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Positive Regulation of Lyn Kinase by CD148 Is Required for B Cell Receptor Signaling in B1 but Not B2 B Cells

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SUMMARY

B1 and B2 B cells differ in their ability to respond to T-cell-independent (TI) antigens. Here we report that the Src-family kinase (SFK) regulator CD148 has a unique and critical role in the initiation of B1 but not B2 cell antigen receptor signaling. CD148 loss-of-function mice were found to have defective B1 B-cell-mediated antibody responses against the T-cell-independent antigens NP-ficoll and Pneumovax 23 and had impaired selection of the B1 B cell receptor (BCR) repertoire. These deficiencies were associated with a decreased ability of B1 B cells to induce BCR signaling downstream of the SFK Lyn. Notably, Lyn appeared to be selectively regulated by CD148 and loss of this SFK resulted in opposite signaling phenotypes in B1 and B2 B cells. These findings reveal that the function and regulation of Lyn during B1 cell BCR signaling is distinct from other B cell subsets.

In Brief

In conventional B cell BCR signaling, CD45 and CD148 are redundant positive regulators of SFKs. Skrzypczynska and colleagues demonstrate a unique requirement for CD148 in B1 B cells due to its selective activation of the SFK Lyn, which appears to have a critical positive regulatory role in B1 BCR signaling.

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SUPPLEMENTAL INFORMATION

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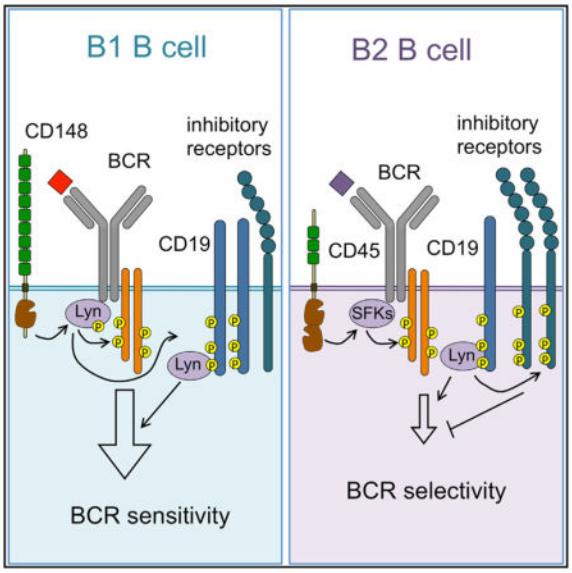
AUTHOR CONTRIBUTIONS

K.M.S. and A.W. designed the research and wrote the manuscript; K.M.S. and J.W.Z. did the experiments and analyzed the data.



INTRODUCTION

Antibody-mediated humoral immunity to T-cell-independent (TI) antigens is orchestrated by distinct pools of peripheral B cells. It has generally been accepted that a division of labor exists between marginal zone (MZ) and B1 B cells, which respond to TI antigens, and follicular B cells, which predominate in the antibody response to T-cell-dependent (TD) antigens. However, the nature of the antigen and presence of additional signals such as inflammatory cytokines or pathogen-associated molecular patterns (PAMPs) can induce the participation of follicular B cells during TI responses (Swanson et al., 2010). How antigen receptor responsiveness may contribute to the recruitment of these different B cell subsets to the TI response independently of additional stimulatory signals is unknown.

B1 B cells are a phenotypically and developmentally distinct population of B cells that make an important contribution to the pre-existing and antigen-induced serum antibodies against TI antigens. The B1 B-cell-derived immunoglobulin repertoire is polyreactive and includes recurrent clonotypes that have been selected by endogenous self-antigens but which can also be reactive against TI antigens such as microbial determinants (Berland and Wortis, 2002). Early studies suggest that B1 B cells exist in a functionally unresponsive state akin to anergy characterized by diminished intracellular calcium mobilization, impaired proliferation, and increased apoptosis upon BCR stimulation (Bikah et al., 1996; Sen et al., 1999). However, the idea of B1 B cell anergy is somewhat incompatible with other studies that report a strong requirement for the presence of endogenous ligand and robust B cell receptor (BCR) signaling for B1 B cell development and maintenance (Berland and Wortis, 2002). Moreover, it has also been demonstrated that B1 B cells are able to proliferate and rapidly generate antibodies in response to bacterial infection, lipopolysaccharide (LPS), or immunization with multivalent synthetic antigens (Martin et al., 2001; Racine et al., 2008; Weber et al., 2014). Therefore, the mechanisms governing B1 BCR signaling in response to endogenous and foreign antigen remain incompletely understood.

BCR signaling is positively regulated by the receptor-like protein tyrosine phosphatases (RPTPs) CD45 (*Ptprc*) and CD148 (*Ptprj*), which promote activation of src-family kinases (SFKs). RPTP-mediated dephosphorylation of the C-terminal inhibitory tyrosine relieves the autoinhibited conformation of SFKs, allowing for a pool of active SFKs that are poised to contribute to the initiation of the BCR signaling cascade. Previous work from our lab has demonstrated high functional redundancy between CD45 and CD148 in SFK regulation in BCR signaling and in the development of conventional B cells (Zhu et al., 2008). However, more recent work on signaling by G protein-coupled receptors (GPCRs) in neutrophils and the observation that elevated expression of CD45 does not appear to affect absolute numbers of B1 B cells in the peritoneum imply that non-overlapping roles for CD45 and CD148 exist (Zhu et al., 2011; Zikherman et al., 2012). 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.

We have previously generated CD148 loss-of-function mice (*Ptprj*$^{TM-/TM-}$ mice), in which the transmembrane (TM) domain of CD148 is deleted, resulting in the presence of secreted, but not surface-bound, CD148. Here, we used *Ptprj*$^{TM-/TM-}$ mice to study the role of SFK regulation by CD148 during B1 B cell development and during antibody responses to both the synthetic antigen NP-ficoll and the clinically relevant vaccine Pneumovax 23. We identified deficiencies in TI antibody responses and B1 BCR repertoire selection that were attributable to decreased B1 cell activation as a result of defective BCR signaling downstream of the SFK Lyn. 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 are critically important for the antibody response against TI antigens such as bacterial polysaccharides (Briles et al., 1981). To study the effects of CD148 deficiency in B1 B cells, we challenged *Ptprj*$^{TM-/TM-}$ mice by intraperitoneal (i.p.) immunization with the TI type 2 polysaccharide antigen Pneumovax 23 and found that *Ptprj*$^{TM-/TM-}$ mice had a profoundly impaired serum IgM response against this vaccine (Figure 1A), despite the fact that CD45 was more highly expressed than CD148 in all B cell subsets, including B1 B cells (Figure S1A). The defective TI response was not due to an overall inability of CD148-deficient B cells to secrete immunoglobulin, as *Ptprj*$^{TM-/TM-}$ mice had normal amounts of preimmune serum immunoglobulin as well as preserved expression of the BCR and other cell surface molecules (Figures S1B–S1D).

To more broadly understand the TI defect in *Ptprj*$^{TM-/TM-}$ mice, we utilized the model antigen 4-hydroxy-3-nitrophenylacetyl (NP) coupled to different carriers to elicit TI type 1 (TI-1), TI type 2 (TI-2), or TD responses. *Ptprj*$^{TM-/TM-}$ mice were immunized i.p. with NP conjugated to the synthetic polysaccharide ficoll, a TI-2 antigen. As with Pneumovax 23, *Ptprj*$^{TM-/TM-}$ mice had an impaired NP-specific IgM response to NP-ficoll (Figure 1B). Paradoxically, production of an anti-NP IgG_3 response was relatively intact in the absence of

a robust IgM response (Figure S1E) and might have been be due to the contribution of IgG_3-producing marginal zone (MZ) B cells to the TI-2 response (Martin et al., 2001).

Toll-like receptor (TLR) engagement can rescue defective BCR signaling in TI-1 responses and augment early antibody production (Rawlings et al., 2012). To determine whether TI-1 responses were also impaired in *Ptprj*$^{TM-/TM-}$ mice, we immunized mice i.p. with NP-LPS and found no defect in NP-specific IgM in *Ptprj*$^{TM-/TM-}$ mice (Figure 1C). To address the possibility that robust antibody production by follicular B cells or MZ B cells was masking a possible defect in B1-mediated TI-1 responses, we assessed B cell activation at the level of Erk phosphorylation in response to stimulation with NP-LPS or LPS and found that CD148 deficiency did not impair B1, follicular, or MZ B cells responses to TI-1 antigens or to TLR ligands (Figures S2A and S2B). This indicated that CD148 probably plays a selective role during TI-2 antigen recognition by the BCR but not to TI-1 antigens, which engage both TLRs and the BCR.

Furthermore, *Ptprj*$^{TM-/TM-}$ mice immunized with the TD antigen NP-keyhole limpet hemocyanin (KLH) had intact NP-specific IgG_1 and IgM responses (Figures 1D and S2C), consistent with previous work showing that CD45 can compensate for CD148 during BCR signaling in follicular B cells (Zhu et al., 2008). 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

Marginal zone B cells and B1 B cells differentially contribute to TI responses depending on the amount and anatomical location of antigen encounter. B1 B cells dominate the response to antigen administered i.p., but MZ B cells respond predominantly to intravenous (i.v.) administration of antigen (Martin et al., 2001). To address whether the B1 B cell compartment is uniquely affected by CD148 deficiency, we immunized *Ptprj*$^{TM-/TM-}$ mice by i.v. injection of NP-ficoll to target the MZ response. In sharp contrast with i.p. immunization, we saw robust production of both IgM and IgG_3 in wild-type and *Ptprj*$^{TM-/TM-}$ mice immunized i.v. (Figure 2A). Splenic B1 B cells make up a small percentage of total splenic B cells and have low expression of CD148. Their ability to signal upon BCR stimulation was unaffected by CD148 deficiency (Figures S2D and S2E). Thus, the small decrease in total serum antibody in *Ptprj*$^{TM-/TM-}$ mice immunized i.v. with NP-ficoll can be attributed to limited involvement of peritoneal B1 B cells that might make a small contribution to antibody responses after i.v. immunization (Figure S2F; Martin et al., 2001).

Myeloid cells such as macrophages and dendritic cells highly express CD148 and can facilitate TI responses through antigen presentation to B cells and by promoting plasmablast differentiation (Balázs et al., 2002; Lin et al., 2004). 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*$^{TM-fl/TM-fl}$) with the *Cd79a-cre* mouse line, which expresses cre recombinase under the control of the Ig-α locus (Hobeika et al., 2006). *Ptprj*$^{TM-fl/TM-fl}$*Cd79a-cre* mice delete the transmembrane region of CD148 during the pro-B cell stage of development, leaving intact

CD148 expression in other hematopoietic cells (Figures S3A–S3E). Expression of cre recombinase or soluble CD148 did not have adverse effects on B cell development (Figure S3F; Hobeika et al., 2006; Zhu et al., 2008). Like the systemic CD148 loss-of-function mice, *Ptprj*$^{TM\text{-}fl/TM\text{-}fl}$*Cd79a-cre* mice had an impaired IgM response to intraperitoneal immunization with Pneumovax 23 (Figure 2B), confirming that the TI-2 defect due to the loss of CD148 is B cell intrinsic. Taken together, these findings indicate that CD148 is required for TI-2 antibody responses in a B1 B-cell-intrinsic manner.

CD148 Is Required for Antigen-Specific Proliferation and IgM Secretion by B1 B Cells

To investigate effects of CD148 deficiency on activation and proliferation of B1 B cells, we challenged wild-type and *Ptprj*$^{TM-/TM-}$ mice i.p. with NP-ficoll and examined the peritoneal cavity and spleen for the presence of dividing Ki-67$^+$ B cells that co-stained with NP-PE, which specifically labels NP-reactive B cells (Figures S4A and S4B). Immunization with NP-ficoll led to a 6.1-fold increase in the number of wild-type NP-PE$^+$Ki67$^+$ peritoneal B1 B cells compared with PBS-treated controls, but only a 3.2-fold increase in *Ptprj*$^{TM-/TM-}$ mice, resulting in nearly three times as many total responding wild-type B1 B cells compared with *Ptprj*$^{TM-/TM-}$ B1 B cells (Figures 3A and 3B). There was also an increase in NP-PE$^+$Ki67$^+$ MZ B cells in mice treated with NP-ficoll compared with PBS, but the proportionate increase was comparable between wild-type and *Ptprj*$^{TM-/TM-}$ mice (2.6- and 2.8-fold increase, respectively) (Figure 3B), further suggesting that MZ B cells do not require CD148 to respond to TI antigens. Although follicular B cells can sometimes be recruited to TI-2 responses, their participation is minor and has been demonstrated in context of additional signals such as TLR ligands or inflammation (Swanson et al., 2010). Because these secondary signals are not present upon NP-ficoll immunization, involvement of peritoneal B2 and splenic follicular B cells was not observed (Figure 3B).

To determine whether the defective proliferative response of *Ptprj*$^{TM-/TM-}$ B1 B cells could account for the diminished serum IgM response, we performed ELISpot analysis of total peritoneal cavity lymphocytes from mice injected i.p. with either NP-ficoll or PBS. There was a robust increase in NP-specific IgM but not IgG_3 antibody-secreting cells (ASCs) in peritoneal lymphocytes from wild-type mice, but few detectable peritoneal cavity-derived ASCs in *Ptprj*$^{TM-/TM-}$ mice (Figures 3C and 3D). However, equal numbers of IgM and IgG_3 ASCs were detectable in the spleens of both WT and *Ptprj*$^{TM-/TM-}$ mice (Figure S4C). Collectively, these results demonstrate that B1 B cells, but not MZ B cells, require CD148 for TI-2 responses.

B1 Cell Repertoire Selection Is 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). Despite normal B1 B cell numbers in *Ptprj*$^{TM-/TM-}$ 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*$^{TM-/TM-}$ mice, which can contribute to the observed defect in TI responses. To investigate this possibility, we stained peritoneal and splenic B cells with NP-PE and detected approximately 50% fewer NP-specific peritoneal B1a B cells and a modest

decrease in B1b B cells, but no differences in NP-specific follicular or MZ B cells in *Ptprj*$^{TM-/TM-}$ mice (Figures 4A, 4B, and S5A). This decrease in frequency of NP-reactive B1 B cells in *Ptprj*$^{TM-/TM-}$ mice was reflected in lower amounts of NP-specific serum IgM in unimmunized mice, and similarly less anti-Pneumovax 23 IgM was detected in the serum of these mice (Figures 1A, 1B, and 2B). However, we did not detect decreased frequencies of B1 B cells or IgM antibodies to autoantigens such as phosphatidylcholine or anti-nuclear antigens (data not shown). This suggests that loss of CD148 affects the selection of B1 B cells cross-reactive against less well-represented or foreign antigens but not against abundantly expressed endogenous ligands, which is consistent with a repertoire shift and preserved numbers of total B1 B cells.

BCR repertoire restriction 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 further explore the effects of CD148 deficiency on B1 B cell development in the context of an NP-restricted repertoire, we crossed *Ptprj*$^{TM-/TM-}$ mice to the B1-8i BCR transgenic mouse line, which produces NP-specific B cells when the rearranged B1-8i transgenic heavy chain pairs with Igλ light chains (Shih et al., 2002). Introduction of the B1-8i heavy chain resulted in a marked skewing away from the B1 lineage in the peritoneum and a respective increase in the percentage of peritoneal B2 B cells (Figures 4C and 4D), with only a mild effect on follicular and marginal zone B cell numbers (Figures S5B and S5C). B1 B cell development is especially sensitive to changes in BCR specificity, availability and recognition of endogenous positively selecting ligand, and strength of BCR-derived signals (Berland and Wortis, 2002; Casola et al., 2004; Lam and Rajewsky, 1999). Therefore, it is not surprising that partially limiting the diversity of the BCR repertoire by fixing the IgM heavy chain led to an overall decrease in B1 B cells in both CD148-sufficient and *Ptprj*$^{TM-/TM-}$ B1-8i mice by affecting B1 B cell selection. The majority of B1 B cells express BCRs that use the Igκ light chain (Figure 4E) 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 (Figure 4D). However, staining for Igκ and Igλ revealed a decrease in Igλ^{+}, and therefore NP-reactive, B1a and B1b B cells in B1-8i-*Ptprj*$^{TM-/TM-}$ mice compared with B1-8i mice (Figures 4E and 4F), which was not evident in splenic follicular or MZ B cells (Figure S5D). The bias away from Igλ usage was not apparent in B1 or B2 B cells on the unrestricted B6 background (Figures S5E and S5F). This suggests that the decrease in NP-restricted B1 B cells in *Ptprj*$^{TM-/TM-}$ mice on both the B1-8i and unrestricted background is indicative of an antigen-specific role for CD148 in establishing and maintaining an appropriate B1 cell repertoire.

BCR Signaling Is Impaired in CD148-Deficent B1 B Cells

To investigate the role of CD148 at the level of B1 BCR signaling, we stimulated peritoneal lymphocytes in vitro with BCR-crosslinking antibody (F(ab′)$_2$ anti-μ) and assessed phosphorylation of Erk by intracellular staining. B1 B cells responded in a bimodal fashion, and at intermediate stimulatory concentrations of F(ab′)$_2$ anti-μ, we observed fewer responding *Ptprj*$^{TM-/TM-}$ B1 B cells (Figures 5A–5C), suggesting that CD148 might regulate the threshold of activation in B1 B cells. Higher concentrations of BCR crosslinking

antibody or longer duration of stimulus were sufficient to overcome this signaling defect, as was bypassing proximal BCR signaling via stimulation with PMA, indicating impaired proximal BCR signaling rather than an insufficiency in downstream components of the BCR signaling pathway (Figures 5A–5C). In contrast, neither splenic follicular nor MZ B cells from *Ptprj*$^{TM-/TM-}$ mice exhibited altered pErk signaling (Figure 5D). Consistent with impaired Erk phosphorylation, there was a decrease in the magnitude of the calcium response in B1 but not B2 cells (Figure 5E). Furthermore, stimulation of splenic and peritoneal B1-8i and B1-8i-*Ptprj*$^{TM-/TM-}$ B cells with the NP-ficoll revealed a markedly decreased ability of B1-8i-*Ptprj*$^{TM-/TM-}$ B1 but not follicular or MZ B cells to phosphorylate Erk after BCR engagement, demonstrating similar responses during stimulation with antigen-receptor crosslinking antibody and bona fide ligand (Figures 5F and S6A).

Src-family kinases are the best described and most proximal substrates of CD148, and impaired SFK activation would account for the impaired BCR signaling in *Ptprj*$^{TM-/TM-}$ B1 B cells. However, other phosphoproteins have been reported as potential substrates for CD148, including those that are downstream of SFKs such as PLCγ1 and Erk (Baker et al., 2001; Takahashi et al., 2012; Whiteford et al., 2011). To address whether depressed BCR signaling in *Ptprj*$^{TM-/TM-}$ B1 B cells could be attributed exclusively to impaired SFK activation, we induced BCR signaling in the presence of the SFK inhibitor PP2. Because Syk can function independently of SFKs when the BCR is clustered to a high degree, PP2 treatment delays but does not eliminate calcium signaling (Mukherjee et al., 2013). Differences in the magnitude of the calcium response between *Ptprj*$^{TM-/TM-}$ and wild-type B1 B cells were lost in the context of SFK-independent BCR signaling (Figure 5G). This suggests that the signaling defect mediated by CD148 is SFK dependent and intact SFK-independent Syk activation explains the ability of B1 B cells to attain near-normal amounts of Erk phosphorylation at late time points or at high concentrations of BCR crosslinking antibody (Figures 5A–5C).

Lyn-Dependent BCR Signaling in B1 Cells Results in Sensitivity to Loss of CD148

To understand the observed defect in distal BCR signaling, we assessed activation of various components of BCR signaling downstream of SFKs. We observed a trend of lower phosphorylation of ITAMs of the alpha chain of the B cell receptor (CD79a) and impaired phosphorylation of the SFK substrate Syk (Figures 6A and 6B). Phosphorylation of PLCγ2 and BLNK, which are upstream of the calcium response, were also decreased in *Ptprj*$^{TM-/TM-}$ B1 B cells (Figures 6C and 6D). These substantial signaling defects were not observed in splenic follicular or MZ B cells (Figures S6B–S6E).

To directly assess SFK activation in B1 B cells, 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*$^{TM-/TM-}$ B1 B cells exhibited decreased basal phosphorylation of Y416 and a lack of subsequent increase in phosphorylation of this residue after stimulation via BCR crosslinking (Figure 6E), 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*$^{TM-/TM-}$ B1 but not B2 B cells and during BCR stimulation (Figure 6F).

Studies of the regulation of GPCR signaling in neutrophils suggest that CD148 preferentially dephosphorylates Lyn kinase (Zhu et al., 2011). It is therefore possible that a similar mechanism is at play in B1 B cells and may account for the defect in Lyn kinase activation in *Ptprj*$^{TM-/TM-}$ B1 B cells. However, the preferential dephosphorylation of Lyn kinase by CD148 would not seem to fully explain the cellular activation and antibody production defects in *Ptprj*$^{TM-/TM-}$ B1 B cells because Lyn is thought to play a predominantly negative regulatory function in B cells through phosphorylation of inhibitory receptors (Chan et al., 1997). If this were similarly the case in B1 B cells, an increase rather than the observed decrease in BCR signaling would be expected in CD148-deficient B1 B cells.

To determine whether the role of Lyn kinase in BCR signaling might be different in B1 B cells than in conventional B cells, we compared BCR responses of peritoneal B1 B cells and splenic B cells from wild-type mice to *Lyn*$^{-/-}$ mice. As expected, follicular B cells from *Lyn*$^{-/-}$ mice exhibited significantly elevated phosphorylation of Erk compared with wild-type B cells. Of note, *Lyn*$^{-/-}$ B1 B cells were hyporesponsive to BCR stimulation (Figures 7A and 7B), leading to a partial phenocopy of the CD148-mediated signaling defect in *Ptprj*$^{TM-/TM-}$ B1 B cells, where *Lyn*$^{-/-}$ B1 B cells had more severely impaired BCR signaling (Figure 7C). Consistent with these findings, *Lyn*$^{-/-}$ mice have been shown to have diminished TI-2 responses (Horikawa et al., 1999).

To further establish whether the signaling defect observed in *Ptprj*$^{TM-/TM-}$ B1 cells was due solely to dysregulation of Lyn kinase, we generated *Lyn*$^{-/-}$*Ptprj*$^{TM-/TM-}$ double-deficient mice. Like the *Lyn*$^{-/-}$ B1 B cells, CD148 and Lyn double-deficient B1 B cells from *Lyn*$^{-/-}$*Ptprj*$^{TM-/TM-}$ mice had a more severe BCR signaling defect than CD148 single-deficient B1 B cells (Figure 7C). In follicular B cells, CD148 and Lyn double deficiency was associated with only a small, partial rescue of the hyperresponsive signaling phenotype (Figure 7C). Importantly, the impaired B1 BCR signaling phenotype of *Lyn*$^{-/-}$*Ptprj*$^{TM-/TM-}$ B1 B cells closely resembled the *Lyn*$^{-/-}$ phenotype in both B1 and follicular B cells (Figures 7C and 7D). The similarity in signaling between *Lyn*$^{-/-}$ and *Lyn*$^{-/-}$*Ptprj*$^{TM-/TM-}$ B1 and follicular B cells was consistent with selective dephosphorylation of Lyn by CD148 and further suggests that dephosphorylation of non-Lyn substrates by CD148 makes a minimal contribution to net BCR signaling outcomes. Thus, Lyn kinase activity appears to be directly regulated by CD148 and has a predominantly positive role during B1 BCR signaling, a function that cannot be compensated for by other SFKs. This positive function in B1 B cells is distinct from Lyn's dominant negative regulatory role in B2 B cells (Chan et al., 1997). These results suggest a unique wiring of BCR signaling in B1 B cells in such a way that a requirement for positive BCR signal regulation by Lyn renders these cells especially sensitive to the loss of CD148.

DISCUSSION

In this study we have described a unique 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 have redundant functions in BCR signaling in MZ and follicular B cells (Zhu et al., 2008). This was notable because CD45 expression was markedly higher than that of CD148 across all B cell subsets. The nonredundant function of CD148 in B1 cells suggests that some intrinsic features of B1 B cells or of the two RPTPs may influence their unique downstream effects on BCR signaling.

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 notable 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 (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 (Bikah et al., 1996; Fujimoto et al., 2001). Although CD5 has been described as a negative regulator of B1 B cell proliferation through its interaction with SHP-1 (Bikah et al., 1996), we found that $CD5^+$ B1a B cells had more robust Erk phosphorylation when compared with $CD5^-$ B1b B cells (data not shown), suggesting that the inhibitory function of CD5 may be to tune BCR signals, rather than maintain hyporesponsiveness in B1 B cells, similar to its function in thymocytes (Azzam et al., 2001).

It is also noteworthy that the negative regulatory function of Lyn in conventional B cells depends upon CD22. Compared with conventional B cells, B1 B cells express lower amounts of CD22 and other inhibitory receptors such as CD72 (http://www.immgen.org). 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). Instead, the inhibitory receptor Siglec-G is highly expressed by B1 B cells and can also bind SHP-1. However, it is unclear how Siglec-G and SHP-1 mediate their inhibitory function in B1 B cells because proximal BCR signaling molecules including PLCγ, Btk, and BLNK show unchanged tyrosine phosphorylation in Siglec-G-deficient B1 B cells (Hoffmann et al., 2007; Jellusova and Nitschke, 2012). Furthermore, Siglec-G has a single ITIM that is permissive for SHP-1 binding, compared with dual ITIMs present on CD22, suggesting that there may exist differences in the quality of inhibitory signaling by the negative regulatory receptors expressed on B1 and B2 B cells. Further work is required to more completely understand how the balance between positive and negative signals is achieved in B1 and B2 B cells, but the present study suggests that the net expression of activating and inhibitory receptors in B1 B cells results in lower inhibitory tone compared with conventional B cells.

Another 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. Although 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., 2004). However, some of these previous observations are based on the inability of B1 cells to proliferate in vitro after stimulation with BCR cross-linking antibody, which does not accurately reflect proximal BCR signaling events. Moreover, the B1-derived BCR transgenic models used in these prior studies relied on heavy and light chain rearrangements derived from endogenous B1 BCRs, which are reactive against a common self-ligand, potentially leading to the induction of an anergic phenotype and thus may not be representative of B1 B cells in an unrestricted repertoire (Bikah et al., 1996; Chumley et al., 2002). Indeed, these transgenic B1 B cells are reported to have elevated expression of the negative regulator CD5, 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., 2004). 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), our results implicate CD148 expression as a mechanism for lowering the threshold of activation through regulation of Lyn kinase activity.

Finally, differences in enzymatic activity of the distinct phosphatase domains of CD45 and CD148 may explain why the loss of CD148 impairs B1 B cell signaling despite the presence of CD45. In unpublished experiments, we tested the ability of the purified cytosolic domains of human CD45 and CD148 to de-phosphorylate phosphopeptides corresponding to the C-terminal tails bearing the inhibitory tyrosine residues of the SFKs Lyn, Blk, and Lck in vitro. There was no apparent selectivity of either RPTP toward any of these substrates at the peptide level (data not shown), probably due to high sequence similarity. However, we did notice that CD148 dephosphorylated its substrates much more rapidly than did CD45 (data not shown). 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 surface of B cells, the higher enzymatic activity of CD148 and its high relative expression in wild-type B1 cells may together account for the signaling defects observed when CD148 function is lost 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 despite lower expression of the phosphatase.

Our studies reveal that the robust BCR signaling in B1 B cells is dependent on positive regulation via the CD148-Lyn axis. In contrast to B2 B cells, whose activation is under strict negative regulation by the Lyn-CD22-SHP-1 pathway and others, 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 BCR and allows B1 B cells to be poised to respond robustly to a broad array of low-affinity ligands.

EXPERIMENTAL PROCEDURES

Mice

Ptprj$^{TM-/TM-}$ and *Ptprj*$^{TM-fl/TM-fl}$ mice were generated and maintained as described (Zhu et al., 2008; Katsumoto et al., 2013). Conditional deletion of CD148 in the B cell lineage was achieved by crossing *Ptprj*$^{TM-fl/TM-fl}$ mice with *Cd79a-cre* 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 the B1-8i transgenic line and mice carrying a single 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 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 UCSF animal care ethics and veterinary committees and are in accordance with NIH guidelines.

Antibodies, FACS Sorting, and Analysis

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, kappa light chain, Ki-67, phospho-BLNK (Y84), phospho-PLCγ2 (Y759), and phospho-Syk (Y352) were from BD Biosciences. Streptavidin-Pacific blue was from Life Technologies. Antibodies against phospho-CD79A (Y182), phospho-Erk (T202/Y204), phospho-Src family (Tyr416), and phospho-Lyn (Tyr507) were from Cell Signaling Technology. Total Lyn antibody (44) was from Santa Cruz Biotechnology. Fluorophore-conjugated anti-mouse IgM Fab and goat anti-mouse F(ab′)$_2$ anti-μ were from Jackson Immunoresearch. Cells were analyzed on a Fortessa (BD Biosciences) and data analyzed with FlowJo software (TreeStar). Quantitation of CD45 and CD148 was performed using BD Quantibrite PE Beads (BD Biosciences).

Immunizations

Mice were immunized i.p. or i.v. with 100 μg/mouse NP-50-ficoll, which is presumed to be the peak amount of antigen, above which an inhibition of the IgM response may occur (Dintzis et al., 1989). Other antigens were used at the following concentrations: 100 μg/mouse NP-33-KLH with Imject alum (Thermo Fisher), 50 μg/mouse NP-0.15-LPS, 10 μg/mouse NP-18-ficoll for ELISpot experiments (Biosearch Technologies), and 10 μg/mouse Pneumovax 23 (Merck) in PBS. Imject Alum was from Thermo Scientific. Blood samples were collected from the tail at days 0, 7, 14, 21, and 28 after immunization.

ELISA and ELISpot

Serum antibody titers were measured by sandwich ELISA. 96-well plates (Costar) were coated with 5 μg/mL NP-23-BSA (Biosearch Technologies) or with 5 μg/mL Pneumovax 23 in PBS. Sera were serially diluted and detected with HRP-conjugated anti-mouse IgM or anti-mouse IgG_3 (Southern Biotech). For total serum Ig ELISA, plates were coated with goat anti-mouse heavy and light chain antibody and detected with HRP-conjugated goat anti mouse Ig antibodies, using mouse Ig as standards (Southern Biotech). ELISAs were

developed using TMB (Sigma) and stopped with 2N sulfuric acid. Absorbance was read at 450 nm. Titers were expressed at half the maximal optical density (OD) for each assay or at an OD in the linear range of the assay (specified in each figure) when maximal optical density was not attainable. For ELISpot, peritoneal lavage cells and splenocytes were collected at days 4 or 7 after immunization. Cells were plated in triplicate at 0.02–0.2 × 10^6 cells/well on mixed cellulose ester MultiScreen filter plates (Miltenyi) coated with 5 μg/mL NP-49-BSA. Spots were detected with goat anti-mouse IgM or IgG_3 followed by SA-AP (Southern Biotech) and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Vector Labs). Scanned images of ELISpots were quantified using ImageJ software.

In Vivo B Cell Proliferation

Mice were immunized i.p. with 10 μg/mouse NP-18-ficoll or PBS; peritoneal lavage cells and splenocytes were collected at day 4. Cells were permeabilized and stained with APC-conjugated anti-human Ki-67 antibody as indicated by manufacturer (BD Biosciences).

Cell Stimulation

For intracellular phosphotyrosine staining, peritoneal and splenic lymphocytes were rested for 30 min and stimulated with $F(ab')_2$ anti-μ (Jackson Immunoresearch) as indicated. Cells were fixed with 2% PFA and methanol permeabilized, followed by surface and intracellular staining. For western blotting, peritoneal B1 B cells were sort-purified by positive staining with anti-IgM Fab and dump staining with antibodies against NK1.1, Gr-1, F4-80, CD3, Ter119, and CD23. Lymph node B cells were purified by negative selection (B Cell Isolation Kit, Miltenyi). Cells were rested and stimulated as above and lysed directly into SDS-PAGE. For calcium mobilization assays, cells were loaded with Indo1-AM (10 μg/mL; Invitrogen), followed by staining with FITC- or APC-conjugated anti-IgM Fab; non-B or non-B1 B cells were excluded by dump staining. Prior to stimulation, cells were rested for 3 min in the presence or absence of 3 μM PP2 (EMD Millipore). Ionomycin was from Calbiochem (EMD Millipore). Phorbol myristate acetate (PMA) was from Sigma.

Statistical Analysis

Prism (GraphPad) was used for statistical analyses as indicated in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- CD148-deficient mice have severely impaired T-cell-independent antibody responses
- B1 B cells require CD148 for development, activation, and antibody production
- CD148 is uniquely required for B1 but not conventional B cell BCR signaling
- The SFK Lyn has a critical positive regulatory role exclusively in B1 B cells

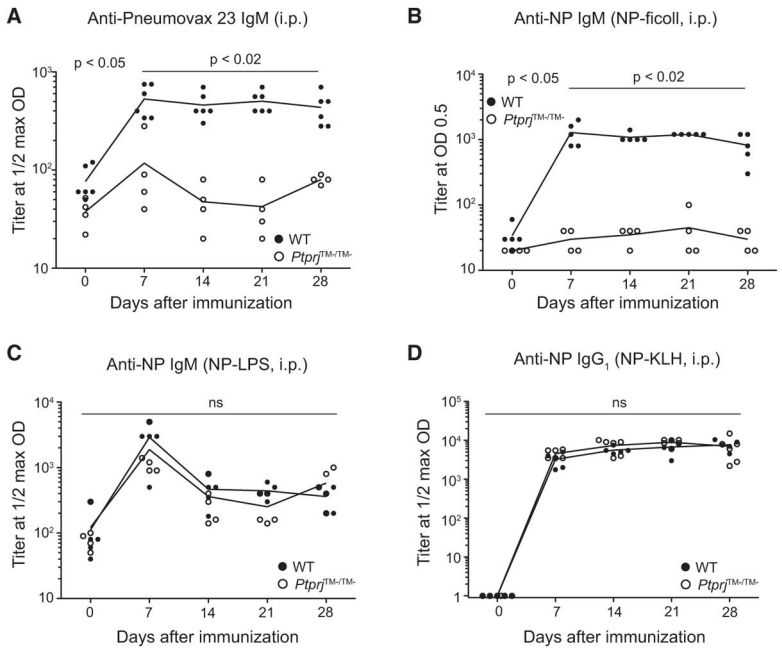

Figure 1. Impaired TI Responses in CD148-Deficient Mice

(A) Pneumovax 23-specific serum IgM titers after i.p. immunization.

(B) NP-specific serum IgM after i.p. immunization with NP-ficoll.

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

(D) NP-specific serum IgG_1 responses in mice immunized i.p. with NP-KLH in alum.

n = 5 wild-type (WT) mice, n = 4–5 *Ptprj*$^{TM-/TM-}$ (*Ptprj*$^{TM-/TM-}$) mice. Each symbol represents an individual. Titer was determined at half of the maximal OD for the assay or in the linear rage of the assay, as stated. Data are representative of two (A), four (B), two (C), and three (D) experiments. p values were calculated using Mann-Whitney t test. See also Figures S1 and S2.

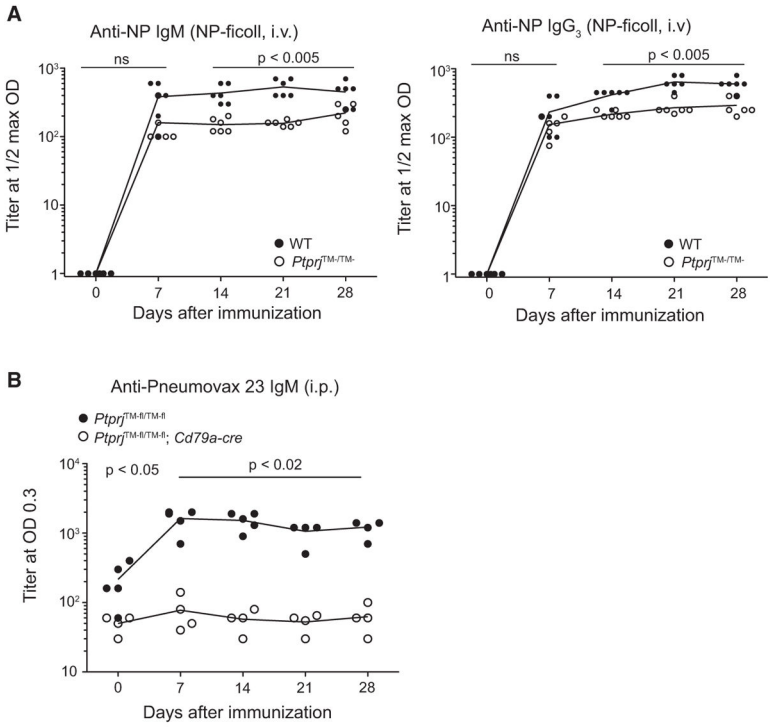

Figure 2. CD148-Mediated TI Defect Is B1 B Cell Intrinsic

(A) NP-specific IgM (left) and IgG_3 (right) in WT and *Ptprj*$^{TM-/TM-}$ mice immunized i.v. with NP-ficoll.

(B) Pneumovax 23-specific IgM titers in *Ptprj*$^{TM-fl/TM-fl}$ and *Ptprj*$^{TM-fl/TM-fl}$*Cd79a-cre* mice after i.p. immunization. Titer was determined at the linear range of the assay (OD 0.3).

For (A), n = 6 wild-type mice, n = 6 *Ptprj*$^{TM-/TM-}$ mice. For (B), n = 5 *Ptprj*$^{TM-fl/TM-fl}$ mice, n = 4 *Ptprj*$^{TM-fl/TM-fl}$*Cd79a-cre* mice. Data are representative of two (A) and three (B) experiments. Mann-Whitney t test was used to calculate p values. See also Figure S3.

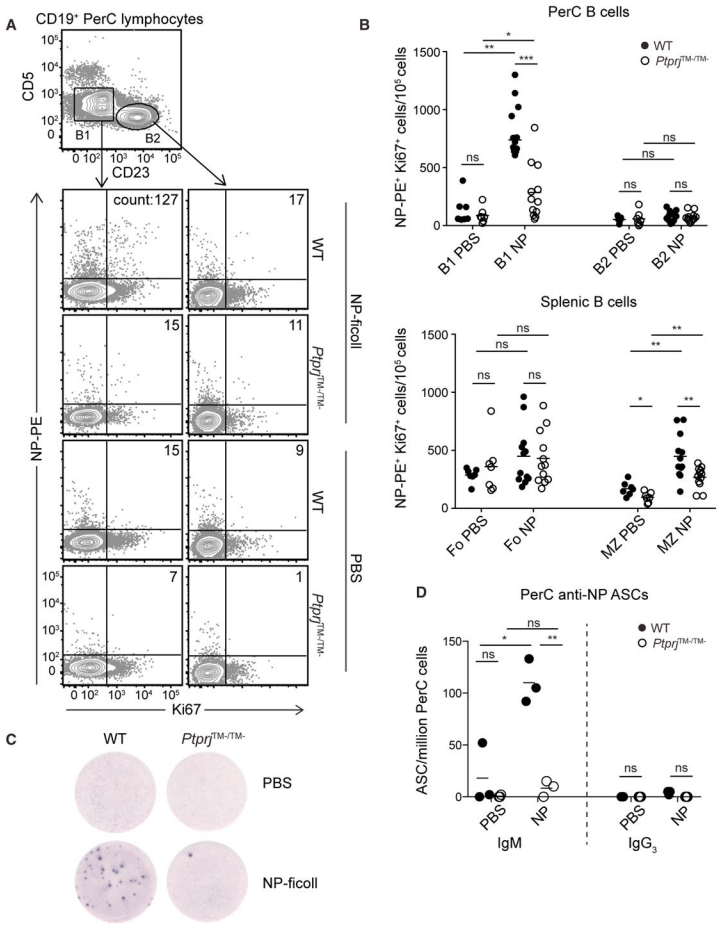

Figure 3. Impaired Antigen-Specific Proliferation of CD148-Deficient B1 B Cells

(A) Intracellular staining of peritoneal B1 ($CD19^+CD23^-CD5^{+/lo}$) and B2 ($CD19^+CD23^+CD5^-$) B cells for Ki-67 and surface NP-PE in mice 4 days after i.p. immunization with NP-ficoll. Cell counts represent the total number of events in the NP $^+$Ki-67^+ gate of each sample.

(B) Quantification of NP-PE^+Ki67^+ peritoneal (PerC) B cells in (A) and splenic follicular ($CD19^+CD23^+CD21^-$) and MZ ($CD19^+CD23^-CD21^+$) B cells. Data depict the number of NP-PE^+Ki-67^+ cells present per 10^5 cells of each B cell subset.

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

(D) Quantification of ASCs in (C). Each symbol represents an individual mouse and is the mean of triplicate samples.

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 from 4 pooled experiments. 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 after immunization, which give similar results. Bars represent the mean. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. See also Figure S4.

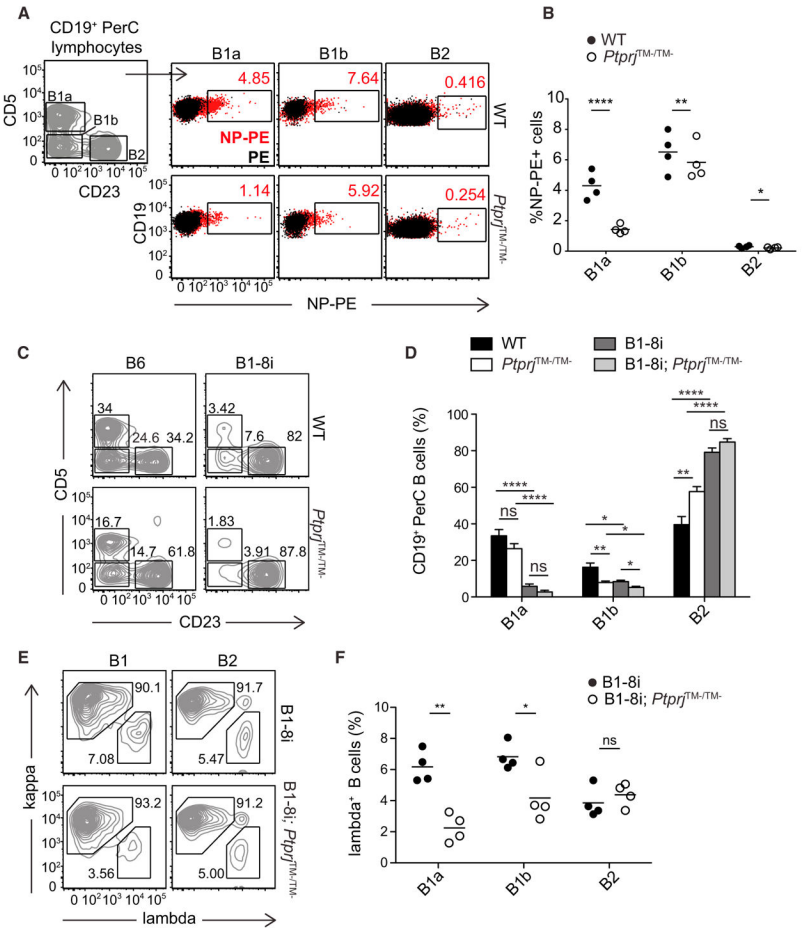

Figure 4. CD148 Is Required for Normal B1 B Cell Repertoire Development

(A) Representative plots of flow cytometric analysis of NP-PE reactive peritoneal B cells.

(B) Quantification of data depicted in (A).

(C) Flow cytometric analysis of peritoneal B1 B cells from B1-8i and B1-8i-*Ptprj*$^{TM-/TM-}$, 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 ± SEM of each B cell subpopulation as a percent of total B cells (CD19^{+} lymphocytes).

(E) Flow cytometric analysis of κ- and λ-expressing B1 and B2 B cells in B1-8i and B1-8i-*Ptprj*$^{TM-/TM-}$ mice.

(F) Quantification of percent κ- and λ-expressing B1a, B1b, and B2 B cells in B1-8i and B1-8i-*Ptprj*$^{TM-/TM-}$ mice.

Data in (A) are representative of four mice of each genotype from two independent experiments; in (B), data show n = 4 of each wild-type and *Ptprj*$^{TM-/TM-}$ mice, representative of two independent experiments. For (C) and (D), data show n = 9 of each B1-8i, B1-8i-*Ptprj*$^{TM-/TM-}$, wild-type, and *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. Student's t test was used to calculate p values. *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001. See also Figure S5.

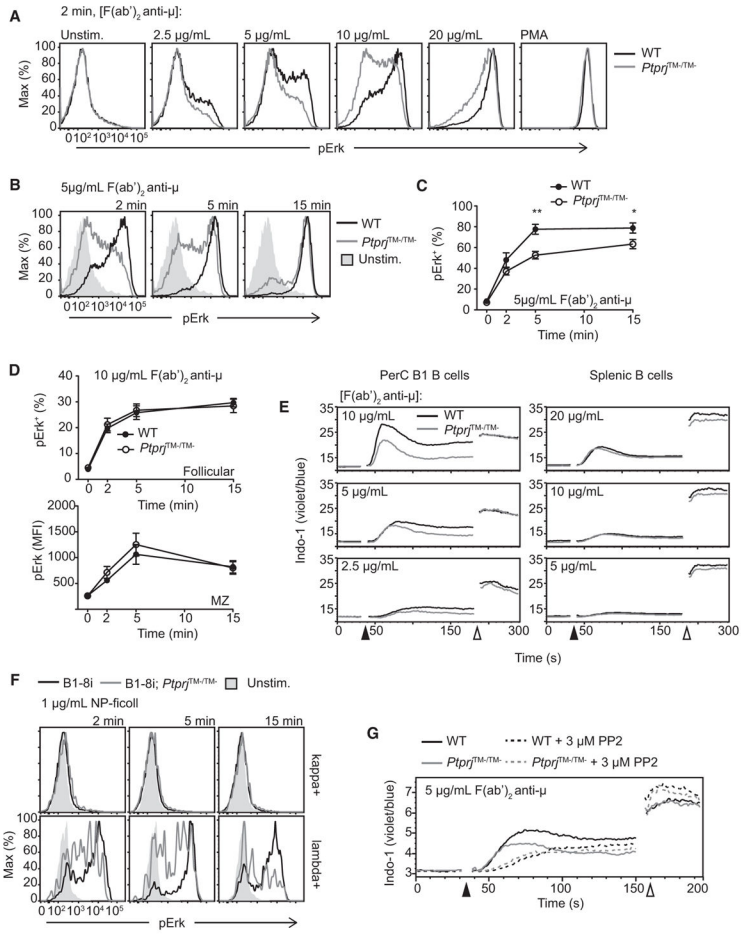

Figure 5. Impaired SFK-Mediated BCR Signaling in CD148-Deficient B1 B Cells

(A) Peritoneal B1 B cells ($B220^{lo}CD23^{-}CD5^{+/lo}$) stimulated for 2 min with indicated concentrations of $F(ab')_2$ anti-μ or 50 ng/mL phorbol myristate acetate (PMA) and stained with anti-phospho-Erk (pErk) antibody.

(B) Peritoneal B1 B cells stimulated with 5 μg/mL of $F(ab')_2$ anti-μ for the indicated times and stained as in (A). Shaded histograms indicate unstimulated cells.

(C) Percentages of $pErk^{+}$ B1 B cells shown over time after stimulation with 5 μg/mL $F(ab')_2$ anti-μ.

(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 $F(ab')_2$ anti-μ.

(E) Total peritoneal lymphocytes or splenocytes loaded with Indo-1 and stained with anti-IgM Fab and dump stain to exclude non-B cells or peritoneal B2 B cells. Peritoneal cells and splenocytes were stimulated with the indicated concentrations of $F(ab')_2$ anti-μ (closed arrow), followed by 1 nM ionomycin (open arrow).

(F) Peritoneal lymphocytes stimulated for the indicated times with 1 μg/mL NP-18-ficoll.

(G) Calcium mobilization of peritoneal B1 B cells stimulated with 5 μg/mL $F(ab')_2$ anti-μ with or without 3 μM PP2.

For (A) and (B), data are representative of seven individual experiments. For (C) and (D), n = 7 each of wild-type and *Ptprj*$^{TM-/TM-}$ mice. B1 and follicular B cells respond in a bimodal

fashion, so percent $pErk^+$ cells are shown in (C) and (D); pErk induction in MZ B cells is associated with a population shift, so mean fluorescence intensity (MFI) of the population is shown. Data in (E), (F), and (G) are representative of three individual experiments with similar results. Data are expressed as mean ± SEM. Student's t test was used to calculate p values. *$p \leq 0.05$, **$p \leq 0.01$. See also Figure S6.

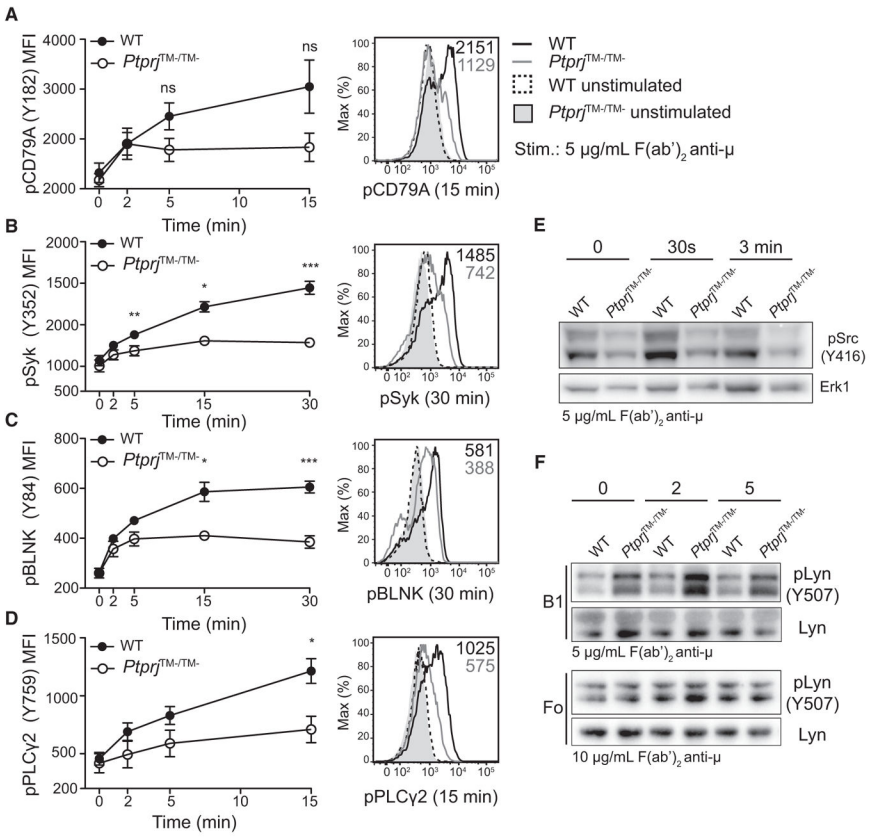

Figure 6. Impaired Lyn Activation in CD148-Deficient B1 B Cells

(A) Peritoneal B1 B cells stimulated with 5 µg/mL of F(ab′)$_2$ anti-µ for the indicated times followed by intracellular staining for phospho-CD79A and surface markers. Data are expressed as mean ± SEM; histograms depict phosphorylation at time of peak signal (indicated); numbers represent MFI.

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

(E) Sort-purified peritoneal B1 B cells stimulated with 5 µg/mL of F(ab′)$_2$ anti-µ. Lysates were blotted with anti-phospho-Src antibody against the activating tyrosine of SFKs (Y416). Total Erk1 was used as a loading control.

(F) Cell lysates from sort-purified peritoneal B1 and purified lymph node follicular B cells stimulated as in (E) and blotted for the inhibitory phosphotyrosine of SFK using 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.

For (A)–(D) and (F), mean MFI ± SEM is shown for each time point. For (A) an average of six experiments is shown, three experiments in (B) and (C), and four experiments in (D). A representative of three individual blots is shown for (E) and (F). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

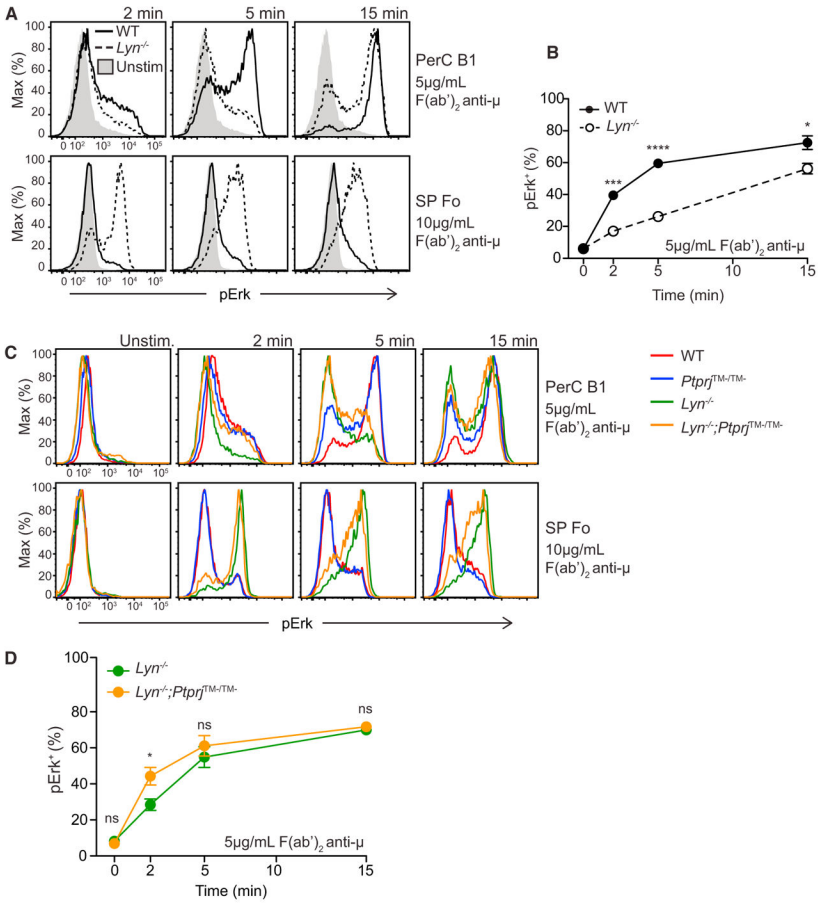

Figure 7. B1 BCR Signaling Requires Lyn Kinase

(A) Intracellular pErk staining of peritoneal B1 B cells and splenic follicular B cells from wild-type and $Lyn^{-/-}$ mice stimulated with 5 and 10 μg/mL F(ab′)$_2$ anti-μ, respectively.

(B) Summary data of percent pErk$^+$ B1 cells shown over time after stimulation with 5 μg/mL of F(ab′)$_2$ anti-μ as in (A).

(C) Intracellular pErk staining of peritoneal B1 B cells and splenic follicular B cells using wild-type, $Ptprj^{TM-/TM-}$, $Lyn^{-/-}$, and $Lyn^{-/-}Ptprj^{TM-/TM-}$ mice stimulated with 5 and 10 μg/mL F(ab′)$_2$ anti-μ, respectively.

(D) Summary data of percent pErk$^+$ B1 B cells from $Lyn^{-/-}$ and $Lyn^{-/-}Ptprj^{TM-/TM-}$ mice over time after stimulation with 5 μg/mL F(ab′)$_2$ anti-μ as in (C).

Data in (A) are representative of four independent experiments. For (B), n = 4 wild-type and n = 4 $Lyn^{-/-}$ mice. Data in (C) are representative of six independent experiments with like results. In (D), n = 6 $Lyn^{-/-}$ and n = 6 $Lyn^{-/-}Ptprj^{TM-/TM-}$. *p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001.