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Reciprocal regulation of B cell antigen receptor signaling by CD148 and Csk

by

Katarzyna Maria Skrzypczynska

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION
To my parents,
Barbara Skrzypczynska and Zbigniew Skrzypczynski,
for their love and support.

With special thanks to my undergraduate mentor, Ned Ruby.
ACKNOWLEDGEMENTS

Throughout my graduate career, on days with exciting data and on hard days alike, I’ve frequently thought how absolutely fortunate I am to have found myself in Art’s lab. Though it certainly hasn’t been an easy road, I know that I could not have asked for a better graduate experience. Art has taught me so much by his example: to push myself to meet his high and fair standards, to question my data and to think critically, to do quality work, and to do the right thing even if it’s the hard thing.

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**Contributions to the presented work**

Chapter 2 of this work is taken directly from a manuscript submitted for publication entitled ‘The receptor-like protein tyrosine phosphatase CD148 selectively regulates B1 B cell antigen receptor signaling.’ The co-author on this manuscript will be Jing Zhu, who generated mouse lines utilized in this study and who identified the phenotype that would become the basis for the described studies. The appendix describes my preliminary studies using a novel mouse line generated by Ying Xim Tan. Arthur Weiss supervised both studies.
Reciprocal regulation of B cell antigen receptor signaling by CD148 and Csk

by

Katarzyna Maria Skrzypczynska

Abstract

B cell antigen receptor (BCR) signaling is tightly regulated by the opposing actions of receptor-like protein tyrosine phosphatases (RPTPs) and C-terminal Src kinase (Csk). The RPTPs CD45 and CD148 positively regulate BCR signaling through their actions on Src-family kinases (SFKs) and have redundant functions in conventional (B2) B cell signaling and activation. However, we found that IgM production in response to T cell-independent type 2 antigens, which is mediated in large part by B1 B cells, was deficient in mice lacking CD148. B1 B cells, which normally express high levels of CD148 compared with B2 B cells, were uniquely affected by the loss of CD148, resulting in impaired proliferation, antibody production, BCR repertoire development, and BCR signaling. Analysis of proximal BCR signaling in mice deficient in CD148 or the SFK Lyn revealed that Lyn has a predominantly positive regulatory role in B1 B cell BCR signaling, compared with its dominant negative regulatory role observed in B2 B cells. This suggests that BCR signaling circuitry is wired differently in B1 B cells resulting in a requirement for positive regulation of Lyn by CD148 for full BCR signaling.

Using novel tools recently generated in the lab, we also investigated the effects of selective chemical inhibition of Csk in primary mouse follicular B cells. This preliminary work reveals mechanisms for the negative regulation of BCR signaling by Csk independent of BCR engagement.
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CHAPTER 1: INTRODUCTION
Overview of B cell development and function

B cells are a phenotypically and functionally diverse population of hematopoietic cells that contribute to the humoral component of the adaptive immune response against infection. The main functions of B cells are performed through antigen presentation to T cells, secretion of cytokines, and critically, through the production of immunoglobulin (Ig). Involved in the generation of protective antibodies are, among others, three important functional subsets: follicular and marginal zone (MZ) B cells, which belong to the B2 lineage, and B1 B cells. These subsets arise through distinct developmental pathways and are specialized to respond to unique types of antigens by the secretion of antibodies. Antibody production is a hallmark of B lymphocytes and is the functional end point of this lineage (LeBien and Tedder, 2008). When secreted, Ig is in the form of antibodies, which serve to neutralize antigens and promote their clearance via complement-mediated opsonization depending on isotype and tissue localization (Xu et al., 2012). When Ig is found anchored in the cell membrane and in association with Igα and Igβ subunits that contain cytoplasmic signal transduction domains, it comprises the antigen-recognition domain of the B cell receptor (BCR) and dictates B cell response to ligand. The structure and mechanism of signaling by the BCR will be discussed in greater detail in the following section.

Immunoglobulins are made up of two heavy (IgH) chains, which define the IgM, IgD, IgG, IgA, and IgE isotypes, and two light (IgL) chains, consisting of either a κ or a λ light chain (Schroeder and Cavacini, 2010; Xu et al., 2012). Determination of antigen specificity and recognition of the ligand occurs at the juxtaposition of the
complementarity-determining region (CDR) of IgH and IgL chains (Martensson et al., 2010). The IgH and IgL chains each contain a variable region and a constant region. Separate multigene families encode these Ig regions: variable (V), diversity (D), and joining (J) segments. The diversity of antibody specificities produced by B cells is achieved through the error-prone process of somatic recombination, also known as V(D)J recombination. This process is executed by enzymes encoded by recombination activating genes (RAG1/2). During V(D)J recombination, individual gene segments for the variable, diversity and joining regions are rearranged and spliced to constant regions to generate complete genes that encode IgH and IgL proteins (Schroeder and Cavacini, 2010). Such gene rearrangement results in the encoding of an infinitely diverse repertoire of secreted or membrane-bound Igs that are capable of recognizing a wide array of foreign and self-antigens. The protective function of B cells is mediated through their ability to recognize a broad array of unique antigens of pathogens via the BCR and to secrete antibodies specific to those antigens.

The development of follicular and MZ B cells begins in the bone marrow from a common lymphoid progenitor (CLP) population and continues in secondary lymphoid tissues such as the lymph nodes and spleen. B lineage commitment and progression through the pro-B, pre-B, new-B, and mature B cell stages is identified by the sequential expression and downregulation of distinct gene products including B220 (CD45R isoform), CD43, HSA, BP-2, c-kit, CD19, and the expression of components of the BCR including Igα and Igβ, mu (IgM) heavy chain and kappa
and lambda light chains, as well as RAG1 and RAG2, whose enzymatic activity is responsible for BCR diversity (Hardy and Hayakawa, 2001).

Antigen independent tonic signaling through the pre-BCR serves as a developmental checkpoint ensuring that the B cell expresses properly rearranged and functional heavy and surrogate light chains. Such signaling induces transcriptional changes that result in pre-B cell proliferation and the expression of new light chains that contribute to BCR diversity (Geier and Schlissel, 2006). Expression of a functional BCR allows for B cells to progress through further developmental stages during which appropriate BCR specificities are selected (Martensson et al., 2010).

Generation of BCR diversity inevitably results in the production of some B cells that are autoreactive. In humans, it is estimated that nearly 85% of early immature B cells are self-reactive but that about one third of these self-reactive B cells are deleted from the repertoire through mechanisms of central tolerance: receptor editing and negative selection (Cambier et al., 2007; Cancro, 2009). Receptor editing is a process by which secondary rearrangements, typically of the light chains, occur in order to alter the antigenic specificity of the BCR in an effort to avoid autoreactivity. Failing this, the BCR repertoire can be further culled of strongly autoreactive B cells by apoptosis through a negative selection process by which high-avidity interaction of the BCR with ligand results in developmental arrest and apoptosis (Pelanda and Torres, 2012; Sindhava and Bondada, 2012). Most B cells successfully complete these developmental checkpoints and leave the bone marrow to continue through the T1 and T2 transitional B cell stages in the
periphery. Self-reactive B cells that escape these processes can be further silenced through the induction of anergy, which is an unresponsive state resulting from chronic antigen engagement by low affinity/avidity ligands in the absence of activation of signaling pathways required for B cell activation (Cambier et al., 2007). Signal strength emanating from the BCR dictates the outcomes of the above described process and continues to be important in determining follicular versus marginal zone (MZ) B cell fates through integration with other survival signals such as BAFF and Notch signaling (Pillai and Cariappa, 2009). BAFF signaling is required for B cell transition from the T2 stage, and for B cell survival by the induction of the anti-apoptotic factors such as Bcl-2 or Bcl-xL (Sindhava and Bondada, 2012).

Upon encounter with a foreign antigen, the antibody responses of mature B cells are classified according to the type of antigen that elicits them, and whether or not T cell help is required for their generation. Follicular B cells are involved in the T cell-dependent (TD) antibody response against protein antigens. The TD response is so called because of the requirement for T cell-derived signals in the generation of high-affinity antibodies to antigens and the subsequent development of memory B cells (Vinuesa et al., 2005). This requirement for presentation of antigen to T cells via MHC-II means that TD responses produce class-switched antibodies that predominantly recognize protein antigens. The TD antibody response is initiated when a naïve, recirculating follicular B cell encounters a protein antigen and is induced to express chemokine receptors which guide it to the interface between the outer edge of T cells zone and the B cell follicle in secondary lymphoid organs (Cyster et al., 2000; Reif et al., 2002). In this location, T cells, B cells, follicular
dendritic cells, and other lymphocytes are in direct contact with one another (Vinuesa et al., 2005). Here, B cells present the peptide antigen via major histocompatibility complex (MHC) to cognate T cells through the formation of dynamic conjugates (Okada et al., 2005). In turn, B cells receive costimulatory signals from T follicular helper (Tfh) cells in the form of CD40 ligation and the release of cytokines (Tobon et al., 2013), and integrin-mediated signals from follicular dendritic cells (Wang et al., 2014). These signals shape B cell differentiation into either short-lived plasma cells or germinal center B cells. Short-lived plasma cells typically produce low affinity antibody, whereas the germinal center response results in the generation of long-lived plasma cells that are capable of producing antibody with high specificity and affinity for the relevant antigen (MacLennan et al., 2003; Vinuesa et al., 2005). Through iterative cycles of B cell clonal expansion, selection, deletion, somatic hypermutation, and class-switch recombination, the specificity and isotype of the BCR and secreted antibodies are fine-tuned (Tobon et al., 2013).

At the expense of such exquisite specificity and affinity is speed, necessitating a more rapid, albeit less precise, antibody response that bridges the gap between innate and adaptive immunity. MZ B cells and B1 B cells contribute to this antibody response in a T cell-independent (TI) manner upon recognition of nonprotein antigens that can be divided into two categories. TI type 1 (TI-1) responses arise from B cell encounter with antigens such as lipopetides, lipopolysaccharide (LPS), and various microbial nucleic acids, which provide additional costimulation via TLR engagement. TI type 2 (TI-2) antigens such as multivalent bacterial capsular...
polysaccharides extensively crosslink the BCR without engaging TLRs (Vinuesa and Chang, 2013). MZ B cells are localized to the perimeter of the splenic white pulp, in an ideal position to quickly produce antibody against blood-borne antigens (Cerutti et al., 2013; Tobon et al., 2013). Like MZ B cells, B1 B cells rapidly produce IgM and IgG3 in response to TI antigens, but their ontogeny and regulation are distinct from B2 B cells.

The distinguishing characteristics of B1 B cells begin with their phenotype and anatomical localization. B1 B cells can be identified by their large size relative to B2 B cells and by their expression of high levels of IgM and CD43, and low levels of IgD, B220, and CD23. Peritoneal B1 B cells can be further subdivided by their expression of CD5: CD5+ B1 B cells are referred to as B1a B cells, and B1b B cells are CD5− (Berland and Wortis, 2002). Functional distinctions between the B1a and B1b subsets will be addressed later. B1 B cells are predominantly found in the peritoneal and pleural cavities and in the omentum from which they relocate to the spleen or draining lymph nodes where they produce antibody after encountering local antigen (Ansel et al., 2002; Choi and Baumgarth, 2008; Ha et al., 2006; Rauch et al., 2012; Weber et al., 2014). B1 cells also exist at very small frequencies in the spleen but are not typically found in peripheral lymph nodes at steady state (Berland and Wortis, 2002).

Compared with the well-described development of B2 B cells in the bone marrow, the origins of B1 cells are less clear. Some studies point to the fetal liver as a source of B1 progenitors (Hardy and Hayakawa, 1991; Tung et al., 2006). In these studies, transfer of fetal liver cells was able to reconstitute both the B1 and B2
compartments in irradiated mice readily, but B1 B cell reconstitution was limited in mice receiving only adult bone marrow (Hayakawa et al., 1986; Herzenberg et al., 1986; Huang et al., 1996). Additional work identified B1 precursors in the fetal omentum and the paraaortic splanchnopleura (Berland and Wortis, 2002). Transfers of B1 cells into the peritoneal cavity of neonatal B cell-depleted mice demonstrated the self-renewal capacity of donor B1 B cells and was associated with very little influx of host-derived B1 cells, suggesting that self-renewal is a mechanism by which the B1 B cell population is maintained throughout adulthood (Lalor et al., 1989). Yet, other studies suggest that B1 and B2 B cells have a common progenitor, and that encounter with antigen and consequent BCR signals determine whether B1 cells adopt the B1 or B2 phenotype (Lam and Rajewsky, 1999; Montecino-Rodriguez et al., 2006). Regardless of the origin, B1 B cell development appears to be sensitive to BCR signal strength and does not appear to require the B cell survival factor BAFF (Montecino-Rodriguez and Dorshkind, 2012).

Studies of transgenic and gene targeted mice have revealed that mutations resulting in impaired BCR signaling lead to a substantial loss of B1 cells, but B2 development remains largely intact. Consistent with this, an expanded B1 compartment develops in mice carrying transgenes or genetic lesions that augment BCR signaling (Berland and Wortis, 2002). It also appears that BCR specificity toward certain types of antigens, specifically those that elicit TI-2 responses, increases the likelihood that a B cell will acquire a B1 phenotype. Several groups have shown that forced expression of a B1-derived transgenic BCR results in an increased presence of B1 B cells, and that this commitment was sensitive to
disruption of BCR signaling (Arnold et al., 1994; Hayakawa et al., 1999; Pennell et al., 1989).

B1 B cells express germline-encoded BCRs specificities with limited heavy chain diversity and show little evidence for somatic hypermutation during development and fewer non-templated nucleotide insertions in their immunoglobulin genes (Baumgarth, 2011; Tornberg and Holmberg, 1995; Weill and Reynaud, 2005). This results in a B1 BCR repertoire that is of low diversity and is weakly polyreactive to a variety of multivalent antigens (Duan and Morel, 2006). Interestingly, B1 BCR specificities are frequently against self-antigens (Baumgarth, 2011). Thus, rather than being subject to negative selection like B2 B cells, the positive selection of autoreactive B1 cells may in fact contribute to the protective properties of B1 B cells (Baumgarth, 2011). Common B1 BCR specificities include those against oxidized lipids, antigens produced by cells undergoing apoptosis, and phosphatidylcholine (PtC) and phosphorylcholine (PC), and some of these antibodies are cross-reactive with bacterial or viral-derived antigens (Baumgarth, 2011; Briles et al., 1982). Thus, such autoreactive antibodies may be beneficial in maintaining tissue homeostasis by promoting clearance of apoptotic debris (Baumgarth, 2011).

In addition to mounting an antibody response to TI-1 and TI-2 antigens, B1 B cells are also involved in the spontaneous production of protective “natural” antibody in the steady state. While MZ B cells and follicular B cells do contribute to the pool of natural IgM, B1 B cells are thought to be responsible for at least 80% of natural antibody in the serum (Baumgarth, 2011; Cerutti et al., 2013). Studies by
Haas and others suggest that B1a, but not B1b, B cells secrete natural antibodies which function to lower bacterial burden and protect the host during nascent infection by *Streptococcus pneumoniae* and do not mount an antigen-specific response (Alugupalli and Gerstein, 2005; Haas et al., 2005). It is therefore thought that the primary role of B1b cells is not to produce natural antibody, but rather to produce antibody in an antigen-dependent manner and to subsequently produce TI memory B cells (Alugupalli and Gerstein, 2005; Alugupalli et al., 2003). However, recent studies have identified a novel population of B1a-derived response activator (IRA) B cells that require GM-CSF in an autocrine manner and are induced upon stimulation with LPS or *S. pneumoniae* infection (Rauch et al., 2012; Weber et al., 2014). Thus, the nature of the B1a and B1b B cell responses appears to be complex and dependent on antigen and other environmental factors.

B1 BCR signaling also appears to be unique. Although B1 B cells have been found to be to be critically dependent on BCR signaling for development, several groups have reported that B1 B cells are relatively unresponsive to BCR crosslinking compared with B2 B cells. In these studies, BCR engagement resulted in modest intracellular calcium mobilization, a lack of proliferation, and the induction of apoptosis (Bikah et al., 1996; Morris and Rothstein, 1993; Murakami et al., 1992; Sen et al., 1999). Expression of the negative regulator CD5 on B1a B cells appears to contribute to B1 cell unresponsiveness and may limit the expansion of autoreactive B1 B cells. In B1a B cells, CD5 has been reported to be constitutively associated with membrane IgM and to negatively regulate BCR signaling (Sindhava and Bondada, 2012). Thus, these incongruous observations regarding the role of BCR signaling
during B1 B cell activation indicate that more work remains to be done to obtain a clearer understanding of the mechanisms regulating B1 BCR signaling, and how they might differ from B2 B cells.

**Overview of BCR signaling**

Signaling through the B cell antigen receptor is integral to B cell development and maturation, maintenance, and antigen responses. Aberrations in BCR signaling may lead to insufficiencies in antibody production during infection or may lead to the development of autoimmune disease (Yu et al., 2003). Therefore, tight regulation of BCR signaling upon encounter with diverse self- and foreign antigens is essential. BCR signaling in response to endogenous ligands during development shapes the functional responsiveness of B cells by influencing the selection of the B cell repertoire and by affecting the expression of BCR signaling machinery in mature B cells (Zikherman et al., 2012b). During foreign antigen encounter by mature B cells, the strength of signal derived from the BCR further determines the ability of B cells to become activated and terminally differentiated. While the precise composition of the pre-BCR and BCR is specific to the developmental and activation state of the B cell, there are many commonalities in the BCR signal transduction machinery used at all stages.

Distinct antigen-recognition and signal transduction domains make up the complex hetero-oligomeric structure of the BCR. In immature B cells, the precursor to the BCR (pre-BCR) consists of a functionally rearranged transmembrane immunoglobulin heavy chain (IgH) of the IgM isotype that is paired with surrogate
light chains. Surrogate light chains are made up of non-polymorphic λ5 and VpreB chains that are replaced as pre-B cells progress through development. Pre-BCR clustering is thought to occur by autoaggregation via λ5 and is independent of ligand engagement (Monroe, 2006). After transitioning from the pre-B cell stage, immature B cells express a BCR that contains an immunoglobulin light chain (IgL) that is covalently paired with the IgM heavy chain, while later in splenic B cell development the IgL can pair with IgM or IgD heavy chains (Monroe, 2006). In contrast with the pre-BCR, clustering of the immature or mature BCR is dependent on antigen ligation. Both ligand-independent autoaggregation and clustering upon antigen recognition result in the spatial reconfiguration of BCRs on the cell surface and subsequent initiation of signaling by mechanisms that will be discussed in greater detail in a later section.

In all cases of BCR signaling, signal transduction is mediated by an Igα-Igβ (CD79α and CD79β) heterodimer, which is non-covalently associated with the transmembrane segment of IgH. Each subunit of the Igα-Igβ heterodimer contains a single immunoreceptor tyrosine-based activation (ITAM) motif (defined by the amino acid sequence YxxI/L-X(6-8)-YxxI/L), which is required for signal transduction by the BCR (Rickert, 2013). Although the Igα and Igβ subunits share significant homology, they are not entirely redundant. Expression of either Igα or Igβ homodimers is sufficient for pre-B cell transition and allelic exclusion, but truncation of either the Igα or the Igβ cytoplasmic domain results in developmental arrest at different stages of B cell development (Papavasiliou et al., 1995; Reichlin et al., 2001).
Aggregation of multiple BCRs by antigen results in the initiation of a signaling cascade that begins with the dual phosphorylation of the ITAM tyrosines by Src-family kinases (SFKs) (Fig. 1.1). In B cells, the predominant SFKs are Lyn, Fyn, Blk, but according to some reports Lck can also be present in B1 B cells (Dal Porto et al., 2004a). Of these kinases, Lyn is the most highly expressed and has a central role in initiating BCR signaling, but is not required for signal propagation (Chan et al., 1998). In resting B cells, the SFK Lyn is in close proximity to the BCR by virtue of weak association between its N-terminal domain and the nonphosphorylated ITAM of Igα (Pleiman et al., 1994). Acylation of Lyn’s unique domain further promotes the recruitment of Lyn to the plasma membrane and stabilizes its interaction with the BCR (Gauld and Cambier, 2004). The association of Lyn with the BCR is also further promoted by preferential localization of clustered, but not monomeric, BCRs to glycosphingolipid-rich microdomains in the plasma membrane called “lipid rafts” (Cheng et al., 2001). Increased concentrations of SFKs, as well as Syk and CD45, have been found in lipid rafts, and thus the spatial localization of these molecules in proximity of clustered antigen receptors may facilitate their interaction (Gupta and DeFranco, 2003).

Upon receptor engagement, Lyn and other SFKs phosphorylate the dual ITAM tyrosine residues of the Igα-Igβ heterodimer, creating docking sites for spleen tyrosine kinase (Syk) (Gauld and Cambier, 2004). Dual ITAM phosphorylation allows Syk to bind Igα and Igβ via its tandem SH2 domains. It is thought that the binding of Syk to the ITAMs induces a conformational change that relieves its autoinhibited state and promotes its activation (Geahlen, 2009). Such activated Syk
**Figure 1.1. B cell antigen receptor signaling.**

B cell antigen receptor signaling is initiated at the plasma membrane by aggregation of multiple BCRs by ligand. BCR-proximal membrane-localized SFKs such as Lyn phosphorylate the intracellular signal transducing elements Igα and Igβ. Dual phosphorylation of the ITAMs allows for the docking of Syk, which initiates the formation of a signaling complex which includes Syk itself, BLNK, PLCγ2, and Btk. PIP₃ in the membrane facilitates the recruitment of Btk and PLCγ2 via their PH domains. Activation of PLCγ2 results in the hydrolysis of PIP₂ resulting in generation of the second inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), which are required for calcium mobilization and Erk signaling.
can go on to phosphorylate ITAMs or other Syk molecules in a cluster of multiple BCRs and promote recruitment of additional Syk molecules (Geahlen, 2009; Mukherjee et al., 2013). Recruitment of Syk to the BCR allows for the formation of multimolecular signaling complexes that further amplify and propagate signals emanating from the BCR via different pathways. Although activation of distal BCR signaling molecules is profoundly impaired in Syk-deficient B cells, the activation of SFKs and the phosphorylation of Igα/β remain largely intact (Takata et al., 1994). This suggests that Syk activity is downstream of SFKs and functions to couple the proximal BCR signaling events to downstream signal transduction components (Dal Porto et al., 2004b). But, as more recent work from the Weiss lab illustrates, Syk can also function independently of SFKs when the BCR is clustered to a high degree by multimeric ligands, suggesting that an important role for SFKs is to increase BCR sensitivity, especially in response to lower-affinity antigens, while greater levels of BCR clustering are required for Syk activation (Mukherjee et al., 2013).

The activation of Syk and its docking to the BCR allows for the assembly of a signaling complex, known as the “signalosome.” Syk nucleates signalosome formation by recruiting several signaling elements, one of which is B cell linker protein (BLNK)/SLP-65, a member the Src homology 2 (SH2)-domain-containing leukocyte protein (SLP) adaptor family (Fu et al., 1998; Wienands et al., 1998). BLNK is a direct phosphorylation target of Syk and is involved in a positive-feedback mechanism in which binding of BLNK to a conserved tyrosine residue in the YxDV motif of the C-terminal sequence of Syk helps activate it (Kulathu et al., 2008). This motif in Syk is preferentially bound by SLP family adaptor proteins via their SH2
domains, and is not found in ZAP-70, a homologous kinase found in T cells (Koretzky et al., 2006; Kulathu et al., 2008). Phosphorylation of BLNK by Syk on multiple tyrosine residues generates docking sites for other SH2 domain-containing molecules, many of which are themselves phosphorylation targets of Syk (Fu et al., 1998).

Lacking intrinsic enzymatic activity, the primary function of BLNK is that of a molecular scaffold for the formation of complexes that include the signaling molecules Bruton’s tyrosine kinase kinase (Btk) and phospholipase C gamma 2 (PLCγ2), which bind to BLNK via their SH2 domains (Dal Porto et al., 2004b). Studies using Lyn-, Syk-, or doubly-deficient B cells revealed that these two kinases are important for tyrosine phosphorylation and activation of Btk in distinct phases (Kurosaki et al., 2000). Lyn is involved in initial activation of Btk by phosphorylation of a tyrosine residue in the activation loop of the catalytic domain of Btk, resulting in increased enzymatic activity (Kurosaki and Kurosaki, 1997). Then, the sustained phase of Btk activity is mediated by Syk, evidenced by delayed kinetics of Btk phosphorylation in B cells lacking Lyn kinase. This is consistent with findings that demonstrate a greater degree of BCR aggregation, occurring at later time points, is required for Syk activation (Mukherjee et al., 2013). The recruitment and activation of Lyn and Syk are required for Btk function, but generation of additional lipid second messengers appears to be the limiting step in Btk activation.

Phosphatidylinositol-3-kinase (PI3K) catalyzes the phosphorylation of the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] to form phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P3]. Interaction of the PH domain
of Btk with PI(3,4,5)P₃ is essential for the recruitment of Btk to the plasma membrane, which influences both the activity and localization of Btk (Saito et al., 2001). The importance of Btk’s ability to localize to the plasma membrane is illustrated by X-linked immunodeficiency (Xid) mice. These mice harbor a point mutation in the PH domain of Btk, resulting in impaired BCR signaling, deficiencies in B cell maturation, and responsiveness to antigen (Rawlings et al., 1993; Takata and Kurosaki, 1996).

Once activated and localized to BLNK, Btk then phosphorylates PLCγ2, which is also bound to BLNK by its SH2 domain and whose localization to the plasma membrane is additionally modulated by the interaction of its PH domain with PI(3,4,5)P₃ in much the same way as Btk (Falasca et al., 1998). Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by activated PLCγ2 results in the generation of soluble inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), two important second messenger molecules. Binding of IP₃ to receptors located on the endoplasmic reticulum (ER) triggers the release of calcium from intracellular stores. This initial burst of intracellular calcium mobilization is transient, as ER calcium stores are quickly depleted (Kurosaki and Tsukada, 2000). Elevated cytosolic calcium concentrations can be sustained after this initial flux through a process called store-operated calcium entry (SOCE). During SOCE, the reduced concentration of calcium within the ER is sensed by proteins of the stromal interaction molecule (STIM) family (Soboloff et al., 2012). STIM family members are transmembrane proteins located in the ER membrane and are activated when depletion of ER calcium stores results in dissociation of calcium ions from their EF-
hand domain allowing for oligomerization of STIM molecules. STIM multimers in the
ER membrane then directly interact with calcium-release-activated (CRAC) channels
made up of one or more ORAI family proteins at the plasma membrane. Interaction
of STIM oligomers with CRAC channels activates the channels resulting in influx of
extracellular calcium into the cell and allows for the maintenance of elevated
intracellular calcium concentrations for periods of time that can be up to many tens
of minutes in duration (Scharenberg et al., 2007; Soboloff et al., 2012). Increases in
intracellular calcium concentrations and DAG activate additional signaling proteins,
including protein kinase C (PKC) family proteins and calcineurin, which contribute
to activation of transcription factors such as NF-κB and N-FAT (Dolmetsch et al.,
1997; Saijo et al., 2002; Trushin et al., 1999).

The other second messenger produced by PLCγ2 lipase activity, DAG, is
involved in B cell activation through its role in the mitogen activated protein kinase
(MAPK) pathway. DAG activates the guanine nucleotide exchange factor (GEF),
RasGRP, which binds to DAG at the plasma membrane and can be further activated
via PKC-mediated phosphorylation and calcium increases (Limnander and Weiss,
2011; Roose et al., 2005). RasGRP exchanges GDP bound to Ras for GTP, and RasGTP
can go on to activate Raf proteins that initiate the MAPK/Erk signaling cascade
(Limnander and Weiss, 2011), which is required for B cell proliferation in response
to antigen, but not for BCR-induced growth arrest or apoptosis (Richards et al.,
2001). RasGTP can also bind the allosteric pocket of another Ras activator, SOS, thus
priming it and enhancing the activity of SOS which results in a positive RasGTP-SOS
feedback loop (Roose et al., 2007). Erk activity has crucial positive and negative
roles in B cells and regulates survival and proliferation, as well as apoptosis and deletion (Limnander et al., 2011; Richards et al., 2001; Rui et al., 2003). Recently, it has been shown that, in addition to the DAG/RasGrp-mediated pathway, Erk can also be activated through a calcium-driven pathway that requires RasGRP and PKC-δ and depends on the concentration of STIM1 (Limnander et al., 2011). Erk activation by this previously unrecognized calcium-mediated pathway results in different functional outcomes in developing B cells by setting a threshold for proapoptotic Erk signaling in developing B cells.

BCR signaling can be amplified through the activation of costimulatory receptors such as CD21 and CD19, found alone or in a complex with each other (Fearon and Carroll, 2000). CD19 is a transmembrane glycoprotein expressed exclusively on B cells that has no known ligand (Weintraub and Goodnow, 1998), while CD21 is more broadly expressed and associates with antigen by binding complement (C3d fragment) that is covalently bound to antigen (Fearon and Carroll, 2000). The extensive intracellular domain of CD19 contains nine tyrosine residues, some of which can be phosphorylated by SFKs, and further regulates their activation in a positive feedback loop of SFK recruitment which facilitates their trans-autophosphorylation (Depoil et al., 2009; Fujimoto et al., 2000; Xu et al., 2002). Phosphorylation of CD19 also promotes the recruitment of intracellular signaling molecules such as Syk, Vav and PI3K, Lyn, and PLCγ2 (Depoil et al., 2008; O’Rourke et al., 1998; Weber et al., 2008), and also contributes to actin cytoskeleton rearrangement, cell spreading, and BCR microcluster formation (Depoil et al., 2008; Harwood and Batista, 2011) Thus, via carefully orchestrated interactions between
the above-described signaling molecules and many others, the B cell integrates signals resulting from antigen recognition into cellular responses.

Initiation of BCR signaling

Although the molecular mechanisms that coordinate proximal and distal signaling are very well described, a definitive model of the early events leading up to BCR signaling is less well established. Given that different BCRs can recognize multitude of diverse ligands in varying modes of presentation, it is likely that the precise molecular events that guide signal initiation are specific to their context.

Nonetheless, the prevailing view is that in resting cells, BCRs can exist on the plasma membrane as highly-mobile monomers as well as in dimers or more ordered oligomers (Depoil et al., 2009; Pierce and Liu, 2010). The actin cytoskeleton and various transmembrane proteins anchored to it are thought to constrain the dynamic diffusion of BCRs on the plasma membrane (Depoil et al., 2009). Imaging studies of individual BCRs and the actin cytoskeleton using single particle tracking support the notion that the actin cytoskeleton is instrumental in preventing BCR cluster formation (Treanor et al., 2010). These experiments revealed that BCRs with different diffusion coefficients are localized to distinct regions of the membrane: actin-poor regions contain faster-moving BCRs, and actin- and ezrin-rich regions are host to relatively immobile BCRs. Chemical stabilization or disruption of the actin cytoskeleton using Jasplakinolide or Cytochalasin D, respectively, each activate BCR signaling pathways in the absence of ligand (Treanor et al., 2010). These findings suggest that steady-state tonic BCR signaling is disrupted by alterations of the actin
cytoskeleton that either concentrate activators of tonic signaling or exclude inhibitors of BCR signaling (Pierce and Liu, 2010; Treanor et al., 2010). While it is largely agreed that the disruption of BCR organization in the plasma membrane is key to signal initiation, some controversy exists regarding the mechanism of signal initiation.

Imaging studies of BCR dynamics provide some evidence that IgM and IgD BCRs are capable of antigen-independent oligomerization and that dissociation rather than the clustering of preaggregated BCRs drives BCR activation (Yang and Reth, 2010b). Such “opening” of BCR clusters was thought to allow for Syk binding to phosphorylated ITAMs and to lead to the further destabilization of the BCR oligomer in an inside-out signaling mechanism that results in a feed-forward amplification process of BCR signaling (Klasener et al., 2014). However, these studies did not clarify what proportion of BCRs is present in such clusters, raising the question of biological significance of their disruption. The authors of these studies argue that B cell activation by BCR dissociation, rather than clustering, is the more probable mechanism of signaling because the flexible hinge region of the membrane Ig (mlg) and the diversity of ligands that can engage the BCR would be unlikely to result in clusters that promote appropriate spacing of BCRs that is conducive to signaling (Yang and Reth, 2010a). However, no evidence exists for steric requirements of BCR organization (Packard and Cambier, 2013). In an alternative and more widely accepted model, ligand-induced BCR clustering is responsible for the induction of BCR signaling.
This model offers several mechanisms by which BCR signaling is triggered upon ligand-induced receptor oligomerization. First, it is possible that BCR aggregation simply brings Lyn, which is already weakly associated with the BCR ITAMs, in close proximity to other BCR molecules allowing for phosphorylation of these molecules in trans. Alternatively, BCR clustering may disrupt the local lipid microenvironment, resulting in the convergence of nearby Lyn-containing lipid rafts with the ITAMs of ligated/oligomerized BCR thus allowing for its phosphorylation. Finally, clustering of the membrane bound immunoglobulin moiety of the BCR may contribute to a conformational change that alters its association with Igα and Igβ in a way that increases accessibility of Lyn to the ITAMs (Cheng et al., 2001; Depoil et al., 2009; Pierce and Liu, 2010).

BCR aggregation results in the rapid formation of microclusters, which mediate intracellular BCR signaling. While it is not completely clear how microclusters form, it has been shown that this is a signaling- and Lyn-independent process, but which does depend on B cell cytoskeleton rearrangement (Harwood and Batista, 2011). Some signaling molecules such as such as CD19, which is required for microcluster formation, are transiently recruited to microclusters, while other molecules like the phosphatases CD45 and CD148 are excluded due to their bulky extracellular domains (Depoil et al., 2008). Furthermore, microcluster formation is associated with carefully orchestrated and sequential recruitment of the intracellular signaling mediators Lyn, Syk, PLCγ2, BLNK, and Vav (Depoil et al., 2009). This implies that the function of microcluster formation may be to aid in the
spatio-temporal regulation of downstream signaling molecules during BCR signaling (Depoil et al., 2009).

The observation that CD45 is excluded from BCR-antigen microclusters is consistent with the kinetic segregation model of T cell receptor (TCR) signaling (Davis and van der Merwe, 2006). This model proposes that CD45 phosphatase activity has a predominantly negative role during antigen receptor signaling and, in order for TCR signals to persist, the phosphatase must be excluded from TCR microclusters. Because binding of the TCR to the peptide-MHC brings the membranes of the T cell and the antigen-presenting cell into close apposition, it is thought that the bulky extracellular domain of CD45 mediates its exclusion from the plasma membrane surrounding the much smaller TCR-peptide-MHC complex (Chakraborty and Weiss, 2014). However, CD45 has concentration-dependent positive and negative regulatory roles in TCR signaling, but a purely positive function in B cells (Zikherman et al., 2012a). This implies that if BCR signaling is modulated by kinetic segregation of CD45 and CD148, it may occur through different mechanisms in B cells than in T cells.

Regulation of SFKs by CD45 and CD148

Due to the central role of SFKs in initiating signaling cascades downstream of the BCR and other receptors such as TCRs and Fc receptors, growth factor receptors, cytokine receptors, and integrins, the activity of these kinases must remain under tight control. Unsurprisingly, dysregulation of SFKs has been associated with various disease states including cancer and autoimmune diseases (Hermiston et al.,
The importance of regulation of SFK activity in immune cell signaling is highlighted by studies using mice that are deficient in the SFK Lyn and those that express Lyn with a gain-of-function mutation (Lyn\textsuperscript{up/up} mice). Lyn-deficient mice develop renal and lymphoproliferative diseases, autoimmunity, and suffer from premature mortality (Chan et al., 1997), but surprisingly, Lyn\textsuperscript{up/up} mice exhibit nearly identical phenotypes (Hibbs et al., 2002). Thus, it appears that regulating the amount of SFK activity, rather than a gross presence or absence of the kinase is critical for maintaining appropriate antigen receptor responses (Lowell, 2004).

Modulation of SFK activity is achieved through the reciprocal actions of receptor-like protein tyrosine phosphatases (RPTPs), covered in this section, and C-terminal Src kinase (Csk), which will be discussed later.

In resting cells, SFKs exist in a dynamic steady state between active and inactive conformations (Fig. 1.2). The relative activity and the transition between active and inactive states are defined by the phosphorylation status of two key phosphotyrosine residues, one in the C-terminal tail and the other in the catalytic pocket of SFKs. Csk-mediated phosphorylation at the tyrosine located in the C-terminal tail of SFKs promotes the interaction of the segment containing this phospho-tyrosine with the SH2 domain, resulting in a closed conformation which stabilizes the inactive conformation of the catalytic domain. In opposition to the actions of Csk are RPTPs, which dephosphorylate this residue and promote the release of SFKs from their autoinhibited state to an open, but not fully active conformation. Trans-autophosphorylation of the tyrosine in the activation loop then
Figure 1.2. SFKs are dynamically regulated by RPTPs and Csk.

SFKs exist in a dynamic equilibrium between inactive and active conformations. Phosphorylation of the C-terminal tyrosine of SFKs by Csk promotes its interaction with the SH2 domain of the SFK, promoting a closed conformation that limits substrate access to the catalytic site of the kinase. Dephosphorylation of this residue allows for the kinase to adopt a more open conformation leaving the SFK “primed” for trans-autophosphorylation. Trans-autophosphorylation at the catalytic site leads to full activation of SFKs.
allows for full kinase activity. The key RPTPs expressed in B cells are CD45, encoded by \textit{PTPRC}, and CD148, encoded by \textit{PTPRJ} (Hermiston et al., 2009).

CD45 has been extensively studied in the immune system, and much is currently known about its expression, function, and regulation. CD45 is expressed exclusively in nucleated hematopoietic cells in relatively high abundance on the surface, comprising up to 10% of cell surface protein (Thomas and Lefrancois, 1988). The importance of CD45 in immune cells is evident by the severe combined immunodeficiency (SCID) phenotype that results in cases of CD45-deficiency in mice and humans (Byth et al., 1996; Kishihara et al., 1993; Kung et al., 2000; Tchilian et al., 2001). The effects of CD45-deficiency are especially profound in mouse T cells, which, unlike B cells, do not express additional RPTPs that could compensate for the loss of CD45. In mice lacking CD45, T cell development is severely blocked due to impaired signaling through the TCR during thymic selection (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999; Pingel et al., 1999). In B cells, the developmental and signaling phenotypes arising from CD45-deficiency are much milder (Byth et al., 1996; Kishihara et al., 1993; Zhu et al., 2008). Expression of both super- and supraphysiological amounts of CD45 in an allelic series of mice demonstrated that CD45 has a purely positive regulatory role during BCR signaling, in contrast to its positive and negative regulatory functions in T cells as a result of its ability to dephosphorylate the activation loop of the T cell SFK Lck, but not that of Lyn (Zikherman et al., 2012a). In other hematopoietic cells, CD45 functions as a critical regulator of signaling thresholds during engagement of antigen, integrin, and cytokine receptors (Hermiston et al., 2003; Holmes, 2006).
CD45 is a glycoprotein consisting of a large extracellular domain, a transmembrane domain, two tandem phosphatase domains, and a 79-amino acid C-terminal tail (Fig. 1.3). Of the two phosphatase domains, only the N-proximal is catalytically active, although the inactive C-terminal domain may have some regulatory function (Hermiston et al., 2009; Nam et al., 2005). At its N-terminus, CD45 contains three alternatively spliced exons (exons 4, 5, and 6), followed by a cysteine-rich domain, and three fibronectin type III domains. Alternative splicing of exons 4, 5, and 6 results in the generation of multiple CD45 isoforms that are differentially expressed on distinct lymphocyte subsets according to their lineage and maturation state (Thomas and Lefrancois, 1988; Zikherman and Weiss, 2008). The alternatively spliced region of CD45 has little protein sequence conservation but does have many sites for O-linked glycosylation and sialylation, which contribute to significant increases in the size and charge of the extracellular domain. Furthermore, the cysteine-rich domain and fibronectin type III domains are subject to various amounts of N-linked glycosylation, depending on the presence of glycosyltransferases expressed in a given cell. The assortment of these glycosyltransferases is also specific to the lineage and developmental or activation state of the cell (Hermiston et al., 2003; Thomas and Lefrancois, 1988). These posttranslational modifications are thought to mediate the interactions of CD45 with other proteins such as lectins and compartmentalization at the cell surface (Chen et al., 2007; Earl and Baum, 2008; Hernandez et al., 2007; van Vliet et al., 2006). Although a bona fide ligand for CD45 has yet to be identified, there is some evidence of glycan-mediated interactions of CD45 with the glycoproteins CD22.
Adapted from Hermiston, M. L., et al., Immunol Rev, 2009
Figure 1.3. Structure of CD45 and CD148.

CD45 and CD148 are structurally distinct phosphatases with some common functional domains. CD45 can exist in multiple splice variants due to alternative splicing of exons 4, 5, and 6 which encode three regions in the extracellular domain (A, B, and C). The extracellular domains of CD45 and CD148 can be modified by the addition of N- and O-linked glycosylation or sialylation. CD148 contains one phosphatase domain, while CD45 contains two (D1 and D2) of which only D1 is active.
(Stamenkovic et al., 1991), galectin-1 (Chen et al., 2007; Walzel et al., 1999), glucosidase II (Baldwin et al., 2000), and the C-type lectin MGL (van Vliet et al., 2006). Spatial localization of CD45 could influence its access to its substrates and consequently alter signaling outcomes in response to stimulation (Hermiston et al., 2009).

In contrast with the high degree of genetic and posttranslational regulation of the extracellular portions, the cytoplasmic domains (D1 and D2) of CD45 are highly conserved. The wedge-like juxtamembrane region of the membrane proximal, functional phosphatase domain (D1) is the most highly conserved region. The crystal structure of PTPRα, a related phosphatase, suggests this juxtamembrane wedge may function in CD45 dimers to inhibit the phosphatase activity of its binding partner, a hypothesis supported by studies of induced dimerization of EGFR-CD45 chimeric proteins (Bilwes et al., 1996; Desai et al., 1993). In vitro and in vivo experiments in which a point mutation was introduced into the tip of the wedge domain also confirmed the importance of the negative regulatory role of the juxtamembrane wedge region of CD45 in B and T cells (Hermiston et al., 2005; Majeti et al., 1998; Majeti et al., 2000). However, the crystal structure of CD45 and other RPTPs have argued against the dimerization model involving the wedge, suggesting that the phosphatase domains may directly interact with each other in a “head-to-toe” orientation (Barr et al., 2009; Bilwes et al., 1996). Instead, some recent genetic studies offer an alternative model for the wedge function as a regulator of substrate specificity and will be discussed below when substrate specificities of CD45 and CD148 are addressed (Zikherman et al., 2013).
In contrast with CD45, CD148 is not universally expressed in all hematopoietic cells and exists at much lower levels on the cell surface. In the immune system, significant CD148 expression is limited to myeloid, erythroid, and B lineage cells, with highest expression of the RPTP on peritoneal B1 B cells (Lin et al., 2004). In mice, CD148 expression is detectable at very low levels in NK cells, γδ T cells, and resting αβ T cells, but can be upregulated following activation of both mouse and human T cells (Lin et al., 2004; Tangye et al., 1998a; Tangye et al., 1998b). In addition to hematopoietic cells, CD148 is also expressed on epithelial and endothelial cells, fibroblasts, platelets, and smooth muscle cells (de la Fuente-Garcia et al., 1998; Katsumoto et al., 2013; Senis et al., 2009; Takahashi et al., 2012; Whiteford et al., 2011). CD148, also called density enhanced protein tyrosine phosphatase-1 (DEP-1), is upregulated in cells cultured at high density or under conditions that induce cellular differentiation (Balavenkatraman et al., 2006; Keane et al., 1996).

While functionally similar, CD148 is structurally distinct from CD45. The extracellular portion of CD148 is larger than that of CD45, containing eight or nine (depending on species), rather than three, fibronectin type II domains which have 34 potential N-linked glycosylation sites (Schraven, 2000) (Fig. 1.3). It lacks a membrane-proximal cysteine-rich domain or additional expression of differentially spliced exons. It is still unclear whether different splice forms of CD148 exist on cells (Hermiston et al., 2009). Unlike CD45, CD148 has a single cytoplasmic phosphatase domain, but it also contains a juxtamembrane wedge. Whether this domain has a role in regulation of CD148 phosphatase activity has not yet been
elucidated (Hermiston et al., 2009). Finally, CD148 contains a C-terminal canonical class II PDZ binding motif, which may mediate its interaction with potential substrates or adapter proteins (Harrod and Justement, 2002).

Due to its importance in non-hematopoietic cells, several studies have been aimed at identifying potential ligands for CD148. One such ligand is thrombospondin-1 (TSP-1), a trimeric glycoprotein that is expressed by megakaryocytes and platelets and is secreted upon platelet activation. By affinity purification and mass spectrometry, soluble TSP-1 was found to bind with high affinity to the extracellular domain of CD148 and act as an agonist of CD148 catalytic activity toward its substrates EGFR and Erk1 and 2 in an epithelial cell line (Takahashi et al., 2012). Syndecan-2, a heparin sulfate proteoglycan, was also found to bind CD148 via its ectodomain in primary and cell line fibroblast models and to promote β1 integrin-mediated adhesion and cytoskeletal organization (De Rossi et al., 2014; Whiteford et al., 2011). Engagement of CD148 by syndecan-2 resulted in CD148-mediated dephosphorylation of the p85 subunit of PI3K in a Src-dependent manner, which resulted in downstream integrin signaling (Whiteford et al., 2011).

The best-described substrates of CD45 and CD148 are SFKs, although other phosphoproteins have been reported as putative targets for CD45. These include the ζ chain of the TCR, the 66 kDa Src-kinase-associated phosphoprotien (Skap66), Jak family kinases, Dap12, and phosphoprotein associated with glycosphingolipid-enriched domains (PAG)/Csk-binding protein (Cbp) (Davidson et al., 2003; Furukawa et al., 1994; Irie-Sasaki et al., 2001; Wu et al., 2002). While these findings are interesting, the biological relevance of these potential substrates remains to be
clarified and is confounded by the possibility that dysregulated SFK activity may partially account for the observations in these studies (Hermiston et al., 2009). Several groups have also identified non-SFK substrates for CD148 in non-hematopoietic cells: Erk1/2 (Sacco et al., 2009), PI3K (Tsuboi et al., 2008), and various growth factor receptors (Kovalenko et al., 2000; Takahashi et al., 2012) earning it the title of a tumor suppressor.

CD45 and CD148 are both promiscuous phosphatases, and until recently, little was known regarding whether the two RPTPs exhibit specificity for any of the SFKs (Barr et al., 2009; Hermiston et al., 2009). Work from the Weiss lab has begun to shed some light on potential mechanisms by which CD45 and CD148 may each exhibit some preference for different SFKs, thereby regulating downstream signaling outcomes. Introduction of a single point mutation into the juxtamembrane wedge of CD45 (E613R) results in the development of B cell-driven autoimmune disease in mice on a susceptible genetic background, possibly due to disruption of dimerization-induced inhibition of phosphatase activity resulting in B cell hyperresponsiveness (Hermiston et al., 2005; Majeti et al., 2000). Later work revealed that the wedge domain of CD45 may play a role in regulating substrate selectivity: expression of the E613R variant results in impaired dephosphorylation of the SFK Lck in T cells and Lyn in B cells, but does not affect the phosphorylation status of Fyn. From these studies, it appears that the juxtamembrane wedge of CD45 influences phosphatase access to specific SFK substrates, adding an additional layer of complexity to the previously established model of dimerization-induced autoinhibition (Zikherman et al., 2013). It is therefore possible that, rather than
simply inhibiting phosphatase activity, the wedge domain may be involved in
mediating interactions with the N-terminal unique domains of SFKs or by affecting
CD45 localization to the membrane or co-receptors (Gervais and Veillette, 1995,
1997; Leitenberg et al., 1996; Zikherman et al., 2013).

It is not known whether CD148, which also contains a membrane-proximal
wedge domain, is subject to such regulation, but studies of chemoattractant
signaling in CD148-deficient neutrophils have revealed distinct functional roles for
CD45 and CD148 that appear to be related to substrate specificity (Zhu et al., 2011).
This work demonstrated redundant roles for CD45 and CD148 in the regulation of
neutrophil adhesion, phagocytosis, and superoxide production leading to bacterial
clearance in an air-pouch model of *Staphylococcus aureus* infection. But
interestingly, examination of chemotactic responses in phosphatase-deficient
neutrophils uncovered distinct functions for these two phosphatases during G-
protein coupled receptor (GPCR) signaling. Loss of CD45 resulted in overall
impaired chemoattraction to N-formyl-methionine-leucine-phenylalanine (fMLP),
while loss of CD148 augmented it. This paradoxical result was explained by the
finding that CD148 exhibits specificity for Lyn, which has both positive and negative
regulatory functions. In contrast, CD45 acts more broadly on all three kinases (Hck,
Fgr, and Lyn) in neutrophils (Zhu et al., 2011). While the exact molecular
mechanisms for the apparent substrate specificities of CD45 and CD148 are unclear,
these experiments point to interesting modes of signal regulation that may be
worthwhile to explore in other cell types.
Regulation of SFKs by Csk

As mentioned above, Csk is an important kinase that opposes the actions of RPTPs to modulate the activity of SFKs before and during antigen receptor signaling. Csk is a ubiquitously expressed non-receptor tyrosine kinase that specifically phosphorylates the negative regulatory sites of SFKs, thus acting as a negative regulator of their activity in a variety of signaling pathways (Okada, 2012).

Csk was first discovered as a novel tyrosine kinase that co-purified with c-Src kinase from cultures of neuronal cells in the late 1980’s (Okada, 2012). Since then, several mouse models have been developed to study the relevance of Csk in vivo. Notably, disruption of Csk led to constitutive activation of Src family kinases in mouse embryos and resulted in neural tube defects and embryonic lethality, demonstrating a critical role for SFK repression in vivo (Imamoto and Soriano, 1993; Nada et al., 1993). To circumvent the limitation posed by embryonic lethality on studying the role of Csk in mature cells, several groups generated mice in which Csk was conditionally deleted. Conditional deletion early during T cell development revealed that loss of Csk bypasses the need for the pre-TCR, the αβ TCR, and MHC class II during T cell development (Schmedt et al., 1998). In granulocytes, conditional mutagenesis of Csk resulted in an increase in spontaneous and ligand-induced degranulation and overexpression and activation of integrins (Thomas et al., 2004). Defects in adhesion and cellular migration were also observed in cell lines derived from Csk-deficient mouse embryos and contributed to development of epidermal hyperplasia in mice that lack expression of Csk in the squamous epithelium (Nada et al., 1994; Thomas et al., 2004; Yagi et al., 2007). Thus, studies of
Csk-deficiency in various model systems all converge on its role as a critical negative regulator of cellular functions mediated by SFKs.

In terms of protein structure, Csk is a 50 kDa cytoplasmic non-receptor tyrosine kinase that is structurally similar to SFKs. It has an N-terminal SH3 and SH2 domain, and a C-terminal kinase domain. However, Csk lacks the N-terminal unique domain containing fatty acylation sites, the autophosphorylation site in the activation loop, and the C-terminal negative regulatory site that is conserved among SFKs, suggesting that the regulation of Csk is distinct from SFKs (Nada et al., 1991; Okada, 2012). For instance, the orientation of the SH2 and SH3 domains of Csk is such that it may enable interactions with other proteins (Okada, 2012). Csk activity appears to be regulated by conformational changes in the molecule. In active Csk molecules, the SH2-kinase and SH2-SH3 linker region is tightly associated with the N-lobe of the kinase domain, stabilizing the active conformation and maintaining direct contact between the SH2 and kinase domain. In inactive molecules, the SH2 domain is rotated outward, disrupting its association with the kinase domain (Ogawa et al., 2002). Therefore, it is likely that active and inactive Csk molecules exist in a dynamic basal state that is regulated by interactions with other receptors and adapter proteins.

The translocation of Csk to the membrane where its substrate is located is an important step in Csk activation. Because Csk does not contain a transmembrane domain or N-terminal myristoylation or palmitoylation to localize it to the plasma membrane, its localization is primarily cytosolic (Cole et al., 2003). As a result, translocation to the membrane is likely mediated through protein-protein...
interactions (Okada, 2012). One such scaffolding protein is PAG/Cbp. This adapter is a tyrosine phosphoprotein found in plasma membrane lipid rafts, where SFKs also localize and phosphorylate PAG. PAG can simultaneously bind Csk and SFKs. Thus, binding of Csk to PAG is an important mechanism by which Csk is brought into close proximity with its substrate. However, PAG-deficient mice develop normally and do not exhibit defects in T cell signaling, suggesting that other mechanisms of Csk regulation must exist (Dobenecker et al., 2005; Xu et al., 2005a). A number of other scaffolding proteins have also been found to anchor Csk to the plasma membrane. These include Dok-1, Lyp/PTPN22, caveolin-1, paxillin, Dab2, VE-cadherin, IGF-1R, IR, LIME, and SIT1 (Okada, 2012). Many of these Csk adapter proteins are also phosphorylated by SFKs, and so recruitment of Csk promotes a negative feedback loop that can tune or terminate antigen receptor signaling (Hrdinka and Horejsi, 2014). Binding of Csk to tyrosine phosphorylated adapter proteins via its SH2 domain has also been shown to induce conformational changes in the catalytic domain and increase its activity toward SFKs, thus providing an additional level of Csk regulation (Manjarrez-Orduno et al., 2012; Ogawa et al., 2002; Zhao et al., 2006). Despite extensive work to understand the function of Csk in T cells and other hematopoietic and non-hematopoietic cells, regulation of B cell antigen receptor signaling by Csk is somewhat poorly studied.

**Negative regulation of BCR signaling**

The strength and duration of signals emanating from the BCR are subject to negative regulation by the activity of inhibitory receptors and their associated
negative regulatory effector molecules. Inhibitory receptors attenuate positive
signaling by ITAM-bearing receptors when they cluster with the activating receptor
at the cell surface (Billadeau and Leibson, 2002). This can occur in *cis* through the
recognition of ligand by the BCR and the inhibitory receptor on the surface of the
same cell, or in *trans* through the recognition of ligands for the inhibitory receptor
expressed on another cell (Lanoue et al., 2002; Tsubata, 2012). The relative strength
of opposing signals by activating and inhibitory receptors ultimately determines the
net signaling outcome. A key feature of an important and growing class of inhibitory
receptors is the presence of an immunoreceptor tyrosine-based inhibitory motif
(ITIM). ITIMs are characterized by the consensus amino acid sequence
S/I/V/LxYxxI/V/L, which is present singly or as multiple motifs in the cytoplasmic
domain of these molecules. B cells of various subsets express a variety of ITIM-
containing inhibitor receptors such as siglecs, CD5, CD72, CD66a, ILT, PIR-B, PD-1,
and LAIR-1 (Ravetch and Lanier, 2000).

Much like ITAM activation, clustering of ITIM-bearing receptors results in
tyrosine phosphorylation by SFKs, which generates docking sites for SH2-containing
cytoplasmic phosphatases (Ravetch and Lanier, 2000). Two families of
phosphatases have been identified in this interaction: the SH2-containing inositol
polyphosphate 5-phosphatases (SHIP-1 and SHIP-2) and SH2 domain-containing
tyrosine phosphatases SHP-1, found predominantly in hematopoietic cells, and SHP-
2, which is more broadly expressed (Billadeau and Leibson, 2002; Tamir et al.,
2000). SHIP and SHP-1 are selectively recruited by different inhibitory receptors,
resulting in the initiation of inhibitory signaling through distinct pathways and thus
result in qualitatively different downstream cellular outcomes (Ono et al., 1997). Specifically, SHIP-1 and SHIP-2 are primarily associated with the low-affinity IgG Fc receptors FcγRIIb and FcγRII. SHP-1 and SHP-2 has been found to associate with phosphorylated ITIMs of inhibitory receptors such as PIR-B, CD22, CD72 and CD5, leading to their recruitment (Adachi et al., 1998; Ono et al., 1997). While the functional role of SHP-1 appears to be inhibitory, the role of SHP-2 is less clear, and some evidence suggests that it may have a positive role downstream of some receptors (Tamir et al., 2000).

SHIP-1 opposes the actions of PI3K by catalyzing the hydrolysis of PIP(3,4,5)P3 into PIP(3,4)P2, causing the release of PH domain-containing molecules such as Btk, Akt and PLCγ2 from the plasma membrane. The result of this is decreased calcium and mTOR signaling (LoPiccolo et al., 2008; Scharenberg et al., 1998). SHP-1 and -2, on the other hand, directly dephosphorylate various protein substrates such as Syk, BLNK, PLCγ2, or the BCR itself, thus reversing some of the early phosphorylation events that take place during proximal antigen receptor signaling (Billadeau and Leibson, 2002; Ravetch and Lanier, 2000).

In B cells, inhibitory receptors are found to be broadly responsive to a wide array of ligands, resulting in the phosphorylation of the ITIMs. It is therefore not surprising that deficiencies in inhibitory receptor signaling are associated with BCR hyperresponsiveness and abnormal B cell development (Ravetch and Lanier, 2000). An important and well-studied example of inhibitory signaling is the Lyn-CD22-SHP-1 pathway. CD22 is an ITIM-containing cytoplasmic member of the siglec family and is expressed exclusively on B cells. This molecule interacts with sialic acid-bearing
ligands expressed on the cell surface (Tedder et al., 1997). Expression of CD22 during B cell development is important for BCR signal attenuation and tolerance induction during B cell development (Gross et al., 2009). Consistent with this, B cells from CD22-deficient mice are hyperresponsive to BCR stimulation, exhibiting elevated calcium mobilization and cellular proliferation at low ligand concentrations. Such elevated BCR signaling in CD22-deficient mice is associated with augmented immune responses and the presence of autoreactive antibodies (O'Keefe et al., 1996). The SFK Lyn is required for constitutive and antigen-induced phosphorylation of CD22 and subsequent recruitment of SHP-1 (Chan et al., 1998; Cornall et al., 1998). Consistent with this, Lyn-deficient mice and SHP-1-deficient mice have B cell phenotypes similar to those in CD22-deficient mice, but which are more extensive and severe, likely due to the fact that the expression of Lyn and SHP-1 is not limited to B cells (Chan et al., 1997; Ravetch and Lanier, 2000).

In addition to CD22, Lyn also phosphorylates other B cell inhibitory receptors including PIR-B, CD72, and CD5 (Gross et al., 2009). Consequently, Lyn plays an important role as both a positive and negative regulator of BCR signaling through phosphorylation of ITAMs and ITIMs alike. As such, Lyn has a particularly important and unique function in negative regulation of BCR signaling.

**Topics to be covered**

Chapter 2 will describe the effects of CD148 deletion on the development, function, and signaling of B1 B cells, revealing a requirement for this phosphatase in B1 but not B2 B cells. A discussion of the these findings in the context of what is
currently known about B cell signaling and considerations of potential future directions follow in Chapter 3. In the Appendix, preliminary data regarding the effects of chemical inhibition of Csk in B cells will be described.
CHAPTER 2

The receptor-like protein tyrosine phosphatase CD148 selectively regulates B1 B cell antigen receptor signaling
Summary

The receptor-like protein tyrosine phosphatases (RPTPs) CD45 (PTPRC) and CD148 (PTPRJ) are thought to have redundant positive functions in the regulation of B cell receptor (BCR) signaling. However, our studies in CD148-loss-of-function mice have revealed a critical role for CD148 in B1 cells that cannot be compensated for by CD45. Mice lacking functional CD148 were found to have defective B1 B cell-mediated antibody responses to immunization with T cell-independent antigens and to have impaired selection of the B1 BCR repertoire. These deficiencies were associated with a decreased ability of B1 B cells to induce BCR signaling downstream of Src-family kinase (SFK) activation, including the SFK Lyn. Surprisingly, we found that in B1 B cells Lyn-deficiency was associated with hyporesponsiveness to BCR stimulation, contrasting with the hyperresponsive phenotype in conventional B2 B cells. These findings suggest that positive regulation of Lyn kinase by CD148 is essential and represents a unique function for this RPTP for B1 B cell BCR signaling and selection.
Introduction

Innate-like B cells such as marginal zone (MZ) B cells and B1 B cells differentially contribute to the TI response depending on their antigen receptor repertoire as well as the amount and anatomical location of antigen encounter (Martin et al., 2001). B1a (CD5hi) and B1b (CD5lo/-) cells each make complementary contributions to the natural and antigen-induced serum immunoglobulin pools, respectively, which are protective against pathogens bearing polymeric polysaccharide antigens (Baumgarth et al., 1999; Haas et al., 2005). The natural antibody repertoire includes recurrent clonotypes such as those against the self-antigens, which are also reactive against microbial determinants present on many pathogenic bacteria such as *Streptococcus pneumoniae* (Briles et al., 1982; Martin et al., 2001; Mercolino et al., 1988).

The development and maintenance of B1 B cells is strongly dependent on antigenic specificity of the BCR, the presence of positively selecting self-ligands, and BCR signal strength. In transgenic mice, expression of B1-derived immunoglobulin genes specific for an endogenous antigen drives the accumulation of B cells with a B1 phenotype (Arnold et al., 2000; Chumley et al., 2000; Hayakawa et al., 1999). Crossing these mice onto a background devoid of the self-ligand or one in which BCR signaling is perturbed results in the lack of B1 B cells (Arnold et al., 2000; Hayakawa et al., 1999). Decreased B1 B cell development and impaired TI responses have been reported in mice that have genetic alterations resulting in decreased BCR signaling, including deletion or inactivation of PLCγ2, Btk, CD19, or BLNK (Hashimoto et al., 2000; Jumaa et al., 1999; Leitges et al., 1996; Pappu et al.,
1999; Rickert et al., 1995), and loss of existing B1 cells upon ablation of surface immunoglobulin via inducible gene targeting (Lam et al., 1997).

Early studies suggest that autoreactive B1 B cells do not cause autoimmune disease due to the induction of a functionally unresponsive state akin to anergy in B1a B cells. This is characterized by a diminished ability to mobilize free intracellular calcium, impaired proliferation, and increased apoptosis upon BCR stimulation with crosslinking antibody (Bikah et al., 1996; Morris and Rothstein, 1993; Sen et al., 1999). The expression of CD5 and its association with negative regulators such as SHP-1 are thought to suppress BCR signaling in B1a cells (Bikah et al., 1996; Sen et al., 1999). However, this model of B1 B cell unresponsiveness is somewhat incompatible with the strong dependence of B1 B cells on BCR signaling for survival and other reports which demonstrate the ability of B1a and B1b cells to proliferate and rapidly generate antibody in response to bacterial infection, lipopolysaccharide (LPS), or immunization with synthetic antigens (Alugupalli et al., 2004; Krljanac et al., 2014; Martin et al., 2001; Weber et al., 2014). Thus, the mechanism of B1 BCR signaling in response to antigen remains incompletely understood.

BCR ligation results in a cascade of signaling events that are dependent on the activity of peripheral membrane Src-family kinases (SFKs), of which Lyn, Blk, Fyn, and Hck predominate in B cells. Following BCR aggregation, SFKs phosphorylate the dual tyrosines in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the cytoplasmic tails of Ig-α and Ig-β (Gauld and Cambier, 2004). These phosphorylation events facilitate the recruitment and activation of the
cytoplasmic kinase Syk via its tandem SH2 domain and the subsequent
phosphorylation of Btk, BLNK, and PLCγ2, in turn leading to the production of
second messengers that promote increases in cytoplasmic free calcium ([Ca^{2+}]) and
activation of the MAPK/Erk pathway (Fu et al., 1998; Saijo et al., 2003). The SFK
Lyn is unique in that it has the ability to phosphorylate receptors bearing ITAMs and
those bearing immunoreceptor tyrosine-based inhibitory motifs (ITIMs), thus
having both positive and negative regulatory roles in antigen receptor signaling.
Lyn appears to have a primarily negative regulatory role in B2 B cells, as Lyn-
deficient mice exhibit B2 cell BCR hyperresponsiveness and develop autoimmunity
that is mediated by B cell autoreactivity (Chan et al., 1997; DeFranco et al., 1998;
Hibbs et al., 1995; Nishizumi et al., 1995).

Due to their central role in immune cell activation, the activity of SFKs must
be tightly regulated to prevent aberrant signaling. This is accomplished by the
opposing actions of receptor-like protein tyrosine phosphatases (RPTPs), and c-src
kinase (Csk). Csk phosphorylates the inhibitory C-terminal tyrosine of SFKs,
thereby promoting an autoinhibited conformation. The action of Csk is opposed by
the RPTPs CD45 and CD148 which dephosphorylate this site, thus relieving the
autoinhibition and promoting some degree of autophosphorylation of the tyrosine
in the activation loop of the catalytic domain (Hermiston et al., 2009). The
constitutive opposing activities of Csk and the RPTPs establish a stable presence of
both active and inactive SFKs in the basal state to provide a pool of active SFK to
participate in and increase during BCR signaling. We have previously shown that
mice harboring either a deletion of Ptprc, encoding CD45, or a functional
inactivation of CD148 via deletion of its transmembrane domain (Ptprj\textsuperscript{TM-\text/-TM-}), have only a partial block in B cell development and mild defects in BCR signaling in follicular B cells, while deletion of both RPTPs results in profound impairments in B cell development resulting from the accumulation of autoinhibitd SFKs (Zhu et al., 2008). Therefore, conventional B cells display a high level of functional redundancy between CD45 and CD148 in SFK regulation. However, more recent work on signaling by ITAM-containing receptors in neutrophils, and the observation that elevated levels of CD45 do not appear to affect absolute numbers of B1 B cells in the peritoneum suggest that non-overlapping roles for CD45 and CD148 may exist (Zhu et al., 2011; Zikherman et al., 2012a). This, along with substantially higher expression of CD148 in B1 B cells compared with B2 B cells (Lin et al., 2004), suggests that CD148 may have a unique role in the regulation of B1 BCR signaling.

Here, we use Ptprj\textsuperscript{TM-\text/-TM-} mice to study the role of SFK regulation by CD148 during B1 cell development and in TI antibody responses. Unlike previous models in which deletion of positive regulators of BCR signaling resulted in an absence of B1 cells, Ptprj\textsuperscript{TM-\text/-TM-} mice have normal numbers of B1 B cells allowing us to study various aspects of B1 cell biology (Berland and Wortis, 2002; Zhu et al., 2008). We identified deficiencies in anti-pneumococcal and anti-NP antibody responses that were attributable to B1 B cells. These defects are due in part to impaired development of an appropriate B1 B cell repertoire and also to decreased B1 cell activation as a result of defective BCR signaling. Furthermore, our findings indicate that, in contrast to B2 B cells, the CD148 substrate Lyn has a predominantly positive
regulatory role in B1 BCR signaling. This suggests that the B1 BCR wiring is distinct from that of B2 B cells and may explain the dependence of B1 B cells on CD148.

**Results**

*CD148 is required for IgM responses to T cell-independent antigens*

B1 B cells make an important contribution to the antibody response against T cell-independent (TI) antigens such as bacterial polysaccharides (Briles et al., 1981). To study the effects of CD148-deficiency in B1 B cells, we challenged *Ptprj*<sup>Tm/-</sup>-/TM-* mice with the TI type 2 antigen Pneumovax 23, a pneumococcal polysaccharide vaccine commonly used in humans to protect against *Streptococcus pneumonia* infection. *Ptprj*<sup>Tm/-</sup>/TM-* mice had a profoundly impaired serum IgM response against Pneumovax 23 after intraperitoneal (i.p.) immunization (**Fig. 2.1 A**). The severity of this defect was surprising because CD45 is so much more highly expressed on B1 B cells compared with CD148 (190,000 molecules of CD45 vs 7,100 molecules of CD148 per B1a B cell; **Fig. 2.2 A**); and, despite this, the loss of CD148 function could not be compensated by CD45. The defective antibody response to the pneumococcal antigens was not due to an overall inability of CD148-deficient B cells to secrete immunoglobulin, since *Ptprj*<sup>Tm/-</sup>/TM-* mice had normal preimmune serum levels of IgM and IgG isotypes as well as preserved expression of the BCR and other cell surface molecules (**Fig. 2.2 B-D**).

In order to more broadly understand the defect in the IgM response to Pneumovax 23 in *Ptprj*<sup>Tm/-</sup>/TM-* mice, we utilized the model antigen 4-hydroxy-3-nitrophenylacetyl (NP), a hapten that can be coupled to different carriers to elicit TI
A) anti-Pneumovax IgM (i.p.)

B) anti-NP IgM (NP-ficoll, i.p.)

C) anti-NP IgM (NP-LPS, i.p.)

D) anti-NP IgG (NP-KLH, i.p.)
Figure 2.1. CD148-deficient mice have impaired TI antibody responses.

Serum antibody responses of wild-type and Ptpri\textsuperscript{TM\textsuperscript{-}/TM\textsuperscript{-}} mice immunized by intraperitoneal (i.p.) injection of TI and TD antigens. Blood samples were taken prior to immunization and at days 7, 14, 21, and 28 following immunization. Immunoglobulin titers were determined by ELISA. Each symbol represents an individual animal followed over the course of 4 weeks. Unless specified, titer was determined at half of the maximal OD for each experiment.

(A) Serum Pneumovax 23-specific IgM titers before and after i.p. immunization with 10 μg Pneumovax 23.

(B) NP-specific serum IgM before and after i.p. immunization with 100 μg NP-50-ficoll. Titer was determined at OD 0.5, which was in the linear range for all samples.

(C) NP-specific IgM responses in mice after i.p. immunization with 50 μg NP-0.15-LPS.

(D) NP-specific IgG\textsubscript{1} responses in mice immunized i.p. with 100 μg NP-33-KLH in alum.

For (A) and (B), n = 5 wild-type mice, n = 4 Ptpri\textsuperscript{TM\textsuperscript{-}/TM\textsuperscript{-}} mice. For (C), n = 5 wild-type mice, and n = 5 Ptpri\textsuperscript{TM\textsuperscript{-}/TM\textsuperscript{-}} mice. Data are representative of two (A), four (B), two (C), and three (D) experiment(s) with similar numbers of animals in each group. Mann Whitney t test was used to calculate p values (A-D).
Figure 2.2. Characterization of B cells from $Ptprj^{TM-/TM-}$ mice.

(A) Quantification of CD45 and CD148 molecules on the surface of B cells.

(B) Total serum immunoglobulin levels in wild-type and $Ptprj^{TM-/TM-}$ mice.

(C) Expression of surface IgM in wild-type and $Ptprj^{TM-/TM-}$ mice.

(D) Expression of B cell surface markers in wild-type and $Ptprj^{TM-/TM-}$ mice.

(A) depicts one mouse of three individual experiments. Data show an average of $n = 5$ wild-type and $n = 5$ $Ptprj^{TM-/TM-}$ mice for (B), and $n = 3$ wild-type and $n = 3$ $Ptprj^{TM-/TM-}$ mice for (C) and (D).
type 1 (TI-1), TI type 2 (TI-2), or T-dependent (TD) responses. *Ptprj*<sup>TM-/TM-</sup> mice were immunized i.p. with NP conjugated to the synthetic polysaccharide ficoll, a TI-2 antigen. As with Pneumovax 23, *Ptprj*<sup>TM-/TM-</sup> mice had an impaired NP-specific IgM response to NP-ficoll (Fig. 2.1 B). Paradoxically, production of IgG<sub>3</sub>, the dominant class-switched isotype during TI-2 immunization (Cunningham et al., 2014), was relatively intact in the absence of a robust IgM response (Fig. 2.3 A). This finding may be explained by the contribution of IgG<sub>3</sub>-producing marginal zone (MZ) B cells to the TI-2 response (Guinamard et al., 2000). Indeed, CD22- and Pyk-2- deficient mice, which have reduced or absent numbers of MZ B cells but intact or increased B1 B cells exhibit a strong defect in anti-NP IgG<sub>3</sub> responses, but only mildly reduced IgM in response to TI-2 antigens (Guinamard et al., 2000; O'Keefe et al., 1996; Otipoby et al., 1996). It is therefore possible that B1 and MZ B cells make different contributions to the total IgM and IgG<sub>3</sub> pools detected in the serum and that the majority of IgG<sub>3</sub> is MZ-derived.

TI-2 responses require Btk-dependent BCR signaling, whereas TI-1 responses occur in the presence of a secondary signal such as toll-like receptor (TLR) ligation, which augments early antibody production and can rescue defective BCR signaling (Rawlings et al., 2012; Vinuesa and Chang, 2013). We therefore asked whether both TI-1 and TI-2 responses were impaired in *Ptprj*<sup>TM-/TM-</sup> mice. We immunized mice i.p. with the TI-1 antigen NP-LPS and found no defect in NP-specific IgM in *Ptprj*<sup>TM-/TM-</sup> mice (Fig. 2.1 C). The ability of in *Ptprj*<sup>TM-/TM-</sup> mice to respond normally to NP-LPS indicated that CD148 likely plays a selective role during TI-2 antigen recognition by the BCR but not TI-1 antigens, which also engage TLRs. Based
A

**anti-NP IgG₃ (NP-ficoll, i.p.)**

titer at OD 0.2

days after immunization

ns  p < 0.05

B

**anti-NP IgM (NP-KLH, i.p.)**

titer at OD 0.5

days after immunization

ns  p < 0.02  ns
Figure 2.3. Intact B2 B cell antibody responses in \( Ptp\text{r}^{\text{Tm-}/\text{Tm-}} \) mice.

(A) Serum titers of NP-specific Ig\( \text{G}_{3} \) from wild-type and \( Ptp\text{r}^{\text{Tm-}/\text{Tm-}} \) mice immunized i.p. with NP-50-ficoll from the same experimental group described in Fig. 2.1 B.

(B) NP-specific IgM from wild-type and \( Ptp\text{r}^{\text{Tm-}/\text{Tm-}} \) mice immunized i.p. with NP-50-KLH form the same experimental group described in Fig. 2.1 D.

Each symbol represents a single animal. Data are representative of four (A) and three (B) individual experiments.
on the observation that CD45 can compensate for CD148 during BCR signaling in follicular B cells, which drive TD responses (Zhu et al., 2008), we hypothesized that Ptprj$^{TM-/TM-}$ mice immunized with the TD antigen NP-keyhole limpet hemocyanin (KLH) would have intact NP-specific IgM and IgG1 responses and, indeed, found this to be the case (Fig. 2.1 D, Fig. 2.3 B). Taken together, these data suggest that CD148 is required for antibody production after immunization with TI-2 but not TI-1 or TD antigens.

**TI defect in CD148-deficient mice is B1 B cell intrinsic**

CD148 is also highly expressed on myeloid cells such as macrophages and dendritic cells, which can facilitate TI responses through antigen presentation to B cells and by promoting plasmablast differentiation (Balazs et al., 2002; Colino et al., 2002; Lin et al., 2004). We therefore wanted to determine whether the TI-2 antibody defect was B cell-intrinsic. CD148 activity was selectively removed from B lineage cells by crossing mice expressing a floxed allele of the CD148 transmembrane region (Ptprj$^{TM-/TM-}$) with the mb1-cre mouse line, which expresses cre recombinase under the control of the Ig-α locus to generate the Ptprj$^{TM-/TM-}; mb1-cre$ mouse line. Ptprj$^{TM-/TM-}; mb1-cre$ mice delete the transmembrane region of CD148 during the pro-B cell stage of development leaving intact CD148 expression in other hematopoietic cells (Fig. 2.4). Like the systemic CD148 knockout, Ptprj$^{TM-/TM-}; mb1-cre$ mice had an impaired IgM response to intraperitoneal immunization Pneumovax 23 (Fig. 2.5 A), confirming that the TI-2 defect due to the loss of CD148 is B cell-intrinsic.
CD19+ PerC lymphocytes

PerC macrophages

- WT
- Ptpn7<sup>Tm1/Tm<sup>de</sup></sup>
- Ptpn7<sup>Tm1/Tm<sup>de</sup> mb1-cre</sup>
- Ptpn7<sup>Tm1/Tm</sup>
- unstained

CD148

% of max
Figure 2.4. B cell-specific deletion of CD148 in Ptprj\textsuperscript{TM-fl/TM-fl}; mb1-cre mice.

(A-D) Bone marrow, splenic, and peritoneal lymphocytes were stained for surface expression of CD148. Proportion of CD148+ cells is shown in the shaded portion of the bar and CD148- cells are represented by the white portion of the bar. Labels are as follows: wt = wild type, mt = Ptprj\textsuperscript{TM-}/\textsuperscript{TM-}; fl/mt = Ptprj\textsuperscript{TM-fl/TM-fl}; mb1-cre, fl/fl = Ptprj\textsuperscript{TM-fl/TM-fl}; mb1-cre. (A) represents bone marrow Hardy fractions A-C; and Hardy fractions D-F are shown in (B); (C) represents mature and transitional splenic B cells; and (D) represents peritoneal B cells.

(E) CD148 expression in peritoneal B1 and B2 B cells, and myeloid cells.

(A-E) depict one mouse of each genotype and are representative of three individual experiments.
A) anti-Pneumovax IgM (i.p.)

B) anti-NP IgM (NP-ficoll, i.v.)
anti-NP IgG3 (NP-ficoll, i.v.)
Figure 2.5. TI antibody response defect in CD148-deficient mice is B1 B cell-intrinsic

Serum antibody responses of mice immunized with the specified antigen either i.p. or by tail vein injection (i.v.) on the indicated days. Each symbol represents an individual animal followed over the course of 4 weeks. Unless specified, titer was determined at half of the maximal OD for each experiment.

(A) Pneumovax 23-specific IgM titers in Ptprj\(^{TM-\text{fl}/TM-\text{fl}}\) and Ptprj\(^{TM-\text{fl}/TM-\text{fl};mb1-cre}\) mice immunized i.p. with 10 μg Pneumovax 23.

(B) NP-specific IgM (left panel) and IgG3 (right panel) in WT and Ptprj\(^{TM-\text{fl}/TM-\text{fl}}\) mice mice immunized by i.v. immunization with NP-ficoll.

For (A) \(n = 5\) Ptprj\(^{TM-\text{fl}/TM-\text{fl}}\) mice, \(n = 4\) Ptprj\(^{TM-\text{fl}/TM-\text{fl};mb1-cre}\) mice. For (B) \(n = 6\) wild-type mice, \(n = 6\) Ptprj\(^{TM-\text{fl}/TM-\text{fl}}\) mice. Data are representative of three (A) and two (B) experiment(s). Mann Whitney t test was used to calculate p values (A) and (B).
We next wanted to address whether the B1 B cell compartment was uniquely affected by CD148 deficiency. Marginal zone and B1 B cells have complimentary functions during TI-2 responses, but the relative contribution of each B cell type to the antibody response depends on the dose and the route of immunization (Martin et al., 2001). B1 B cells dominate the response to antigen introduced i.p. but MZ B cells respond predominantly to intravenous (i.v.) administration of antigen (Martin et al., 2001). Therefore, we immunized Ptprj<sup>TM-/-</sup> mice by i.v. injection of NP-ficoll to target the MZ responses and measured serum IgM and IgG3 over time. In sharp contrast with i.p. immunization, we saw robust production of both immunoglobulin isotypes in wild-type and Ptprj<sup>TM-/-</sup> mice (Fig. 2.5 B). The statistically significant, but relatively small decrease in serum IgM and IgG3 in Ptprj<sup>TM-/-</sup> mice after i.v. immunization could be attributed to potentially impaired function of a minor population of B1 B cells residing in the spleen (2% of splenic B cells) (Stall et al., 1996). These findings indicate that that CD148 is required for TI-2 antibody responses to antigens encountered i.p. in a B1 B cell-intrinsic manner.

*CD148 is required for antigen-specific proliferation and IgM secretion by B1 B cells*

Antibody secretion in response to TI antigens is associated with antigen-specific proliferation of B1 B cells (Haas et al., 2009; Krljanac et al., 2014). To investigate whether the observed defect in IgM production in Ptprj<sup>TM-/-</sup> mice was due to impaired activation and proliferation of peritoneal B1 B cells, we challenged wild type and Ptprj<sup>TM-/-</sup> mice i.p. with NP-ficoll and examined the peritoneal cavity and spleen for the presence of dividing NP-specific B cells. Dividing cells were
identified by intracellular staining for the proliferation marker Ki-67 and by surface staining with NP-phycoerythin (PE) to detect cells with BCRs specific for NP. A small number of NP-PE⁺, Ki67⁺ B1 cells were present in the PBS control, and likely represent self-renewing cells that are precursors of responding B1 B cells. Immunization with NP-ficoll led to an increase in the number of NP-PE⁺, Ki67⁺ peritoneal B1 B cells in both wild-type and Ptprj⁻/⁻ mice compared with PBS-treated controls (Fig. 2.6 A). However, the fold increase of responding B1 B cells in the NP-ficoll condition compared with the PBS control was much lower in Ptprj⁻/⁻ mice compared with wild type (3.2-fold vs. 6.1-fold) and the absolute number of NP-PE⁺, Ki67⁺ wild type B1 cells in NP-ficoll-immunized was several times higher than that of Ptprj⁻/⁻ B1 B cells under the same conditions (Fig. 2.6 A and B). There was also an increase of NP-PE⁺, Ki67⁺ MZ B cells in mice treated with NP-ficoll compared with PBS, but this increase was comparable between wild type and Ptprj⁻/⁻ mice (2.6 and 2.8 fold increase, respectively) (Fig. 2.6 B). This is consistent with the notion that MZ B cells do not require CD148 to respond to T1 antigens.

We next asked whether the defective proliferative response of peritoneal B1 B cells in Ptprj⁻/⁻ mice was associated with decreased numbers of antigen-specific antibody producing B1 cells, which would be consistent with the observed diminished IgM serum response. We performed ELISpot analysis of total peritoneal cavity (PerC) lymphocytes from mice injected i.p. with either NP-ficoll or PBS. There was a robust increase in NP-specific IgM but not IgG₃ antibody secreting cells (ASCs) in peritoneal lymphocytes from wild-type mice, but few detectable peritoneal
A CD19+ PerC lymphocytes

B PerC B cells

C WT Ptprj\textsuperscript{TM/\textsuperscript{TM}}

D PerC anti-NP ASCs

- IgM
- IgG

# ASC/million PerC cells

** ns

PerC anti-NP ASCs

# ASC/million PerC cells

** ns

PerC anti-NP ASCs

# ASC/million PerC cells

** ns

PerC anti-NP ASCs

# ASC/million PerC cells

** ns
Figure 2.6. CD148-deficient peritoneal B1 B cells fail to proliferate and produce IgM in an antigen-dependent manner.

(A) Intracellular staining of NP-specific peritoneal B1 (CD19*, CD23; and CD5*/lo) and B2 (CD19*, CD23*, CD5*) cells for Ki67 in mice 4 days after i.p. immunization with 10 μg NP-18-ficoll. Cell count represents the number of events in the NP+, Ki67+ gate.

(B) Quantification of NP-PE+, Ki67+ peritoneal B cells in (A) and splenic follicular (CD19*, CD23*, CD21-) B cells and splenic MZ (CD19*, CD23; CD21+) B cells. Cell counts were normalized between experiments based on the number of total events collected. Data represent the number of NP-PE+, Ki67+ cells present per 10^6 cells of each B cell subset. Each symbol represents an individual mouse.

(C) ELISpot analysis of NP-specific IgM secreting cells from mice 7 days after i.p. immunization with NP-18-ficoll.

(D) Quantification of NP-specific IgM and IgG3 secreting B cells in (C). Each symbol represents an individual mouse and is the mean of triplicate samples. Data represent the number of ASCs per million peritoneal cells.

For (A) and (B), data are representative of n = 12 wild-type mice and n = 12 Ptprj^{TM-}/^{TM-} NP-ficoll immunized mice, and n = 7 wild-type mice and n = 7 Ptprj^{TM-}/^{TM-} PBS control mice. For (C) and (D), data are representative of 5 independent experiments with n = 3 mice in each experimental group at either day 4 or day 7 post immunization, which give similar results. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.
Figure 2.7. Anti-NP responses by splenic B cells are normal in Ptprj\textsuperscript{TM-/-TM-} mice.

Quantification of NP-specific IgM and IgG\textsubscript{3} secreting B cells in the spleens of PBS or NP-ficoll-treated mice at 7 days post immunization as described in Fig. 2.6 (C) and (D). Each symbol represents an individual mouse and is the mean of triplicate samples. Data represent the number of ASCs per million splenocytes. Data are representative of 5 independent experiments done at days 4 or 7, which give similar results.
cavity-derived ASCs in Ptprj\textsuperscript{TM-/TM-} mice, while equal numbers of IgM and IgG\textsubscript{3} ASCs were detectable in the spleens of WT and Ptprj\textsuperscript{TM-/TM-} mice (Fig. 2.6 C and D; Fig. 2.7). Collectively, these results demonstrate that B1 B cells, but not MZ B cells, are unable to become activated in response to TI-2 antigens.

**B1 cell development altered in the absence of CD148**

Development and maintenance of B1 B cells is dependent on the presence of endogenous BCR ligands, and is sensitive to the strength of signal derived from these antigens (Rawlings et al., 2012). The lower frequency of NP-PE\textsuperscript{*}, Ki67\textsuperscript{*} B1 B cells in unimmunized Ptprj\textsuperscript{TM-/TM-} mice (Fig. 2.6 A and B) led us to ask whether CD148-deficiency is associated with a failure to establish a normal NP-responsive BCR repertoire in B1 B cells. Although total B1 B cell numbers were normal in Ptprj\textsuperscript{TM-/TM-} mice (Zhu et al., 2008), it is possible that a BCR repertoire shift may compensate for impaired BCR signaling in the absence of CD148, leading to the underrepresentation of NP-reactive cells in the B1 repertoire of Ptprj\textsuperscript{TM-/TM-} mice. To investigate this possibility, we stained peritoneal lymphocytes and splenocytes with NP-FITC-ficoll, and detected an approximately 50 percent fewer NP-specific peritoneal B1a, B1b, and B2 B cells in the peritoneum but no differences in splenic NP-specific follicular or MZ B cells in Ptprj\textsuperscript{TM-/TM-} mice (Fig. 2.8 A and B, Fig. 2.9 A). The presence of NP-specific serum IgM in preimmune mice and B1 B cells that stain positively for NP in preimmune wild-type mice suggests that NP-crossreactive autoantigens or environmental antigens such as those from gut flora may be sufficient to drive the development of NP-reactive precursors. While the identity of
A

CD19+ PerC lymphocytes

CD5

CD19

NP-FITC-ficol

NP-FITC-ficol

B

WT

PtprjTM-/TM-

B1a

B1b

B2

# NP+ B cells/10^6

% of B cells

C

CD5

CD23

NP-FITC-ficol

NP-FITC-ficol

D

WT

PtprjTM-/TM-

B1-8i

B1-8i

PtprjTM-/TM-

E

kappa

lambda

B1

B1-8i

PtprjTM-/TM-

F

% of B cells

B1a

B1b

B2

% of B cells

B1-8i

B1-8i; PtprjTM-/TM-
**Figure 2.8. CD148 is required for normal B1 B cell repertoire development.**

(A) Total peritoneal lymphocytes were stained with NP-FITC-ficoll. Frequencies of NP+ cells are represented in the gates as a percentage of the parent population (B1a, B1b, or B2) that stains positively for NP above background.

(B) Quantification of data depicted in (A). Each symbol represents an individual animal. Data are shown as number of NP+ cells per million B cells of the parent population.

(C) Flow cytometric analysis of B1a, B1b, and B2 B cells from peritoneal lavage of B1-8i and B1-8i; Ptprj^{TM/-TM-} mice compared with wild-type and Ptprj^{TM/-TM-} mice.

(D) Quantification of peritoneal B cell subsets in B1-8i and B1-8i; Ptprj^{TM/-TM-}, wild-type, and Ptprj^{TM/-TM-} mice. Bars represent the mean frequencies of each B cell subpopulation as a percent of total peritoneal (PerC) B cells (CD19+ lymphocytes).

(E) Flow cytometric analysis of kappa and lambda light chain expressing B1 and B2 B cells in B1-8i and B1-8i; Ptprj^{TM/-TM-} mice.

(F) Quantification of percent lambda light chain expressing B1a, B1b, and B2 B cells in B1-8i and B1-8i; Ptprj^{TM/-TM-} mice.

For (A) and (B) data are representative of n = 3 of each wild-type and Ptprj^{TM/-TM-} mice. For (C) and (D) data are representative of n = 4 B1-8i mice, n= 4 B1-8i; Ptprj^{TM/-TM-} mice, n = 4 wild-type, and n = 6 Ptprj^{TM/-TM-} mice. For (E) and (F), data are representative of n = 4 of each B1-8i and B1-8i; Ptprj^{TM/-TM-} mice. Error bars represent standard error of the mean. Student’s t test was used to calculate p values.

* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.
Figure 2.9. Normal B2 B cell development in \( Ptp\rangle^{\text{TM-/-TM-}} \) mice.

(A) Total splenocytes were stained with NP-FITC-ficoll. Frequencies of NP+ cells are represented in the gates as a percentage of the parent population (follicular or MZ) that stains positively for NP above background.

(B) Flow cytometric analysis of follicular and MZ B cell populations in wild-type and \( Ptp\rangle^{\text{TM-/-TM-}} \) mice on the B1-8i background.

(C) Quantification of splenic B cell subsets in B1-8i and B1-8i; \( Ptp\rangle^{\text{TM-/-TM-}} \); wild-type, and \( Ptp\rangle^{\text{TM-/-TM-}} \) mice. Bars represent mean frequencies of follicular and MZ B cells of total splenic B cells (CD19+ lymphocytes).

(D) Quantification of flow cytometric analysis of lambda and kappa light chain usage by follicular and MZ B cells in B1-8i and B1-8i;\( Ptp\rangle^{\text{TM-/-TM-}} \) mice.

(E and F) Quantification of flow cytometric analysis of lambda and kappa expressing B cells in wild-type and \( Ptp\rangle^{\text{TM-/-TM-}} \) mice in the peritoneal cavity (PerC) (E) and in the spleen (F).

For (A) \( n = 3 \) wild-type and \( n = 3 \) \( Ptp\rangle^{\text{TM-/-TM-}} \) mice. For (B) and (C) data are representative of at least 4 wild-type mice and 4 \( Ptp\rangle^{\text{TM-/-TM-}} \) mice and \( n = 4 \) B1-8i mice, \( n = 4 \) B1-8i; \( Ptp\rangle^{\text{TM-/-TM-}} \) mice, \( n = 4 \) wild-type, and \( n = 6 \) \( Ptp\rangle^{\text{TM-/-TM-}} \) mice. For (D) \( n = 4 \) B1=8i mice, \( n = 4 \) B1-8i; \( Ptp\rangle^{\text{TM-/-TM-}} \) mice. For (E) and (F) \( n = 4 \) wild-type, and \( n = 6 \) \( Ptp\rangle^{\text{TM-/-TM-}} \) mice. Error bars represent standard error of the mean.

Student’s t test was used to calculate p values.
NP-BCR reactive autoantigens is unclear, reactivity against NP in the normal B1 repertoire has previously been observed (Hsu et al., 2006).

We further probed the role of CD148 in B1 cell development by using a transgenic BCR system, which may reveal developmental defects that are otherwise masked in an unrestricted B cell repertoire when BCR signaling is only partially impaired (Cyster et al., 1996). To explore the effects of CD148-deficiency on B1 B cell development in the context of limited BCR diversity, we crossed \( Ptprj^{TM-/-TM-} \) mice to the B1-8i mouse knock-in line to generate B1-8i-\( Ptprj^{TM-/-TM-} \) mice. The B1-8i mice express a rearranged heavy chain that produces NP-specific BCRs when paired with Ig\( \lambda \) light chains (Shih et al., 2002). Introduction of the B1-8i heavy chain had no effect on the development of follicular B cells and was associated with a small increase in the percentage splenic MZ B cells (Fig. 2.9 B and C). There was, however, a marked skewing away from the B1 lineage in the peritoneum and an increase in the percentage of peritoneal B2 B cells (Fig. 2.8 C and D). Because BCR specificity is known to influence the acquisition of the B1 phenotype, it is not surprising that limiting the diversity of the heavy chain might lead to an overall decrease in B1 B cells in both CD148-sufficient and \( Ptprj^{TM-/-TM-} \) B1-8i mice (Berland and Wortis, 2002). The majority of B1 B cells express BCRs that use the Ig\( \kappa \) light chain (Fig. 2.8 E) and for this reason only a minor population is NP-restricted. Consequently, the overall percentages of B1a and B1b B cells in the peritoneum of B1-8i-\( Ptprj^{TM-/-TM-} \) mice were comparable to those in CD148-sufficient B1-8i mice (Fig. 2.8 D). However, staining for Ig\( \kappa \) and Ig\( \lambda \) revealed a decrease in Ig\( \lambda + \), and therefore NP-reactive, B1a and B1b cells in B1-8i-\( Ptprj^{TM-/-TM-} \) mice compared with B1-
8i mice (Fig. 2.8 E and F). This CD148-mediated developmental defect in NP-restricted B cells was not evident in splenic follicular or MZ B cells (Fig. 2.9 D). The bias away from Igλ usage was not apparent in B1 or B2 B cells on the unrestricted B6 background (Fig. 2.9 E and F), suggesting that the decrease in NP-restricted B1 B cells in Ptprj<sup>TM-/-</sup> mice on both the B1-8i and unrestricted repertoires is antigen specific and is likely due to impaired BCR signaling. Thus, BCR repertoire restriction via introduction of the B1-8i transgene unmasked a developmental requirement for CD148 exclusively in the B1 compartment of B cells. Taken together, these results suggest an important role for CD148 in establishing and maintaining an appropriate B1 cell repertoire.

*BCR signaling is impaired in CD148-deficient B1 B cells*

In the absence of CD148 activity, CD45 is sufficient to promote SFK activation and initiate ITAM-mediated BCR signaling (Zhu et al., 2008). However, the effects of CD148-deficiency on B1 B cells were not studied, and the above data suggest a unique requirement for this phosphatase by B1 BCR signaling. To investigate the role of CD148 at the level of B1 BCR signaling, we stimulated peritoneal lymphocytes *in vitro* with BCR-crosslinking antibody and assessed phosphorylation of Erk by intracellular staining. At intermediate stimulatory concentrations (5 and 10 μg/mL) of α-IgM F(ab')<sub>2</sub>, we observed a defect in the activation of the Erk pathway in Ptprj<sup>TM-/-</sup> B1 B cells at 2 minutes after stimulation (Fig. 2.10 A). Higher concentrations of BCR crosslinking antibody or longer duration of stimulus were sufficient to overcome this signaling defect, as was bypassing proximal BCR
Figure 2.10. Impaired BCR signaling in CD148-deficient B1 B cells

(A) Peritoneal lymphocytes were stimulated for 2 minutes with the indicated concentrations of anti-IgM F(ab’)2 or 50 ng/ml phorbol myristate acetate (PMA), fixed, then permeabilized and stained with anti-phospho-Erk antibody and surface markers. B1 cells were identified as B220lo, CD23-, CD5+/lo. Wild-type are shown in black lines and PtpriTM-/TM- are shown in grey lines.

(B) Peritoneal lymphocytes were stimulated with 5 μg/mL of anti-IgM F(ab’)2 or 50 ng/ml PMA for the indicated times and stained as in (A). Shaded histograms indicate unstimulated cells.

(C) Percentages of pErk+ B1 cells shown over time after stimulation with 5 μg/mL of anti-IgM F(ab’)2.

(D) Percentages of pErk+ follicular B cells (B220+, CD23+, CD21-) and MFI of pErk+ MZ B cells (B220+, CD23-, CD21+) over time after stimulation with 10 μg/mL of anti-IgM F(ab’)2.

(E) Total peritoneal lymphocytes or splenocytes were loaded with Indo-1 and stained with FITC anti-IgM Fab and a dump gate to exclude non-B cells in peritoneal and splenic cells as well as CD23+ B2 B cells in peritoneal lymphocytes. Peritoneal cells were stimulated with 10 μg/mL and splenocytes with 20 μg/mL anti-IgM F(ab’)2 indicated by the closed arrow, and then with 1 nM ionomycin indicated by the open arrow.

(F) Peritoneal lymphocytes were stimulated for the indicated times with 1 μg/mL NP-18-ficoll. Prior to stimulation B cells were stained with APC-anti-IgM Fab to gate
for B cells, and then stimulated and stained as in (A) with the addition of antibodies against the lambda and kappa light chains.

For (A) and (B) data are representative of 7 individual experiments. For (C) and (D) n = 7 each of wild-type and Ptpri\textsuperscript{TM-/-}\textsuperscript{TM-} mice. Because B1 and follicular B cells respond in a bimodal fashion % pErk+ cells were shown in (C) and (D), whereas pErk induction in MZ B cells is associated with a population shift; therefore mean fluorescence intensity (MFI) of the population is shown. (E) and (F) are representative of 3 individual experiments. Error bars represent standard error of the mean. Student’s t test was used to calculate p values. * p ≤ 0.05, ** p ≤ 0.01.
signaling via stimulation with PMA. These results indicated an impairment in BCR signaling rather than an insufficiency in downstream components of the BCR signaling pathway (Fig. 2.10 A-C). In comparison with B1 cells, neither splenic follicular nor MZ B cells from Ptpri\textsuperscript{TM/-TM-} mice exhibited altered pErk signaling (Fig. 2.10 D). We also analyzed BCR-induced intracellular calcium mobilization in peritoneal Ptpri\textsuperscript{TM/-TM-} B1 and splenic B cells after BCR crosslinking. Consistent with impaired Erk phosphorylation, there was a decrease in the magnitude of the calcium response in B1 but not conventional B cells (Fig. 2.10 E). To confirm that B1 B cell signaling was also defective in response to bona fide antigen such as NP-ficoll, we examined induction of pErk in peritoneal and splenic B1-8i and B1-8i-Ptpri\textsuperscript{TM/-TM-} B cells stimulated with NP-ficoll and gated on Ig\textgreeklambda+ and Ig\textkappa+ B1, follicular, and MZ B cells. As expected, only Ig\textgreeklambda+ B1-8i cells were responsive to stimulation with NP-ficoll. However, in contrast to robust responses of follicular or MZ B cells from B1-8i-Ptpri\textsuperscript{TM/-TM-} mice, peritoneal B1 B cells had a markedly decreased ability to phosphorylate Erk after BCR engagement (Fig. 2.10 F, Fig. 2.11 A).

\textit{Lyn-dependent BCR signaling in B1 cells results in sensitivity to loss of CD148}

Calcium mobilization and Erk phosphorylation are distal BCR events that are downstream of the BCR signaling cascade initiated by Src-family kinases, whose activity is positively regulated by CD45 and CD148. To understand the defect in calcium and Erk responses in B1 cells, we again stimulated peritoneal and splenic B cells by antibody-mediated BCR crosslinking and assessed activation of various components of the proximal BCR signaling cascade. We observed a trend of lower
phosphorylation of ITAMs of the alpha chain (pCD79A) of the B cell receptor, and impaired phosphorylation of substrates of SFKs and Syk (Fig. 2.11 A and B). Phosphorylation of PLCγ2 and BLNK, which are upstream of the calcium response, were also decreased in Ptprj\textsuperscript{Tm-/Tm-} B1 B cells (Fig. 2.12 C and D). Therefore, CD148 was required for full proximal BCR signaling downstream of SFK activation in B1 cells. These substantial signaling defects were not observed in splenic follicular or MZ B cells (Fig. 2.11 B-E). To assess SFK activation in resting B1 B cells and following stimulation of the BCR, we sort-purified peritoneal B1 cells and blotted cell lysates for phosphorylation of the activation loop tyrosine (pY416) of SFKs. In the resting state, Ptprj\textsuperscript{Tm-/Tm-} B1 B cells exhibited decreased basal phosphorylation of Y416, and a lack of subsequent increased phosphorylation of this residue after stimulation via BCR crosslinking (Fig. 2.12 E), demonstrating impaired SFK basal activity and activation. In sharp contrast with follicular B cells, the decreased phosphorylation of the activating tyrosine of SFKs coincided with hyperphosphorylation of the inhibitory tyrosine (Y507) of the SFK Lyn in resting Ptprj\textsuperscript{Tm-/Tm-} B1 B cells and during BCR stimulation (Fig. 2.12 F).

The SFK Lyn is highly expressed in B cells and has been described to have both positive and negative regulatory roles in BCR signaling due to its ability to phosphorylate both ITAMs and ITIMs (Gauld and Cambier, 2004). The observation that, in B1 B cells, SFKs including Lyn kinase had lower activity in the absence of CD148 is distinct from the previously reported high level of redundancy of CD45 and CD148 in regulating SFKs and BCR signaling in follicular B cells (Zhu et al., 2008). However, studies of the regulation of G-protein coupled receptor signaling
**Figure 2.11. Normal BCR signaling in follicular and MZ B cells.**

(A) Splenocytes were stimulated for the indicated times with 1 μg/mL NP-18-ficoll. Prior to stimulation B cells were stained with APC anti-IgM Fab to gate for B cells, and then stimulated, fixed and permeabilized, followed by intracellular staining for phospho-Erk and staining for surface markers as well as lambda and kappa light chain expression. Top panel: splenic follicular (B220⁺, CD23⁺, CD21⁻) B cells, and bottom panel: MZ (B220⁺, CD23⁻, CD21⁺) B cells.

(B) Splenocytes were stimulated for the indicated times with 10 μg/mL anti-IgM F(ab′)₂, fixed, then permeabilized and stained with anti- phospho-CD79A (Y182) and surface markers. Mean MFIs over time of stimulated follicular (B220⁺, CD23⁺, CD21⁻), top panel; and MZ B cells (B220⁺, CD23⁻, CD21⁺), bottom panel.

(C) Cells treated as in (B) stained with anti-phospho-Syk (Y352),

(D) phospho-BLNK (Y84), and

(E) phospho- PLCγ2 (Y759).

For (A), data are representative of at least 3 independent experiments. For (B-E) average MFI and error bars depicting standard error of the mean are shown. For (B) an average of 3 experiments is shown, 3 experiments in (C) and (D), and 5 experiments in (E). For (B-E) Student’s t test was use to calculate p values and no statistical significance was found between wild-type and Ptprj<sub>TM⁻/TM⁻</sub>.
Figure 2.12. Lyn has a predominantly positive role in B1 BCR signaling.

(A) Peritoneal lymphocytes were stimulated with 5 μg/mL of anti-IgM F(ab’)2 for the indicated times fixed, then permeabilized and stained with phospho-CD79A and surface markers. B1 cells were identified as B220lo, CD23-, CD5+/lo. Graphs depict the mean MFI of multiple experiments; histogram depicts one representative experiment at the time point when peak signal is achieved.

(B) B1 cells as in (A) stained with phospho-Syk (Y182),
(C) phospho-BLNK (Y352),
(D) and phospho-PLCγ2 (Y759).

(E) Sort-purified peritoneal B1 B cells were stimulated with 5 μg/mL of anti-IgM F(ab’)2 for the indicated times. Lysates were blotted with anti-phospho-Src antibody against the activating tyrosine of SFKs (Y416). Total Erk1 was used as a loading control.

(F) Sort-purified peritoneal B1 and lymph node follicular B cells were stimulated as in (E) and blotted for the inhibitory phosphotyrosine of SFK using commercially available anti-phospho-Lyn (Y507), which may cross react with other SFKs. Total Lyn was used as a loading control, for B1 B cells anti-Lyn 44 (Santa Cruz), which recognizes both Lyn isoforms was used, and anti-Lyn (Cell signaling) was used for follicular B cells.

(G) Peritoneal lymphocytes were stimulated with 5 μg/mL anti-IgM F(ab’)2 for the indicated times, fixed, then permeabilized and stained with anti-phospho-Erk antibody and surface markers. B1 cells were identified as B220lo, CD23-, CD5+/lo. Wild-type are shown in black lines and Lyn-/- are shown in dashed lines.
(H) Percentages of pErk+ B1 cells shown over time after stimulation with 5 μg/mL of anti-IgM F(ab’)2 as in (G).

For (A-D) average MFI and error bars depicting standard error of the mean are shown. For (A) an average of 6 experiments is shown, 3 experiments in (B) and (C), and 4 experiments in (D). A representative of 3 individual blots is shown for (E) and (F). (G) is representative of at least 4 independent experiments. For (H) n = 4 wild-type and n = 4 Lyn−/− mice. Error bars represent standard error of the mean. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.
in neutrophils suggested that CD45 and CD148 may differentially target different SFKs, and that CD148 preferentially dephosphorylates Lyn kinase (Zhu et al., 2011). The molecular basis of this selectivity of CD148 for Lyn kinase is not fully understood, but it is possible that a similar mechanism is at play in B1 B cells and would explain the defect in Lyn kinase activation in Ptpτ/−/− B1 B cells. However, the preferential dephosphorylation of Lyn kinase by CD148 would not seem to fully explain the activation and antibody production defects in Ptpτ/−/− B1 B cells. This is because Lyn plays a predominantly negative regulatory function in conventional B cells; indeed, its deficiency results in hyperresponsiveness to BCR stimulation, rather than impaired signaling due to compensation by other B cell SFKs such as Blk and Fyn (Chan et al., 1997). If this were similarly the case in B1 B cells, we would expect increased, rather than decreased BCR signaling.

We therefore asked whether the role of Lyn kinase in BCR signaling might be different in B1 B cells than in conventional B cells. To address this, we compared the BCR responses of peritoneal B1 B cells and splenic B cells from wild type mice to Lyn−/− mice after stimulation through the BCR. As expected, follicular B cells from Lyn−/− mice exhibited significantly elevated phosphorylation of Erk compared with wild type B cells. Remarkably, Lyn−/− B1 B cells were hyporesponsive to BCR stimulation (Fig. 2.12 G and H). Thus, Lyn-deficiency is associated with a BCR signaling defect that partially phenocopies the CD148-mediated signaling defect in Ptpτ/−/− B1 B cells. We conclude that that Lyn may be preferentially regulated by CD148 and that the role of Lyn kinase appears to have a predominantly positive regulatory role in B1 B cells that cannot be compensated for by other SFKs. These
results suggest a unique wiring of BCR signaling in B1 B cells in such a way that renders these cells especially sensitive to the loss of CD148, which appears to be an important positive regulator of Lyn.

Discussion

In this study we describe a previously unappreciated, nonredundant role for the RPTP CD148 in the regulation of B1 B cell BCR signaling. This is a striking contrast to our previous studies in which we reported that CD45 and CD148 had redundant functions in BCR signaling in MZ and follicular B cells (Zhu et al., 2008). Consequences of this non-redundant function of CD148 were evident in functional responses and in B1 B cell development. *Ptpj*−/− mice had a marked B cell-intrinsic defect in their ability to generate antigen-specific IgM in response to immunization with the TI-2 antigens Pneumovax 23 and NP-ficoll. This activation defect was associated with altered development of the endogenous BCR repertoire in B1, but not B2 B cells. The severity of the antibody response defect against TI-2 antigens in *Ptpj*−/− mice was unexpected for two reasons. First, CD45 and CD148 were demonstrated to have overlapping functions during ITAM-mediated signaling in conventional B cells and macrophages. Second, CD45 expression is many-fold higher than that of CD148 across all B cell subsets. Therefore, the nonredundant function of CD148 in B1 cells suggests that some intrinsic features of B1 cells or of the two RPTPs may influence their unique regulation, activity, or downstream effects on BCR signaling.
A distinguishing feature of our findings relating to B1 BCR signaling is the strong phosphorylation of both proximal and distal BCR signaling components and robust calcium mobilization after BCR engagement in wild type mice. While highly reproducible in our studies, these results contrast with previously published findings that describe B1 B cells as being anergic or hyporesponsive to BCR stimulation (Bikah et al., 1996; Chumley et al., 2002; Dal Porto et al., 2004a; Wong et al., 2002). However, some of these previous observations are based on the inability of B1 cells to proliferate in vitro after stimulation with BCR crosslinking antibody, which does not accurately reflect proximal BCR signaling events. Another possible explanation is that the B1-derived BCR transgenic models used in these studies rely on heavy and light chain arrangements derived from endogenous B1 BCRs, which are reactive against the known self-ligand phosphatidylcholine (Bikah et al., 1996; Chumley et al., 2002). Forced expression of a self-reactive BCR could promote the induction of an anergic phenotype that may not be representative of B1 B cells in an unrestricted repertoire. In one such system, transgenic B1 B cells had elevated expression of the negative regulator CD5 compared with non-transgenic B1 B cells, which has been shown to downregulate BCR signaling in B1 B cells and maintain tolerance in anergic follicular B cells (Bikah et al., 1996; Dal Porto et al., 2004a). Taken together with other studies describing antigen-induced proliferation of B1 cells in vivo (Alugupalli et al., 2003; Baumgarth et al., 1999; Haas et al., 2005; Krljanac et al., 2014), our results provide a new perspective on B1 B cell BCR responsiveness and B1 B cell physiology.
Our work to uncover the molecular mechanism by which CD148 exerts an important role in activation of B1 but not B2 B cells also led us to the surprising finding that Lyn appears to have a prominent positive rather than negative regulatory role in B1 BCR signaling. A complete understanding of how the role of Lyn kinase differs in B1 and B2 BCR signaling requires further investigation. However, an attractive candidate for study is the association of Lyn with the co-receptor CD19, which is expressed more highly on B1 B cells than conventional B cells and is required for their development and self-renewal (Fig. 2.2 D) (Haas et al., 2005; Krop et al., 1996; Sato et al., 1996). Lyn has been shown to amplify BCR signal strength through phosphorylation of CD19 and recruitment of further downstream signaling components in a positive amplification loop (Gauld and Cambier, 2004). Recruitment of Lyn to CD19 not only serves to amplify BCR signaling, but may also prevent negative regulation of BCR signaling by sequestering Lyn away from inhibitory receptors such as CD5, which was implicated in the negative regulation of B1a signaling (Bikah et al., 1996; Fujimoto et al., 2001). It is also noteworthy that the negative regulatory function of Lyn in conventional B cells depends upon CD22. B1a B cells express lower levels of CD22 than conventional B cells (Fig. 2.2 D), and unlike MZ and follicular B cells, deletion of CD22 does not affect B1 cell numbers or BCR signaling (Lajaunias et al., 2002; O'Keefe et al., 1996; Samardzic et al., 2002). Thus, the positive and negative regulatory circuitry may be wired differently in B1a cells compared to conventional B2 cells. In this regard, the activation of Lyn kinase by CD148 appears to have a predominantly positive effect on B1 BCR signaling.
Structural and catalytic differences between CD45 and CD148 may also contribute to the selective regulation of B1 BCR signaling by the latter. CD148 has a large extracellular domain with eight (or nine, depending on species) fibronectin type III domains that has multiple sites for N-linked glycosylation. CD45 has only 3 extracellular fibronectin domains, but its N-terminus contains a large number of O-glycosylated sites encoded in protein segments that are the products of alternatively spliced exons. The glycosylation status of RPTPs can influence their spatial localization on the cell surface and alter their ability to access substrate or modulate their interaction with inhibitory receptors such as CD22 thereby modulating cellular responses (Hermiston et al., 2009; Kelm et al., 2002). In macrophages, the large ectodomains of CD45 and CD148 caused them to be excluded from the immunologic synapse during signaling in response to the hemi-ITAM-containing receptor Dectin-1, thus allowing for discrimination between soluble and particulate antigens (Goodridge et al., 2011). It is conceivable that B1 cells, which preferentially respond to polyvalent antigens and to large, particulate antigens such as encapsulated bacteria, may employ CD148 via a similar mechanism of kinetic segregation to ensure an appropriate response to antigen. However, the fact that CD148-deficient B1 B cells have impaired BCR signaling after stimulation with the soluble antigen NP-ficoll and with crosslinking antibody implies that the role of CD148 in B1 signaling is not limited to antigen size discrimination.

Our previous studies provide strong evidence that CD148 exhibits substrate specificity for Lyn. In the context of G-protein coupled receptor signaling by chemoattractant receptors in neutrophils, different substrate specificities appear to
govern nonredundant functions of CD45 and CD148. That study showed that CD148 preferentially dephosphorylated Lyn, while CD45 had broader activity on the other SFKs Hck and Fgr expressed in neutrophils. In those experiments, neutrophils from Lyn-deficient mice phenocopied the signaling defect observed in Ptprj<sup>T</sup>-<sup>T</sup>-/T<sup>-</sup>-neutrophils (Zhu et al., 2011). Whereas limiting cell number precluded us from performing similar biochemical analyses in B1 B cells, the signaling phenotype in Lyn<sup>-/-</sup> B1 B cells was qualitatively similar to that in Ptprj<sup>T</sup>-<sup>T</sup>-/T<sup>-</sup>- B1 B cells, supporting the notion that CD148 also exhibits some specificity for Lyn in this cell type.

It is also possible that differences in enzymatic activity of the distinct phosphatase domains of CD45 and CD148 may explain why the loss of CD148 significantly impairs B1 signaling despite the presence of CD45. CD148 has only a single phosphatase domain, whereas CD45 has two tandem phosphatase domains, albeit the second one is inactive and is presumed to have a regulatory function (Hermiston et al., 2009). We tested the ability of the purified cytosolic domains of human CD45 and CD148 to dephosphorylate phosphopeptides corresponding to the C-terminal tails bearing the inhibitory tyrosine residues of the SFKs Lyn (FTATEGQpYQQQP) and Blk (FTATEGQpYELQP) in vitro. There was no apparent selectivity of either RPTP toward any of these substrates at the peptide level (Fig. 2.13 A), likely due to high sequence similarity. However, we did notice that CD148 dephosphorylated peptide and non-peptide substrates much more rapidly than did CD45 (Fig. 2.13 A and B). This is consistent with a previously published assessment of enzymatic activities of CD45 and CD148 against a panel of diverse phosphopeptides (Barr et al., 2009). Thus, despite being underrepresented on the
**A**  
Free phosphate production from peptide dephosphorylation

![Graph showing free phosphate production](image)

**B**  
Nitrophenol production from pNPP dephosphorylation

![Graph showing nitrophenol production](image)
**Figure 2.13. CD45 and CD148 have different phosphatase activities.**

(A) Free phosphate production as a result of dephosphorylation of synthetic C-terminal phosphopeptides of Lyn and Blk by the intracellular portion of human CD45 and CD148 proteins measured by colorimetric malachite green assay.

(B) Dephosphorylation of the synthetic substrate p-nitrophenyl phosphate by CD45 and CD148 measured by the production of p-nitrophenyl by colorimetric assay.

(A) depicts a single experiment and (B) is representative of three independent experiments.
surface of B cells, the higher enzymatic activity of CD148 and high relative expression in B1 cells may each account for the signaling defects observed in $Ptprj^{TM-/TM}$ B1 B cells. It is therefore tempting to speculate that the high phosphatase activity of CD148 in B1 cells may promote activation of the critical SFK Lyn in B1 B cells.

Our studies reveal that robust B1 BCR signaling is dependent on positive regulation via the CD148-Lyn axis. In contrast with B2 B cells, whose activation is stringently regulated by inhibition via the negative regulatory Lyn-CD22-SHP-1 pathway, CD148 may serve to tip the balance toward positive signaling by the B1 BCR, which has characteristically weak affinity for its ligands. Thus, at the expense of selectivity, CD148 increases the sensitivity of B1 B cells to weak ligand and allows them to be poised to respond robustly to a broad array low-affinity ligands such as Ti-2 antigens.

**Experimental procedures**

**Mice**

$Ptprj^{TM-/TM}$ and $Ptprj^{TM-il/TM-il}$ mice were generated and maintained as described (Zhu, 2008, Katsumoto 2013). Conditional deletion of CD148 in the B lineage was achieved by crossing $Ptprj^{TM-il/TM-il}$ mice with $mb1-cre$ knockin mice (Hobeika et al., 2006). B1-8i mice were obtained from Jackson Laboratory. B1-8i-$Ptprj^{TM-/TM}$ mice were generated by crossing $Ptprj^{TM-/TM}$ with B1-8i transgenic line and only mice carrying one copy of the transgene were used for experiments. $Lyn^{-/-}$ ($Lyn^{tm1Sor}$)
mice were obtained from Clifford Lowell at UCSF (Chan et al., 1997). All animals used in the experiments were at 8–16 weeks of age. All animals were housed in a specific-pathogen-free facility at UCSF and were treated according to protocols that were approved by university animal care ethics and veterinary committees and are in accordance with NIH guidelines.

**Antibodies and reagents**

Anti-mouse CD148 antibody was generated as previously described (Lin et al., 2004) and directly conjugated to PE or used with goat anti hamster FITC secondary antibody (Invitrogen). Antibodies to murine B220, CD19, CD5, CD23, CD21, IgM, IgD, CD69, CD11b, CD22, CD45, CD86, lambda light chain, and kappa light chain, conjugated to biotin, FITC, PE, PerCP, APC, PE-Cy7, or Pacific Blue were from BD Biosciences. Antibodies to Ki-67, phospho-BLNK (Y84), phospho-PLCγ2 (Y759), and phospho-Syk (Y352) that were directly conjugated to APC were purchased from BD Biosciences. Streptavidin-Pacific blue was from Life Technologies. Antibodies reactive with Phospho-CD79A (Y182), phospho-Erk (T202/Y204), phospho-Src family (Tyr416), phospho-Lyn (Tyr507), and total total Lyn antibody (44) was from Santa Cruz Biotechnology. Goat anti-mouse IgG, goat anti-mouse IgM F(ab’)2 fragments was from Jackson ImmunoResearch. NP-ficoll, NP-KLH, NP-LPS, NP-BSA, NP-PE, and NP-FITC-ficoll were from Biosearch Technologies. Imject Alum was from Thermo Scientific. Ionomycin was from Calbiochem (EMD Millipore). Phorbol myristate acetate (PMA) was from Sigma.
**Immunizations**

Mice were immunized i.p. or i.v. with 100 μg/mouse NP-50-ficoll, 100 μg/mouse NP-33-KLH in alum, 50 μg/mouse NP-0.15-LPS (Biosearch Technologies) or with 10 μg/mouse Pneumovax 23 (Merck) in PBS. Blood samples were collected from the tail at days 0, 7, 14, 21, and 28 after immunization.

**ELISA Assays**

Serum antibody titers were measured by sandwich ELISA. Briefly, 96-well vinyl assay plates (Costar) were coated overnight with 5 μg/ml NP-23-BSA (Biosearch Technologies) or with 5 μg/ml Pneumovax 23 in PBS. Plates were blocked for 1 h with PBS containing 0.2% Tween-20 and 1% BSA. Sera were serially diluted and incubated for 2 h at 37 °C, followed by a 2-h incubation with horseradish peroxidase-conjugated anti-mouse IgM or anti-mouse IgG₃ (Southern Biotech) at room temperature. For total serum Ig ELISA, plates were coated with goat anti-mouse heavy and light chain antibody, and various Ig isotypes detected with HRP-conjugated goat anti mouse IgG, IgG1, IgG₂a, IgG₃, and IgM using mouse Ig standards of known concentrations of the appropriate isotype (Southern Biotech). ELISA plates were developed using TMB (Sigma) and stopped with 2N sulfuric acid. Absorbance was read at 450 nm. Titers were expressed at ½ the maximal optical density for each assay or at an optical density in the linear range of the assay (specified in each figure) when maximal optical density was not achievable.

**In vivo B cell proliferation**
Mice were immunized i.p. with 10 μg/mouse NP-18-ficoll or PBS and peritoneal lavage cells and splenocytes were collected at 4 days. Antigen-specific proliferating B cells were permeabilized with BD Cytofix/Cytoperm reagent and stained in Perm/Wash buffer (BD Biosciences) with Alexa Fluor 647-conjugated anti-human Ki-67 antibody (BD Biosciences) according to manufacturers instructions.

**ELISpot**

Mice were immunized as described for antigen-specific proliferation assays, and peritoneal lavage cells and splenocytes were collected at days 4 or 7 under sterile conditions. Mixed cellulose ester MultiScreen filter plates (Miltenyi) were pre-wetted with PBS for 5 min and then incubated overnight with 5μg/mL NP-49-BSA. Plates were blocked with 5% FBS in PBS for 30 min at 37 °C. Cells were plated overnight in triplicate at 0.02-0.2 10⁶ cells/well. Spots were detected with goat anti mouse IgM or IgG₃ followed by SA-AP (all from Southern Biotech) and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate (Vector Labs). Scanned images of ELISpots were quantified using ImageJ software. For detection of total IgM or IgG₃ spots, plates were coated with 10μg/mL goat anti mouse heavy and light chain antibody (Southern Biotech).

**Cell isolation and stimulation**

Peritoneal lymphocytes were obtained by lavage of the peritoneal cavity with 7 mL PBS containing 2% FBS and 2 mM EDTA followed by 3 mL air. After gentle agitation,
lavage fluid was drained into a 50 mL conical tube. A single-cell suspension of splenocytes or lymph node cells was obtained by passing cells through a 40um nylon mesh filter. For intracellular phosphotyrosine staining, peritoneal and splenic lymphocytes were rested in RPMI1640 supplemented with 1% BSA and 20mM HEPES and then stimulated with goat anti-mouse IgM F(ab’)2 (Jackson Immunoresearch) at concentrations and times indicated in the text. Cells were fixed with 2% PFA and permeabilized with 90% ice-cold methanol followed by staining of cell surfaces and intracellular staining with anti-phospho-ERK1/2 (T202/Y204) and anti-phospho-CD79A (Y182) (Cell Signaling Technology), anti-phospho-Syk (pY542), anti-phospho-BLNK (Y84), and anti-phospho-PLCγ2 (Y759) (BD Biosciences). For Western blotting, peritoneal B1 cells were sort-purified by positive staining with FITC-conjugated anti-IgM Fab and dump staining with APC-conjugated antibodies against NK1.1, Gr-1, F4-80, CD3, Ter119 and CD23. Follicular B cells were isolated from lymph nodes and purified by negative selection (B Cell Isolation Kit, Miltenyi). Cells were rested in non-supplemented RPMI1640 at 37 °C for 30 min prior to stimulation with IgM F(ab’)2. At specified time points cells were lysed directly into SDS-PAGE.

*Flow cytometry and quantitation of CD45 and CD148*

Single-cell suspensions were prepared from peritoneal lavage, lymph nodes, spleen, and bone marrow. Fc receptors were blocked with rat anti-CD16/CD32 (2.4G2). 0.5-2 x 10⁶ cells were stained with the indicated antibodies and cell were analyzed on a Fortessa (BD Biosciences) and data analyzed with FlowJo software (TreeStar).
Quantitation of CD45 and CD148 surface expression was performed using BD Quantibrite PE Beads (BD Biosciences).

**Calcium-Sensitive Fluorescence Assay**

Total peritoneal lavage lymphocytes and splenocytes were loaded for 30 min at 37 °C with the fluorescent Ca²⁺ indicator dye Indo 1–AM (10µg/mL; Invitrogen) in RPMI1640 medium with 5% FBS and washed. B cells were then stained with FITC-conjugated anti-IgM Fab and non-B cells were excluded by dump staining (NK1.1, Gr-1, F4-80, CD3, Ter119; including CD23 for peritoneal cells only). Cells were kept on ice in 5% FBS in RPMI1640 medium. Prior to stimulation, cells were warmed to 37 °C for 3 min.

**Phosphatase assays**

Dephosphorylation of phosphopeptides was determined by malachite green phosphatase assay using purified protein corresponding to intracellular portion of human CD148 and CD45 proteins. Synthetic phosphopeptides containing the C-terminal inhibitory tyrosine of murine Lyn (FYTATEGQpYQQQP) and Blk (FYTATEGQpYELQP) were purchased from Biomatik. Briefly, protein at a 2x concentration of 4 nM (2nM desired final assay concentration) was diluted in assay buffer [150mM NaCl, 25mM HEPEs, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), 1% Tween20, at pH7.2]. Phosphopeptide was diluted in assay buffer at a 2x concentration of 100mM (50mM desired final assay concentration) and aliquoted into a 96-well vinyl assay plate (Costar). Reaction was started by addition of 25 µL
protein to 25 μL peptide and then stopped at the indicated times by the addition of 50 μL hydrogen peroxide. Free phosphate concentration was determined by generating a standard curve of known phosphate concentration (Millipore). Colorimetric analysis of free phosphate was done by adding malachite green solution [0.034% malachite green (Sigma), 10mM Ammonium Molybdate (Sigma), 1 N HCl, 3.4 % ethanol in water], and incubated at room temperature for 15 minutes. Absorbance was read at 620-660 nM on a spectrophotometer.

Dephosphorylation of the synthetic substrated p-nitrophenyl phosphate (pNPP, Sigma) was done by preparing CD148 and CD45 protein as described for the malachite green assay. CD45 and CD148 proteins were diluted in assay buffer at a 2x concentration of 80nM (40 nM desired final concentration) and aliquotted in a 96-well assay plate. Assay was started by addition of 2x concentration of pNPP substrate at 20 mM (10 mM pNPP final desired). At desired timepoints, assay was stopped with 1 M NaOH to convert the reaction product (p-nitrophenyl) into p-nitrophenolate, which can be detected colorimetrically by reading absorbance at 405 nM on the spectrophotometer. Concentration of dephosphorylated product p-nitrophenyl in the samples was determined by generating a standard curve of known concentrations of 4-nitrophenol (Sigma).

Statistical analysis

Prism (GraphPad) was used for statistical analyses with Mann Whitney and paired t-tests as indicated in the figure legends.
CHAPTER 3: DISCUSSION
Our current understanding of the role of BCR signal strength during B1 B cell development and antibody responses relies heavily on the use of genetic knockout or transgenic mouse models, which in many cases lead to the deletion of the B1 lineage or result in potentially abnormal B1 cell development. In this work, we use \textit{Ptp}\textsubscript{rj}\textsuperscript{TM-/-TM-} mice, which have intact numbers of phenotypically normal B1 B cells, to ask questions about the role of this PTP in regulating B1 B cell function. Defective responses to TI-2 antigen stimulation in the deficient mice led us to study the regulation of BCR signal strength by the phosphatase CD148 during B cell development and antibody responses. The data presented in Chapter 2 reveal that CD148 has a previously unappreciated nonredundant positive regulatory function in B1 B cells and suggest unique wiring of the B1 BCR pathway. These findings raise additional questions about how strength of BCR signaling affects different aspects of B1 cell physiology: 1) What are the implications of altered BCR repertoire development during B1 responses to infection or the development of autoimmune disease? 2) What unique properties of CD148 result in its requirement by B1 but not B2 B cells? 3) What new insights into the wiring of B1 B cells can be gained from these studies? Future lines of investigation that may lend themselves to answering some of these questions are discussed in this chapter.

**B1 BCR repertoire development**

We have demonstrated that \textit{Ptp}\textsubscript{rj}\textsuperscript{TM-/-TM-} mice are unable to mount a normal IgM response to the TI-2 antigen NP-ficoll. This was partially explained by a decrease in the presence of NP-reactive B1 B cells but not follicular or MZ B cells,
suggesting that CD148-mediated regulation of BCR signal strength is involved in establishing an appropriate B1 BCR repertoire.

As discussed in Chapter 1, B1 development is highly sensitive to the presence of positively-selecting autoantigen and the strength of BCR signaling. As such, the B1 repertoire appears to be enriched for weak autoreactivity, and these weak interactions with self-ligands result in the positive selection of specific B1 clonotypes (Berland and Wortis, 2002). This is illustrated by the SM/J transgenic mice, which express a BCR specificity for the thymocyte cell surface glycoprotein Thy-1 cloned from a B1 B cell-derived hybridoma. B1 B cells from SM/J mice were able to develop and produce anti-thymic antibodies only in mice where the Thy-1 antigen was expressed and not in Thy-1 null mice (Hayakawa et al., 1999).

We found that NP-specific peritoneal B1 B cells can be identified in wild-type by staining with fluorophore-conjugated NP. We also detected NP-specific IgM in the serum of unimmunized mice. Work from other groups that described the presence of endogenous NP-reactive IgM in the sera of normal mice is consistent with this, suggesting that NP-reactivity is a component of a normal B1 BCR repertoire and that NP-reactive BCRs of various affinities are endogenously encoded (Hsu et al., 2006; Maizels et al., 1988). These observations provide support for the notion that there exists an endogenous antigen that is cross-reactive with the NP-restricted BCR. Although the identity of this autoantigen is yet unknown, it is likely that the decreased BCR signal strength in the absence of CD148 resulted in impaired selection of B1 cells into the pool of NP-reactive B1 cells in PtprjTM-/-TM- mice. Given that the overall number of peritoneal B1 B cells and total serum IgM levels are
normal in \textit{Ptprj}^{TM/-TM} mice compared with wild-type, this result raises the possibility that there is a general skewing of the B1 BCR repertoire toward other specificities in mice with weaker B1 BCR signaling. An early study of transgene-expressing fetal liver and bone marrow B cell precursors described a mechanism for B cell repertoire maturation that demonstrates that very strong pre-BCR signaling may actually lead to the loss of B1 B cells. In that study, expression of an Ig transgene resulting in strong pre-BCR signaling resulted in the inhibition of expansion of fetal precursors, but not bone marrow-derived pre-B cells (Wasserman et al., 1998). In light of this, it is possible that decreased BCR signal strength is permissive for the persistence of B1 cell clones that are otherwise edited or deleted during development such as those reactive against single-stranded DNA (ssDNA) or those which produce rheumatoid factor (Rf). Increased numbers of ssDNA-reactive B1 B cells have been reported in patients with autoimmune disease and in mouse models of systemic lupus erythematosus (SLE) (Zhong et al., 2009). However, B1 cell development is restricted in knock-in transgenic mice expressing anti-ssDNA-specific BCRs (Fossati-Jimack et al., 2008). This suggests that B1 B cells are capable of expressing BCRs specific for ssDNA, but do not predominate in non-disease states. Increased production of Rf has been shown to be associated with stronger BCR signaling and an expansion of B1 B cells as a result of CD19 overexpression (Sato et al., 1996), and with the presence of bacterial factors which stimulate B1 B cells (Yamanishi et al., 2006). A targeted screen for IgM that reacts with various nuclear antigens and Rf production may begin to address whether the B1 repertoire is shifted toward or away from autoreactivity in CD148-deficient B1 B cells. As CD148-
deficiency also affects B1 B cell ability to respond to antigens a direct examination of
the B1 BCR repertoire sequences would complement such studies. A skewing
toward usage of certain heavy chains, specifically V_{H}11 and V_{H}12 appears to be
predetermined during B1 selection by signaling through the pre-BCR and generates
both antimicrobial and autoreactive specificities (Holodick and Rothstein, 2013;
Yoshikawa et al., 2009). Therefore, it would be interesting to determine by
sequencing of the B1 BCR repertoire whether specific common heavy and light chain
rearrangements that encode specificity for known self-antigens are under- or over-
represented in Ptprj^{TM/-TM} B1 B cells.

In addition to impaired IgM responses against the synthetic antigen NP-ficoll,
we also found that Ptprj^{TM/-TM} mice did not respond to immunization with
Pneumovax 23, which is also a TI-2 antigen made up pneumococcal polysaccharides
(PPS) derived from the capsules of 23 different serotypes of *Streptococcus
pneumoniae*. Due to lack of appropriate reagents, we were not able to directly detect
the presence of B1 B cells bearing BCRs specific for these antigens. However, it is
likely that Ptprj^{TM/-TM} mice also have a decreased presence of PPS-specific B1 B cells
prior to immunization, indicated by the small, but statistically significant, difference
in Pneumovax 23-specific IgM in the serum of unimmunized mice. This defect in
natural antibody indicates that Ptprj^{TM/-TM} mice would likely be more susceptible to
infection with *S. pneumoniae*. CD19-deficient (CD19^{-/-}) mice, which lack B1a B cells,
have decreased titers of natural IgM against heat killed *S. pneumoniae*, the
pneumococcal polysaccharide type 3 (PPS), and phosphocholine (PC). Consequently,
CD19^{-/-} mice are also more susceptible to peritoneal infection with live *S.
pneumoniae (Haas et al., 2005). Haas et al., and others, also showed that immunization of mice against S. pneumoniae, results in the production of specific antibodies that are protective against infection. Given that Ptprf^TM-/TM^- mice have decreased anti-pneumococcal natural IgM titers and are unable to mount an antibody response against Pneumovax 23, it is likely that these mice would be particularly susceptible to infection with S. pneumoniae.

There is evidence that B1 B cells, which reside in both the pleural and peritoneal cavities, are important for clearance of bacterial, fungal, and viral infection in the lung (Baumgarth et al., 1999; Baumgarth et al., 2000; Choi and Baumgarth, 2008; Szymczak et al., 2013; Weber et al., 2014). In collaboration with the laboratory of Dr. Mitchell Kronenberg at LIAI, we attempted to determine whether CD148 is important in the B cell-mediated clearance of pulmonary infection by S. pneumoniae. Because CD148 has been shown to play an important role in bacterial clearance by neutrophils (Zhu et al., 2011), Ptprf^TM-fl/TM-fl;mb1-cre mice were infected intratracheally with live S. pneumoniae to specifically address the role of B cells in the clearance of this pathogen. However, our preliminary studies were not sensitive enough to detect meaningful differences in the ability of Ptprf^TM-fl/TM-fl;mb1-cre and Ptprf^TM-fl/TM-fl mice to clear bacterial infection in the lung or in serum titers of anti-pneumococcal IgM (data not shown). Therefore, further studies using carefully titrated infectious doses of intratracheal or intraperitoneal infection may be informative. We have also previously attempted to assess the role of CD148 during lung infection by influenza as it has been shown that B1 B cells play a role in the immune response against this type of infection (Baumgarth et al., 1999;
Similarly to *S. pneumoniae* infection, we did not see differences in survival, weight loss and recovery, or anti-hemagglutinin IgM in the serum of infected mice (data not shown). However, unlike during sterile immunization with NP-ficoll or Pneumovax 23, various molecules containing pathogen-associated molecular patterns (PAMPs) such as peptidoglycan in the case of *S. pneumoniae* infection and nucleic acids in the case of viral infection may be released and engage toll-like receptors (TLRs) allowing for B1 B cells to overcome defects in BCR signaling during activation. Furthermore, the release of type 1 interferon (IFN-1), which is associated with influenza infection, may also influence and augment B1 cell responses (Li et al., 2012; Priest and Baumgarth, 2013). In support of this, we saw that immunization of mice with the TI-2 antigen NP-LPS, which engages TLR4 resulted in an intact IgM and IgG3 response in *Ptprj*<sup>Tm-/+</sup> mice, suggesting that additional signals in the form of TLR engagement are sufficient to rescue BCR signaling and activation of CD148-deficient B1 B cells.

B1 B cells are also found in the gut lamina propria and peyers patches and produce IgA, which can contribute to maintenance of normal gut flora (Kroese et al., 1996; Roy et al., 2013). It may therefore be interesting to investigate whether CD148-deficiency in B1 B cells alters gut homeostasis in mice. Finally, B1 B cells have been shown to produce various cytokines, including IL-10, and may have regulatory roles during diseases such as chronic colitis (Shen and Fillatreau, 2015; Shimomura et al., 2008; Szymczak et al., 2013). This suggests a potential role of CD148 in the regulation of immunomodulatory functions of B1 B cells in models of inflammatory bowel disease (IBD) and colitis.
CD148 regulation and phosphatase activity

Our immunization and signaling studies revealed that CD148 has a unique role in the specific regulation of B1 B cells since follicular and MZ B cell signaling, proliferation, and antibody production were unaffected in in Ptprj<sup>TM-/-</sup> mice, but impaired in B1 B cells. This finding was unexpected in light of previous work from our lab that demonstrated that CD45 is functionally redundant with CD148 and can compensate for the loss of the latter RPTP. In these studies, B cell development and proximal BCR signaling were minimally affected by the loss of either CD45 or CD148. The redundancy of the phosphatases was demonstrated by deletion of both CD45 and CD148, which resulted in a severe block in B cell development during the pre-B cell stage and was associated with markedly reduced tyrosine phosphorylation, Erk phosphorylation, and intracellular calcium mobilization (Zhu et al., 2011). The precise reasons why CD148 is uniquely required by B1 but not B2 B cells remain unclear, but the unique structure and phosphatase activity of CD148 offer some clues.

Recently, two ligands for CD148 have been described in studies of epithelial and endothelial cells: thrombospondin-1 and syndecan-2 (Takahashi et al., 2012; Whiteford et al., 2011). In the case of B cells, no ligand for CD148 has yet been identified, but it is possible that either one of the aforementioned ligands may play a role in regulation of B1 B cell responses through its interaction with CD148. Thrombospondin-1 (TSP-1) is a matricellular glycoprotein that is expressed by a variety of cells and has several known ligands including CD36 and CD47 (Kaur et al.,...
2014; Li et al., 2013). In endothelial cells, TSP-1 was found to interact with high affinity with the extracellular domain of CD148 resulting in the inhibition of cell growth, associated with reduced phosphorylation of Erk kinase (Lopez-Dee et al., 2011; Takahashi et al., 2012). TSP-1 is also expressed on monocytes and macrophages, which, along with dendritic cells, have been shown to interact with B1 and MZ B cells during TI responses (Balazs et al., 2002; Colino et al., 2002). We therefore wondered whether TSP-1 might be involved in the TI-2 response to NP-ficoll, which would suggest a potential regulatory mechanism via CD148. Our preliminary studies did not reveal any differences in anti-NP IgM production in mice lacking TSP-1. However, this result is complicated by the fact that TSP-1 belongs to a family of 5 thrombospondin proteins (1-5), which are structurally similar and may have overlapping functions (Lopez-Dee et al., 2011). It is not clear whether the other thrombospondin family members bind to CD148. The other known CD148-ligand, syndecan-2, has been shown to be inducibly expressed on the cell surface of human activated macrophages and to regulate cell growth by sequestering growth factor receptors by direct binding (Clasper et al., 1999). Thus, it remains to be determined whether syndecan-2 on mouse splenic or peritoneal macrophages can similarly regulate B1 B cell activity through ligand-dependent binding of CD148.

As described in Chapter 1, the bulky extracellular domains of CD45 and CD148 are excluded from the immunologic synapse during T cell engagement with an APC in the kinetic segregation model of TCR signal initiation (Chakraborty and Weiss, 2014; Lin and Weiss, 2003). The effects of CD148 exclusion from the site of antigen receptor engagement of ligand has different effects depending on cell type:
in CD148-expressing T cell lines, CD148 exclusion from the immunologic synapse resulted in down-regulated signaling to soluble TCR stimuli but prolonged receptor signaling to TCR ligands that were immobilized or to cell surface ligands (Baker et al., 2001; Cordoba et al., 2013; Lin and Weiss, 2003); in bone marrow-derived macrophages CD148 had a positive regulatory role when excluded from the phagocytic synapse (Goodridge et al., 2011). While it is known that the balance of tyrosine phosphorylation in B cells is important during signal initiation, it remains unclear whether the kinetic segregation model can be used to describe BCR signal initiation (Harwood and Batista, 2011). Although technically challenging, confocal microscopy of B1 B cells interacting with an APC or intact microorganisms could begin to address this. In the case of B1 B cells, this is a particularly interesting question, because unlike B2 B cells, which recognize protein antigens, B1 B cells are capable of directly binding large polymeric polysaccharide antigens such as those associated with whole bacterial or fungal microorganisms. Therefore, exclusion of CD45 and CD148 from the immunologic synapse may be a mechanism employed by B1 B cells for discrimination between soluble and particulate antigens, the latter of which might be representative of pathogen associated antigens and indicate a more immediate threat to the host (Goodridge et al., 2011). However, CD148-deficiency resulted in impaired BCR signaling in B1 B cells in response to soluble polymeric antigens or BCR crosslinking with soluble antibody, suggesting that other mechanisms of BCR signaling by CD148 must exist.

The observation that CD45 cannot compensate for the loss of CD148 in B1 B cells is puzzling since CD45 is far more highly expressed on the surface of B1 B cells.
than CD148. We hypothesized that some aspect of CD148 phosphatase activity may
account for this surprising phenotype. In previous work from our lab, it was
demonstrated that CD45 and CD148 have distinct functions in regulating
chemoattractant-mediated neutrophil migration during infection with
*Staphylococcus aureus* (Zhu et al., 2011). In this work, it was found that CD45 had an
exclusively positive regulatory role in signaling through G protein-coupled
receptors, while CD148 had both positive and negative regulatory functions. These
functional differences in GPCR regulation appear to be due to distinct selectivities of
CD45 and CD148 toward the negative regulatory tyrosine phosphorylation site of
different SFKs: CD45 is able to dephosphorylate the neutrophil SFKs Lyn, Hck, and
Fgr equally well, but CD148 appeared to be more selective for Lyn. Thus, the
dephosphorylation and activation of Lyn resulted in the activation of ITIM-mediated
inhibitory signaling leading to the emergence of a negative regulatory role for
CD148 in neutrophils. We therefore wondered whether similar specificity could be
apparent in B1 B cells. In *Ptprj* 

B1 B cells we detected decreased
phosphorylation of the activation loop in the kinase domain of SFKs and increased
phosphorylation of the inhibitory site, including Lyn. These changes were
associated with impaired phosphorylation of Lyn substrates (CD79A, Syk, PLCγ2),
suggesting that Lyn kinase may play an especially important role in B1 B cell
signaling. In light of this, we asked whether CD148 preferentially dephosphorylated
the inhibitory tyrosine of Lyn, compared with that of Blk or Hck, the main B1 B cell
SFKs. We performed *in vitro* phosphatase assays using purified recombinant
cytosolic portions of human CD45 and CD148 and found no differences in
dephosphorylation of the C-terminal phosphopeptide of Lyn (FYTATEGQpYQQQP) compared with that of Blk (FYTATEGQpYELQP). This suggests that substrate specificity of CD45 and CD148 is not determined at the C-terminal peptide level. However, it is still possible that substrate localization or overall SFK protein structure may contribute to the phosphatase specificity observed in neutrophils and B1 B cells. Thus, more advanced biochemical and imaging studies could offer some insight.

Although we did not notice any differences in peptide dephosphorylation between the two phosphopeptide substrates, we did find that the rate of dephosphorylation of synthetic phosphopeptide substrates as well as the alkaline phosphatase substrate PNPP was much more rapid by CD148 than by CD45 recombinant proteins that encompassed their cytoplasmic domains. This is consistent with previously reported phosphatase activity against a range of phosphopeptides (Barr et al., 2009). This finding is significant because it provides an explanation as to why CD45 phosphatase activity cannot compensate for the loss of CD148 in B1 B cells: despite much higher expression on the cell surface, the catalytic activity of the CD45 phosphatase domain is substantially lower. Thus, the combination of relatively high CD148 expression, its high phosphatase activity, and potential substrate specificity may explain the preferential dephosphorylation of Lyn in wild-type B1 B cells. A teleological explanation for the presence of this type of regulation of BCR signaling by CD148 and Lyn in B1 B cells is that B1 BCRs are reactive toward low-affinity ligands. Therefore, in order to be able to respond to their ligands, B1 B cell BCRs may need to be hyperresponsive compared with B2
BCRs. Furthermore, because B1-derived antibodies are of relatively low affinity, inappropriate activation of B1 B cells in response to autoantigens may be less detrimental to the host than aberrant production of high-affinity antibodies against self-antigen by follicular B cells, which must therefore be subject to strong negative regulation by ITIM-bearing inhibitory receptors.

**Unique BCR signaling in B1 B cells**

During our investigation of B1 B cells from *Ptprj*<sup>TM-/TM-</sup> mice and comparison with follicular and MZ B cells, we made the unexpected observation that peritoneal B1 B cells respond with very robust tyrosine phosphorylation of proximal BCR signaling molecules and increases in [Ca<sub>2+</sub>]. In fact, B1 B cells responded more robustly than follicular B cells when stimulated with low concentrations of α-IgM F(ab')<sub>2</sub>. This result was very reproducible, but somewhat inconsistent with earlier studies in which B1 B cells were reported to be hyporesponsive to BCR crosslinking. Current literature suggests that the hyporesponsiveness of B1 B cells is due to the expression of CD5 in B1 B cells and the interaction of this negative regulatory receptor with the inhibitory molecule SHP-1 (Bikah et al., 1996; Ochi and Watanabe, 2000; Sen et al., 1999; Wong et al., 2002). However, this model of B1 cell unresponsiveness or anergy is incomplete for a number of reasons. First, CD5 is only expressed on B1a and not B1b B cells. Therefore, BCR signaling and proliferation by B1b cell are not taken into account. In our analyses of B1 B cell signaling in Chapter 2, we did not distinguish between B1a and B1b cells. However, in retrospective reanalysis of phospho-flow data, it appears that B1a B cells did not induce Erk
phosphorylation to a lesser extent or more slowly than B1b cells from the same sample, and in fact, pErk induction was somewhat more robust in B1a B cells. This argues against a CD5-mediated mechanism of inhibition of B1a B cells because they signaled similarly to B1b cells, which do not express this inhibitory receptor. Another indicator of B cell anergy is elevated basal [Ca\textsuperscript{2+}]\textsubscript{i} in unstimulated cells and impaired mobilization of intracellular free calcium upon BCR crosslinking (Zikherman et al., 2012b). In peritoneal B1 B cells, the majority of which are B1a, we did not observe elevated [Ca\textsuperscript{2+}]\textsubscript{i} in resting cells and saw robust calcium mobilization upon stimulation, which provides further evidence against B1 B cell anergy. Finally, B1 B cells express high levels of surface IgM, which is also inconsistent with an anergic phenotype (Zikherman et al., 2012b). In T cells, CD5 expression is associated with increased TCR signal strength (Azzam et al., 2001). Therefore, it is likely that CD5 expression on B1a B cells, which express high levels of IgM, may reflect a high level of tonic BCR signaling in response to endogenous ligand and might serve to tune BCR signal strength rather than act as a strong inhibitor of BCR signaling after stimulation.

Another novel and important observation revealed by our studies was the dependence of B1 B cells on Lyn for BCR signaling. In studies of Lyn-deficient mice, this kinase was dispensable for positive BCR signaling in follicular B cells due to compensation by other SFKs and resulted in BCR hyperresponsiveness as a result of the loss of Lyn-mediated activation of ITIM-containing inhibitory receptor signaling by CD22 (Chan et al., 1998; Chan et al., 1997; Cornall et al., 1998; Gross et al., 2009). Interestingly, B1 B cells express low levels of CD22 in wild type mice and its deletion
has little to no effect on B1 development and BCR signaling (Lajaunias et al., 2002; O'Keefe et al., 1996; Samardzic et al., 2002). This strongly suggests that the same inhibitory circuits as B2 B cells do not regulate B1 B cells.

Instead, B1 B cells express relatively high levels of the inhibitory receptor Siglec-G, a member of the CD33-related sialic acid-binding immunoglobulin-like lectin family. The expression of this protein is restricted to B cells, with high expression in B1a B cells. Inhibition of calcium signaling was observed when overexpressed in the chicken B cell line DT40 (Hoffmann et al., 2007). Deletion of Siglec-G in mice (Siglecg−/−) results in the expansion of the B1a B cell population in the peritoneum, increased serum IgM levels, and augmented calcium mobilization after stimulation with BCR-crosslinking antibody. Interestingly, antibody responses to trinitrophenol (TNP)-ficoll or α1,3-dextran were not significantly affected in Siglecg−/− mice (Hoffmann et al., 2007). Instead, it is thought that the interaction of Siglec-G with its ligands (sialylated carbohydrates) may promote the induction of antigen-specific B cell tolerance (Muller and Nitschke, 2014). The mechanism by which Siglec-G mediates its inhibitory function is has not been fully elucidated, but Siglec-10, a related protein, is thought to bind SHP-1 via a conserved tyrosine in its cytoplasmic tail (Muller and Nitschke, 2014; Nitschke, 2014; Whitney et al., 2001). It is worthwhile to note that, while CD22 and Siglec-G both carry ITIMs in their intracellular domains, CD22 has three ITIMs and Siglec-G has a single ITIM. Thus, SHP-1 can bind two of the three ITIMs in CD22, but can only interact with one such ITIM in Siglec-G (Muller and Nitschke, 2014). This raises the possibility that the mechanism of inhibitory signaling by SHP-1 interaction with Siglec-G may be
different compared with CD22. It is known that the ITIMs of CD22 are phosphorylated by Lyn leading to recruitment of SHP-1, SHP-2 and SHIP, but it is unclear whether Siglec-G is regulated by a similar mechanism (Nitschke, 2014). A comparison of activation of negative regulatory molecules such as SHP-1 downstream Siglec-G and CD22 in B1 B cells and B2 B cells may be helpful in addressing why B1 BCR signaling is unique.

The activities of inhibitory receptors such as CD22 and Siglec-G are also regulated by their interactions with ligand in cis and in trans (Hutzler et al., 2014; Kelm et al., 2002). CD22 binds α2,6-linked sialic acid (α2,6Sia) residues on N-linked oligosaccharides, which are a component of glycoproteins on the surface of cells. IgM and CD45 have been identified as cis binding partner for CD22 (Sgroi et al., 1995; Stamenkovic et al., 1991; Zhang and Varki, 2004). In B1 B cells, expression of CD45 is comparable to that of B2 B cells, as identified by a “pan” CD45 antibody, which does not distinguish between splice variants of the molecule. However, staining with an antibody specific for the CD45R (B220) isoform reveals that B1 B cells express much lower levels of this epitope than do B2 B cells. This raises the possibility that much less of this potential cis ligand for CD22 is present on the surface of B1 B cells. The lower expression of this putative ligand for CD22 might offer a potential explanation for decreased dependence on CD22 for inhibitory signaling in B1 B cells.

The CD45R molecule can be identified by monoclonal antibodies against different epitopes: Lp-2, 2C2, and 62B, the latter of which is used as the B220 B cell marker due to restricted expression on this cell type; is also expressed at low levels.
on B1 B cells (Nishimura et al., 1992). However, the 6B2 epitope is resistant to treatment with sialidases, but is sensitive to treatment with chymotrypsin, suggesting that the peptide structure, rather than sugar modifications is responsible for the structure of the 6B2 epitope recognized by anti-B220 antibodies (Nishimura et al., 1992). The identity of the 6B2 epitope is unclear, but its resistance to sialidase treatment suggests that this portion of CD45 is not a ligand for CD22.

Instead, it is possible that the ability of CD22 and Siglec-G to interact with IgM defines their roles in BCR signal regulation in B1 and B2 B cells. Mutation of the ligand-binding domain of CD22 did not appear to affect its association with CD45 or IgM (Nitschke, 2005). In contrast, mutation of the ligand-binding region of Siglec-G resulted in a loss of its interaction with IgM and was associated with B1 B cell phenotypes similar to Siglec-G knockout mice. As a result, B1 cell-specific inhibitory function of Siglec-G is thought to be due to differential sialic acid binding (Hutzler et al., 2014). CD22 only binds $\alpha 2,6$Sia ligands, while Siglec-G can bind $\alpha 2,6$Sia and $\alpha 2,3$Sia and is the only B cell siglec that does so. The authors of these studies hypothesize that this feature of Siglec-G may be responsible for its ability to bind directly to surface IgM and could explain is selective inhibitory function in B1 B cells (Hutzler et al., 2014). Further work regarding the precise identity of inhibitory receptor ligands may be useful in gaining a better understanding of how B1 B cell signaling differs from that of B2 B cells. Due to the paucity of B1 B cells in the mouse, the use of B1-like B cell lymphoma lines such as BCL-1 or the CH lymphoma series might aid such studies (Haughton et al., 1986; Nishimura et al., 1992).
Instead of negative regulation of BCR signaling via ITIM-containing inhibitory receptors as in B2 B cells, we found that Lyn has a predominantly positive regulatory role in B1 antigen receptor signaling. B1 B cells express higher levels of CD19 compared with B2 B cells, and are sensitive to the loss or overexpression of this positive regulator of BCR signaling (Haas et al., 2005). CD19−/− mice have impaired B1a B cell development and are hyporesponsive to BCR signals, but interestingly they do not lack B1b cells, nor do they have impaired B1b responses (Engel et al., 1995; Haas et al., 2005; Rickert et al., 1995). Conversely, overexpression of CD19 results in augmentation of B1a development (Sato et al., 1996). CD19 is a positive regulator of BCR signaling through its interactions with, Vav, PI3K, and PLCγ1, and SFKs (Hasegawa et al., 2001; Sato et al., 1997). Moreover, Lyn is the SFK that is essential for its phosphorylation. In vitro kinase assays using purified Lyn suggest that its coincubation with purified CD19 decreases kinase activity, and that interaction of Lyn with CD19 could limit the availability of the kinase for interaction with inhibitory receptors such as CD22 (Fujimoto et al., 2000; Fujimoto et al., 2001). Thus, it is conceivable that through preferential engagement of the Lyn/CD19 positive regulatory loop and decreased activation of CD22-mediated inhibitory signaling, B1 B cells have unique wiring of BCR signaling that results in a predominantly positive regulatory role for Lyn in this lineage (Fig. 3.1).

Concluding remarks

In humans, CD148 is found in both hematopoietic and non-hematopoietic tissues and has implications in a variety of disease states. Differential CD148
Figure 3.1. Model of proposed mechanism of selective regulation of B1 BCR signaling by CD148.

High expression of CD148 in B1 B cells, as well as greater CD148 phosphatase activity and potentially preferential dephosphorylation of Lyn kinase by CD148 may lead to enhanced activation of Lyn in B1 B cells, compared with the less biased activation of all B cell SFKs by CD45 in B2 B cells, where CD148 expression is lower. Relative to B2 B cells, B1 B cells have high levels of the co-receptor CD19, and lower expression of the inhibitory receptor CD22, each of which are phosphorylated by Lyn to promote the recruitment of positive and negative effectors, respectively. This may result in wiring of the B1 BCR signaling pathway in such a way that positive BCR signaling outcomes are favored upon BCR crosslinking and subsequent Lyn activation, thus revealing a predominantly positive regulatory role for Lyn. This highlights a critical role for CD148 as a positive regulator of Lyn in B1 B cell signaling.
expression and phosphatase activity were observed in cases of rheumatoid arthritis where CD148 expression could be dynamically regulated by the presence of proinflammatory stimuli, synovial fluid, and various cytokines (Dave et al., 2013a; Dave et al., 2013b). CD148 is also found to be a useful marker for various diseases including chronic lymphocytic leukemia (CLL), and in distinguishing between different types of B-cell chronic lymphoproliferative disorders (B-CLPDs) (Fan et al., 2015). Importantly, CD148 is the only RPTP expressed on platelets and has a dominant role in regulating platelet activation via GPVI, the collagen receptor, and thrombosis in mice (Ellison et al., 2010; Mori et al., 2012; Senis et al., 2009). Therefore, drug targeting of phosphatases such as CD148 could prove to be effective in the treatment of arterial thrombosis via antiplatelet therapy (Tautz et al., 2015), and in the treatment of B cell lymphomas.

The presence of human B1 (CD20+CD27+CD43+) B cells has only recently been described; their ontogeny, identity, and function are under continued investigation (Griffin et al., 2011; Griffin and Rothstein, 2011; Rothstein et al., 2013). Recent studies have highlighted the clinical importance of human B1 B cells in bone marrow transplantation, multiple sclerosis, and common variable immunodeficiency (CVID) (Rothstein and Quach, 2015). Vaccination of humans with polysaccharide vaccines against Streptococcus pneumoniae results in the rapid expansion of a B1-like antigen-specific memory B cell population, drawing some parallels to the already present body of work in mice (Leggat et al., 2013a; Leggat et al., 2013b). Thus, the function and regulation of human B1 B cells appears to be an emerging and clinically relevant field.
APPENDIX

Regulation of activating and inhibitory BCR signaling by Csk
**Summary**

Activation of Src family kinase (SFKs) is a critical step in the initiation of the B cell antigen receptor (BCR) signaling cascade. Csk regulates SFK activity in the basal state and during BCR ligation. To understand how Csk regulates SFK activity, we took advantage of the CskAS mice, which express a Csk variant allele that can be selectively inhibited using an analog of the kinase inhibitor PP1. Inhibition of CskAS resulted in robust activation of proximal BCR signaling in the absence of receptor ligation, but did not result in receptor-independent activation of the distal BCR signaling pathway. Furthermore, CskAS inhibition had a negative overall effect on ligation-induced BCR signaling. CskAS inhibition was also associated with robust activation of inhibitory pathways, including CD22, Dok-1, SHP-1, and SHIP-1.

Deletion of the SFK Lyn, which is a positive regulator of inhibitory signaling resulted in only partial rescue of BCR signaling resulting from CskAS inhibition, suggesting that other SFKs can participate in inhibitory signaling if sufficiently activated. These studies indicate that inhibitory pathways are dominant over positive BCR signaling, and suggest that physical events associated with BCR ligation are required for shifting the balance toward productive BCR signaling.

**Introduction**

Csk is a ubiquitously expressed cytosolic protein that has a critical role in the negative regulation of SFKs (Okada, 2012). Due to the profound alterations in T cell
development in mice with a conditional deletion of Csk, existing studies regarding
the function and regulation of Csk during antigen receptor signaling had been
limited to cell lines (Schmedt et al., 1998). In studies using siRNA-mediated
knockdown of Csk in Jurkat T cells, decreased Csk expression resulted in augmented
TCR signaling and cytokine secretion (Vang et al., 2004), while overexpression of
Csk had the opposite effect on T cell signaling and activation (Chow et al., 1993). In
the DT40 chicken B cell line, deletion of Csk was associated with constitutive
phosphorylation of Lyn at its autophosphorylation site, as well as constitutive
activation of Syk. However, spontaneous calcium mobilization, IP₃ production, and
phosphotyrosine induction did not occur without BCR crosslinking, suggesting that
activation of Lyn and Syk in the absence of Csk is insufficient for full BCR signal
transduction in DT40 cells (Hata et al., 1994). However, a caveat of these
knockdown and gene deletion studies is the possibility that cells may develop
compensatory mechanisms in the absence of a critical negative regulator, thus
altering the normal signaling networks that exist in wild-type cells. Given these
relatively limited studies, the role of Csk in the dynamic regulation of positive and
negative pathways during antigen receptor signaling in primary B cells remains
relatively poorly understood.

Recently, in collaboration with the laboratory of Kevan Shokat, the Weiss
laboratory has taken a chemical-genetic approach to overcome some of the
described limitations associated with studying the role of Csk-mediated regulation
of antigen receptor signaling. To do this, Schoenborn and colleagues developed an
analog-sensitive variant of Csk (CskAS) in which the conserved gatekeeper residue
was mutated (T266G) to accommodate a larger analog of the kinase inhibitor PP1 (Fig. A1) (Tan et al., 2013). This allows for the selective inactivation of the mutant kinase in vitro. These studies revealed that a balance between the effects of Csk and CD45 dynamically controls the activity of the SFK Lck. Rapid inhibition of Csk was sufficient to activate canonical TCR signaling in the absence of TCR engagement by ligand (Schoenborn et al., 2011). In contrast with T cells stimulated through the antigen receptor, T cells activated through inhibition of CskAS had sustained and enhanced phosphorylation of TCR pathways suggesting that Csk has a role in signal termination. Upon inhibition of CskAS, rapid phosphorylation of the inhibitory adaptor molecule downstream of kinase 1 (Dok-1) was observed. Dok-1 is an adapter molecule, which binds to the SH2 domain of Csk and promotes its localization to the plasma membrane allowing it to participate in a negative feedback circuit to modulate TCR signaling (Yasuda et al., 2007). Analogously, it has been found that the inhibitory receptor leukocyte-associated Ig-like receptor (LAIR)-1 may exert a negative regulatory function in DT40 B cells through its interaction with Csk via the kinase’s SH2 domain (Verbrugge et al., 2006).

In further studies by Tan et al., the CskAS allele was introduced into Csk−/− mice in order to facilitate the study of Csk function in unmanipulated primary cells (Tan et al., 2014). In thymocytes derived from these mice, inhibition of CskAS resulted in robust proximal phosphorylation of TCR signaling components up to phospholipase C-γ1 (PLCγ1), but impaired downstream Erk phosphorylation and inositol phosphate production were observed. Similar to the earlier cell line data, Csk also had a critical role in preventing TCR signaling. The block in signaling
WT Csk  Csk$^{AS}$\textsuperscript{(T266→G)}

Reused with permission from Tan, et al., Cold Spring Harb Symp Quant Biol, 2013
Figure A1. Generation of the analog sensitive Csk variant.

Modification of the gatekeeper residue from threonine to the smaller glycine (T226G) in the catalytic pocket of Csk results in the generation of the CskAS variant. The enlarged ATP-binding pocket of CskAS can accommodate a modified, bulkier analog of the nonselective kinase inhibitor PP1, thus allowing for specific inhibition of CskAS but not wild type kinases.
downstream of PLCγ1 was relieved by disruption of the actin cytoskeleton using
actin depolymerizing drugs or by costimulation via CD28 in immature thymocytes
with varying results in more mature T cells (Tan et al., 2014). Thus, CskAS inhibition
revealed requirement for remodeling of the actin cytoskeleton mediated by
engagement of costimulatory molecules such as CD28 for the potentiation of full
TCR signaling (Tan et al., 2014). These results in T cells expanded upon findings in B
cells that implicated actin cytoskeleton dynamics in regulation of BCR signaling
(Treanor et al., 2010). In these latter studies, perturbation of the actin cytoskeleton
via actin stabilizing or actin depolymerizing drugs was sufficient to induce calcium
mobilization in B cells, presumably a consequence of disrupting BCR diffusion
dynamics (Treanor et al., 2010). Whether and how the regulation of antigen
receptor signaling by Csk differs in B and T cells remains unclear.

In the Appendix to this thesis, I take advantage of the CskAS model system to
study the dynamic regulation of SFKs in B cells and their activation of ITAM- and
ITIM-mediated signaling. This section describes emergent findings in the regulation
of the balance between positive and negative signals in B cells using selective
inhibition of Csk in the CskAS transgenic system.

Results

CskAS mice were generated by introducing a variant allele of Csk that is
sensitive to the PP1 kinase inhibitor analog 3IB-PP1 via bacterial artificial
chromosome (BAC) transgenesis into a Csk-null (Csk\(^{-/-}\)) background (Tan et al., 2014). The Csk\(^{AS}\) mouse line expresses approximately 2.5-fold as much Csk as wild-type mice, but exhibits grossly normal T and B cell development (Fig. A2 A-C) (Tan et al., 2014).

Treatment of purified B cells with 3IB-PP1 resulted in increased global tyrosine phosphorylation in Csk\(^{AS}\) B cells but not wild-type B cells (Fig. A3 A). The overall tyrosine phosphorylation pattern in Csk\(^{AS}\) B cells treated with 3IB-PP1 is similar to the phosphorylation pattern that results from stimulation of wild-type or Csk\(^{AS}\) B cells via antibody-mediated BCR crosslinking in the absence of inhibitor (Fig. A3 B). Simultaneous inhibition of Csk\(^{AS}\) and BCR crosslinking via anti-IgM F(ab')\(_2\) appears to augment the level of tyrosine phosphorylation above that which is achieved with BCR crosslinking alone (Fig. A3 B). Thus, inhibition of Csk\(^{AS}\) appears to result in the activation of BCR proximal signaling components, presumably as a result of SFK activation.

To test directly for activation of SFKs we blotted for the activating (pSrc Y416) and inhibitory (pLyn Y507) tyrosines of SFKs. Inhibition of Csk\(^{AS}\) resulted in marked dephosphorylation of the SFK inhibitory C-terminal tyrosine within 30 seconds after addition of the inhibitor. Accordingly, the activating tyrosine was strongly hyperphosphorylated (Fig. A4 A). However, at late timepoints (10 minutes) phosphorylation of pSrc Y416 was not detectable, suggesting potential negative regulation due to the activation of negative regulatory phosphatases. Alternatively, the loss of phosphorylation at pSrc Y416 could be explained by ubiquitin-mediated degradation of the SFK Lyn, which has been observed in Csk\(^{AS}\) mice.
A

% SP B cells

% B220+ lymphocytes

WT ×

CskAS

B

B cell subsets

% of cells

FM ×

MZ ×

T1/T2 ×

C

CD86

CD5

MHCII

CD23

IgM

WT ×

CskAS

% of max

MFI
Figure A2. B cell development is grossly normal in Csk\textsuperscript{As} mice.

(A) Flow cytometric analysis of the percentage of total B cells in the spleens of wild-type and Csk\textsuperscript{As} mice, gated on the percent of B220\textsuperscript{+} splenocytes.

(B) Flow cytometric analysis of development of B cell populations in the spleens of wild-type and Csk\textsuperscript{As} mice, gated on follicular (CD23\textsuperscript{+}CD21\textsuperscript{−}) marginal zone (MZ) (CD23\textsuperscript{−}CD21\textsuperscript{−}) and transitional (T1/T2) (CD23\textsuperscript{−}CD21\textsuperscript{−}) B cells as a percent of B220\textsuperscript{+} splenocytes.

(C) Flow cytometric analysis of surface markers expressed on wild type and Csk\textsuperscript{As} follicular B cells

For (A) and (B), the average of three animals in one experiment is shown, error bars represent standard error of the mean. For (C) one animal of each genotype is depicted from a single experiment.
Figure A3. Inhibition of Csk<sup>AS</sup> results in increased global tyrosine phosphorylation.

(A) Purified splenic B cells were treated with 10 µM of the 3IB-PP1 inhibitor (i) or vehicle (DMSO, D) for the indicated times and cell lysates were analyzed by immunoblotting with anti-phosphotyrosine antibody clone 4G10.

(B) B cells were treated as in (A) but 10 µg/mL anti-IgM F(ab’)<sub>2</sub> ("αIgM") was added simultaneously with 10 µM 3IB-PP1.

(A) and (B) depict a single experiment.
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Figure A4. Inhibition of Csk$^{AS}$ results in hyperactivation of SFKs and phosphorylation of proximal BCR signaling molecules.

(A) Purified splenic B cells were treated with 10 µM 3IB-PP1 (i) for the indicated times and cell lysates were analyzed by immunoblotting with the indicated phospho-specific antibodies.

(B) B cells were treated as in (A) but 10 µg/mL anti-IgM F(ab')2 (IgM) was added simultaneously with 10 µM 3IB-PP1.

(A) and (B) are representative of at least two independent experiments.
macrophages treated with 3IB-PP1 (Freedman, et al., manuscript in press), and following aggregation of the high affinity IgE receptor (FceRI) in mast cells (Kyo et al., 2003). Inhibition of CskAS in combination with BCR crosslinking appeared to result in somewhat enhanced phosphorylation of pSrc Y416 compared with 3IB-PP1 treatment alone at early timepoints, and was associated with a more rapid loss of pSrc Y416 phosphorylation, which was visible as early as 2 minutes after treatment and was nearly absent by 10 minutes. Phosphorylation of the SFK targets CD79A, Syk, and PLCγ2 corresponded to SFK activation upon inhibition of CskAS (Fig. A4 A and B, Fig. A5). These data indicated that inhibition of CskAS results in the rapid dephosphorylation the C-terminal inhibitory tyrosine of SFKs and their robust activation. This allowed for the initiation of early BCR proximal signaling events in the absence of BCR crosslinking.

Activation of PLCγ2 by BCR stimulation results in the hydrolysis of PtdIns(4,5)P₂ (PIP₂) and the formation of the second messengers IP₃ and diacylglycerol, which are necessary for the initiation of calcium-mediated signaling and activation of the MAPK/Erk pathway (Gauld and Cambier, 2004). We therefore predicted that inhibition of CskAS would result in activation these downstream signaling pathways. Surprisingly, despite robust activation of proximal signaling molecules, inhibition of CskAS did not result in increased Erk phosphorylation (Fig. A6 A). Furthermore, CskAS inhibition in combination with a range of concentrations of anti-IgM F(ab’)₂ resulted in abrogated Erk phosphorylation (Fig. A6 B). Similarly, treatment of CskAS B cells with 3IB-PP1 resulted in only mild and transient mobilization of intracellular calcium, and dampened calcium signaling in response
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**pCD79A (Y182)**

**Total ERK**

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**pCD79A (Y182)**

**Total ERK**
Figure A5. Inhibition of Csk<sup>A5</sup> results in phosphorylation of the ITAMs of the BCR.

(A) Purified splenic B cells were treated with 10 µM 3IB-PP1 (i) for the indicated times and cell lysates were analyzed by immunoblotting with anti-phospho Igα (pCD79A). Total Erk was used as a loading control. Asterisks mark lanes with degraded sample and do not represent meaningful data.

(B) B cells were treated as in (A) but 10 µg/mL anti-IgM F(ab’)<sub>2</sub> (αIgM) was added simultaneously with 10 µM 3IB-PP1.

(A) and (B) depict a single experiment.
A. 

- **WT** and **Csk\textsuperscript{AS}** cell lines treated with DMSO or 3IB-PP1, followed by PMA at different time points (0, 2 min, 5 min, 15 min).

B. 

- Concentration of anti-IgM f(ab\textsuperscript{'})\textsubscript{2} in μg/mL for DMSO, 2.5, 5, 10, 20, and PMA.
- **WT** and **Csk\textsuperscript{AS}** cell lines treated with 3IB-PP1, showing the percentage of max pErk at different time points (0, 50, 100, 150 s).

C. 

- Indo-1 (violet/blue) intensity over time (s) for 3IB-PP1, WT, Csk\textsuperscript{AS}, 3IB-PP1 + anti-IgM f(ab\textsuperscript{'})\textsubscript{2}, DMSO + anti-IgM f(ab\textsuperscript{'})\textsubscript{2}.
Figure A6. Inhibition of Csk<sup>AS</sup> results in impairment of distal BCR signaling.

(A) Flow cytometric analysis of intracellular phospho-Erk (pErk) staining of splenic follicular B cells (B220<sup>+</sup>CD23<sup>+</sup>CD21<sup>-</sup>) treated for the indicated times with 10 µM 3IB-PP1 or vehicle (DMSO).

(B) Flow cytometric analysis as in (A), in follicular B cells treated with the indicated concentrations of anti-IgM F(ab')<sub>2</sub> at two minutes with and without 3IB-PP1.

(C) Flow cytometric analysis of intracellular calcium mobilization splenic B cells (gated on IgM Fab<sup>+</sup> and dump<sup>-</sup>) loaded with Indo-1 and treated with 10 µM 3IB-PP1, 10 µg/mL anti-IgM F(ab')<sub>2</sub> (αIgM), or a combination of both.
to BCR crosslinking (Fig. A6 C). Thus, despite SFK-mediated phosphorylation of proximal signaling components, downstream BCR signaling was markedly impaired.

Previous work from our lab in Csk\textsuperscript{AS} has shown that disruption of the actin cytoskeleton by the actin depolymerizing drug cytochalasin D was able to restore impaired calcium and Erk signaling which occurs upon Csk\textsuperscript{AS} inhibition in immature CD4+CD8+ (DP) thymocytes. This rescue in signaling was likely due either to an increase in accessibility of PLC\textgamma{}1 to membrane-localized PIP\textsubscript{2} or to the release of PIP\textsubscript{2} from actin-binding or actin-regulatory proteins with which it associates (Tan et al., 2014). We therefore wondered whether disruption of the actin cytoskeleton in B cells might similarly restore calcium mobilization upon Csk\textsuperscript{AS} inhibition, or whether Csk\textsuperscript{AS} inhibition-induced SFK activation might augment the spontaneous increase in intracellular calcium induced by cytochalasin D that has been reported by others (Treanor et al., 2010). To test this, we treated B cells with cytochalasin D alone, or with simultaneous addition of 3IB-PP1. In our studies, cytochalasin D treatment resulted in the release of intracellular calcium (Fig. A7), albeit with slower kinetics and lesser overall magnitude of signaling in comparison with the calcium mobilization that results from BCR crosslinking. Strikingly, in contrast with the published findings in thymocytes, we found that Csk\textsuperscript{AS} inhibition had an overall inhibitory effect on the spontaneous calcium mobilization that occurs as a result of actin depolymerization (Fig. A7). A major difference between B and T cell antigen receptor signaling is the presence of a variety of ITIM-containing inhibitory receptors which can be phosphorylated by the SFK Lyn (Billadeau and Leibson, 2002). Hence, it is possible that equally robust activation of ITIM-mediated
A

3IB-PP1

WT

CsK\textsuperscript{AS}

Cytochalasin D + DMSO

Cytochalasin D + 3IB-PP1

anti-IgM f(ab')\textsubscript{2} + DMSO

\text{Indo-1 (violet/blue)}

\text{time (s)}

0 50 100 150

0 3 4 5 6

3IB-PP1

WT

CsK\textsuperscript{AS}

Cytochalasin D + DMSO

Cytochalasin D + 3IB-PP1

anti-IgM f(ab')\textsubscript{2} + DMSO

\text{Indo-1 (violet/blue)}

\text{time (s)}

0 50 100 150

0 3 4 5 6
Figure A7. Actin depolymerization does not rescue impaired BCR signaling upon CskAS inhibition.

Flow cytometric analysis of intracellular calcium mobilization in splenic B cells (gated on IgM Fab⁺ and dump⁻) loaded with Indo-1 and treated with 10 µM 3IB-PP1, 10 µM cytochalasin D, vehicle (DMSO), or 10 µg/mL anti-IgM F(ab’)₂.

Data are representative of at least 2 independent experiments.
inhibitory receptors is responsible for impaired ITAM-mediated downstream signaling that is induced by CskAS inhibition, BCR crosslinking, or disruption of the actin cytoskeleton.

CD22 is a B cell-specific inhibitory protein that quickly becomes tyrosine phosphorylated by Lyn on its intracellular ITIM-containing domain following BCR engagement due to its association with surface IgM. ITIM phosphorylation of CD22 allows for the recruitment, phosphorylation and activation of the tyrosine phosphatase SHP-1, which is able to directly dephosphorylate various proximal signaling molecules with which it is co-aggregated (Nitschke, 2005; Waterman and Cambier, 2010). We detected increased phosphorylation of CD22 in B cells after BCR stimulation and substantially more CD22 phosphorylation upon CskAS inhibition (Fig. 8A). BCR stimulation and CskAS inhibition had an additive effect on CD22 phosphorylation when used in combination (Fig. A8 A). This might imply that in resting B cells, CD22 is not as readily accessible to Lyn, which associates with the BCR, and that BCR crosslinking allows for spatial reorganization of CD22 on the cell membrane and increases its proximity to Lyn. In accordance with increased CD22 phosphorylation, we also detected increased phosphorylation of the CD22-associated inhibitory molecule SHP-1 at Y564 after CskAS inhibition (Fig. A8 B). Phosphorylation of SHP-1 at this residue has been shown to increase SHP-1 phosphatase activity (Yoshida et al., 1999). However, unlike what was observed in other SFK phosphorylation targets, BCR stimulation did not appear to have an additive effect on SHP-1 phosphorylation during CskAS inhibition, suggesting that receptor crosslinking is sufficient for maximal activation of SHP-1 (Fig. A8 C). This
Figure A8. Inhibition of Csk\textsuperscript{AS} results in activation of ITIM-mediated inhibitory pathways.

(A) Purified splenic B cells were treated with 10 µM 3IB-PP1 (i), DMSO (D), or 10 µg/mL anti-IgM F(ab')\textsubscript{2} (αIgM), or both for the indicated times and cell lysates were analyzed by immunoblotting with anti-phospho CD22 antibody. Total Erk was used as a loading control.

(B and C) B cells were treated as in (A), and lysates were blotted for phosphorylated SHP-1. Asterisks mark lanes with degraded sample and do not represent meaningful data.

(A) and (B) depict a single experiment.
might imply that the amount of activated SHP-1 rather than inhibitory receptor abundance is the limiting factor in ITIM-mediated inhibitory signaling.

Another phosphatase that is involved in negative regulation of BCR signaling is the inositol phosphatase SHIP-1, which is involved in the hydrolysis of PI(3,4,5)P$_2$ (PIP$_3$) to PI(3,4)P$_3$. PIP$_3$ is essential for the activation of various positive BCR signaling components (Bolland et al., 1998; Okada et al., 1998). Strong SHIP-1 phosphorylation was observed upon Csk$^{AS}$ inhibition, but unlike SHP-1, its phosphorylation upon 3IB-PP1 treatment was greater than that achieved with BCR crosslinking alone, and did not appear to be induced to a greater degree after treatment with both anti-IgM F(ab')$_2$ and 3IB-PP1 (Fig. A9). The adapter molecule downstream of kinase 1 (Dok-1) is also phosphorylated by Lyn, and has been shown to associate with SHIP-1 in anergic B cells (O’Neill et al., 2011; Xu et al., 2005b). We observed inducible phosphorylation of Dok-1 in B cells when Csk$^{AS}$ was inhibited (Fig. A9). This might implicate phosphorylation of Dok-1 in the recruitment of SHIP-1 via its SH2 domains to the plasma membrane, localizing it to its substrate PI(3,4,5)P$_2$. Because SHP-1 phosphorylation upon Csk$^{AS}$ inhibition occurs to a similar degree as during BCR crosslinking in the absence of 3IB-PP1, but SHIP-1 is significantly more phosphorylated by Csk$^{AS}$ inhibition than by BCR activation, we reasoned that the inhibitory mechanism of Csk$^{AS}$ inhibition is mediated in large part by SHIP-1.

SHIP-1 exerts its negative regulatory function through depletion of PIP$_3$, which is necessary for the recruitment of various BCR signaling molecules to the plasma membrane, including Btk, SOS, Vav, and Akt (Bolland et al., 1998; Gold et al.,
Figure A9. Inhibition of Csk<sup>AS</sup> results in activation of the Dok-1—SHIP-1 inhibitory pathway.

Purified splenic B cells were treated with 10 μM 3IB-PP1 (i), DMSO (D), or 10 μg/mL anti-IgM F(ab′)<sub>2</sub> (αIgM) for the indicated times and cell lysates were analyzed by immunoblotting with the specified antibodies. Total Erk was used as a loading control.

Data depict a single experiment.
Recruitment of Akt to the plasma membrane induces a conformational change that allows it to become phosphorylated by upstream kinases, leading to its activation (Gold et al., 1999). To test whether the inhibitory effects of CskAS inhibition were due at least in part to depletion of PIP₃, we measured Akt phosphorylation by intracellular staining in the presence and absence of CskAS inhibition and BCR crosslinking. Inhibition of CskAS led to a small and temporary increase in Akt phosphorylation by 2 minutes of treatment with inhibitor and subsided fully by 10 minutes. This early and transient activation of Akt is similar to the small amount of intracellular calcium mobilization that occurs upon inhibition of CskAS and is indicative of BCR signaling initiation and potential subsequent downregulation by inhibitory signaling (Fig. A10 A). In contrast with calcium mobilization and Erk phosphorylation, significant Akt phosphorylation was induced by BCR crosslinking even in the presence of CskAS inhibition (Fig. A10 A). However, the inducible Akt response was dampened in CskAS B cells compared with wild-type B cells, which are not sensitive to inhibition of Csk (Fig. A10 A). Furthermore, increased BCR signaling by crosslinking with higher concentrations of anti-IgM F(ab')₂ appeared to be able to overcome the inhibitory effects of CskAS inhibition on Akt phosphorylation (Fig. A10 B). These data provide further evidence of BCR signaling initiation through SFK activation during inhibition of CskAS, and are suggestive of rapid engagement of inhibitory mechanisms, one of which is the depletion of PIP₃ by SHIP-1.

The SFK Lyn has been shown to have both positive and negative regulatory functions through its phosphorylation of both ITIMs and ITAMs, with the net effect
Figure A10. Akt signaling is impaired upon Csk\textsuperscript{A5} inhibition.

(A) Flow cytometric analysis of intracellular Akt (pS 473) staining in splenic follicular B cells treated with 10 μM 3IB-PP1, DMSO, 10 μg/mL anti-IgM F(ab')\textsubscript{2} or both for the indicated times.

(B) B cells treated as in (A) for 2 minutes with 10 μM 3IB-PP1, DMSO, or the indicated concentrations of anti-IgM F(ab')\textsubscript{2}.

Data are representative of two individual experiments.
of Lyn activity on BCR signaling being predominantly negative (DeFranco et al., 1998; Gross et al., 2009). In consideration of this, and the apparent activation of Lyn-mediated inhibitory signaling in Csk²⁻ B cells treated with 3IB-PP1, we wanted to know whether deletion of Lyn kinase would rescue positive signaling in the presence of Csk²⁻ inhibition. To test this, we crossed Csk²⁻ mice onto a Lyn deficient (Lyn⁻⁻) background to generate Lyn⁻⁻ Csk²⁻ mice. We found that deletion of Lyn resulted in somewhat greater calcium mobilization upon Csk²⁻ inhibition, but that this response was similarly transient as in Lyn-sufficient Csk²⁻ B cells (Fig. A11 A). Furthermore, disruption of the actin cytoskeleton only mildly augmented calcium mobilization upon Csk²⁻ inhibition in Lyn⁻⁻ Csk²⁻ B cells. The rapid decrease in intracellular calcium that is in Lyn⁻⁻ Csk²⁻ B cells treated with 3IB-PP1 suggests that activation of some inhibitory signaling pathways could still occur in the absence of Lyn (Fig. A11 A).

Partially intact negative regulation of BCR signaling during Csk²⁻ inhibition was also evident in Lyn⁻⁻ Csk²⁻ B cells when these cells were stimulated by BCR crosslinking in the presence of 3IB-PP1. In control conditions, BCR stimulation of Lyn⁻⁻ Csk²⁻ or Lyn⁻⁻ B cells resulted in enhanced calcium mobilization compared with Csk²⁻ B cells, which respond like wild-type in the absence of 3IB-PP1 (Fig. A11 B, Fig. A6 C). These findings are consistent with previous reports that describe BCR hyperresponsiveness in Lyn⁻⁻ mice (Chan et al., 1997; Fujimoto et al., 2001). However, upon Csk²⁻ inhibition, Lyn deficiency restored calcium mobilization of Lyn⁻⁻ Csk²⁻ to levels similar to those of control Csk²⁻ B cells that were not treated with 3IB-PP1 (Fig. A11 B). Thus, even in the absence of Lyn, Csk²⁻ inhibition had a
Figure A11. Lyn-deficiency results in a partial rescue of impaired BCR signaling upon Csk\textsuperscript{A5} inhibition.

(A) Flow cytometric analysis of intracellular calcium mobilization in splenic B cells (gated on IgM Fab\textsuperscript{+} and dump\textsuperscript{−}) of the indicated genotypes were loaded with Indo-1 and treated with 10 \( \mu \)M 3IB-PP1, 10 \( \mu \)M cytochalasin D, or vehicle (DMSO).

(A) B cells as in (A) treated with 10 \( \mu \)M 3IB-PP1, vehicle (DMSO), or 10 \( \mu \)g/mL anti-IgM F(ab\textsuperscript{'})\textsubscript{2}. Closed arrow indicates treatment described in each panel, open arrow indicates addition ionomycin.

(A) depicts a single experiment and (B) is representative of two individual experiments. Pink line provides a reference point between conditions.
negative effect on calcium mobilization induced upon BCR crosslinking. Because the concentrations of 3IB-PP1 that we used result in supraphysiological activation of SFKs, it is possible that under these conditions, SFKs other than Lyn might be able to induce inhibitory signaling in B cells.

Indeed, SFK phosphorylation at Y416 was prominent in lysates of Lyn−/−CskAS B cells treated with 3IB-PP1 (Fig. A12). This corresponded with enhanced phosphorylation of, CD79A, and SHIP-1, and possibly Syk, although the phosphorylation of these signaling components appeared to be somewhat less than in Lyn-sufficient CskAS B cells. Consistent with a partial rescue of calcium signaling in Lyn−/−CskAS B cells treated with 3IB-PP1, we also detected Erk phosphorylation in Lyn−/−CskAS but not CskAS B cells. This suggests that even in the absence of Lyn, strong phosphorylation of other SFKs such as Fyn and Blk might also be able to phosphorylate ITIM-containing inhibitory receptors.

Discussion

In these preliminary studies, we made use of mice expressing CskAS allele on a Csk−/− background to study the effects of rapid and highly selective inhibition of Csk kinase activity in primary B cells. Similar to published studies in thymocytes and mature T cells, we observed rapid dephosphorylation of C-terminal tail of SFKs in CskAS B cells treated with 3IB-PP1 (Tan et al., 2014). This was associated with equally rapid phosphorylation of the activating SFK tyrosine, leading to hyperactivation of SFKs and subsequent activation of proximal BCR signaling components beginning with SFK-mediated phosphorylation of the BCR ITAMs. We
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- **pSHIP-1 (Y1020)**
- **pSyk (Y352)**
- **pSrc (Y416)**
- **pErk (T202/Y204)**
- **pCD79A (Y182)**
Figure A12. Activation of inhibitory signaling in the absence of Lyn during CskAS inhibition.

Activation of inhibitory signaling persists in Lyn-deficient B cells after CskAS inhibition.

Purified splenic B cells were treated with 10 µM 3IB-PP1 (i) or DMSO (D) for 2 minutes and cell lysates were analyzed by immunoblotting with the specified antibodies.

Data are representative of three individual experiments.
also observed concurrent phosphorylation of ITIM-dependent inhibitory signaling in the form of CD22 phosphorylation and activation of SHP-1, and ITIM-independent inhibitory signaling in the form of Dok-1 and SHIP-1 phosphorylation. In the absence of BCR ligation, SFK-mediated inhibitory signaling was dominant over ITAM-mediated positive BCR signaling, leading to inhibited Erk phosphorylation and weak and transient intracellular calcium mobilization and Akt phosphorylation despite robust phosphorylation of PLCγ2. When coupled with stimulation of the BCR through antibody-mediated crosslinking, CskAS inhibition continued to have an overall negative effect on signaling, but could be partially overcome with higher doses of crosslinking antibody, suggesting that physical clustering is critical for B cell antigen receptor signaling. Thus, live-cell imaging studies of B cell interactions with lipid bilayers may elucidate the role of spatial localization of various ITIM-containing inhibitory receptors and phosphatases relative to the BCR and provide insight into how the degree of BCR clustering shifts the balance from negative to positive signaling in the presence of CskAS inhibition. Additionally, it was shown that in thymocytes engagement of the co-receptor CD28 by antigen presenting cells induces actin remodeling and restores TCR signaling following inhibition of CskAS. Therefore, an investigation of the effects of engagement of various B cell co-receptors such as CD40, CD19, and CD21 may be informative.

Unlike in thymocytes, interfering with actin dynamics via cytochalasin D, which may mimic physical changes that occur upon BCR crosslinking, was not sufficient to rescue impaired BCR signaling upon CskAS inhibition. Impaired BCR signaling in CskAS B cells treated with 3IB-PP1 was likely due to the strong activation
of inhibitory molecules such as SHP-1 and SHIP-1 downstream of ITIM-containing inhibitory receptors or SFK-dependent phosphorylation of adapter proteins. This is in contrast with thymocytes, which express few inhibitory receptors, and is more consistent with the effects of Csk<sup>AS</sup> inhibition in mature CD4<sup>+</sup> T cells, which might have different expression of such phosphatases or inhibitory receptors than do immature T cells (Tan et al., 2014). Decreased phosphorylation of Akt was indicative that the mode of BCR signal inhibition was through depletion of PIP<sub>3</sub> by hyperactive SHIP-1. However, direct measurement of the production of inositol phosphates and phosphatidylinositol bisphosphate species will help clarify the mechanism of BCR signal down-modulation by Csk<sup>AS</sup> inhibition.

In order to test the role of ITIM signaling induces upon Csk<sup>AS</sup> inhibition, we attempted to rescue signaling in Csk<sup>AS</sup> B cells by genetic deletion of Lyn kinase. Surprisingly, we found that deletion of Lyn, which is thought to be unique among SFKs in that it phosphorylates both ITIMs and ITAMs, only partially rescued signaling in Csk<sup>AS</sup> B cells treated with 3IB-PP1 and anti-IgM F(ab’)<sub>2</sub> or cytochalasin D. This finding suggests that SFKs other than Lyn are capable of initiating inhibitory signaling when sufficiently activated. Thus, further studies using a dose titration of lower 3IB-PP1 concentrations may be a useful approach toward understanding the contribution of Lyn activity under conditions that result in more physiological levels of SFK activation.

Because of the strong influence of inhibitory signaling in Csk<sup>AS</sup> B cells, the direct role of Csk in signal termination in B cells is unclear. However, the phosphorylation of Dok-1 does suggest that recruitment of cytosolic Csk to the
plasma membrane is a possible mechanism for negative regulation of BCR signaling in addition to SHP-1 and SHIP-1. Further work to tease apart the relative contributions of SHP-1 and SHIP-1 phosphatase activity and Csk kinase activity remains to be done.

Much as in T cells, it is likely that Csk activity opposed by CD45 and CD148 phosphatase activity functions to set a threshold for the activation of BCR signaling, albeit through the engagement of different signaling pathways (Hermiston et al., 2002). It will therefore be interesting to investigate how Csk functions to modulate B cell responsiveness in vivo. A chemical genetic system similar to CskAS mice has been developed to study the effects of inhibiting receptor tyrosine kinases of the EphB family in which the PP1 analog 1-NA-PP1 was used in vivo, with the caveat that this inhibitor is known to have off-target effects (Soskis et al., 2012). This suggests that potential exists for optimizing the in vivo use of CskAS inhibition in various models of disease or antigen responses. This is of relevance because Csk polymorphisms in humans have been found to be associated with systemic lupus erythematosus, suggesting that Csk may prove to be a valuable drug target in the control of a variety immune cell-mediated malignancies (Manjarrez-Orduno et al., 2012).

**Experimental procedures**

*Mice*

CskAS mice were generated as described (Tan et al., 2014), and were bred with Lyn−/− (Lyn\tm\nm\nSor) mice to generate Lyn−/−CskAS mice (Chan et al., 1997). Mice used were
between 6 and 12 weeks of age. All mice were housed in a specific pathogen-free facility at UCSF according to the guidelines of the University Animal Care Committee and the US National Institutes of Health.

Inhibitors

3IB-PP1 was a gift from the Kevan Shokat laboratory and has been previously described (Schoenborn et al., 2011). Cytochalasin D was from Sigma.

Antibodies and reagents

Flow cytometry reagents used were from BD and are described in Chapter 2. Antibody to phosphorylated tyrosine (4G10) was from Upstate. Antibodies to phospho-PLCγ2 (Y1217), phospho-Syk (Y352), phospho-Lyn (Y507), phospho-Src (Y416), phospho-CD79A (Y182), phospho-Erk (T202/Y204), phospho-SHP-1 (Y564), phospho-SHIP-1 (Y1020), and phospho-Akt (S473) were from Cell Signaling Technology. Phospho-CD22 antibody (Y822) was from Epitomics. Phospho-Dok-1 (Y362) and total Erk1 and Erk2 antibodies were from Santa Cruz Biotechnology.

Cell stimulations, flow cytometry, and Western blotting

Characterization of surface marker expression, cell purification, cell stimulation, intracellular phospho-flow staining, intracellular calcium mobilization, and Western blotting were performed as described in Chapter 2.
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