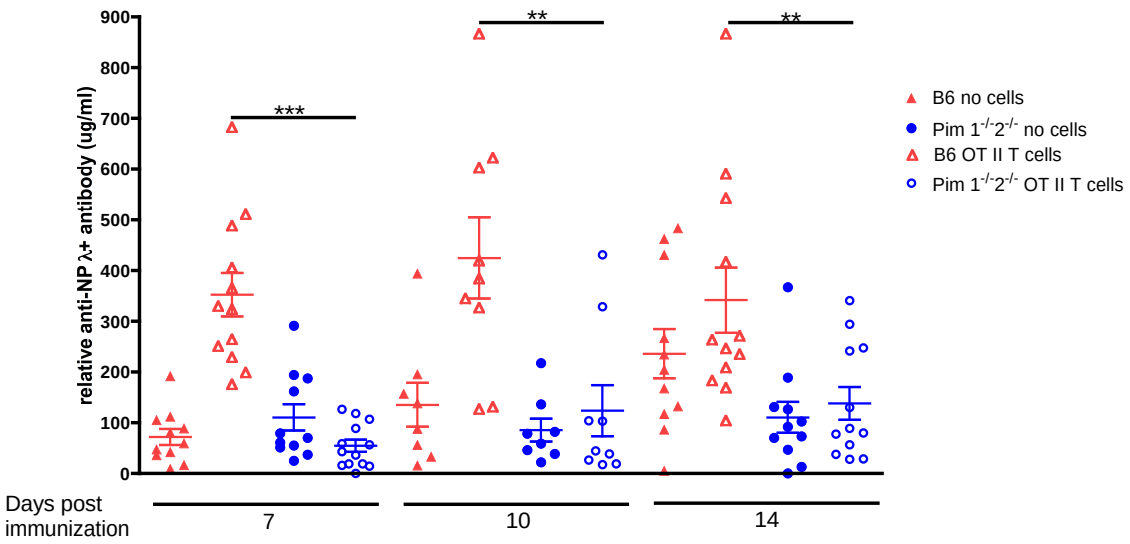


**WT T cell help cannot compensate for the loss of Pim 1 and 2 in B cells**

B cell activation and antibody production in TD responses depends upon T cell help. It is therefore possible that the impairment we have observed in the TD response is due to T cell defects. To determine whether WT T cell help would correct the TD response in Pim $1^{-/-}2^{-/-}$ mice, we used spleen cells from OT II $rag2^{-/-}$ mice, which do not contain any B cells. T cells in these mice are specific to OVA, so transferring a small number of these T cells into Pim $1^{-/-}2^{-/-}$ mice was sufficient for T cell help during an immune response to NP-OVA. WT or Pim $1^{-/-}2^{-/-}$ mice receiving 1-2 million OT II splenocytes were immunized with 100 µg NP-OVA absorbed to Imject Alum and NP specific antibody was measured at 7 and 14 days after immunizations. A significant difference was observed between WT and Pim $1^{-/-}2^{-/-}$ mice given OT II T cells as early as day 7 as anti-NP $\lambda^{+}$ antibody in WT mice averaged 352 ± 43 µg/ml versus 54.2 ± 11.96 µg/ml in Pim $1^{-/-}2^{-/-}$ mice ($p<.0001$). A significant difference was also observed 10 (250.8 ± 72 µg/ml vs. 51.9 ± 18 µg/ml) and 14 (341.5 ± 64.2 µg/ml vs. 137.7 ± 32.4 µg/ml) days after immunization ($p<.01$). The addition of WT T cell help did not correct the deficiency in the TD response in Pim $1^{-/-}2^{-/-}$ mice indicating that a T cell defect is not solely responsible for the TD impairment though we can not rule it out as a contributing factor. These data were consistent with the hypothesis that Pim deficiency is manifest in B cells impairing a TD response. Experiments in Chapter V test this hypothesis.

**Figure 4.12. WT T cell help does not repair the TD response in Pim $1^{-/-}2^{-/-}$ mice.** Unirradiated B6 (red triangles) or Pim $1^{-/-}2^{-/-}$ (blue circles) mice received OT II T cells (specific to OVA) (open symbols) or not (closed symbols) and were immunized with 100 µg NP-OVA in Imject alum. Sera was collected and measured for anti-NP $\lambda^{+}$ antibody at days indicated. Data are pooled from 3 experiments, lines represent mean and SEM for each group, each symbol is an individual mouse. Day 7 no cells n=11, WT OT cells n=12, Pim $1^{-/-}2^{-/-}$ OT cell n=13, day 10 no cells n=8, OT cells n=9, day 14 no cells n=11, OT cells n=7.

**WT T cell help does not repair the TD response in Pim $1^{-/-}2^{-/-}$ mice.**



# Chapter V

# Thymus Independent Responses In Pim $1^{-/-}2^{-/-}$ Mice

## Introduction

Antigens can be classified as TD or TI based on whether or not they required T cell help to elicit an immune response. TI antigens produce an immune response without T cell help and can then be further subdivided into TI-1 or TI-2. The separation of these two categories was originally defined by the lack of a TI-2 response generated in CBA/N mice, which have an X-linked immune deficient gene, xid, (67, 68). TI antigens have since been defined by their method of activating a B cell. TI-1 antigens have intrinsic non-specific B cell activating activity, such as LPS, which activates B cells by binding to TLRs. TI-2 antigens have repeating epitopes, which cross-link BCRs on the B cell surface, leading to activation.

Another difference in TI antigens is the observation that they activate different B cell subpopulations. TI-1 antigens are characterized by an expansion of conventional B2 B cells (6). The primary site for follicular B2 B cells is the spleen and these are found in normal numbers in the spleens of Pim $1^{-/-}2^{-/-}$ knockout mice. The response to TI-2 antigens, however, is controlled largely by the MZ and B1 B cell subsets (6, 69). We have already shown that the number of Peritoneal B1 B cells is diminished by the loss of Pim 1 or 2, which may affect

our TI-2 immunization results in these mice, however, marginal zone B2 cells are found in normal number in the spleens of Pim $1^{-/-}2^{-/-}$ mice.

Studies indicate that i.p. administration of TI-2 antigen relies primarily on peritoneal B cells for a robust response while i.v. administration relies on the splenic MZ and Bl a B cell populations (70). For example, i.p. administration of the R36A streptococcal vaccine (a TI-2 antigen) to $CXCL13^{-/-}$ mice, which have cell migratory defects and diminished numbers of peritoneal B cells, results in a significantly reduced antibody response relative to normal controls. In contrast, the same vaccine administered i.v. elicits a normal antibody response because intravenous delivery to the spleen can activate the intact MZ population (70). These experiments indicate that we can use the route of administration of antigen to target different populations of B cells.

Despite the route of immunization, B cells activated by a TI-2 antigen differentiate into ASCs. This differentiation involves a tightly regulated specific genetic program, which has been partly elucidated. The most critical ASC associated transcription factor is B Lymphocyte induced maturation protein 1 (BLIMP-1), the loss of which severely impacts ASC generation (17) whereas over expression can spontaneously induce ASC differentiation (18). Immunization of conditional BLIMP-1 knockout mice resulted in a 3-fold decrease and 10-fold decrease in ASCs to both TD and TI-2 antigens, respectively (17). In vitro, BLIMP-$1^{-/-}$ B cells proliferate normally but do not generate ASCs after LPS activation. In addition to BLIMP-1, IRF4 has been identified as another key transcription factor in ASC differentiation. IRF4 is the first transcription factor t

induced upon B cell commitment to the ASC lineage followed by BLIMP-1 although it is not clear if there is a direct linear relationship between IRF4 and BLIMP-1 expression (19, 20). Consistent with its role in ASC development, $IRF4^{-/-}$ B cells generate 95% fewer ASCs when activated in vitro and few $CD138^{+}$ B cells after in vivo TD immunization (19, 20). A third transcription factor involved in ASC generation is XBP-1 (21). XBP-1 is downstream of IRF4 and BLIMP-1; B cells lacking either IRF4 (19) or BLIMP-1 (17) have no induction of XBP-1, demonstrating that XBP-1 expression depends on the expression of either IRF4 and/or BLIMP-1. Although in vitro experiments revealed that survival and proliferation were unaffected in $XBP\text{-}1^{-/-}$ B cells, in vivo immunization produced 70-fold fewer ASCs and little IgM and $IgG_3$ response to TI-2 and TD antigens, respectively (21).

Chapter IV of this dissertation investigated the affect of losing Pim 1 and 2 on a TD immune response. Providing antigen specific WT T cell help before immunizing Pim $1^{-/-}2^{-/-}$ mice with a TD antigen did not improve the response, consistent with an intrinsic B cell defect. To more closely examine the B cells, we evaluated immune response of Pim $1^{-/-}2^{-/-}$ mice to TI antigens. We found that the loss of Pim 1 or 2 negatively impacts the response to both TI-1 and TI-2 antigens and in the case of the TI-2 response even i.v. antigen delivery to Pim $1^{-/-}2^{-/-}$ to the intact MZ B cells does not produce a response. In vitro experiments revealed that Pim $1^{-/-}2^{-/-}$ B cells proliferate and survive normally to TI antigens but generate reduced ASC numbers, supporting the notion that differentiation to an ASC was blocked. Taking advantage of a mouse model containing a GFP marker for

BLIMP-1 expression, a key regulator in ASC development, we were able to determine that Pim $1^{-/-}2^{-/-}$ B cell are unable to induce BLIMP-1 normally, resulting in the significant drop in the number of ASCs and antibody.

## Results

### Pim 1 or 2 are required for TI-2 responses

The humoral response against NP-CGG described in the previous chapter requires T cell help and the reduced antibody response could be due to a defect in the T and/or B cell function. We have shown that addition of wild type T cells does not correct the TD defect, consistent with a B cell intrinsic defect. To test the ability of Pim $1^{-/-}2^{-/-}$ B cells to respond in vivo, we immunized control and Pim $1^{-/-}2^{-/-}$ mice with NP-Ficoll and TG4-Ficoll, TI-2 antigens that do not require T cell help.

B6x129 Pim $1^{-/-}2^{-/-}$ and control mice were immunized with 50 µg NP-Ficoll in PBS i.p.; lambda positive antibody to NP, the dominant antibody light chain used in this response, was measured from serum samples taken at 3-13 days post-immunization. Antibody to NP in Pim $1^{-/-}2^{-/-}$ mice was significantly decreased compared to control wild type mice on day 3 ($1 \pm .2$ vs. $10.3 \pm 3.6$ µg/ml, $p<.05$), day 5 ($1.2 \pm .4$ vs. $44.8 \pm 5.2$ µg/ml, $p<.001$), day 7 ($2.1 \pm 1.1$ vs. $210.8 \pm 17.3$ µg/ml, $p<.001$), and day 10 ($7.2 \pm 4.3$ vs. $222.9 \pm 44.1$, $p<.001$) after immunization (Figure 5.1 A). These data demonstrated that Pim $1^{-/-}2^{-/-}$ mice were unable to respond to a TI-2 antigen, consistent with the idea of an intrinsic B cell defect.

Further studies using fully backcrossed C57BL/6 Pim1$^{-/-}$2$^{-/-}$ and control mice confirmed our initial findings. After immunization with NP-Ficoll, total $\lambda^+$ NP-specific antibody from Pim 1$^{-/-}$2$^{-/-}$ mice was lower than B6 mice on all days examined (Figure 5.1 C). By three days after immunization, a 5-fold difference in NP specific antibody was observed between Pim 1$^{-/-}$2$^{-/-}$ and WT mice (2.2 ±1.2 vs. 10 ± 1.4 μg/ml, p<.001) and by 5 days after immunization the difference increased to 11-fold (13.26 ± 2.8 vs. 145.9 ± 20.3 μg/ml, p<.001). The greater than 10-fold antibody levels between Pim 1$^{-/-}$2$^{-/-}$ and WT responses to NP-Ficoll continued through out the response; 10-fold at day 7 (20.8 ± 4.3 vs. 206.7 ± 19.7 μg/ml, p<.001), 14-fold at day 10 (18.85 ± 3.6 vs. 270 ± 33.9 μg/ml, p<.001), and 25-fold at day 13 (16.17 ± 2.5 vs. 410.2 ± 44.6 μg/ml, p<.001). We looked at longer times post-immunization to assess whether the Pim 1$^{-/-}$2$^{-/-}$ response was just delayed, however, the NP specific antibody did not increase to wild type levels. These results are consistent with a B cell intrinsic functional defect in Pim 1$^{-/-}$2$^{-/-}$ mice.

To assess the role of the individual Pim kinases in the TI-2 response, we immunized single knockout mice with 50 μg of NP-Ficoll in saline i.p.. Responses of Pim 1$^{-/-}$ mice immunized with NP-Ficoll were similar to that of B6 mice up until day 10 (250 ± 68 μg/ml vs. 270 ± 33.9 μg/ml) following immunization (Figure 5.1 B). By day 13, however, the response of Pim 1$^{-/-}$ mice had declined to below 200 ± 12 μg/ml (p<.01) whereas B6 levels continued to increase suggesting that Pim 1 is important for the maintenance of the TI-2 response. Pim 2$^{-/-}$ mice also responded to NP-Ficoll and their response

displayed delayed kinetics and a reduced magnitude relative to B6 controls at each time point. Therefore single knockouts respond to challenge with NP-Ficoll with altered duration or magnitude of antibody production suggesting separate roles for Pim 1 and 2 during the TI-2 response. These individual mutations synergize to ablate the response to TI-2 antigen in the double knockout.

Pim 2 deficiency has been shown to result in decreased numbers of $\lambda^+$ light chain B cells in bone marrow populations (60). Because the bulk of the NP-response is produced by $\lambda^+$ B cells, deficiencies in the development of this population might account for the lower response to NP-Ficoll we observed. We assessed this possibility by immunizing with two other TI-2 antigens whose response is not dominated by the $\lambda$ light chain, TG4-Ficoll and TNP-Ficoll and then measuring the $\kappa^+$ specific response. B6x129 control and Pim $1^{-/-}2^{-/-}$ mice were immunized with 50 μg of TG4-Ficoll. At the peak of the response (day 5) the mean antibody titer from WT mice was 51.1 ± 12.4 μg/ml and Pim $1^{-/-}2^{-/-}$ mice was 4.4 ± 1.2 μg/ml ($p<.01$), which was not above the unimmunized background (Figure 5.1 C). Although the antibody titer in WT mice had declined by 10 days (23.9 ± 4.6 mg/ml) after immunization it was still significantly higher than Pim $1^{-/-}2^{-/-}$ mice, which had not increased relative to day 5 (5.2 ± 1.5 μg/ml, $p<.01$). The response to TNP-Ficoll by Pim $1^{-/-}2^{-/-}$ mice was reduced about 3.5-fold compared to B6 mice on both days ($p<.001$) (Figure 5.1 D). Pim $1^{-/-}$ sera averaged 142.4 ± 32 μg/ml at 5 days ($p<.05$) and 96.75 ± 11.6 μg/ml at 10 days ($p<.001$) after TNP-Ficoll immunization, significantly less than WT on both days. Sera from Pim $2^{-/-}$ was significantly less than WT mice averaging 138.6 ± 23.2

μg/ml ($p<.05$) 5 days after immunization, however, by 10 days after immunization the mean anti-TNP antibody in WT decreased enough that Pim $2^{-/-}$ sera was no longer significantly different. TNP specific antibody levels in Pim $2^{-/-}$ mice did not differ significantly from day 5 to day 10. Thus responses to all 3 TI-2 antigens were depressed in Pim $1^{-/-}2^{-/-}$ mice, demonstrating that this phenomenon is not specific to NP or the result of a diminished lambda$^{+}$ B cell population.

The number of NP specific ASCs was also measured by ELISpot. For B6x129 mice immunized with NP-Ficoll, Pim $1^{-/-}2^{-/-}$ had over 10-fold fewer ASCs on day 3 ($127.4 \pm 50$ vs. $1416 \pm 49.8$ ASCs/$10^6$ splenocytes, $p<.001$) and 20-fold fewer on day 7 ($54.3 \pm 25$ vs. $1112 \pm 185$ ASCs/$10^6$ splenocytes, $p<.01$) compared to WT (Figure 5.2 A). NP specific ASCs in C57BL/6 Pim $1^{-/-}2^{-/-}$ mice were also decreased significantly compared to B6 controls at day 5 ($16.47 \pm 2.8$ vs. $49.44 \pm 11.7$ ASCs/$10^6$ splenocytes, $p<.05$) and day 10 ($8.4 \pm 2.8$ vs. $48.4 \pm 14.7$ ASCs/$10^6$ splenocytes, $p<.05$) (Figure 5.2 B). Although each ELISpot can vary based upon differences between individual mice, incubation times, and counting, the difference observed between the scales of parts A and B of figure are due to optimization of the ELISpot assay over time. Note that the background is far less in part B than in part A.

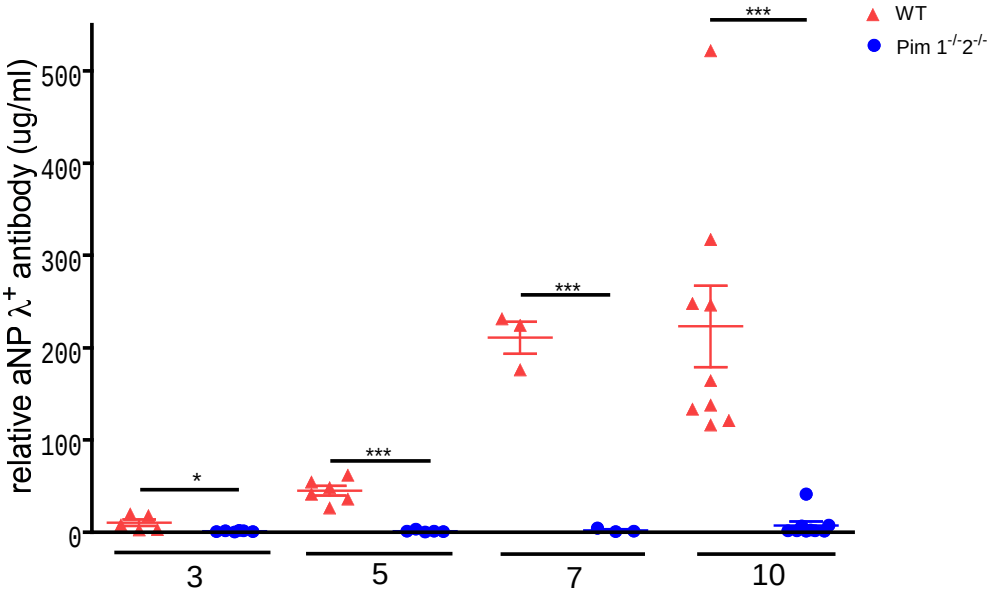
A diminution in splenic ASCs during the immune response is not unexpected. Considering the significant decrease in antibody observed during the short time of the TI-2 response, we asked whether this ASC defect extended to the long-lived ASC population by aging mice immunized with NP-Ficoll. More

than 6 months after the NP-Ficoll immunization mice were sacrificed and NP-specific $\lambda^+$ ASCs in the spleen were enumerated by ELISpot. ASCs in Pim $1^{-/-}2^{-/-}$ mice were 3.5-fold lower compared to WT (6.2 ± 1.1 vs. 21.8 ± 4.2 ASCs/$10^6$ splenocytes, $p<.05$) while the mean ASCs in Pim $1^{-/-}$ mice was a little more than half of WT (13.26 ± 2.2 vs. 21.8 ± 4.2 ASCs/$10^6$ splenocytes) this was not a statistically significant difference (Figure 5.2 C). Long-lived ASCs in the bone marrow after a TI immunization have been reported by some groups but not others. We measured bone marrow samples from these mice as well but did not detect any NP specific ASCs. These data reveal a defect in ASCs associated with the most severe antibody defect found in Pim $1^{-/-}2^{-/-}$ mice.

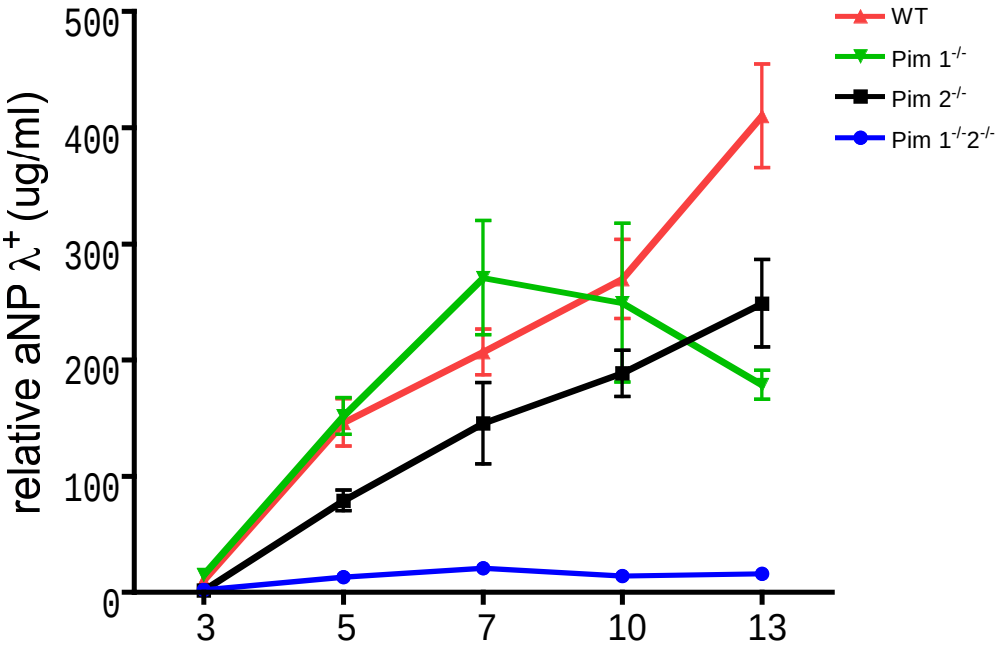
**Figure 5.1 Pim $1^{-/-}2^{-/-}$ mice do not respond to TI-2 antigens. (**A) B6x129 Pim $1^{-/-}2^{-/-}$ and control mice were immunized i.p. with 50 µg of NP-Ficoll; sera was measured for anti-NP $\lambda^+$ antibody at days indicated. Data includes 2 experiments WT: day 3 n=5, day 5 n=6, day 7 n=3, day 10 n=9 Pim $1^{-/-}2^{-/-}$: day 3 n=6, day 5 n=5, day 7 n=3, day 10 n=9. (B) C57BL/6 Pim deficient and control mice were immunized i.p. with 50 µg NP-Ficoll, mean anti-NP $\lambda^+$ antibody is given for each time point over 3 experiments, bars represent SEM, For days 3 and 7: WT n=8, Pim $1^{-/-}$ n=6, Pim $2^{-/-}$ n=7, Pim $1^{-/-}2^{-/-}$ n=10, for day 5 WT n=12, Pim $1^{-/-}$ n=6, Pim $2^{-/-}$ n=12, Pim $1^{-/-}2^{-/-}$ n=16, for day 10 WT n=16, Pim $1^{-/-}$ n=6, Pim $2^{-/-}$ n=17, Pim $1^{-/-}2^{-/-}$ n=20, for day 13 WT n=4, Pim $1^{-/-}$ n=4, Pim $2^{-/-}$ n=7, Pim $1^{-/-}2^{-/-}$ n=3. (C) B6x129 Pim $1^{-/-}2^{-/-}$ and control mice were immunized i.p. with 50 µg TG4-Ficoll, sera was measured for anti-TG4 $F(ab)_2^+$ antibody at days indicated, graph includes 2 experiments, lines indicate mean and SEM, WT/Pim 1-/-2-/- unim n=2 at days 5 and 10 n=6. (D) C57BL/6 mice of indicated genotypes were immunized i.p. with 50 µg of TNP-Ficoll, anti-TNP $F(ab)_2^+$ antibody was measured on days 5 and 10. Graph includes 1 experiment, for day 5 WT n=6, for day 10 WT n=5, on both days Pim $1^{-/-}$ n=4, Pim $2^{-/-}$ n=4, Pim $1^{-/-}2^{-/-}$ n=5. For A, C, and D each symbol represents an individual mouse. Statistics were determined by student's t test *=p<.05, **=p<.01, ***=p<.001. In all experiments unimmunized background sera was below 20 µg/ml.

**Pim $1^{-/-}2^{-/-}$ mice do not respond to TI-2 antigens**

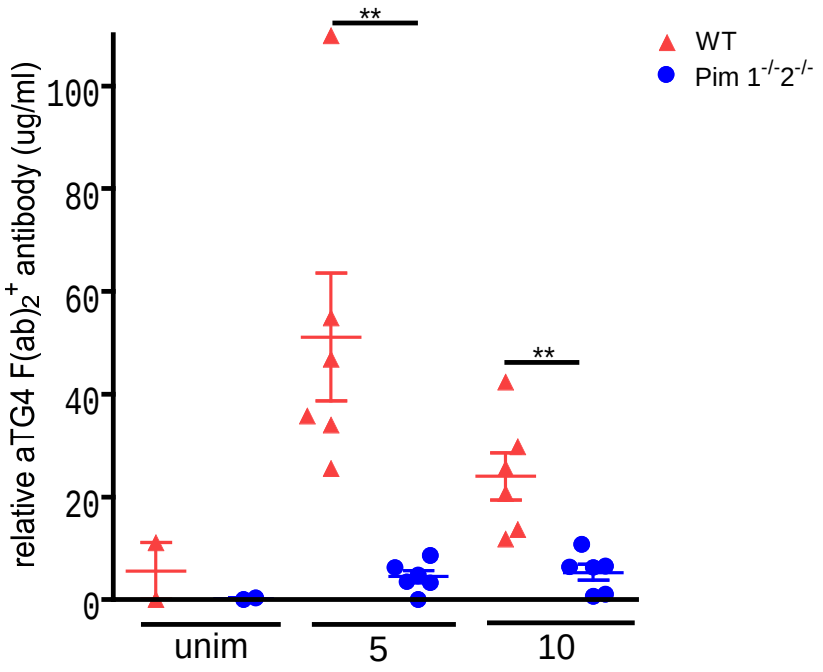
**A. NP-Ficoll (B6x129)**




**B. NP-Ficoll (C57BL/6)**



**C. TG4-Ficoll**




**D. TNP-Ficoll**

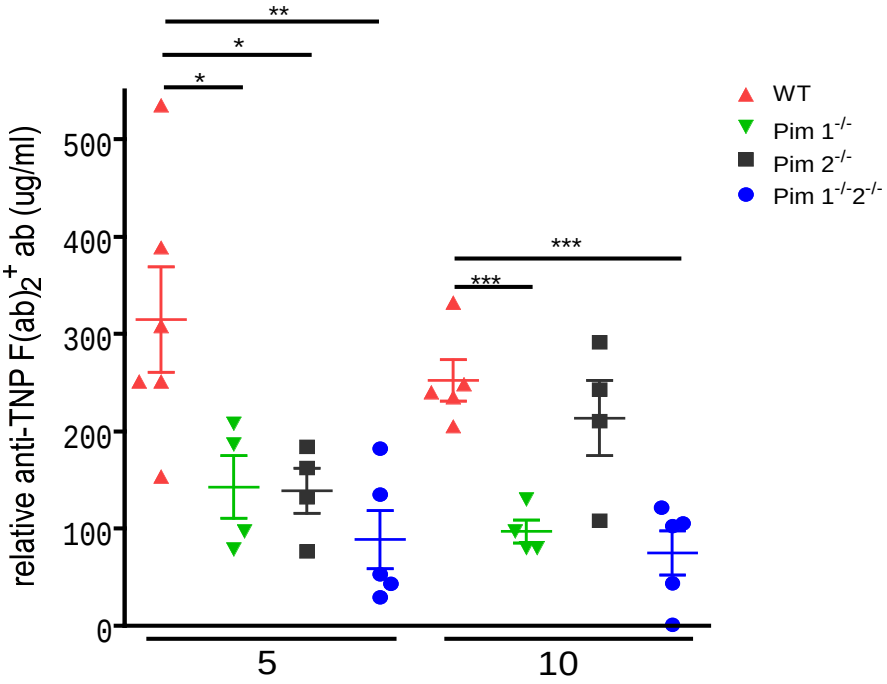
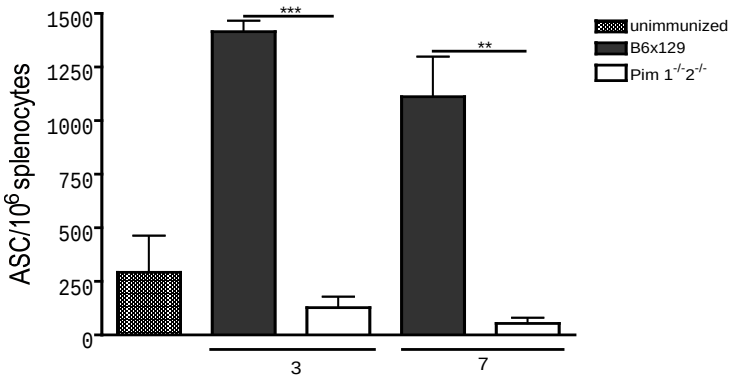


**Figure 5.2. Pim $1^{-/-}2^{-/-}$ mice have reduced ASCs after immunization with NP-Ficoll**. ASCs in the spleen of B6x129 (A) or B6 (B) control and Pim deficient mice were enumerated by ELISpot at days indicated after i.p. immunization with 50 μg of NP-Ficoll. (A, n=3) (B, n=5) C. ELISpots were performed on spleen samples from some mice in Figure 5.1 part B >6 months after immunization with NP-Ficoll. WT n=4, Pim $1^{-/-}$ n=6, Pim $1^{-/-}2^{-/-}$ n=4. Unimmunized WT splenocytes were used as a background control. Background on part C was zero. ELISpot was performed with an NP-BSA plate coat and anti-mouse λ detecting antibody. Statistics were determined by student's t test *=p<.05, **=p<.01, ***=p<.001

## Pim $1^{-/-}2^{-/-}$ mice have reduced ASCs after immunization with NP-Ficoll

**A.**

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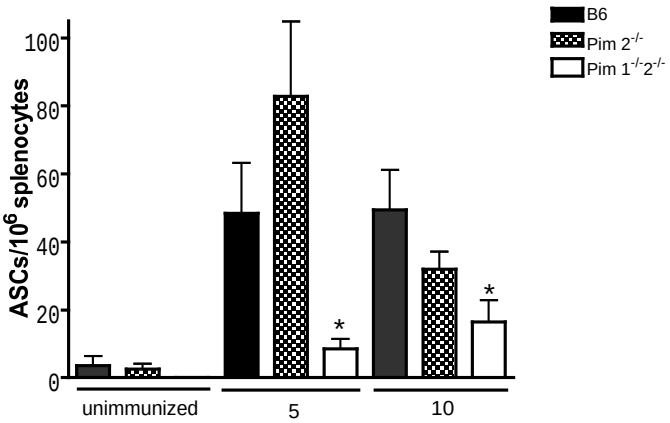


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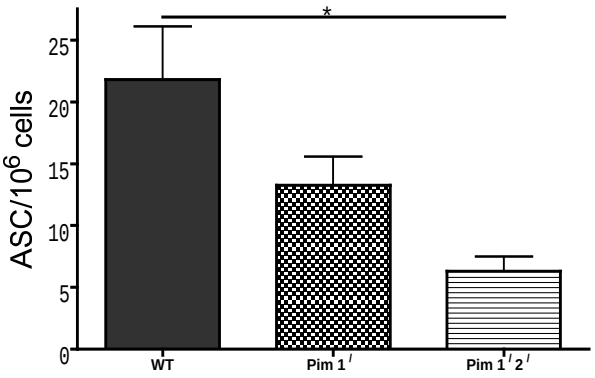


**Splenic B cells do not respond to TI2 antigen in Pim $1^{-/-}2^{-/-}$ mice**

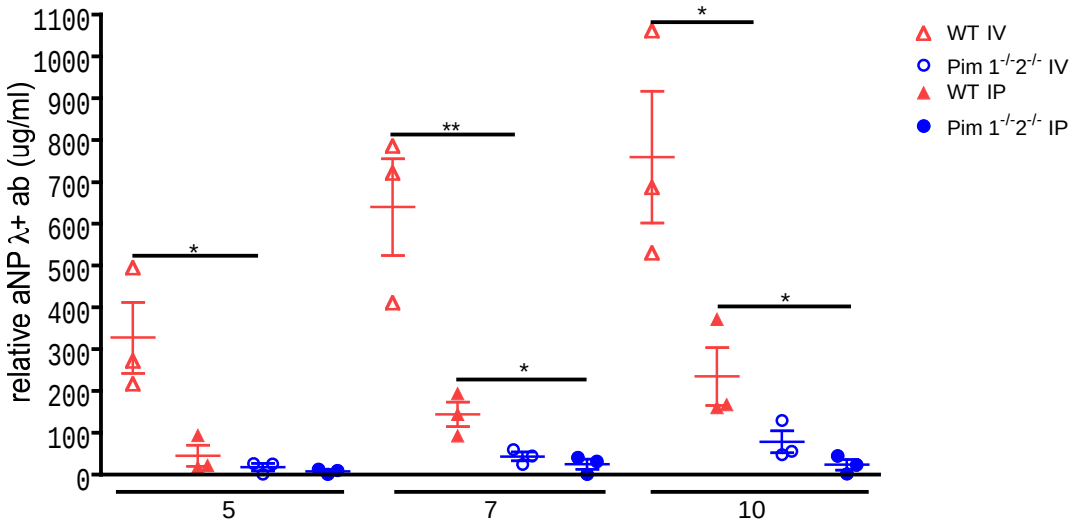
There is evidence in the literature that i.p. administration of an antigen relies primarily on peritoneal B cells for a robust response whereas i.v. administration relies on the splenic MZ and Bla B cell populations. In $CXCL13^{-/-}$ mice, which have cell migration defects (70), i.p. administration of the R36A streptococcal vaccine (a TI-2 antigen) results in a significantly reduced antibody response relative to normal controls. In contrast the same vaccine administered i.v. produces a normal robust response. This occurs because intravenous delivery activates the intact and functioning MZ population while an i.p. injection relies on B1 B cells in the peritoneal cavity, which are diminished in $CXCL13^{-/-}$ mice due to the migratory defect (70). We wanted to eliminate the possibility that the decreased response to TI-2 antigen was due to the diminution of peritoneal B cells and not a functional defect in the splenic B cells. Pim $1^{-/-}2^{-/-}$ and B6 mice were immunized with 50 µg of NP-Ficoll in PBS by i.p. or i.v. routes then bled 5, 7, and 10 days post immunization. Although the TI-2 response was more robust when B6 mice were immunized i.v., Pim $1^{-/-}$ $2^{-/-}$ mice did not show an improved response to NP-Ficoll (Figure 5.3 A). Pim $1^{-/-}$ and Pim $2^{-/-}$ mice showed a divergent antibody response from Pim $1^{-/-}2^{-/-}$ when immunized i.p with NP-Ficoll (Figure 5.1). To determine if a defect in the B1 B cell population could be responsible for the impaired TI-2 responses in Pim single knockout mice, we immunized control and Pim deficient mice with 50 µg NP-Ficoll i.v. and monitored the antibody response. Both Pim $1^{-/-}$ and Pim $2^{-/-}$ displayed normal TI-2 antibody titers after i.v. immunization (Figure 5.3 B) supporting our notion that

the defect observed with i.p. immunization was due to the diminution of B1 B cells in the peritoneal cavity and not due to an inability in Pim single knock-out B cells to response to TI-2 antigens. Pim $1^{-/-}2^{-/-}$ mice continued to generate a significantly impaired TI-2 response ($p<.05$, Figure 5.3 B) when antigen is delivered i.v. directly to the intact MZ population so we conclude that a migratory or developmental defect of the B1 B cells is not responsible for the loss of the TI-2 response in Pim $1^{-/-}2^{-/-}$ mice.

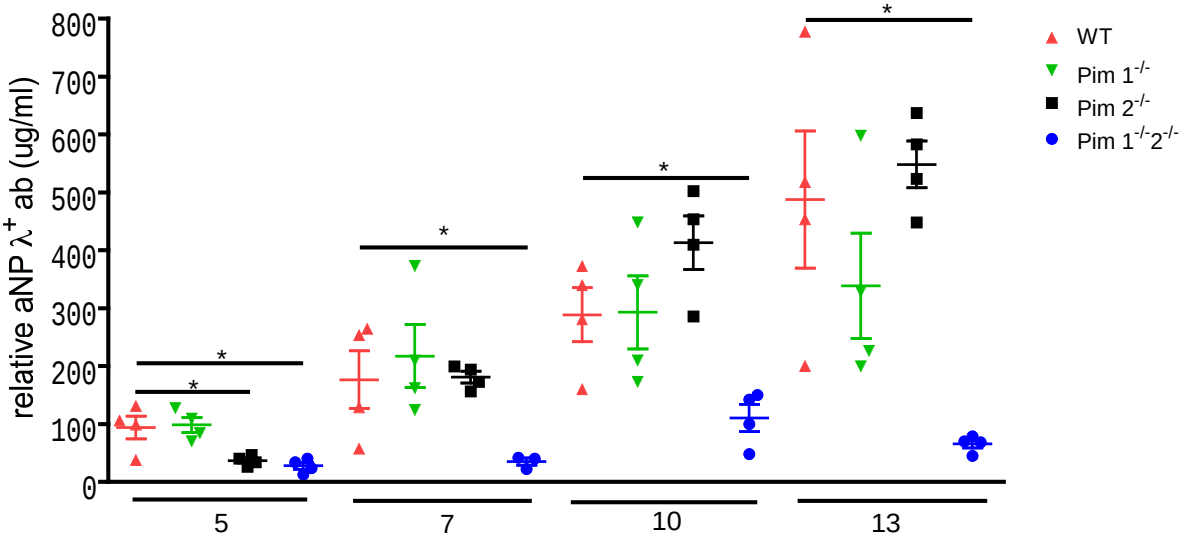
**Figure 5.3 Pim $1^{-/-}2^{-/-}$ MZ B cells do not respond to TI-2 antigen.** C57BL/6 mice of indicated genotypes were immunized with 50 μg NP-Ficoll, sera was collected at indicated time points and measured by ELISA for anti-NP $\lambda^{+}$ antibody. Each symbol represents an individual mouse, lines indicate the mean with SEM, statistics were determined by student's t test *=p<.05, **=p<.01, ***=p<.001. (A) I.p. and i.v. immunizations were compared (B) I.v immunization among the 4 genotypes.

## Pim $1^{-/-}2^{-/-}$ MZ B cells do not respond to TI2 antigen

**A.**




**B.**



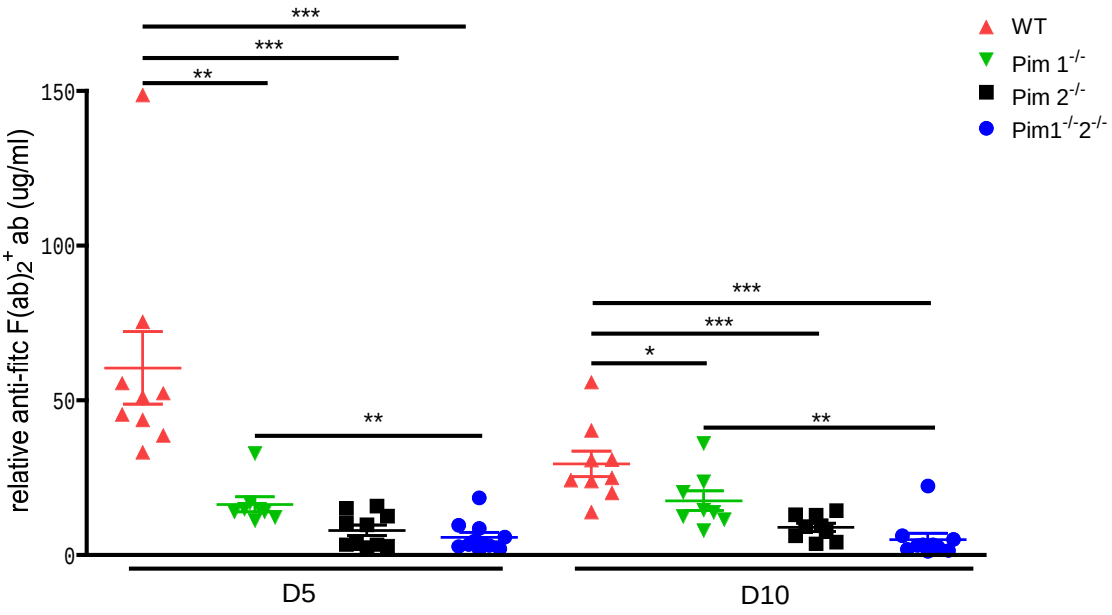
**TI-1 responses are deficient in Pim $1^{-/-}2^{-/-}$ mice**

Our TI-2 results above are consistent with a response defect in MZ B cells, which are normal in number and location in Pim $1^{-/-}2^{-/-}$ mice. TI-1 antigens also activate B cells without T cell help but are characterized by an expansion of conventional follicular B2 B cells not MZ B cells (6). As shown in chapter III, follicular B cells are also normal in proportion and number in Pim $1^{-/-}2^{-/-}$ mice (Figure 3.1). To determine if impaired antibody responses also extended to TI-1 antigens, we immunized Pim $1^{-/-}2^{-/-}$ and B6 mice i.p. with 25 µg of FITC-LPS. FITC specific antibody was measured by ELISA. TI-1 responses were significantly impaired in Pim 1 and 2 deficient mice relative to B6 controls on days 5 and 10 post immunization (Figure 5.4). The mean antibody in Pim $1^{-/-}2^{-/-}$ mice was found to be 10 fold less than that in B6 mice (5 ± 1.5 vs. 60.5 ± 11.7 µg/ml, $p<.001$) on day 5 and 6-fold less on day 10 as Pim $1^{-/-}2^{-/-}$ anti-Fitc antibody remained the same 5 ± 1.9 µg/ml but decreased in WT to 29.5 ± 4.1 µg/ml ($p<.001$). We examined single knockout mice to determine if this phenotype could be attributed to the loss of a particular Pim kinase. At day 5 post-immunization anti-FITC antibody levels in Pim $1^{-/-}$ mice were below 20 µg/ml, less than one-third the average in B6 mice. It is also noteworthy that while the response of Pim $1^{-/-}$ mice was reduced relative to control mice, their antibody response was significantly higher than that of Pim $1^{-/-}2^{-/-}$ mice ($p<.01$). The mean antibody level in Pim $2^{-/-}$ mice was found to be 8 ± 5.3 µg/ml approximately one-tenth that of the response of control mice ($p<.001$) (Figure 5.4). Ten days after immunization Fitc specific antibody in both Pim 1 and Pim 2 knock out mice did

not change from day 5 at 17 ± 3.1 μg/ml and 9 ± 1.2 μg/ml, respectively. These data indicate that the loss of Pim 1 has a more modest affect on the TI-1 immune response than Pim 2. Taken together, these results confirm a B cell intrinsic defect for Pim $1^{-/-}2^{-/-}$ mice and show distinctions in Pim control of the TI-1 and TI-2 responses.

**Figure 5.4. The loss of Pim 1 or 2 impairs TI-1 responses.** Mice of indicated genotypes were immunized i.p. with 25 µg of Fitc-LPS. Sera was measured for anti-Fitc $F(ab)_2^+$ antibody at 5 and 10 days after immunization. Each symbol represents an individual mouse, lines indicate the mean and SEM, Wt n=9, Pim $1^{-/-}$ n=8, on day 5 Pim $2^{-/-}$ n=10 and Pim $1^{-/-}2^{-/-}$ n=11, on day 10 Pim $2^{-/-}$ n=9, and Pim $1^{-/-}2^{-/-}$ n=10. Statistics were determined by student's t test *=p<.05, **=p<.01, ***=p<.001.

**The loss of Pim 1 or 2 impairs TI-1 responses**



**Despite normal activation and proliferation in vitro ASC generation is impaired in Pim $1^{-/-}2^{-/-}$ B cells**

The defects observed in antibody generated in vivo to TD and TI antigens as well as the amount of natural antibody in unimmunized mice pointed to an underlying deficiency in antibody production. Consistent with the antibody analysis, ELISpot results revealed significantly fewer ASCs in Pim $1^{-/-}2^{-/-}$ mice after immunization with a TD or TI-2 antigen. The diminished number of ASCs could be due to a defect in differentiation of B cells into ASCs or survival of these cells once they were generated.

First we determined if Pim $1^{-/-}2^{-/-}$ B cells were becoming activated when they encountered a mitogen in vitro. B cells isolated from Pim $1^{-/-}2^{-/-}$ or control mice were cultured with the mitogens LPS, anti-IgM and anti-CD40 for 24 or 48 hours then analyzed by flow cytometry for surface expression of early activation markers. Pim $1^{-/-}2^{-/-}$ cultures displayed equivalent induction of activation markers CD69, CD71, and CD95 as wild type, indicating that early signaling events in these 3 pathways were intact (Figure 5.5). To examine ASC differentiation more closely, we used an in vitro system to generate ASCs by culturing purified B cells for 4 days with LPS. This system allows us to compare Pim $1^{-/-}2^{-/-}$ to B6 control B cells in survival, proliferation, and the generation of ASCs. After 4 days in culture cell counts and proliferation were comparable (Figure 5.6 A/B). Equivalent numbers of viable cells coming out of culture and rounds of proliferation are strong evidence that survival is not compromised by the loss of Pim 1 and 2; however, it could be difficult to detect a survival difference within the small

subpopulation of ASCs that are produced. To definitively look at cell survival within the ASC population, we would need a marker to identify ASCs among other cells in the culture. This will be addressed in the next section.

Differentiation into ASCs in these cultures was determined by ELISpot and revealed a significant deficiency in Pim $1^{-/-}2^{-/-}$ mice, which generated only one-third the number of ASCs as control mice (Figure 5.6 C). Loss of either Pim 1 or 2 alone did not reduce ASC generation. The loss of IRF4 negatively impacts ASC generation (19, 20). To compare the defective ASC generation between $IRF4^{-/-}$ and Pim $1^{-/-}2^{-/-}$ mice, B cells were isolated from spleens ($IRF4^{-/-}$ generously provided by Dr. Ren, Brandeis University) and cultured for 4 days with LPS. $IRF4^{-/-}$ B cells generated almost 6-fold fewer ASCs than WT, displaying a more severe impairment than Pim $1^{-/-}2^{-/-}$ B cells (Figure 5.6 D). The defect in ASC generation despite equal induction of activation markers, cell counts, and proliferation indicate that Pim 1 and/or 2 play an important role the differentiation pathway of an activated B cell to an antibody-secreting cell.

**Figure 5.5. Induction of early activation markers is unimpaired in Pim $1^{-/-}2^{-/-}$ B cells.** Splenic B cells isolated from WT and Pim $1^{-/-}2^{-/-}$ mice were cultured with LPS, anti-CD40, or anti-IgM for 24 hours then analyzed by flow cytometry for CD69 and CD71 expression or 48 hours before analysis for CD95 expression. Plots represents 3 separate experiments. Red lines are unstimulated controls, blue lines are stimulated cells.

**Induction of early activation markers is unimpaired in Pim $1^{-/-}2^{-/-}$ B cells**

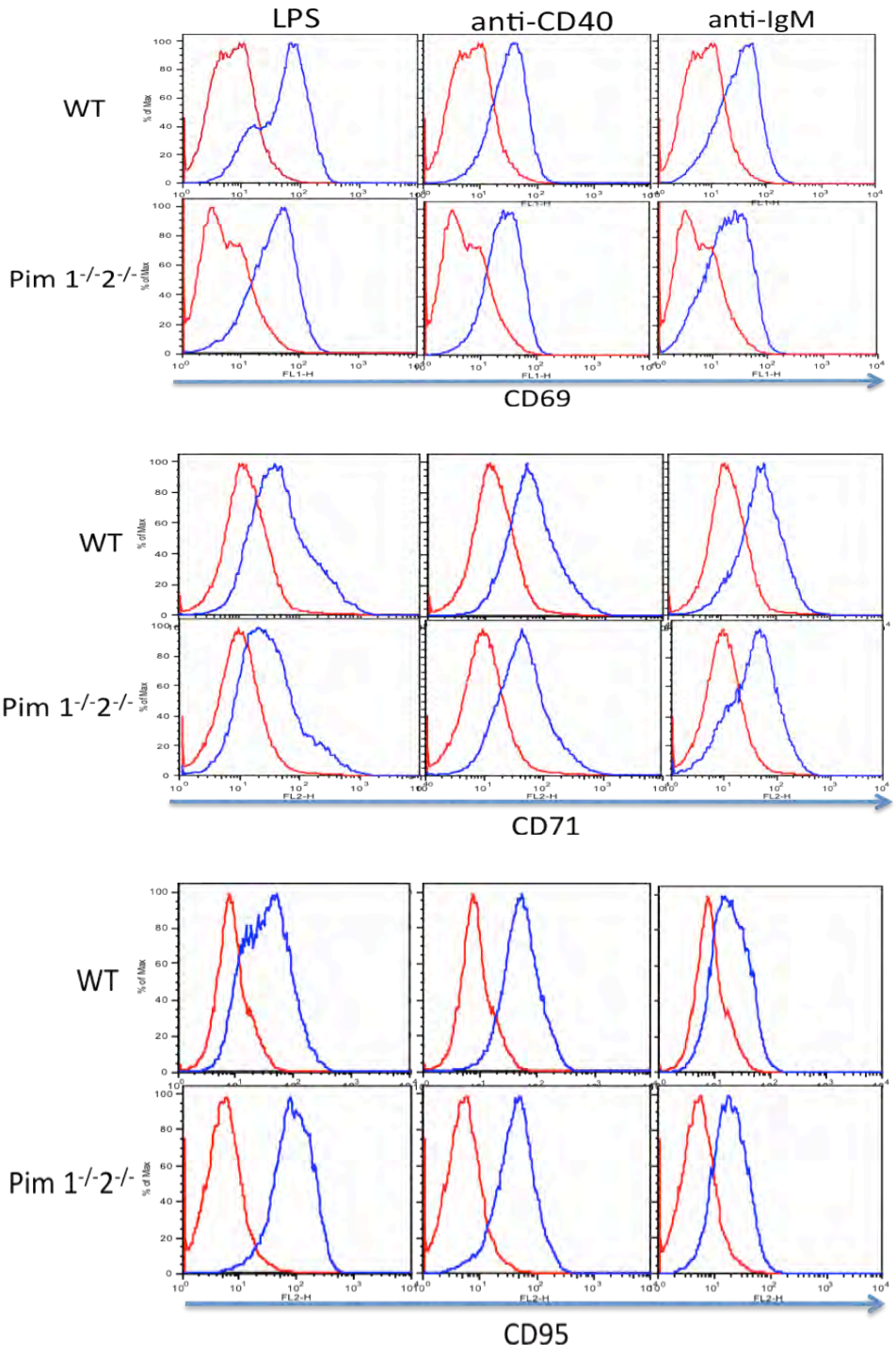
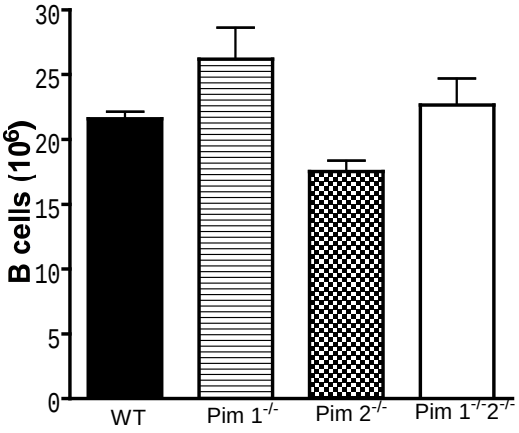


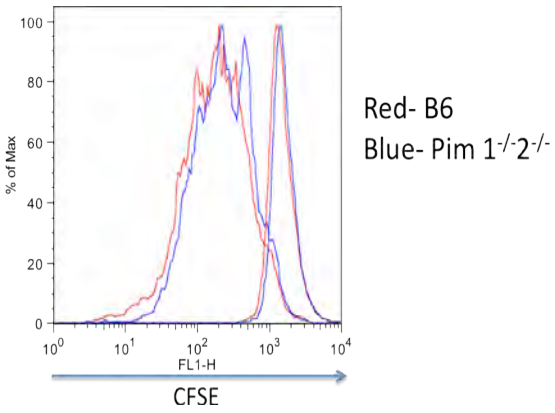
**Figure 5.6. Despite normal live cell counts and proliferation Pim $1^{-/-}2^{-/-}$ B cells generate significantly fewer ASCs.** Splenic B cells isolated from WT, Pim $1^{-/-}$, Pim $2^{-/-}$, and Pim $1^{-/-}2^{-/-}$ mice were cultured with LPS for 4 days. (A) Live cells were counted in each culture using trypan blue exclusion. Data includes 3 experiments. (B) WT and Pim $1^{-/-}2^{-/-}$ B cells were stained with CFSE on day zero. Proliferation was monitored by CFSE dilution; day 4 with LPS is shown. Data represents 3 experiments. (C) ASCs were enumerated by ELISpot. Graph includes 3 experiments; statistics were determined by student's t test *=p<.05, **=p<.01, ***=p<.001. (D) $IRF4^{-/-}$ and WT B cells were cultured for 4 days with LPS, ASCs were enumerated by ELISpot. Graph represents 2 separate experiments.

**Despite normal live cell counts and proliferation Pim $1^{-/-}2^{-/-}$ B cells generate significantly fewer ASCs**

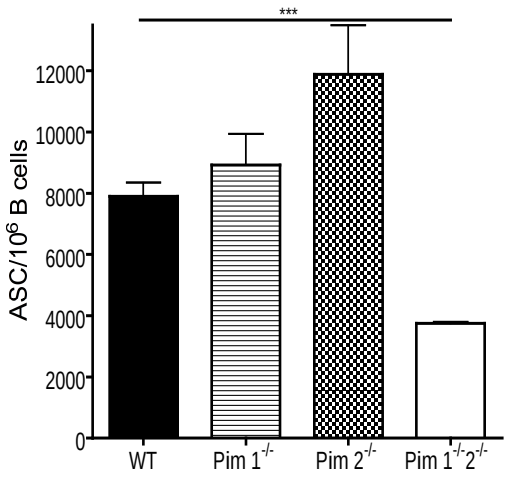
**A.**



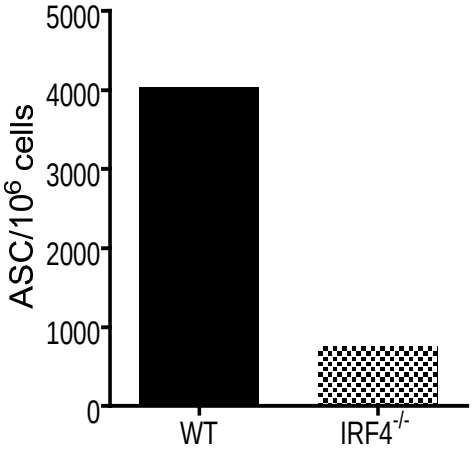

**B.**




**C.**




**D.**



**Fewer Pim $1^{-/-}2^{-/-}$ B cells express BLIMP-1**

Upon commitment to the ASC pathway, many genes are turned on or off in the B cell. Several key transcription factors have been identified that contribute to the ability of a B cell to differentiate into an ASC. In predicted order of expression, IRF-4, BLIMP-1 and XBP-1 are important for a B cell to differentiate into an ASC. A loss of any of these transcription factors decreases the ASC number found in the mouse (17, 19, 21, 46). BLIMP-1 in particular has been called the master regulator because its loss impairs ASC development and its over-expression pushes B cells to become ASCs.

To further investigate where Pim 1 and 2 affect the ASC pathway, we measured the expression of these key transcription factors in ASC differentiation. IRF4 protein expression was measured using intracellular staining and FACS analysis. Pim $1^{-/-}2^{-/-}$ and wild type B6 B cells were cultured with LPS for the indicated times, harvested and stained for IRF4. B cells cultured with BLyS alone were used as negative controls. Pim $1^{-/-}2^{-/-}$ and B6 B cells showed the same levels of IRF4 over the time course tested (Figure 5.7 A) demonstrating that the loss of Pim 1 and 2 does not affect IRF-4 induction.

To look further downstream in the ASC differentiation pathway, we measured BLIMP-1 induction. For this analysis we took advantage of a BLIMP-$1^{gfp/+}$ mouse and crossed it to our Pim $1^{-/-}2^{-/-}$ mouse. The resulting B cells, BLIMP-$1^{gfp/+}$ Pim $1^{-/-}2^{-/-}$, mice allow us to measure the number of cells expressing BLIMP-1 by measuring the GFP expression by flow cytometry. B cells were isolated from BLIMP-$1^{gfp/+}$ Pim $1^{-/-}2^{-/-}$ and control mice and cultured with BLyS

with or without LPS (Figure 5.7B). Three days after activation with BLyS+LPS control cultures contained an average of 6.2% $GFP^+$ B cells while Pim $1^{-/-}2^{-/-}$ cultures contained on average only 1.3% $GFP^+$. The difference was greatest on day 4; control cultures were 17.9% $GFP^+$ more than 3-fold higher than Pim $1^{-/-}2^{-/-}$ cultures at 5.4% $GFP^+$. By day 5 the GFP+ percentage in both control and Pim $1^{-/-}2^{-/-}$ cultures increased to 29.5% and 20.63% respectively. Both cultures plateau on days 6 and 7. Despite the increase on day 5 the percent of B cells expressing BLIMP-1 was significantly depressed in Pim $1^{-/-}2^{-/-}$ cultures compared to B6 controls on each day measured after activation with BLyS + LPS (Figure 5.7 B). These results indicate the step, at which ASC generation is restricted in Pim $1^{-/-}2^{-/-}$ B cells, is induction of BLIMP-1. We also analyzed the geometric mean fluorescent intensity (MFI) of the $GFP^+$ cells from Pim $1^{-/-}2^{-/-}$ and control cultures as a means of comparing how much blimp protein was made on a per cell basis. With the exception of day 3 in culture, the geometric MFI of the Pim $1^{-/-}2^{-/-}$ $GFP^+$ B cells was significantly less than WT. Recent publications have suggested that increased expression of IRF4 induces BLIMP-1 expression and the Pim 2 is a target of IRF4 (71). These new insights suggest a mechanism by which Pim 1 and/or 2 are critical for IRF4 induction of BLIMP-1.

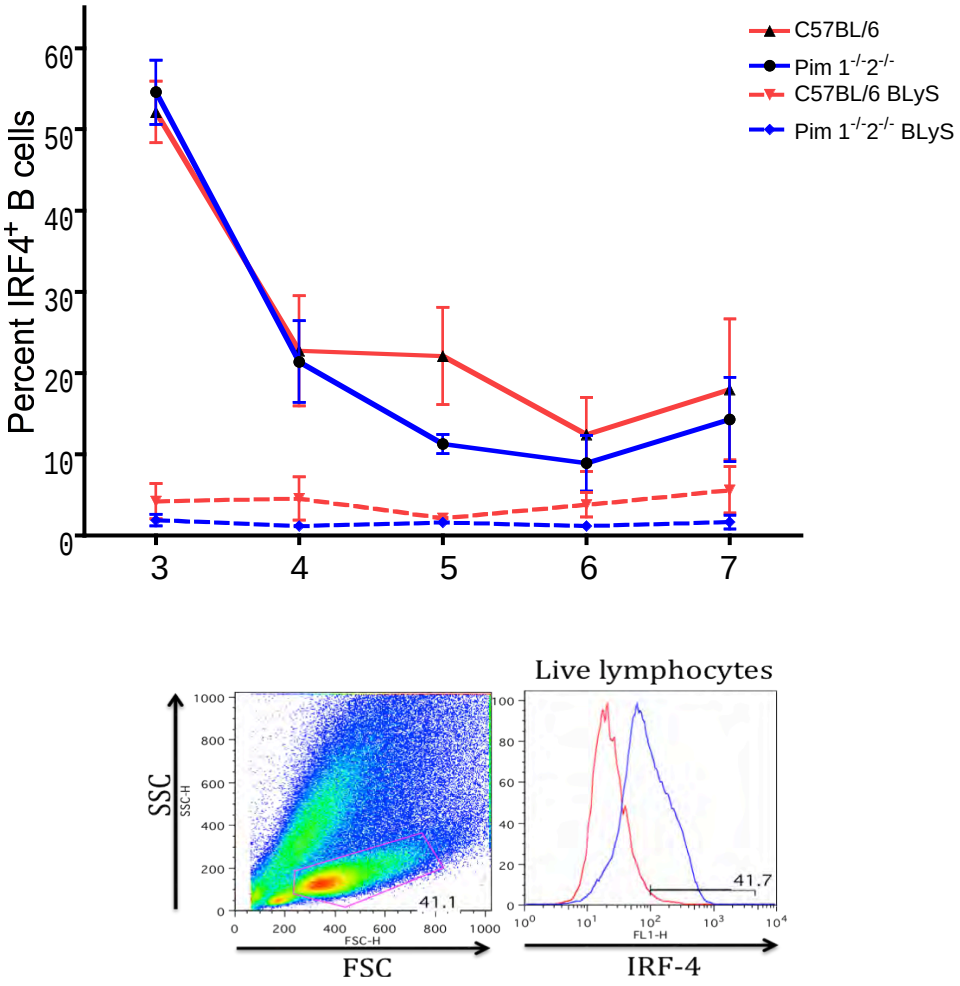
Although previous experiments had shown that cell counts on day 4 were equivalent in control and Pim $1^{-/-}2^{-/-}$ B cell cultures, we could not explicitly rule out the possibility that a survival impairment only within the small ASC subpopulation in each culture might be missed by this method. Because we now had the ability to identify ASCs in the culture using GFP expression, we revisited the survival

question. In each culture we measured the percent of dead cells with propidium iodide, combined with GFP expression we were able to identify dead cells within and outside the ASC population. We found that both the $GFP^{+}$ and $GFP^{-}$ dead cell percentages were equal in control and Pim $1^{-/-}2^{-/-}$ cultures (Figure 5.7 C and D). Consistent with our previous data, this is consistent with the notion that there is not a survival defect in these cultures and the diminished number of ASCs is due to an inability to induce BLIMP-1 expression.

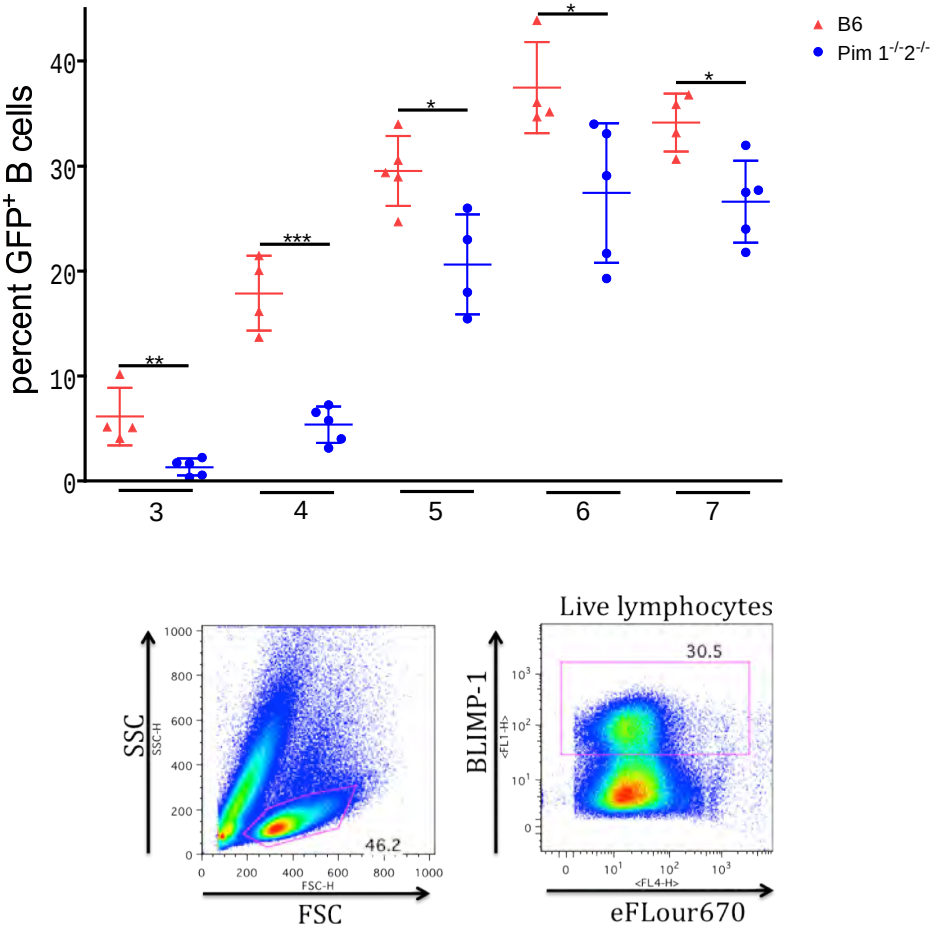
**Figure 5.7. BLIMP-1 but not IRF-4 expression is impaired by the loss of Pim 1 and 2.** B cells isolated from BLIMP-$1^{gfp/+}$ Pim $1^{-/-}2^{-/-}$ or control BLIMP-$1^{gfp/+}$ mice were cultured in BLyS with or without LPS (A) The mean percent of IRF-4 positive cells is given for each day (WT n=4 except day 5 when n=5; Pim $1^{-/-}2^{-/-}$ n=5 except day 5 when n=6) (B) The percent of $GFP^+$ B cells in each culture is given, each symbol in a given day represents cells from one mouse, B cells isolated from a single mouse were measure over the 7 day time course, lines indicate mean and SEM, (WT n=4 except day 5 when n=5; Pim $1^{-/-}2^{-/-}$ n=5 except day 5 when n=4) (C) Cell death was also measured in $GFP^+$ and $^-$ populations by propidium iodide staining. Plots shown are from 4 days with LPS+BLyS and are representative of 3 experiments. The same results were obtained at each time point. (D) Geometric mean fluorescent intensity of $GFP^+$ cells was determined for WT and Pim $1^{-/-}2^{-/-}$ samples at each time point. Pim $1^{-/-}2^{-/-}$ geometric MFI is plotted as a percent of WT controls. Graph includes 3 experiments, lines indicate the mean and SEM, n=5 except day 5 when n=4. Statistics were determined by student's t test *=p<.05, **=p<.01, ***=p<.001

**BLIMP-1 but not IRF-4 expression is impaired by the loss of Pim 1 and 2**

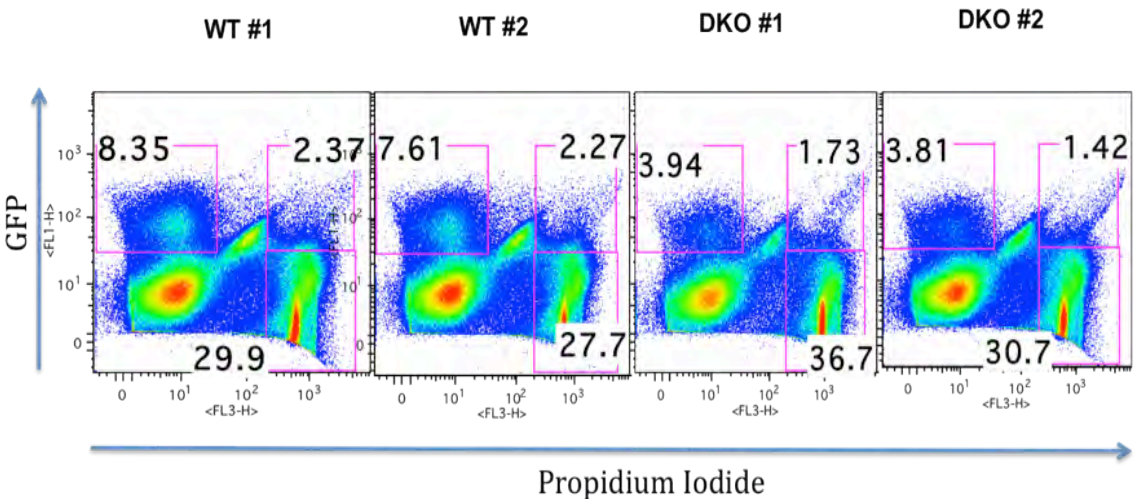
**A.**



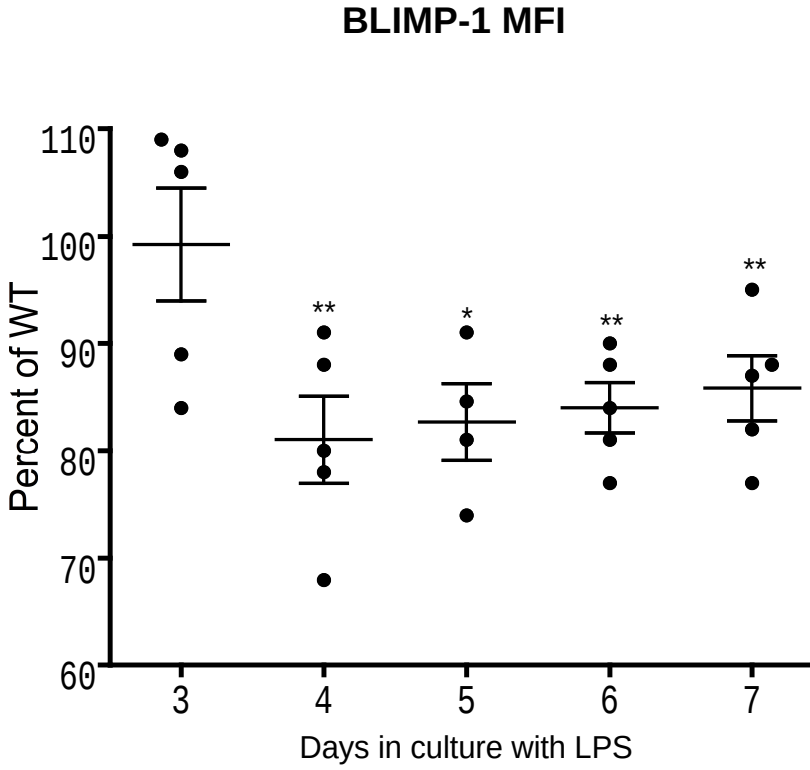
**B.** **BLIMP-1**


B6
Pim 1-/-2-/-
percent GFP+ B cells
0
10
20
30
40
3
4
5
6
7
**
***
*
*
*
Live lymphocytes
SSC
SSC-H
FSC-H
FSC
46.2
BLIMP-1
30.5
eFLour670

**C.**


WT #1
WT #2
DKO #1
DKO #2
GFP
8.35
2.37
29.9
7.61
2.27
27.7
3.94
1.73
36.7
3.81
1.42
30.7
Propidium Iodide

**D.**


BLIMP-1 MFI
Percent of WT
110
100
90
80
70
60
3
4
5
6
7
Days in culture with LPS
**
*
**
**

**Pim $1^{-/-}2^{-/-}$ $GFP^+$ B cells behave as WT**

To examine the number of ASCs from the BLIMP-$1^{gfp/+}$ Pim $1^{-/-}2^{-/-}$ or control BLIMP-$1^{gfp/+}$ B cell cultures, $GFP^+$ or $GFP^-$ cells were sorted by FACS and the number of ASCs was measured in each population. $GFP^-$ B cells were used as background controls and were always below 200 ASC/$10^6$ cells. ASCs/$10^6$ $GFP^+$ cells are plotted in figure 5.8. No significant difference was observed in the number of ASCs comparing WT and Pim $1^{-/-}2^{-/-}$ $GFP^+$ cells at day 4 or 6, demonstrating that once a Pim $1^{-/-}2^{-/-}$ B cell is able to induce BLIMP-1, they are able to secrete antibody like the WT cells.

To further examine different transcription factors required for ASC differentiation, RNA was also isolated from each sorted (GFP + or -) sample. We measured BLIMP-1 RNA using qPCR. No significant difference in RNA levels between Pim $1^{-/-}2^{-/-}$ $GFP^+$ and control $GFP^+$ cells was seen (Figure 5.9 A). From this we concluded that although the number of cells able to induce BLIMP-1 in Pim $1^{-/-}2^{-/-}$ cultures was significantly reduced the amount of BLIMP-1 mRNA expressed in $GFP^+$ B cells was not. Though this seems to contradict our earlier observation of a depressed geometric MFI in the Pim $1^{-/-}2^{-/-}$ $GFP^+$ B cell population, this could be due to translational control of BLIMP-1.

We also analyzed these samples for expression of a third key transcription factor, XBP-1. XBP-1 is downstream of BLIMP-1 and IRF4 in the ASC differentiation pathway and experimental evidence suggests that losing either will block its expression (17, 19). XBP-1 expression levels were unaffected in the Pim

$1^{-/-}2^{-/-}$ $GFP^{+}$ B cells (Figure 5.9 B). This is not surprising considering that IRF4 was unaffected in Pim $1^{-/-}2^{-/-}$ B cells and $GFP^{+}$ B cells also displayed normal BLIMP-1 expression.

Mcl-1 is a pro-survival molecule in the bcl-2 family that has been shown to be induced in antibody secreting cells (72) and by our lab to be downstream of Pim 2 (58) in naïve B cells. Consistent with our previous data on survival and $GFP^{+}$ B cells, we found equal expression of Mcl-1 mRNA in control and Pim $1^{-/-}2^{-/-}$ $GFP^{+}$ B cell populations which was higher than that found in $GFP^{-}$ populations. The Mcl-1 expression in the $GFP^{-}$ populations was also comparable between controls and Pim $1^{-/-}2^{-/-}$ (Figure 5.9 C). Although significantly fewer Pim $1^{-/-}2^{-/-}$ B cells are able to induce BLIMP-1 expression compared to WT these data demonstrate that those that do express BLIMP-1 are completely normal.

We began this chapter with the hypothesis that a B cell intrinsic defect was responsible for the impaired innate and TD humoral immunity described in chapters III and IV. Each experiment described here was consistent with this idea but not definitive. Culturing B cells alone allowed us to evaluate their intrinsic ability to differentiate into ASCs without outside influence. These experiments revealed a strong but incomplete block at BLIMP-1 induction in Pim $1^{-/-}2^{-/-}$ B cells cultured with LPS and demonstrated a B cell intrinsic defect with the loss of Pim 1 and 2.

**Figure 5.8. GFP$^{+}$ WT and Pim 1$^{-/-}$2$^{-/-}$ populations produce the same number of ASCs.** WT BLIMP-1$^{gfp/+}$ and Pim 1$^{-/-}$2$^{-/-}$ BLIMP-1$^{gfp/+}$ B cells isolated from individual mice were cultured with LPS and BLyS for 4 (WT n=3, Pim 1$^{-/-}$2$^{-/-}$ n=4) or 6 (WT n=4, Pim 1$^{-/-}$2$^{-/-}$ n=5) days before being sorted into GFP$^{+}$ and GFP$^{-}$ populations. ASCs were measured by ELISpot, the GFP$^{+}$ populations are shown, GFP$^{-}$ cells were used as background and ranged between 0 and 200 ASCs/10$^{6}$ GFP$^{-}$ cells. Graph includes 3 experiments, lines indicate the SEM.

**GFP$^+$ WT and Pim 1$^{-/-}$2$^{-/-}$ populations produce the same number of ASCs**

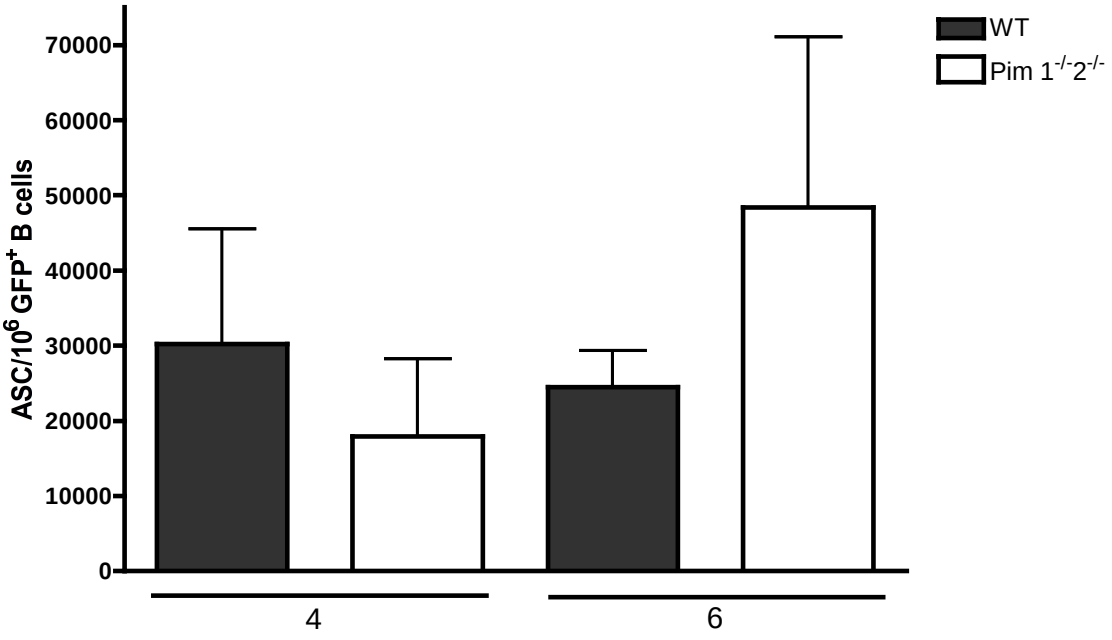
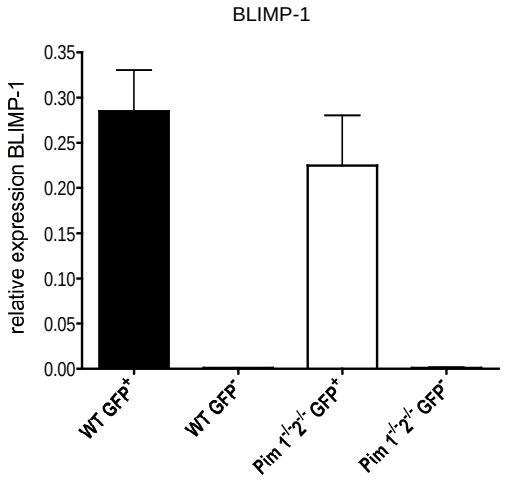


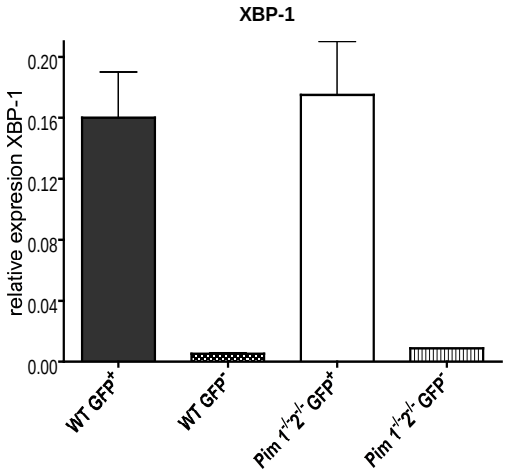
**Figure 5.9. GFP$^{+}$ Pim 1$^{-/-}$2$^{-/-}$ B cells express normal levels of BLIMP-1, XBP-1, and Mcl-1.** Splenic B cells isolated from BLIMP-1$^{gfp/+}$ control and Pim 1$^{-/-}$2$^{-/-}$ mice were cultured in LPS for 4 days then sorted based on GFP expression. RNA isolated from GFP$^{+}$ and GFP$^{-}$ samples was measured by qPCR for relative expression of BLIMP-1 (A), XBP-1 (B), and Mcl-1 (C). Graphs are of one experiment but represent similar results from 3 separate experiments, WT n=2, Pim 1$^{-/-}$2$^{-/-}$ n=2 lines indicate SEM.

**GFP$^{+}$ Pim 1$^{-/-}$2$^{-/-}$ B cells express normal levels of BLIMP-1, XBP-1, and Mcl-1**

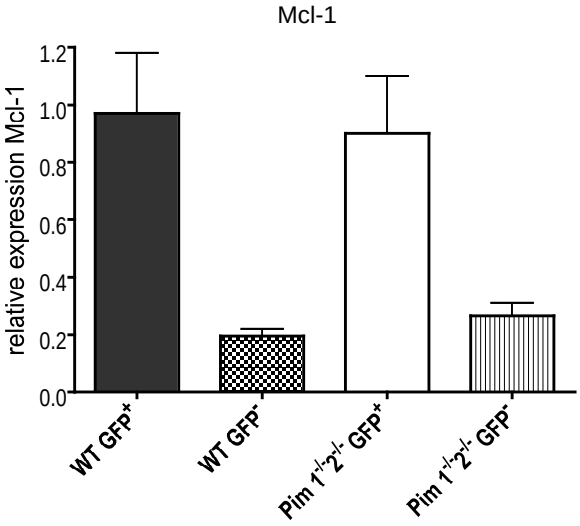
A.




B.




C.



# Chapter VI

# Migration Of Pim $1^{-/-}2^{-/-}$ Cells

## Introduction

Experiments in Chapter IV revealed impairment in TD responses due to the loss of Pim 1 and 2 kinases. The function of both T and B lymphocytes are important in mounting a TD response so a defect in either population would result in an impaired response. In Chapter IV we demonstrated that Pim sufficient T cell help was unable to rescue the TD response in Pim $1^{-/-}2^{-/-}$ mice, consistent with B cell defect. Another way to look at the function of T and B lymphocytes separately is to create a chimera.

Bone marrow chimera mice have been established as a useful tool for evaluating immune mechanisms (73). To produce chimeric mice, recipients are lethally irradiated to deplete host bone marrow stem cells before receiving transplant hematopoietic stem cells from bone marrow or fetal liver from one or more donor mice. Stem cells from bone marrow or fetal liver will home to the bone marrow microenvironment that sustains them then begin expanding. In the weeks to months following a bone marrow transfer the engrafted stem cells replace all the cells lost to the host mouse and can continue hematopoiesis for the rest of the mouse's life. By using multiple donors it is possible to examine the behavior of specific deficient cell populations, such as Pim $1^{-/-}2^{-/-}$ B cells, in a WT

environment. This approach would allow us to assess the individual contributions of B and T cell to the impaired TD response.

By using donors that differ at the CD45 locus or Igh locus (allotype) marker we are able to identify the source of the peripheral lymphoid populations. In the work presented in this chapter, bone marrow or fetal liver from WT or Pim $1^{-/-}2^{-/-}$ mice were transferred into a depleted host at various ratios, which enables us to observe the behavior of Pim $1^{-/-}2^{-/-}$ B cells after immunization in a completely sufficient environment created by the engraftment of WT stem cells.

While examining the immune response in chimera mice 12 weeks or less after reconstitution, we observed that WT cells seemed to be outcompeting Pim $1^{-/-}2^{-/-}$ cells during reconstitution. In addition we found that lethally irradiated mice reconstituted with Pim $1^{-/-}2^{-/-}$ bone marrow or fetal liver did not live past 2 months suggesting that Pim $1^{-/-}2^{-/-}$ stem cells are unable to migrate normally preventing engraftment and survival.

At the same time a paper was published showing that Pim $1^{-/-}$ bone marrow cells are unable to reconstitute irradiated recipients due to a migratory defect. This migratory defect was attributed to deregulation of CXCR4 on the surface of these cells (44). CXCR4 is involved in early B cell development and organization of the germinal center as well as homing of bone marrow cells. Considering our observations that germinal centers in Pim $1^{-/-}2^{-/-}$ mice were impaired we asked whether homing of Pim $1^{-/-}2^{-/-}$ mature lymphocyte populations might also be affected. By transferring WT and Pim deficient splenocytes into

irradiated recipients we found that homing of mature Pim deficient splenocytes was unimpaired.

Previously published work had shown that despite impairments in homing and engraftment fetal liver chimeras created by transferring a high number of CXCR4 fetal liver cells overcome this deficiency (45). We tried this approach with Pim $1^{-/-}2^{-/-}$ fetal liver cells but found that this did not overcome the impaired engraftment. These data suggest that other chemokines may be downstream of Pim 1 or 2 contributing along with deregulation of CXCR4 to the impaired engraftment of both bone marrow and fetal liver stem cells.

## Results

### Pim $1^{-/-}2^{-/-}$ reconstitution of peripheral lymphocyte populations is inefficient

The first set of chimeras was created by injecting mixtures of Pim $1^{-/-}2^{-/-}$ (Ly5.2) and B6 wild type (Ly5.1) into lethally irradiated B6 wild type recipients (Ly5.1). Bone marrow from B6 Ly5.1 control and B6 Ly5.2 Pim $1^{-/-}2^{-/-}$ mice were combined at 3 different ratios (10:90, 50:50, and 90:10). A total of 6 chimeras were generated for each ratio. For preliminary analysis of the chimeras at 12 weeks after reconstitution peripheral blood from one mouse of each ratio was examined for Ly5.1 (WT) or Ly5.2 (Pim $1^{-/-}2^{-/-}$) B cells. Very few $B220^+$ cells in the mouse given 90% Pim $1^{-/-}2^{-/-}$ bone marrow expressed either Ly marker; $B220^+$ cells from the 50:50 recipient were equally distributed (47% $Ly5.2^+$ and 46% $Ly5.1^+$) and $B220^+$ cells from the 10% Pim $1^{-/-}2^{-/-}$ recipient were over 97% $Ly5.1^+$. To assess function in TD responses each recipient was immunized with 50 µg of

NP-CGG in Imject Alum. Ten days after immunization mice were sacrificed and spleens, lymph nodes, and Peyer's patches were analyzed for reconstitution and germinal center B cells ($CD95^{+}GL7^{+}$) by flow cytometry.

The fraction of B cells derived from the Pim $1^{-/-}2^{-/-}$ bone marrow was highly variable but consistently less than the percent of Pim $1^{-/-}2^{-/-}$ in the bone marrow mix for almost every recipient. Out of 5 recipients who received 90% Pim $1^{-/-}2^{-/-}$ bone marrow only 1 had a splenic B cell pool with close to 90% Pim $1^{-/-}2^{-/-}$ B cells. The other individuals ranged from 56 to 60% bringing the mean percent Pim $1^{-/-}2^{-/-}$ B cells to 63% ± 5. B cells derived from Pim $1^{-/-}2^{-/-}$ bone marrow in the Peyer's Patches was slightly higher ranging from 62 to 85%. Pim $1^{-/-}2^{-/-}$ B cell reconstitution of the lymph nodes varied considerably with 90% Pim $1^{-/-}2^{-/-}$ recipients ranging from 12 to 14% Pim $1^{-/-}2^{-/-}$ B cells (Figure 6.1 A). In individuals who received 50% Pim $1^{-/-}2^{-/-}$ bone marrow the percent of Pim $1^{-/-}2^{-/-}$ derived B cells in the lymph nodes ranged from 2 to 66% with 3/5 recipients below 20% Pim $1^{-/-}2^{-/-}$ B cells. In Peyer's Patches of the same mice receiving 50% Pim $1^{-/-}2^{-/-}$ bone marrow the fraction of Pim $1^{-/-}2^{-/-}$ derived B cells was also variable, ranging from 11 to 52.7% with a mean of 26.3% ± 6. Pim $1^{-/-}2^{-/-}$ B cells percentages in the spleens of 5 mice receiving 50% bone marrow mixtures ranged from 6.9 to 44.1% with a mean of 18.27% ± 5, a noticeable decrease from the 50% Pim $1^{-/-}2^{-/-}$ bone marrow transferred (Figure 6.1 A). Pim $1^{-/-}2^{-/-}$ B cells in the spleens of mice receiving 10% Pim $1^{-/-}2^{-/-}$ bone marrow contained less than 5% Pim $1^{-/-}2^{-/-}$ B cells (Figure 6.1 A). Peyer's Patches in the same individuals varied greatly with 3 individuals having 5% or fewer, 1 individual

having 19%, and 2 individual slightly above 30% Pim $1^{-/-}2^{-/-}$ B cells. Lymph nodes isolated from these mice averaged 6.5% ± 2 of their B cells being Pim $1^{-/-}2^{-/-}$ where 3 mice were 2% or less, one was 7%, and 2 were more than 10% at 12 and 13% (Figure 6.1 A). With few exceptions the contribution of Pim $1^{-/-}2^{-/-}$ cells to the B cell pool in these recipients fell below the percent of Pim $1^{-/-}2^{-/-}$ cells in the transferred bone marrow. Out of these results we must consider two things, one that poor reconstitution of Pim $1^{-/-}2^{-/-}$ B cells will skew the proportion of WT:Pim $1^{-/-}2^{-/-}$ B cells participating in a germinal center response in our bone marrow chimeras after immunization and second that WT bone marrow cells may have an advantage over Pim $1^{-/-}2^{-/-}$ bone marrow cells during reconstitution.

Earlier in this dissertation we demonstrated a defect in the ability of Pim $1^{-/-}2^{-/-}$ mice to generate germinal centers in response to NP-CGG. To examine the function of Pim $1^{-/-}2^{-/-}$ B cells during a TD response in a WT environment we immunized the bone marrow chimeras and identified GC B cells by flow cytometry. In the reconstituted chimeras B cells that were participating in a germinal center reaction could be determined by cell Ly surface markers. In chimeras reconstituted with 50% and 10% Pim $1^{-/-}2^{-/-}$ mixtures WT GC B cells were above the unimmunized control (Figure 6.1 B). In chimeras given 90% Pim $1^{-/-}2^{-/-}$ bone marrow mixtures, however, WT B cells were not above unimmunized levels most likely due to the low reconstitution of WT B cells (Figure 6.1 B). None of the chimeras contained Pim $1^{-/-}2^{-/-}$ GC B cells in numbers above the unimmunized controls (Figure 6.1 B). In all cases the percent of Pim $1^{-/-}2^{-/-}$ B cells in the periphery were less than the percent of Pim $1^{-/-}2^{-/-}$ bone marrow in their

representative mixture transferred, accordingly only mice reconstituted with 90% Pim $1^{-/-}2^{-/-}$ bone marrow had enough Pim $1^{-/-}2^{-/-}$ peripheral B cells to allow us to draw a conclusion about the behavior of Pim $1^{-/-}2^{-/-}$ B cells during a TD response in a WT environment. In these mice reconstituted with a 90:10 Pim $1^{-/-}2^{-/-}$:WT mixture, despite the majority of peripheral B cell originating from Pim $1^{-/-}2^{-/-}$ bone marrow, germinal center B cells were almost entirely wild type. This supported the idea that there is a B cell intrinsic defect in the Pim $1^{-/-}2^{-/-}$ B cells.

One impediment to a full interpretation of our previous chimera results using WT Ly5.1 bone marrow was our inability to identify the contribution from the irradiated hosts that were also $Ly5.1^{+}$. To circumvent this problem we produced a second set of chimeras in which we replaced the WT Ly5.1 bone marrow with μMT bone marrow, in these chimeras stem cells from μMT donors should reconstitute all cell types except the B cell pool, T cells derived from μMT bone marrow can provide normal T cell help to the Pim $1^{-/-}2^{-/-}$ B cells upon immunization 8-12 weeks after the bone marrow transfer.

Our initial strategy was to reconstitute Ly5.1 WT mice receiving 600 rads 2 times with $1.5x10^{6}$ bone marrow cells mixed at 80% μMT and 20% Pim $1^{-/-}2^{-/-}$ or WT, the high percent of μMT bone marrow transferred ensures that all cells other than B cells originated from the μMT bone marrow while the B cell compartment is reconstituted by either Pim $1^{-/-}2^{-/-}$ or control WT cells in the two sets of chimeras. This, however, resulted in poor reconstitution of the mature splenic B cell pool in both Pim $1^{-/-}2^{-/-}$ and WT chimeras. To try and improve the efficiency of reconstitution we increased the percent of Pim $1^{-/-}2^{-/-}$ and WT bone

marrow cells and transferred a total of $3x10^6$ cells at a 90% Pim $1^{-/-}2^{-/-}$ or WT and 10% μMT into lethally irradiated (600 rads, 2 times) B6 Ly5.1 recipients. We found consistently good reconstitution of peripheral WT B cells but not Pim $1^{-/-}2^{-/-}$ B cells. Of the $B220^+$ B cells we did identify in 90% Pim $1^{-/-}2^{-/-}$ 10% μMT chimeras only a fraction were $Ly5.2^+$ and therefore identified as originating from Pim $1^{-/-}2^{-/-}$ bone marrow (Figure 6.2).

We also mixed Pim $1^{-/-}2^{-/-}$ bone marrow and bone marrow from allotype congenic B6 ($Ig^a$ $Thy1^a$) mice at various ratios and increased the total number of cells transferred into irradiated B6 Ly5.1 mice (600 rads, 2 times) from $3x10^6$ to $5x10^6$. Increasing the number of cells transferred was an attempt to overcome any difficulties in reconstitution by Pim $1^{-/-}2^{-/-}$ bone marrow. We consistently found, however, that peripheral B cells were not derived from the Pim $1^{-/-}2^{-/-}$ bone marrow even if 90% of the cells transferred were obtained from Pim $1^{-/-}2^{-/-}$ donors. Furthermore, we began to observe that these mice would become dehydrated and have a hunched posture 7-12 weeks after transfer and had to be euthanized. Although these chimeras did not die from radiation sickness, which takes about 10 days, the results do indicate a defect in Pim $1^{-/-}2^{-/-}$ done marrow reconstitution, which became more apparent when we increased the percent of Pim $1^{-/-}2^{-/-}$ cells transferred.

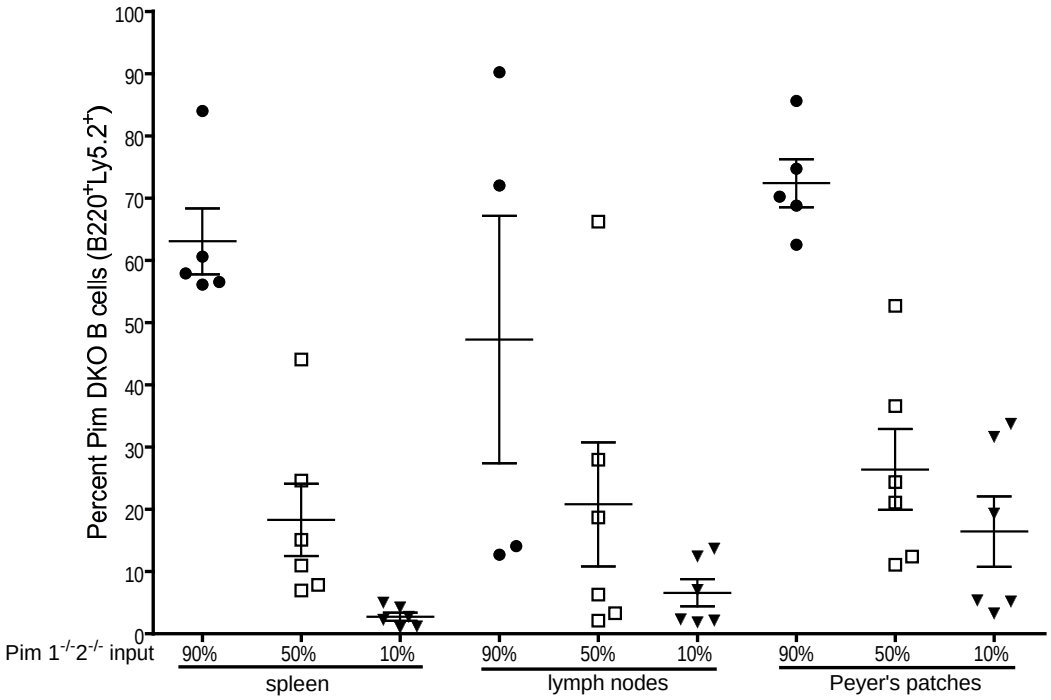
We employed several strategies in trying to overcome this deficiency in reconstitution. Table 6.1 illustrates the different chimeras that were attempted with the ratio and recipients; none resulted in consistent reconstitution of the Pim $1^{-/-}2^{-/-}$ B cell pool. Together these data support the conclusion that Pim $1^{-/-}2^{-/-}$

derived stem cells were unable to compete with WT stem cells in the generation of peripheral B cells. This may reflect a broader defect in Pim $1^{-/-}2^{-/-}$ cell homing.

**Figure 6.1. Pim $1^{-/-}2^{-/-}$ peripheral B cells are less than expected and not found in the germinal center.** (A) Lethally irradiated mice (600 rads, 2 times) were given WT (Ly5.1) or Pim $1^{-/-}2^{-/-}$ (Ly5.2) bone marrow i.v., the ratio of Pim $1^{-/-}2^{-/-}$ bone marrow to WT bone marrow cells transferred is indicated on the x-axis. 8-10 weeks later mice were sacrificed, the percent of Pim $1^{-/-}2^{-/-}$ B cells was determined by flow ctometry ($B220^{+}Ly5.2^{+}$) (y-axis) for each organ (x-axis) is indicated. 90% Pim $1^{-/-}2^{-/-}$ n=5 (spleen and Peyer's Patches) n=4 (lymph nodes), 50% Pim $1^{-/-}2^{-/-}$ n=6, 10% Pim $1^{-/-}2^{-/-}$ n=6 (B) The same mice from part A were immunized with 50 µg of NP-CGG absorbed to Imject Alum 10 days before sacrifice. The number of Pim $1^{-/-}2^{-/-}$ ($B220^{+}Ly5.2^{+}$) or WT ($B220^{+}Ly5.1^{+}$) GC B cells ($CD95^{+}GL7^{+}$) found in the spleen is depicted (y-axis) for each ratio transferred (x-axis). Different symbols were used to distinguish between imput Pim $1^{-/-}2^{-/-}$ percentage. 90% Pim $1^{-/-}2^{-/-}$ unimmunized n=1, immunized n=4, 50 and 10% Pim $1^{-/-}2^{-/-}$ unimmunized n=2, immunized n=4. Lines represent mean and SEM, each symbol represents an individual mouse, data from 2 separate experiments.

**Pim $1^{-/-}2^{-/-}$ peripheral B cells are less than expected and not found in the germinal center.**

**A.**




**B.**

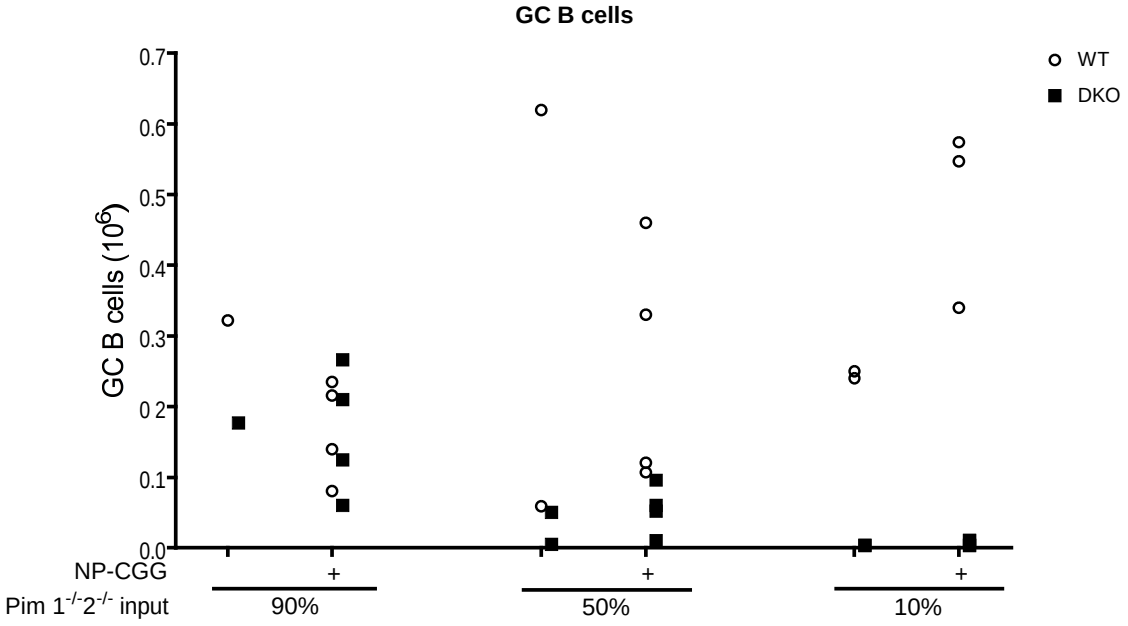


**Figure 6.2. Reconstitution of the peripheral B cell compartment in Pim $1^{-/-}2^{-/-}$ :µMT bone marrow chimeras is impaired**. Lethally irradiated (600 rads, 2 times) B6 Ly5.1 mice received $3x10^6$ bone marrow cells at a 90% Pim $1^{-/-}2^{-/-}$ or WT and 10% µMT mixture. 12 weeks after cell transfer chimeras were sacrificed and the percent of B cells originating from Pim $1^{-/-}2^{-/-}$ and WT bone marrow were identified as $B220^+Ly5.2^+$ by flow cytometry. Plots are representative of 5 Pim $1^{-/-}2^{-/-}$:µMT chimeras and 3 WT:µMT chimeras.

**Reconstitution of the peripheral B cell compartment in Pim $1^{-/-}2^{-/-}$:µMT bone marrow chimeras is impaired**

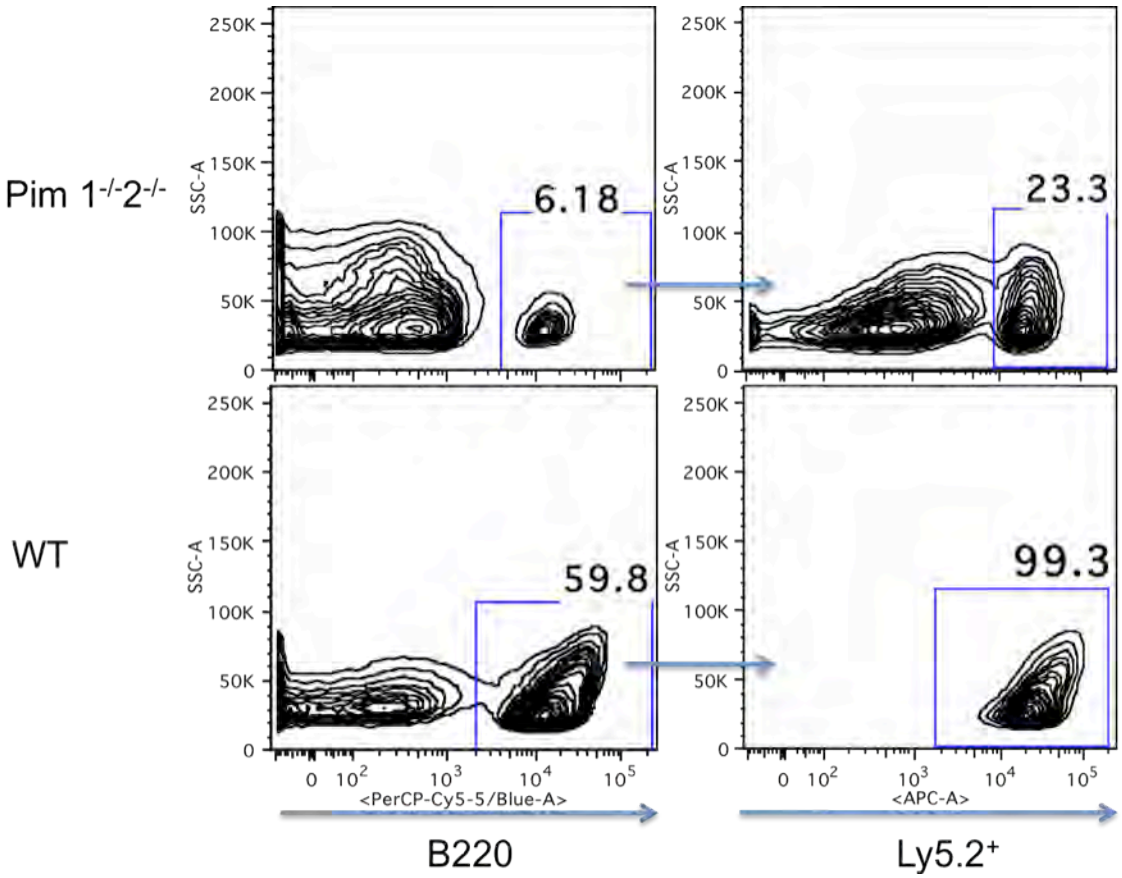


**Table 6.1. Various combinations of donors and hosts were used with multiple ratios to create Pim $1^{-/-}2^{-/-}$ / WT mixed bone marrow chimeras.** Table 6.1 depicts from left to right the source(s) of transferred bone marrow (donors), the ratio(s) at which the transferred bone marrow was mixed, and the recipients from all bone marrow chimera experiments.

**Various combinations of donors and hosts were used with multiple ratios to create Pim $1^{-/-}2^{-/-}$ / WT mixed bone marrow chimeras.**

| Bone Marrow Donors | Ratio | Recipients | Cell source | Outcome |
|---|---|---|---|---|
| B6.Ly5.1\ Pim $1^{-/-}2^{-/-}$ | 90:10, 50:50, 10:90, or 100% | B6.Rag$^{-/-}$ or B6 | Bone marrow | Poor/no reconstitution |
| µMT\Pim $1^{-/-}2^{-/-}$ | 80:20, 10:90, or 30:70 | B6 or B6.Ly5.1 | Bone marrow | No reconstitution or death |
| B6.Igh$^{a}$\ Pim $1^{-/-}2^{-/-}$ | 90:10, 70:30, or 50:50 | B6.Ly5.1 | Bone marrow and/or fetal liver | No reconstitution or death |

**Pim $1^{-/-}2^{-/-}$ bone marrow cannot reconstitute lethally irradiated recipients**

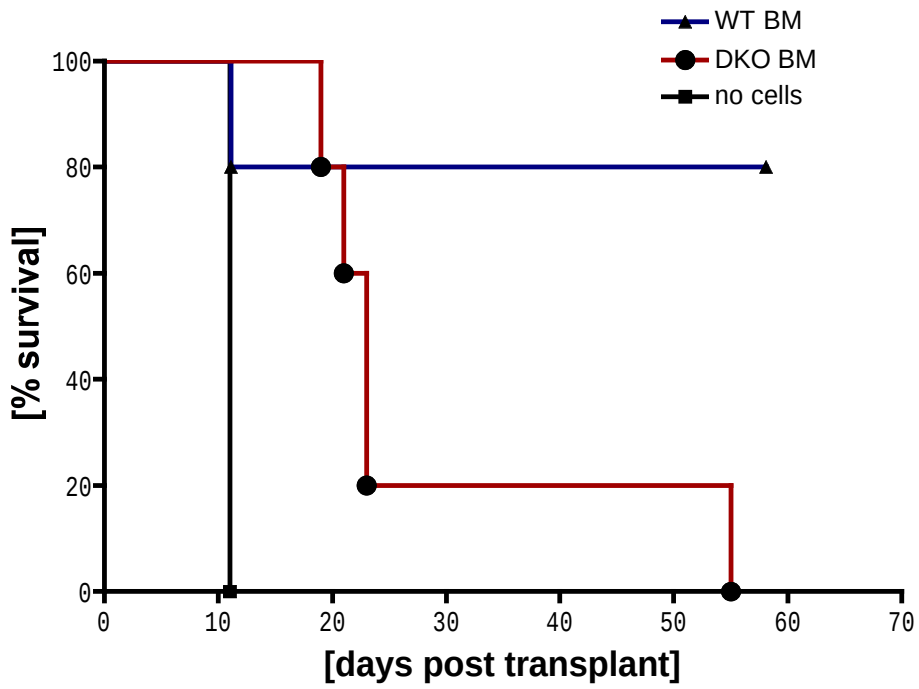
The pathology observed previously during chimera studies indicated that Pim stem cells competed poorly in the generation of peripheral B cells and that chimeras became ill or died weeks after transfer. Work by Grundler and colleagues demonstrated in an alternative system that bone marrow from Pim $1^{-/-}$ donors was unable to reconstitute lethally irradiated mice due to a defect in sustained CXCR4 expression, a chemokine receptor critical for homing of the cells to the bone marrow (44). This impairment may explain our difficulties in generating a reliable Pim $1^{-/-}2^{-/-}$ / WT mixed bone marrow chimera. We tested the ability of Pim $1^{-/-}2^{-/-}$ bone marrow to reconstitute lethally irradiated recipients by transferring $3x10^6$ Pim $1^{-/-}2^{-/-}$ bone marrow cells without wild type bone marrow support. Indeed Pim $1^{-/-}2^{-/-}$ bone marrow cells were unable to rescue lethally irradiated recipients (Figure 6.3). Mice receiving Pim $1^{-/-}2^{-/-}$ bone marrow had a mean life expectancy of 23 days whereas 4 of the 5 mice receiving WT bone marrow cells lived until the experiment was terminated at 60 days.

These results closely matched those previously published concerning Pim $1^{-/-}$ bone marrow studies, which suggested that a defect in CXCR4 was responsible for these observations (44). This suggested that our difficulties in creating a Pim $1^{-/-}2^{-/-}$ bone marrow chimera might also be due to a CXCR4 defect. In addition to its role in homing of hematopoetic stem cells CXCR4 is essential for proper organization of the GC (45). CXCR4 migration defects may also be present in mature peripheral B cells. Considering the similarities between our observations and the published results by Grundler et al with the continued

role of CXCR4 in mature B cells we tested the homing efficiency of mature Pim $1^{-/-}2^{-/-}$ B cells.

**Figure 6.3. Pim $1^{-/-}2^{-/-}$ bone marrow cannot rescue lethally irradiated recipients.** C57BL/6 mice were lethally irradiated receiving 900 rads followed by transfer i.v. of $3x10^6$ bone marrow cells from either WT or Pim $1^{-/-}2^{-/-}$ or no cells 24 hours later. Survival is plotted over 70 days.

**Pim $1^{-/-}2^{-/-}$ bone marrow cannot reconstitute lethally irradiated recipients**



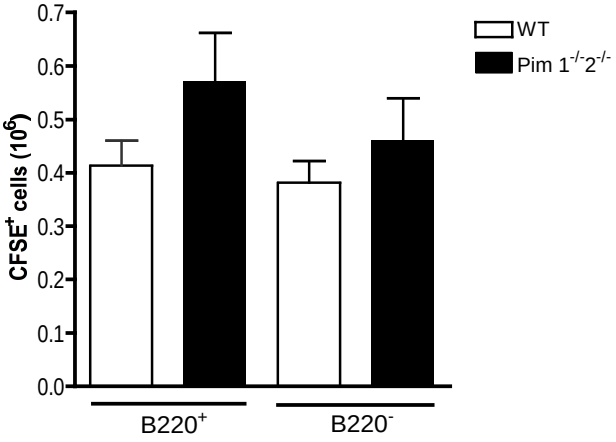
**Pim 1 and/or 2 deficient mature splenocytes migrate normally**

Pim $1^{-/-}2^{-/-}$ B cells developing in chimeras in what should be a supportive environment for TD responses fail to participate in a GC response (Figure 6.1 B). Given the published CXCR4 impairment in Pim $1^{-/-}$ bone marrow cells (44) which closely matched our own reconstitution results, and the role for CXCR4 in the germinal center (45) it is possible that CXCR4 migration defects may also present in mature peripheral B cells. To investigate the homing capacity of mature B cells from Pim deficient mice we transferred mature splenocytes from Pim $1^{-/-}2^{-/-}$ or WT mice labeled with CFSE into lethally irradiated recipients. Twenty-four hours later spleens were harvested and the number of CFSE positive B cells determined by flow cytometry. No significant difference was observed in recipients of Pim $1^{-/-}2^{-/-}$ cells (Figure 6.4 A). We were concerned that by irradiating the recipients we had created space for the cells to flow freely regardless of possible homing defects. Therefore the experiment was repeated using un-irradiated recipients. Splenocytes from Pim $1^{-/-}$, $2^{-/-}$, $1^{-/-}2^{-/-}$, and control mice were labeled with CFSE and transferred into un-irradiated recipients. Twenty-four hours later we examined the spleen and bone marrow for CFSE labeled B cells as before. No difference was observed in the ability of mature splenic B cells of any genotype to home to the spleen or bone marrow (Figure 6.4 B). Although the inability of Pim $1^{-/-}2^{-/-}$ bone marrow to rescue lethally irradiated recipients is consistent with a homing deficiency in those cells we have found no evidence of a homing defect in mature splenic Pim $1^{-/-}2^{-/-}$ B cells.

**Figure 6.4. Pim 1 and/or 2 deficient mature splenocyte migrate normally. (**A) CFSE labeled splenocytes from Pim $1^{-/-}2^{-/-}$ or control mice were transferred i.v. into B6 mice exposed to 900 rads 24 hours previous. 24 hours after transfer recipients were sacrificed and the number of $CFSE^{+}$ cells in the spleen determined by flow cytometry. For each bar n=5 (B) CFSE labeled splenocytes from Pim $1^{-/-}2^{-/-}$, Pim $1^{-/-}$, Pim $2^{-/-}$ or WT mice were transferred into intact B6 mice, 24 hours later recipients were sacrificed. The number of $CFSE^{+}$ cells in the bone marrow and spleen were determined by flow cytometry. For each bar n=3. No significant difference was found in either experiment.

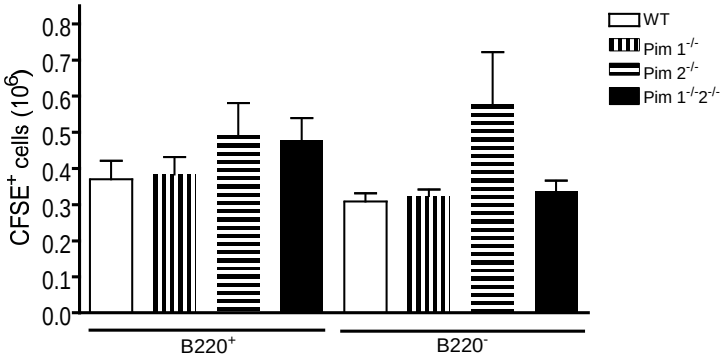
**Pim 1 and/or 2 deficient mature splenocyte migrate normally**

**A.**

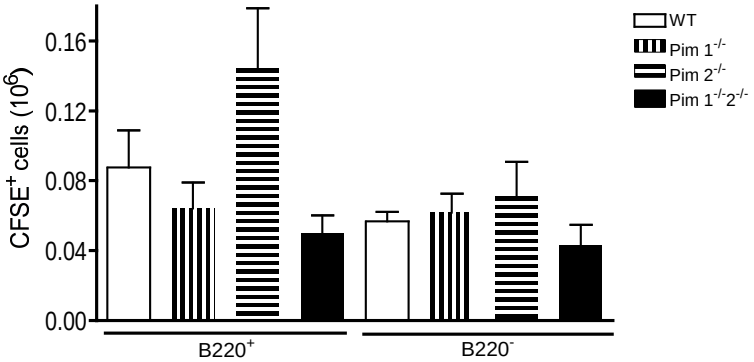



**B.**

Spleen




Bone marrow



**Pim $1^{-/-}2^{-/-}$ fetal liver cells do not reconstitute peripheral populations**

Work on CXCR4 deficient mice had shown that their inability to reconstitute irradiated recipients could be overcome by using high numbers of fetal liver cells, which successfully results in viable chimeras (45). We hypothesized that if Pim $1^{-/-}2^{-/-}$ bone marrow cells were unable to reconstitute lethally irradiated recipients due to a CXCR4 functional defect as in Pim $1^{-/-}$ bone marrow cells described previously (44) then transferring a high number of fetal liver cells would allow us to create WT:Pim $1^{-/-}2^{-/-}$ chimeras.

We transferred $10x10^6$ fetal liver cells collected from control $Ig^a$ $Thy1^a$ or Pim $1^{-/-}2^{-/-}$ $Ig^b$ $Thy1^b$ mice at a 20:80 or 50:50 WT:Pim $1^{-/-}2^{-/-}$ ratio into B6 $Rag^{-/-}$ mice. 12 weeks after cell transfer the percent of peripheral B cells originating from WT or Pim $1^{-/-}2^{-/-}$ fetal liver stem cells were determined by flow cytometry. Our results show the emergence of $IgM^a$ (WT) but not $IgM^b$ (Pim $1^{-/-}2^{-/-}$) B cells in the spleen (Figure 6.4). T cell reconstitution was more variable, Pim $1^{-/-}2^{-/-}$ fetal liver derived (Thy1.2) T cells were the only T cells detected in one chimera while the majority of T cells in the other chimeras originated from WT (Thy1.1) fetal liver (Figure 6.4).

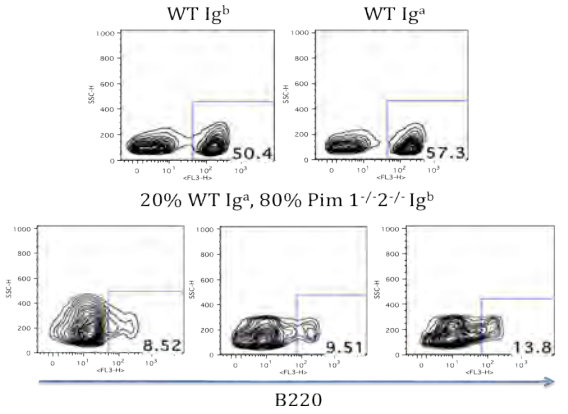
We attempted to create fetal liver chimeras again using only a 80:20 Pim $1^{-/-}2^{-/-}$:WT mixture to favor Pim $1^{-/-}2^{-/-}$ reconstitution, we also allowed more time for reconstitution than in the first experiment and irradiated (600 rads, 2 times) the B6 $Rag^{-/-}$ recipients. Pim $1^{-/-}2^{-/-}$ $Ig^b$ fetal liver cells and (due to the unavailability of $Ig^a$ fetal liver) WT $Ig^a$ bone marrow cells were transferred into lethally irradiated recipients at an 80:20 ratio. Despite the high number of cells

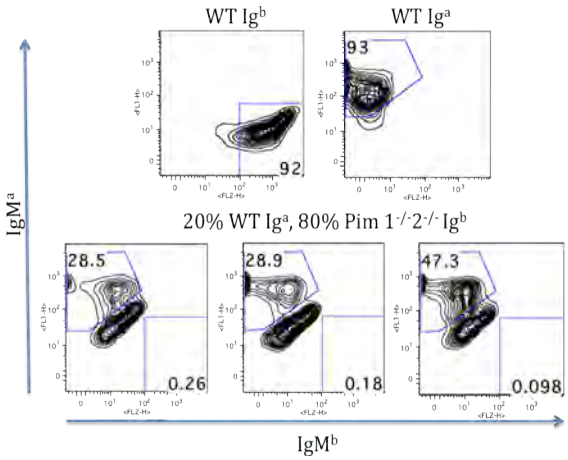
transferred and the WT bone marrow cells included in the mixture these chimeras died within 2 months (Figure 6.5). Furthermore many of these mice demonstrated symptoms we saw in the Pim $1^{-/-}2^{-/-}$ bone marrow chimeras described earlier in this chapter including dehydration, and hunched posture and had to be euthanized as a result. These results lead us to conclude that Pim $1^{-/-}2^{-/-}$ fetal liver and bone marrow cells are unable to reconstitute recipients. Our observations when transferring Pim $1^{-/-}2^{-/-}$ bone marrow into lethally irradiated recipients closely mimicked those published by Grundler and colleagues demonstrating impaired Pim $1^{-/-}$ bone marrow reconstitution due to a CXCR4 defect. However, transferring Pim $1^{-/-}2^{-/-}$ fetal liver cells, even in high numbers, which has been shown to overcome the loss of CXCR4, was insufficient to rescue the defect preventing reconstitution by Pim $1^{-/-}2^{-/-}$ stem cells. This suggests that either a defect in CXCR4 is not responsible for the impairment in Pim $1^{-/-}2^{-/-}$ stem cells or it is only one of multiple defects caused by the loss of both Pim 1 and 2, which prevent reconstitution by stem cells from either bone marrow or fetal liver.

**Figure 6.5. Pim $1^{-/-}2^{-/-}$ fetal liver cells do not reconstitute peripheral B cells.** Control $Ig^a$ and Pim $1^{-/-}2^{-/-}$ $Ig^b$ fetal liver cells collected 14 days into gestation were transferred i.v. into B6 $Rag^{-/-}$ mice at a 20:80 ratio. At 12 weeks post transfer mice were sacrificed and splenic B cell (A) and T cell (B) populations were examined by flow cytometry.

**Pim $1^{-/-}2^{-/-}$ fetal liver cells do not reconstitute peripheral B cells.**

**A.**




**B.**

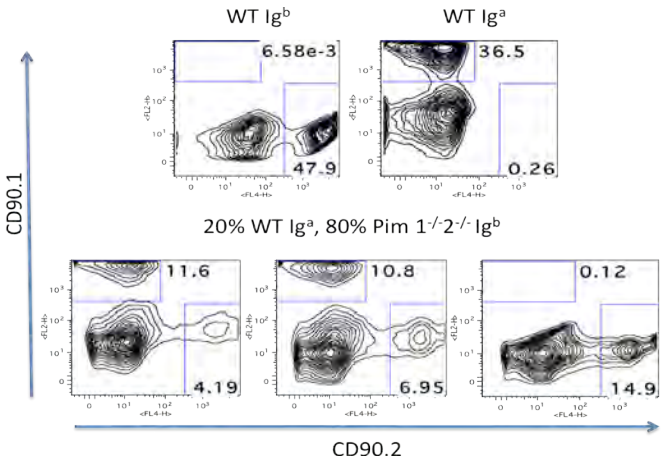
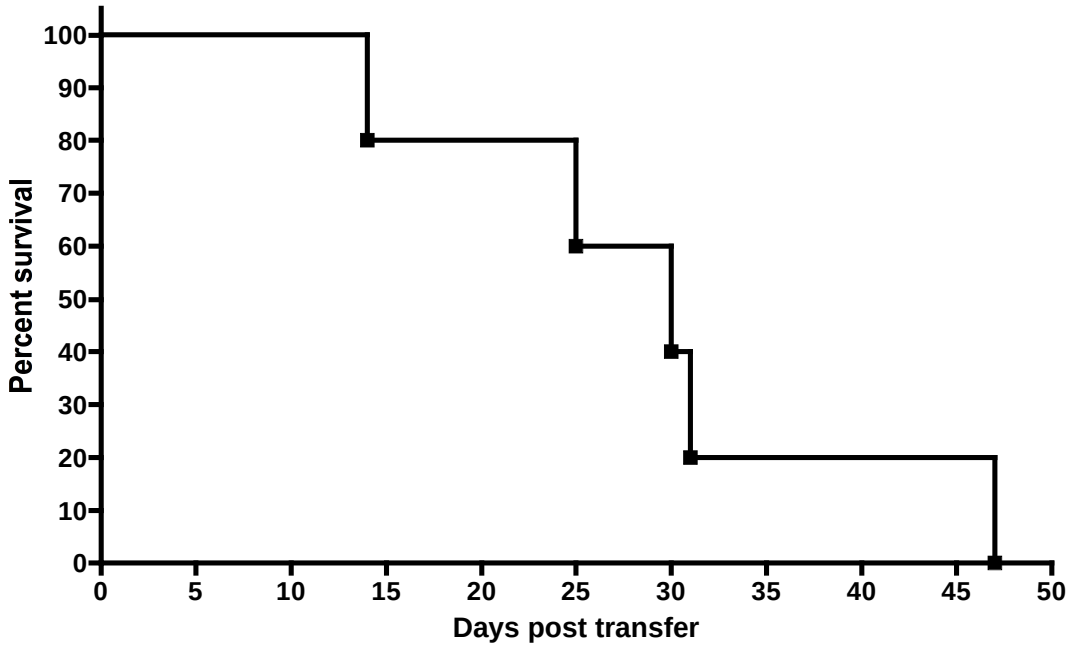


**Figure 6.6. Pim $1^{-/-}2^{-/-}$ fetal liver cells do not reconstitute lethally irradiated recipients.** Pim $1^{-/-}2^{-/-}$ fetal liver cells collected on day 14 of gestation and WT $Ig^{a}$ bone marrow cells at an 80:20 ratio were transferred i.v. into lethally irradiated B6 $rag^{-/-}$ mice. The percent of surviving mice (y-axis) is plotted for day after reconstitution (x-axis) (n=12).

**Pim $1^{-/-}2^{-/-}$ fetal liver cells do not reconstitute lethally irradiated recipients**



# Chapter VII

## Discussion

Despite the many roles of Pim 1, 2, and 3, mice deficient in all three kinases did not show any gross abnormalities (52). This may be due to redundancy in signaling pathways. For example, Pim $1^{-/-}2^{-/-}$ T lymphocytes behave normally in vitro until the Akt pathway is blocked with rapamycin, which reveals a role for Pim 1 and 2 in T cell activation and proliferation (42). Pim $1^{-/-}2^{-/-}$ and $2^{-/-}$ naive B lymphocytes survival normally in culture in the presence of BLyS unless treated with rapamycin. When Akt was inhibited, Pim 2 was defined as the critical kinase for BLyS dependent B cell survival (58). Given the role of Pim 1 and 2 in T cell activation and the survival of naïve B cells, we asked whether B cells required Pim 1 and/or 2 after activation for differentiation to antibody secretion or memory cells. Consistent with a role for Pim 1 and 2 in activated B cells, we demonstrated that Pim 1 and 2 were induced in B cells activated in culture with various mitogens. The immune response of Pim $1^{-/-}2^{-/-}$ mice had not previously been investigated. After immunization with both T cell dependent and independent antigens, we found a significant impairment in antigen specific antibody, germinal center formation, and ASCs in the Pim DKO mice. This is the first time a phenotype has been described for the loss of Pim 1 and 2 when Akt activity is intact. To further pursue the mechanism(s) for this phenotype, we have examined the humoral immunity in naïve and immunized Pim deficient mice. Functions of the humoral immune response, which we have found to rely on Pim

1, Pim 2, or both kinases are summarized in figure 7.1. All our observations in Pim single and double deficient mice are summarized in table 7.1. The evidence presented in this dissertation has led us to a model in which Pim 1 or 2 are critical for the induction of BLIMP-1 interrupting the antibody secreting cell differentiation pathway which effects several facets of humoral immunity (Figure 7.2).

Induction of Pim 1 and 2 was observed after B cell activation by LPS, CpG, anti-delta dextran, anti-CD40, anti-IgM, and the TI-2 antigen TG4. This diverse group of stimuli mimic, as best we can in vitro, activation pathways used by TI-1 (LPS and CpG), TI-2 (TG4, anti-delta dextran, anti-IgM) and TD (anti-IgM, anti-delta-dextran, and anti-CD40) antigens during B cell activation in vivo. These activators bind either TLR4, TLR9, BCR, or CD40 whose downstream signaling includes both overlapping and divergent targets. The fact that these receptors take part in different immune responses - for example TLRs are involved in TI-1 responses while CD40 is involved in TD responses - highlights their differences and separate roles in the B cell. It also raises the possibility that although Pim 1 and 2 are induced by activation of each receptor, it may play a different role in the cell depending on which receptor was activated. This idea is further supported by our observation that the TD and TI responses in Pim 1-/-2-/- mice are not equal. While both responses signal through the BCR, only TD antigens elicit signals through CD40, suggesting that Pim 1 and 2 may play different roles downstream of these 2 receptors. Furthermore, the defects we observed in the single knockout mice after immunization with a TI-2 or TI-1 antigen were not the

same. Although Pim 1-/- mice produced significantly fewer antibodies than WT early after a TI-1 immunization, however, the early stages of the TI-2 response were unperturbed. This would be consistent with divergent roles for Pim 1 downstream of TLRs, involved in TI-1 responses, and the BCR, required for TI-2 responses. The signaling pathways which connect these receptors to Pim 1 and 2 induction remains an area of active investigation but will likely shed some light on our differing observations during in vivo immune responses.

In response to immunization with a TD antigen mice deficient in either Pim 1 and/or 2 have significantly decreased antigen specific antibody, antibody secreting cells, and germinal centers. To determine if this was due to an impaired mature lymphocyte pool we analyzed the splenic B and T cell subpopulations. Since the number of both marginal zone and follicular splenic B cell populations are intact in the fully B6 backcrossed Pim KO mice, the problem with the immune response is more likely intrinsic to the B cells. When we analyzed mature splenic T cell numbers in the C57BL/6 Pim $1^{-/-}2^{-/-}$ mice, a slight decrease in the $CD8^{+}$ but not the CD4+ T cell number was observed. Therefore, a T cell effect on humoral immunity in these mice is less likely. These observations do not explain the significant impairment observed in antibody titer. We also observed that the serum antibody in naïve Pim $1^{-/-}2^{-/-}$ mice was consistently lower than that of naïve WT controls, which suggests that there may be a B cell intrinsic defect in antibody generation due to this genetic deficiency.

When we examined the B1 B cell populations in the peritoneal cavity that are the source of natural antibody, we did find a significant decrease in B1 B cell numbers that may explain the reduced levels of IgM, $IgG_3$, and IgA in naïve Pim $1^{-/-}2^{-/-}$ mice. One possible explanation for this diminished peritoneal B cell population, which includes B1a and B1b, as well as some B2 cells, is that the generation of these cells from a common precursor is impaired by the loss of Pim 1 or 2. Fetal liver preferentially reconstitutes the peritoneal B cell compartment while bone marrow preferentially reconstitutes the splenic B cell compartment. This suggests that these two groups of B cells can originate from separate precursor cells so the loss of Pim may impair the generation of peritoneal B cells but not splenic B cells. Another possibility is that the loss of Pim 1 or 2 disrupts signaling downstream of the BCR and other receptors required for the commitment of B cell precursors to become peritoneal B cells. Mutations in positive regulators of BCR signaling such as btk and CD19 as well as some B cell transcription factors, for example NFATc, have been shown to decrease the number of B1 cells (9). These mutations generally results in the diminution of only one subset of peritoneal B cells but our in vitro experiments demonstrated that Pim 1 and 2 were induced upon stimulation by many receptors suggesting that the loss of either of these kinases could interrupt a number of the signaling pathways thereby affecting multiple peritoneal B cell subpopulations.

B1 B cells in the peritoneal cavity produce natural $IgG_3$ and IgM but migrate to the intestine before producing IgA. IgA is mainly attributed to mucosal immunity; in particular the gut associated lymphoid tissue (GALT) including

Peyer's Patches. When we examined Peyer's Patches from Pim $1^{-/-}2^{-/-}$ mice, we found a significant decrease in the number of B cells from $3.5x10^6 \pm .4$ to $1.3x10^6 \pm .2$ and about half as many $IgA^+$ B cells compared to controls. Interestingly, we also found significantly fewer germinal center B cells by FACS analysis, matching our observation that the generation of germinal centers in the spleen was impaired during a TD response. So during antibody response to both environmental antigens and immunization Pim $1^{-/-}2^{-/-}$ mice have deficient germinal centers.

Germinal centers in the spleen are a hallmark of a TD response. We observed that under most circumstances Pim $1^{-/-}2^{-/-}$ mice produced few if any GCs though this defect could be overcome in some mice with the use of a stronger antigen or adjuvant. Using Imject Alum as an adjuvant, Pim-deficient animals consistently did not produce GC B cell populations as examined by flow cytometry, nor did they have PNA+ GCs in spleen sections examined by immunohistochemistry. A few Pim deficient mice immunized with NP-CGG that had been precipitated in alum produced some GCs although there were non-responders with no GC B cells or PNA+ GCs in the same group. Those mice that did generate GCs produced normal numbers compared to wild type. These "all or nothing" observations have led us to propose a few possibilities to explain our GC data.

One hypothesis is that signaling cascades that include Pim 1 and 2 kinases downstream of the BCR and CD40 lower the threshold of activation in a B cell. The loss of Pim, therefore, would prevent efficient B cell activation unless

there is increased signal strength either downstream of BCR, CD40, or another receptor pathways were able to compensate. This would potentially allow for the normal formation of GCs in some mice where there may be an environmental stimulus in conjunction with the immunization allowing the B cell to reach a threshold of activation despite the loss of Pim 1 and 2. This hypothesis is consistent with our observation that early in a TD response the affinity of antibody from Pim $1^{-/-}2^{-/-}$ mice is significantly higher than WT suggesting that only the high affinity B cells present in the Pim $1^{-/-}2^{-/-}$ pool are being activated because their higher affinity BCR produces a stronger signaling. Our in vitro data demonstrates that Pim $1^{-/-}2^{-/-}$ B cells are capable of normal activation and proliferation, which argues that the in vitro conditions used for those experiments were very strong, unlike an antigen in vivo, which can only activate B cells with BCR specificity to that antigen. Indeed, preliminary in vitro experiments using sub-optimal activator concentrations shows that Pim DKO B cells have delayed proliferation, determined by CFSE dilution, compared to wild type B cells. Further studies to confirm this observation are ongoing.

FACS analysis of splenic B cells from the in vitro cultures also demonstrated that Pim $1^{-/-}2^{-/-}$ B cells increased expression of CD95 normally upon activation. During a TD response, signaling downstream of CD95 results in cell death unless B cells receive appropriate survival signals from the BCR or CD40 receptors. Considering that Pim 1 and 2 are downstream of both of these receptors and that Pim 2 has been shown to play a role in survival of naïve B cells another possibility problem with GC formation is the survival of activated

Pim 1 and 2 KO B cells. The loss of Pim 1 and 2 may render the activated B cell (which is now expressing CD95 on its surface) more susceptible to CD95 killing, preventing the B cells from living long enough to form a GC.

It is also possible that GC formation is impaired due to defective B cell migration. There have been several examples of this in the literature. Recently $S1P_2$ was shown to promote clustering and migration of GC B cells to retain normal size and shape of the GC structure. Although GCs do form in $S1P_2$-deficient mice, deficient GC B cells were found at or outside the GC perimeter more often than were WT B cells (74). In CXCL13-deficient mice, GC can also be found, though they are smaller and less organized than those found in WT mice (45). Combining these defects by transferring $S1P_2$-deficient B cells into CXCL13-deficient mice followed by TD immunization reveals a loss in GC formation (74). Pim $1^{-/-}$ bone marrow cells have impaired migration due to decreased cell surface expression of CXCR4 (44), a receptor, which is also important in the organization of germinal centers (45). Cell transfer experiments revealed that homing of mature splenic Pim DKO B cells was normal (Figure 6.4). These data suggest that GC formation and organization are dependent on multiple signals both intrinsic and extrinsic to the B cell, which may explain why we do not see a complete ablation but a decrease in GCs in Pim $1^{-/-}2^{-/-}$ mice.

So how does GC formation occur in some Pim DKO mice when they receive a stronger antigenic signal? The response to a TD antigen requires the interaction of B and T cells so it is possible that a T cell, and not B cell, defect is responsible for the impaired GC response. To ask if WT T cell help would

alleviate the impaired TD response, we transferred WT T cells specific to OVA into Pim $1^{-/-}2^{-/-}$ or control mice then immunized with NP-OVA. The response in Pim $1^{-/-}2^{-/-}$ mice was not improved by the addition of WT T cell help demonstrating that Pim $1^{-/-}2^{-/-}$ T cells are not the likely cause of the deficiency. This is consistent with our idea that Pim DKO B cells have an intrinsic B cell defect but does not rule out the possibility of defects in both T and B lymphocytes exacerbate the problem in GC formation.

To study the possibility of an intrinsic B cell defect, we determined if B cells could respond normally to antigen when T cell help was not needed. Pim $1^{-/-}2^{-/-}$ mice immunized with TI-1 or TI-2 antigens had impaired antibody responses. Pim 1 and 2 seem to play diverging roles in TI responses as the antibody responses of the single knockouts were different from WT, Pim $1^{-/-}2^{-/-}$, and each other. All these antigens were delivered i.p., therefore the responses are influenced by peritoneal B cells in TI-2 responses (70). To circumvent the peritoneum and determine if the TI-2 impairment was due to the decreased peritoneal cavity B cell numbers, we immunized Pim deficient mice with NP-Ficoll i.v. which is delivered directly to the splenic marginal zone B cells. The response of Pim single knockout mice was the same as wild type under these conditions; however, the TI-2 response in Pim $1^{-/-}2^{-/-}$ mice was still decreased. The observation that antigen delivered to the marginal zone B cell population which we know to be numerically intact did not induce an immune response again supported our hypothesis of an intrinsic B cell defect.

A B cell's response to stimulation can be tested directly in vitro. WT and Pim $1^{-/-}2^{-/-}$ B cells were isolated and cultured with various mitogens to examine activation. Induction of activation markers, proliferation, and cell survival were normal in activated Pim $1^{-/-}2^{-/-}$ B cells, however, Pim $1^{-/-}2^{-/-}$ cultures produced significantly fewer ASCs. In the future we would like to examine these parameters in vivo. Using NP-APC to detect NP-specific B cells by flow cytometry in naïve vs. Pim $1^{-/-}2^{-/-}$ mice, we can ask whether the number of precursors has been altered. NP-APC will also allow us to detect NP-APC specific B cells during a TD response to NP-CGG and track their proliferation by administering BrdU. If WT and Pim $1^{-/-}2^{-/-}$ NP specific B cells are dividing equally from a comparable number of precursors than the number of $BrdU^{+}$ cells will be equal. Activation markers can also be measured by flow cytometry on this same population. By performing these experiments on BLIMP-$1^{gfp/+}$ mice we can also measure the number of NP specific BLIMP-$1^{+}$ B cells, which have committed to the plasma cell lineage by flow cytometry. Alternatively, ASCs can be measured ex-vivo by ELISpot. Survival can be measured within a NP specific B cell population at each stage using P.I. or one of many cell survival dyes made for flow cytometry. If consistent with our in vitro results we would expect to see no difference in the proliferation, induction of activation markers, or survival concurrent with a drop in $GFP^{+}$ Pim $1^{-/-}2^{-/-}$ B cells by facs and of Pim $1^{-/-}2^{-/-}$ ASCs by ELISpot.

Having observed that Pim $1^{-/-}2^{-/-}$ B cells were able to recognize antigen and proliferate but that antibody and ASC numbers were decreased in response

to TI antigens and in vitro activation, we hypothesized that the B cells were unable to differentiate to the antibody secreting cell lineage. Impairment in the ability of B cells to differentiate into ASCs would impact any humoral response in naïve and immunized mice. WT and Pim $1^{-/-}2^{-/-}$ B cells were cultured with LPS and the number of ASCs was measured by ELISpot. A clear deficiency was observed in Pim $1^{-/-}2^{-/-}$ but not single knock out B cells, matching our previous observation using i.v. immunization.

To try to identify which step in ASC differentiation Pim $1^{-/-}2^{-/-}$ B cells were blocked, we took advantage of a BLIMP-$1^{gfp/+}$ mouse. Pim $1^{-/-}2^{-/-}$ BLIMP-$1^{gfp/+}$ and control B cells were cultured in vitro with LPS and induction of 2 key transcription factors, IRF-4 and BLIMP-1, were measured by flow cytometry. These experiments revealed that Pim $1^{-/-}2^{-/-}$ B cells were able to express normal levels of IRF4 but not BLIMP-1, which is the master regulator of ASC generation. A recent publication identified Pim 2 as a target of IRF4 in plasma cells (71); our data suggests that Pim 1 is able to compensate for this loss as single knock out mice generated normal ASC numbers in vitro. Taken together these data reveal a model where Pim 1 or 2 is required downstream of IRF4 for the induction of BLIMP-1 (Figure 7.2). BLIMP-1 is critical for the complete differentiation to the plasma cell lineage due to its ability to induce genes necessary for plasma cell function and extinguish genes associated with mature B cells (18). This block in the generation of ASCs inhibits the B cell response to TI and TD antigens as well as the production of natural antibody as BLIMP-1 is required for antibody production by B1 cells as well as B2 cells (43). Our current model depicting how

Pim 1 and 2 are involved in BLIMP-1 induction is depicted in figure 7.2. The model also includes several other players, which must be considered when thinking about ASC generation. MicroRNA-155 (75) for example has been shown to down regulate PU.1 a transcription factor that positively regulates BLIMP-1 (76). No direct connection of miRNA155 to Pim kinases has been shown to date, however, we are currently testing RNA isolated from WT and Pim $1^{-/-}2^{-/-}$ ASCs to determine if regulation of either of these factors is disrupted with the loss of Pim 1 and 2. Deregulation of c-myc impairs ASC generation (77), under normal conditions c-myc is repressed by BLIMP-1 (6). Bcl-6 and Pax-5 are also known to be repressed by BLIMP-1 (6) and would therefore be expected to be higher in Pim $1^{-/-}2^{-/-}$ B cells unable to induce BLIMP-1 though this has not yet been tested.

The inability of Pim $1^{-/-}2^{-/-}$ B cells to differentiate into ASCs does not explain why Pim $1^{-/-}2^{-/-}$ B cells do not isotype switch efficiently despite normal levels of sterile transcripts and AID mRNA levels. A number of AID co-factors have been implicated in CSR though biochemical evidence is scarce (65). Indeed the aberration of any one of these components due to the loss of Pim 1 and 2 may be responsible for the decrease in isotype switching we have observed. However, because AID is unquestionably required for both isotype switching and affinity maturation, which is also impaired in Pim $1^{-/-}2^{-/-}$ mice, we have focused our attention there. Even focusing on AID alone there are several steps, which could be impacted. Although AID expression in Pim $1^{-/-}2^{-/-}$ B cells, which fail to switch, appears at WT levels we have yet to determine where it is localized. AID is transported to the nucleus after BCR stimulation (which also induces Pim 1

and 2) but the mechanisms regulating this transport are not fully elucidated and so could involve Pim 1 and/or 2 (65). In addition to transcriptional and localization control, post-translational control is critical to AID regulation. Phosphorylation at serine and threonine residues regulates AID activity and mutations at these residues have been shown to interfere with isotype switching and somatic hypermutation, critical to affinity maturation (66). Given the requirement for serine/threonine phosphorylation of AID for normal isotype switching and somatic hypermutation and the established role for Pim 1 and 2 as serine/threonine kinases we hypothesized that Pim 1 or 2 kinases modify AID protein thereby affecting the level of isotype switching and affinity maturation. We are currently pursuing this idea. In this scenario it seems likely that the same mechanism is responsible for the loss of isotype switching and affinity maturation we observed in the Pim $1^{-/-}2^{-/-}$ TD response. However, unlike our observations on isotype switching, our observation of impaired affinity maturation was only in vivo and cannot be definitively linked to a B cell intrinsic defect. Alternatively affinity maturation during the TD response to NP may have been absent because of the impaired germinal center response, where affinity maturation generally takes place. However it has been demonstrated that affinity maturation can occur normally without germinal centers (78), suggesting that like isotype switching the defect in affinity maturation is intrinsic to the Pim $1^{-/-}2^{-/-}$ B cell.

This work is the first demonstration of impaired function due to the loss of Pim 1 and/or 2 when Akt is intact. We have also elucidated a previously unknown and critical role for Pim kinases in humoral immunity. The novel discoveries in

this dissertation expands our basic understanding of B cell function and impacts both the investigation of immunodeficiencies in humans as well as the field of cancer research where Pim 1 and 2 have been suggested to be good drug targets in cancer therapy.

**Figure 7.1. Humoral immune functions that rely on Pim 1 alone, Pim 2 alone, or either.** This ven diagram depicts functions of the humoral immune system, which we have determined to rely on Pim 1 (left), Pim 2 (right), or to be intact when either Pim 1 or Pim 2 (center) is present.

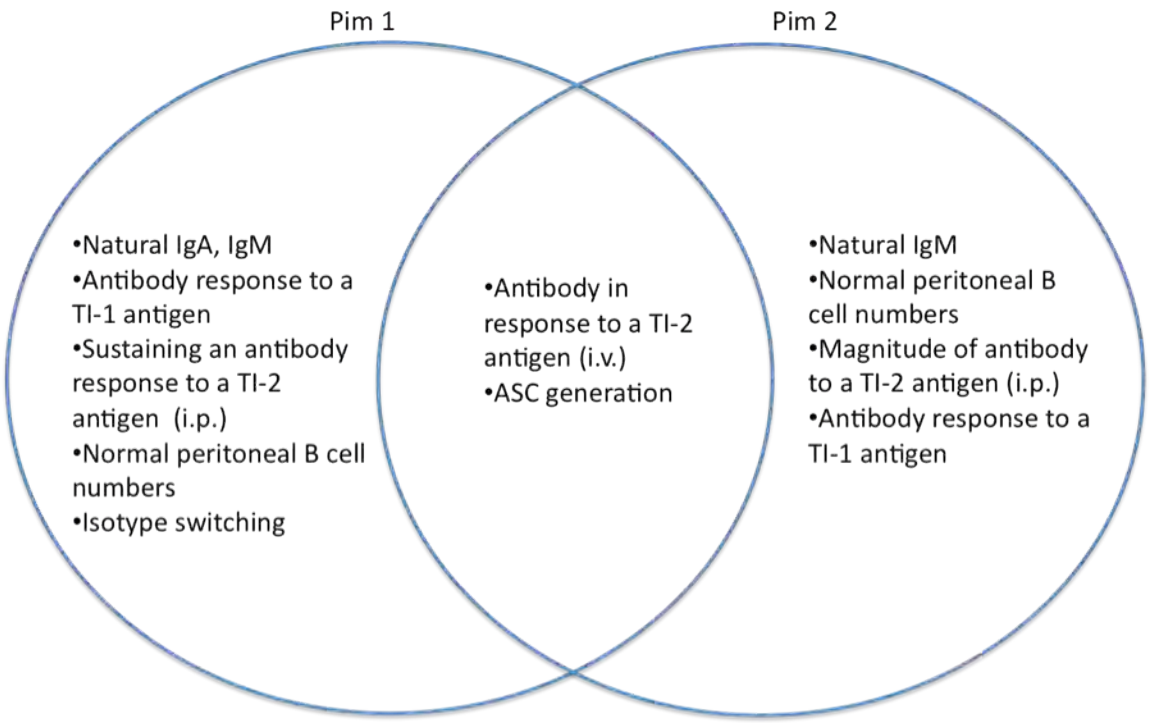


**Figure 7.2. Current model for Pim 1 and 2 involvement in BLIMP-1 induction.** Activation signals increase IRF4 expression early in ASC commitment, which we know to be intact in Pim $1^{-/-}2^{-/-}$ B cells. Pim 2 has been demonstrated to be a target of IRF4 in antibody secreting cells while our data indicate that Pim 1 is a suitable substitute. Pim 1 and 2 are required for normal BLIMP-1 induction though the molecular mechanism behind this remains unclear. Other components implicated in ASC differentiation are depicted as described in the literature. Pim 1 and 2 may also regulate MicroRNA-155. MircroRNA-155 represses PU.1, which binds directly to BLIMP-1 to increase its expression. BLIMP-1 represses c-myc, Pax5, and Bcl-6 while inducing XBP-1 as part of the transition from GC to antibody secreting cell. XBP-1 induction relies on both BLIMP-1 and IRF4 increased expression. In this model we propose that Pim 1 and/or 2 are required for BLIMP-1 induction as a direct target of IRF-4 and/or for the repression of miRNA155. Red lines indicate that a direct relationship has been demonstrated; dashed line was determined by our data.

**Current model for Pim 1 and 2 involvement in BLIMP-1 induction**

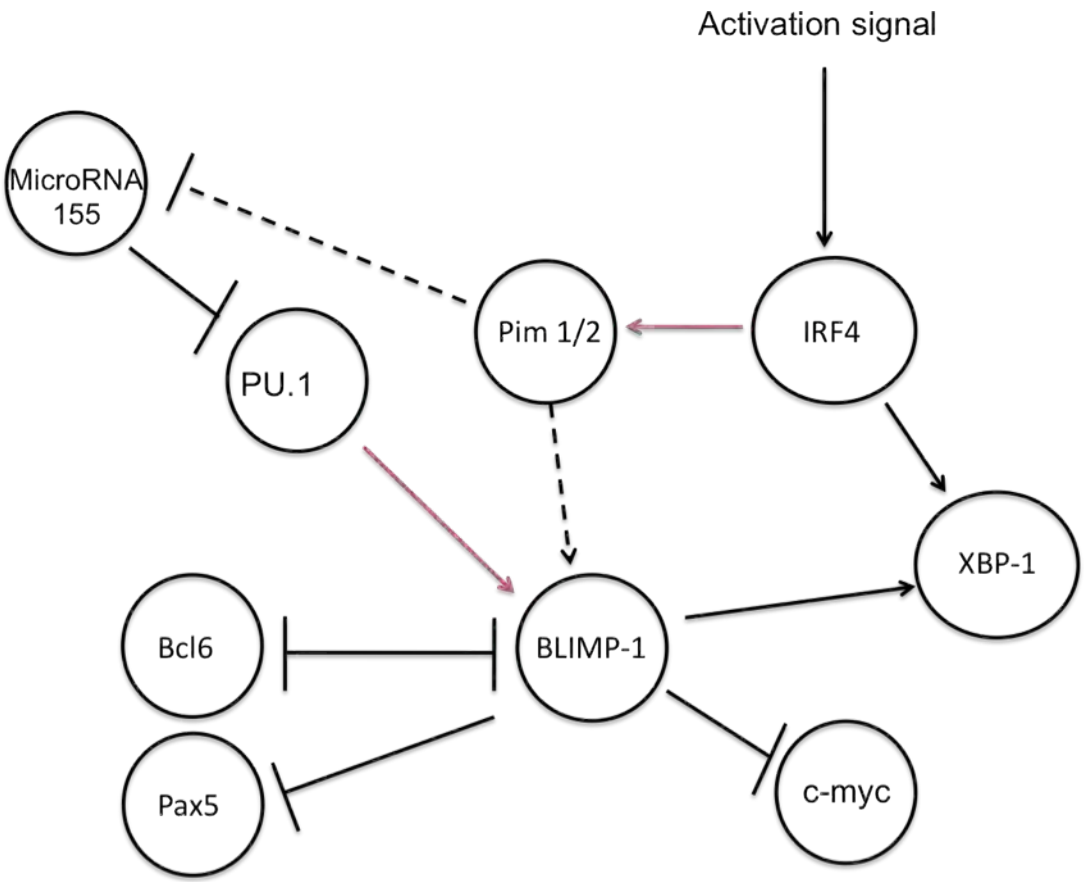


**Table 7.1 A summary of our observations in Pim single and double knock out mice.** Percent is of the mean relative to WT, no difference is indicated as “100%”, Not done=ND.

**A summary of our observations in Pim single and double knock out mice**

| | Pim $1^{-/-}$ | Pim $2^{-/-}$ | Pim $1^{-/-}2^{-/-}$ |
|---|---|---|---|
| Splenic B cells | 100% | 100% | 100% |
| Splenic T cells | 100% | 100% | 60% |
| Peritoneal B2 B cells | 20% | 35% | 20% |
| Peritoneal B1a B cells | 37% | 45% | 20% |
| Peritoneal B1b B cells | 30% | 50% | 25% |
| “Natural” IgM | 8% | 6% | 3% |
| “Natural” $IgG_3$ | ND | ND | 16% |
| “Natural” $IgG_1$ | 100% | 300% | 100% |
| “Natural” IgA | 25% | 100% | 45% |
| “Natural” IgE | 100% | 100% | 100% |
| GC B cells in Peyer’s Patches | 13% | 47% | 40% |

| | | | |
|---|---|---|---|
| $IgA^+$ B cells in Peyer's Patches | 2% | 100% | 23% |
| $IgM^+$ B cells in Peyer's Patches | 11% | 1% | 25% |
| TI-2 antibody | Shortened response (i.p.)<br>100% (i.v.) | 60% (i.p.)<br>100% (i.v.) | 10% i.p.<br>20% i.v. |
| TI-2 ASCs | SLPCs-ND<br>LLPCs-60% | SLPC-100%<br>LLPCs-ND | SLPCs-25%<br>LLPCs-30% |
| TI-1 antibody (Day 5) | 30% | 13% | 10% |
| Early Activation markers | ND | ND | 100% |
| Proliferation | 100% | 100% | 100% |
| In vitro ASC generation | 100% | 100% | 40% |
| IRF4 induction | ND | ND | 100% |
| BLIMP-1 induction | ND | ND | 20% |
| In vitro survival | 100% | 100% | 100% |
| Bone marrow or fetal liver Reconstitution | ND | ND | No |
| Homing of mature splenocytes | 100% | 100% | 100% |
| Germinal Center | ND | 100% | 30% |

| | | | |
|---|---|---|---|
| formation | | | |
| IgM response to a TD antigen | 100% | 20% | 20% |
| λ response to a TD antigen | 30% | 30% | 20% |
| IgG1 antibody to a TD antigen | 25% | 10% | 10% |
| ASC numbers in response to a TD antigen | ND | SLPCs- 50% | SLPCs- 30%<br>LLPCs- 8% |
| Isotype switching | 45% | 80% | 35% |
| Sterile transcripts | 100% | ND | 100% |
| AID mRNA | 100% | ND | 100% |
| Affinity (> 6 months) | ND | ND | 58% |
| Response to secondary TD challenge | ND | ND | 5% |

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