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Previous studies demonstrated that BCR signaling in GC
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enhanced proliferation in the DZ (Luo et al., 2018). Expression
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its absence, antibody affinity maturation is perturbed (Sander
et al., 2015; Dominguez-Sola et al., 2015). BCR triggering in-
duces inactivation of Foxo1 by phosphorylation (Yusuf et al.,
2004; Herzog et al., 2009; Srinivasan et al., 2009), and there-
fore, it is expected that antigen engagements in the LZ
would restrain transition to the DZ. Together, these findings
suggest that an additional unknown mechanism is involved in
BCR signal transduction that allows both Foxo1 inactivation
and interzonal migration.

Germinal centers (GCs) are sites at which B cells proliferate and mutate their antibody-encoding genes in the dark zone (DZ), followed by affinity-based selection in the light zone (LZ). B cell antigen receptor (BCR) signals induce Syk activation followed by rapid phosphatase-mediated desensitization; however, how degradation events regulate BCR functions in GCs is unclear. Here, we found that Syk degradation restrains plasma cell (PC) formation in GCs and promotes B cell LZ to DZ transition. Using a mouse model defective in Cbl-mediated Syk degradation, we demonstrate that this machinery attenuates BCR signaling intensity by mitigating the Kras/Erk and PI3K/Foxo1 pathways, and restricting the expression of PC transcription factors in GC B cells. Inhibition of Syk degradation perturbed gene expression, specifically in the LZ, and enhanced the generation of PCs without affecting B cell proliferation. These findings reveal how long-lasting attenuation of signal transduction by degradation events regulates cell fate within specialized microanatomical sites.

**Introduction**

Effective enduring protection from invading pathogens depends on formation of long-lived plasma cells (PCs) that secrete high-affinity antibodies, and memory B cells that rapidly differentiate into antibody-forming cells upon secondary exposure (Victora and Nussenzweig, 2012; Weisel and Shlomchik, 2017). These cells are generated in microanatomical sites known as germinal centers (GCs) that form within secondary lymphoid organs in response to invading microbes or vaccination (Berek et al., 1991; MacLennan, 1994). GCs are divided into two distinct functional zones, a dark zone (DZ) in which B cells proliferate and introduce mutations into their immunoglobulin genes, and a light zone (LZ), where B cells encounter antigen on the surface of follicular dendritic cells (FDCs), and are subjected to affinity-based selection (MacLennan, 1994; Allen et al., 2007a; Victora and Nussenzweig, 2012). Following cell division in the DZ, B cells migrate to the LZ, where their newly mutated B cell receptors (BCRs) interact with and capture antigen for processing and presentation to cognate T cells as peptides on surface MHCII molecules. These specialized T cells, known as T follicular helper cells, physically interact with cognate B cells and deliver help signals in the form of secreted cytokines and surface-bound molecules (Victora and Nussenzweig, 2012). Furthermore, several studies demonstrated that in addition to antigen uptake (Batista and Neuberger, 2000; Kwak et al., 2018), BCR affinity and triggering of downstream signals play important roles in the GC functions (Phan et al., 2003; Kräutler et al., 2017; Suan et al., 2017; Luo et al., 2018, 2019; Ise and Kurosaki, 2019; Shlomchik et al., 2019); however, how modulation of signal transduction intensities regulates B cell fate within specific GC zones and promotes generation of PCs is incompletely understood.

Previous studies demonstrated that BCR signaling in GC B cells is rewired and is significantly less efficient in trigger phosphorylation events of most downstream factors than in their naive counterparts (Khalil et al., 2012). B cells that receive T cell help up-regulate the transcription factor Myc, which is required for reentry of LZ B cells into the DZ and for subsequent clonal expansion (Dominguez-Sola et al., 2012; Calado et al., 2012; De Silva and Klein, 2015). Combination of BCR and CD40 signals leads to maximal expression of Myc in GC B cells, indicating that B cell selection in GCs depends on synergistic signals from T cells and the BCR for enhanced proliferation in the DZ (Luo et al., 2018). Expression of Foxo1 is critical for acquisition of the DZ phenotype, and in its absence, antibody affinity maturation is perturbed (Sander et al., 2015; Dominguez-Sola et al., 2015). BCR triggering induces inactivation of Foxo1 by phosphorylation (Yusuf et al., 2004; Herzog et al., 2009; Srinivasan et al., 2009), and therefore, it is expected that antigen engagements in the LZ would restrain transition to the DZ. Together, these findings suggest that an additional unknown mechanism is involved in BCR signal transduction that allows both Foxo1 inactivation and interzonal migration.
The BCR complex includes the two amplifying adaptors, Igα and Igβ, that contain immuno-tyrosine activating motifs (ITAMs) in their cytoplasmic domains (Reth and Wienands, 1997; Dal Porto et al., 2004). Receptor ligation induces rapid phosphorylation of these sites and recruitment of the key kinase, spleen tyrosine kinase (Syk), which binds the phosphorylated ITAMs via its SH2 domains (Mócsai et al., 2010; Satpathy et al., 2015). These events lead to rapid Syk autophosphorylation at multiple tyrosines, most of which were shown to play an important role in BCR signal transduction (Reth and Wienands, 1997; Kulathu et al., 2009; Song et al., 2016). Subsequent rapid down-regulation of the immunoglobulin signals depends on its inactivation by several distinct mechanisms (Pao et al., 1997; Kulathu et al., 2009; Mócsai et al., 2010; Luo et al., 2019). Phosphatases dephosphorylate Syk and its downstream targets, and attenuate the BCR signaling pathway in both naive and GC B cells (Dustin et al., 1999; Adachi et al., 2001; Khalil et al., 2012). Furthermore, Akt phosphorylation activates a negative feedback loop that attenuates the magnitude of BCR signaling (Luo et al., 2019). An additional and less explored mechanism that restricts BCR signaling is through degradation of key signaling molecules. Phosphorylation of Syk at tyrosine 317 leads to recruitment of the E3 ubiquitin ligase Casitas B-lineage lymphoma (Cbl), which tags Syk for degradation through its ubiquitylation (Lupher et al., 1998; Rao et al., 2002; Sohn et al., 2003). However, how this mechanism regulates B cell fate within the GC, including interzonal transitions and generation of PCs, remains unknown.

Here, we found that BCR triggering and extended signaling through Syk promote PC generation and impede B cell LZ to DZ transition, whereas rapid Syk degradation in the LZ attenuates signal transduction intensity and promotes B cell progression to a DZ cell state. Inhibition of Syk degradation enhanced the activation of the Kras and PI3K pathways and modulated gene expression specifically in the LZ. Collectively, our findings describe a mechanism of signal transduction attenuation in GCs that is different from the previously described kinase- and phosphatase-mediated desensitization processes.

**Results**

**A mouse model for analysis of Cbl-mediated Syk degradation**

BCR engagement by cognate antigen triggers a series of phosphorylation events with many targets, including Syk, that amplify the receptor signals and propagate downstream signaling cascades (Mócsai et al., 2010). Syk phosphorylation at tyrosine 317 (Y317) is essential for its interaction with the E3 ligase Cbl (Yankee et al., 1999) followed by rapid Syk ubiquitylation and degradation (Geahlen, 2009; Lupher et al., 1998; Sohn et al., 2003; Fig. 1 A). To explore the physiological role of this molecular mechanism in BCR signaling and GC dynamics, we generated a new mouse strain using CRISPR/Cas9 mutagenesis to introduce a point mutation into the SYK gene to abolish Cbl interaction with phosphorylated Syk protein (Syk<sup>Y317F</sup>; Fig. 1 B; Mócsai et al., 2010). The advantage of this new model over conditional activation of the BCR pathway in transgenic mice is that it allowed us to examine the role of Syk functions specifically upon Syk activation, whereas nonphosphorylated Syk is expected to be unaffected by this perturbation. To verify whether Syk degradation is indeed interrupted in our new model, freshly isolated follicular B cells derived from Syk<sup>Y317F</sup> and WT mice were stimulated by anti-IgM for 2 and 5 min, and total Syk protein levels were examined by Western blot analysis. Consistent with previous findings that show Syk ubiquitylation 2 min after BCR triggering and Cbl-mediated degradation in cell lines (Lupher et al., 1998; Sohn et al., 2003; Geahlen, 2009; Satpathy et al., 2015), Syk levels declined in WT B cells by ∼50% after 2 and 5 min of immunoglobulin stimulation, whereas almost no reduction in Syk levels was observed in Syk<sup>Y317F</sup> B cells (Fig. 1, C and D).

To assess how inhibition of Syk degradation affects the magnitude of BCR signaling, we examined the changes in intracellular calcium levels in response to BCR triggering by loading the cells with the ratiometric calcium indicator, Indo-1, followed by stimulation with a high or low dose of anti-IgM antibody. While WT B cells showed a typical transient increase followed by a decrease in intracellular calcium levels in response to stimulation at either dose, Syk<sup>Y317F</sup> B cells showed elevated intracellular calcium levels for up to 10 min after BCR triggering (Fig. 1 E). To examine how prolonged BCR signaling through inhibition of Syk degradation affects B cell activation, we stimulated B cells with anti-IgM for 16 h and examined the expression of the typical activation marker, CD86, by flow cytometry. We found that in stimulated Syk<sup>Y317F</sup> B cells, CD86 expression was twofold higher compared with WT B cells (Fig. 1 F). Thus, Syk degradation restrains the duration of BCR signaling period and attenuates B cell activation.

Ligation of the BCR triggers downstream signaling followed by internalization of the receptor. High levels of immunoglobulin expression on the surface of the B cells, as well as defects in BCR internalization can lead to enhanced immune-receptor signaling (Gazumyan et al., 2006). To rule out this possibility, we stimulated cells with biotinylated anti-IgM antibody, and tracked cell surface BCR by staining the stimulating antibody with fluorescent streptavidin. Flow cytometry analysis of non-stimulated B cells revealed that Syk<sup>Y317F</sup> B cells expressed ∼30% less IgM receptor compared with WT B cells (Fig. S1). However, in response to anti-IgM stimulation, we did not detect defects in global BCR internalization over time (Fig. 1 G). Furthermore, we found that the BCR-Induced Syk autophosphorylation of tyrosine 348, which forms a binding site for downstream effectors (Kulathu et al., 2009; Mócsai et al., 2010), was unaffected by deletion of the tyrosine at position 317 (Fig. S1). Collectively, we conclude that prolonged BCR signaling in Syk<sup>Y317F</sup> B cells was a result of the disruption in Syk degradation rather than enhanced immunoglobulin expression or defects in its internalization.

**Syk<sup>Y317F</sup> mice contain abnormally high levels of PCs and serum immunoglobulins**

BCR signaling regulates B cell development in the bone marrow (BM), and a defect in downstream signals, including those of Syk, leads to severe deficiency in the generation of mature B cells (Turner et al., 1995; Cheng et al., 1995). Furthermore, it was demonstrated that Cbl-deficient mice show altered B cell development; however, it remains unclear which Cbl targets are...
involved in this process, and whether Syk plays a role (Kitaura et al., 2007). To address the role of Syk degradation under physiological conditions, we first examined the different B cell developmental stages in WT and Syk<sup>Y317F</sup> mice within different organs. Flow-cytometric analysis of B cells derived from the BM compartment of these mice revealed no defect in formation of pre- and pro-B cells (B220<sup>+</sup>IgM<sup>−</sup>) or in the generation of immature and mature B cells (IgM<sup>+</sup>IgD<sup>−</sup> and IgM<sup>+</sup>IgD<sup>+</sup>), and whether Syk plays a role (Kitaura et al., 2007). To address the role of Syk degradation under physiological conditions, we first examined the different B cell developmental stages in WT and Syk<sup>Y317F</sup> mice within different organs. Flow-cytometric analysis of B cells derived from the BM compartment of these mice revealed no defect in formation of pre- and pro-B cells (B220<sup>+</sup>IgM<sup>−</sup>) or in the generation of immature and mature B cells (IgM<sup>+</sup>IgD<sup>−</sup> and IgM<sup>+</sup>IgD<sup>+</sup>),
respectively; Fig. S2 A). The different developmental stages of B cells in the circulation were intact as well (Fig. S2 B). Furthermore, the frequency of T1 (CD21+CD24+), T2 (CD21+CD24+CD23+), follicular mature (CD21midCD24int), and marginal zone (CD21-CD24+CD23+) B cell subpopulations in the spleen was largely normal in SykY317F mice (Fig. S2 C). Most importantly, the frequencies of mature B cells as well as cells that typically do not express Syk, such as mature CD4 and CD8 T cells in SykY317F mice, were similar to those in WT mice (Fig. 2 A). Thus, B cell development in the BM and maturation in the spleen, as well as generation of T lymphocytes, are not defective in SykY317F mice.

Enhanced BCR signaling can lead to the development of autoimmune responses, which include generation of spontaneous GCs and antibody-forming cells in response to self-proteins, as well as to an exaggerated response to foreign antigens (Rawlings et al., 2017). Flow-cytometric analysis of unimmunized SykY317F mice revealed that spontaneous GCs were slightly smaller in the spleen compared with their WT counterparts, suggesting that these mice do not develop T cell–dependent autoimmunity (Fig. 2 B). Nevertheless, we found an abnormally high frequency of CD138+ PCs in the spleen of SykY317F mice (Fig. 2 C). Accordingly, analysis of serum immunoglobulins by ELISA revealed that SykY317F mice show high titers of IgM and IgA in their serum compared with WT mice (Fig. 2 D). IgG1, an antibody isotype that highly depends on T cell functions for its generation, was also elevated in the SykY317F mice (Fig. 2 D). Although B cells are the main cell type expressing Syk, other immune cells express this kinase as well (Mócsai et al., 2010). To examine whether the increase in the presence of PCs in SykY317F mice is a result of an intrinsic defect in Syk degradation, specifically in B cells, we generated mixed BM chimeric mice, wherein irradiated WT mice were reconstituted with ~70% GFP WT and ~30% SykY317F BM-derived cells. In spite of the WT frequency advantage in the follicular compartment, flow-cytometric analysis revealed that most of the PCs in the spleen and BM of the chimeric mice were derived from the SykY317F BM cells (Fig. 2 E). Normalization of the PC frequency to the follicular compartment revealed that SykY317F PCs were over-represented in both the spleen and the BM compartments (Fig. 2 E). GCs give rise to both plasma and memory cells. Strikingly, as opposed to the increase in PCs and IgG1 serum antibodies, we did not find a similar increase in memory B cells in the BM (IgG1+CD38hi) of chimeric mice (Fig. 2 F). We conclude that disruption of Syk degradation specifically in B cells results in enhanced PC generation, whereas B cell development and memory cell formation are largely unaffected by this perturbation.

B cell persistence in GCs depends on Syk degradation

Our findings demonstrate that serum IgG1 as well as the pool of BM-resident antibody forming cells, which includes long-lived PCs, are increased in SykY317F mice, suggesting that Syk degradation plays a role in T cell–dependent immune responses. To examine this hypothesis, we generated mixed BM chimeric mice (~50:50 WT and SykY317F) and induced a T cell–dependent immune response by subcutaneous immunization with 4-hydroxy-3-nitrophenylacetyl (NP)–KLH in alum into the hind footpads. Flow-cytometric analysis of the GC compartment in popliteal LNs, 7 d later, revealed that the frequency of SykY317F and WT B cells was similar to their initial proportions in the follicular compartment (Fig. 3 A). Nonetheless, analysis of the GC compartment after an additional 7 or 14 d revealed that the frequency of SykY317F B cells was significantly reduced in the GC reaction (Fig. 3 A). This suggests that Syk degradation is required for B cell persistence and/or retention within the GC.

To further examine the role of Syk degradation in the early stages of the response, we examined the clonal composition of the GC B cells by single-cell immunoglobulin sequencing. We found that the V-region usage was similar between WT and SykY317F B cells in the GC (Fig. 3 B). In addition, clonal analysis based on the CDR3 sequence clustering did not reveal abnormal clonal diversity and expansion in SykY317F B cells 7 d after the immunization (Fig. 3 C). The proportion of GC SykY317F B cells that express IgG1 and the amount of their surface BCR were similar to those observed for WT GC B cells (Fig. S1 B). Staining of GC cells with an antigen (NP-PE) revealed that the proportion of total NP-specific IgG1+ B cells was similar between WT and SykY317F B cells in the GCs of the chimeric mice (Fig. 3 D). Together, we conclude that Syk degradation did not play a significant role in initial GC seeding; however, retention in the GC reaction was severely impaired in SykY317F B cells.

Our findings raise the possibility that affinity maturation might be defective in SykY317F B cells. To test this hypothesis, we compared the ability of WT and SykY317F GC B cells to acquire somatic hypermutations (SHM) and affinity-enhancing nucleotide changes in the IgH of single GC B cells. Immunization of C57BL/6 mice with NP haptent conjugated to a protein carrier triggers an immune response that is dominated by IgA B cells expressing NP-specific BCR (Cumano and Rajewsky, 1986). Typically, these cells express the V186.2 VH gene, and introduction of either of two mutations at specific locations (W33L and K59R) dramatically increases the affinity of their BCR and promotes clonal expansion (Allen et al., 1988; Furukawa et al., 1999). To examine whether SykY317F B cells can acquire affinity-enhancing SHM, we immunized WT and SykY317F mice with NP-OVA, and sorted single IgG1+ IgM- GC B cells for IgH mRNA sequencing. Analysis of the V186.2 mRNA transcripts revealed that SykY317F GC B cells accumulate SHM in their IgH variable regions to a similar extent as WT GC B cells (Fig. 3 E), and that the frequency of WT and SykY317F B cells that acquired one of the affinity-enhancing mutations (W33L or K59R) was similar (Fig. 3 F). We next examined whether the decline in the frequency of SykY317F cells in the GCs is a result of ineffective cell division. Immunized chimeric mice were injected with the nucleoside analog 5-ethynyl-2′-deoxyuridine (EdU), which is incorporated into newly formed DNA strands. Flow-cytometric analysis of the GC cells 2.5 or 24 h after EdU injection revealed that WT and SykY317F B cells incorporated EdU to a similar extent (Fig. 3 G). Together, we conclude that the dilution of SykY317F B cells from the GC cannot be explained by reduced proliferation rate or inability to acquire affinity-enhancing mutations.
目的，SykY317F 小鼠与表达 B1-8hi 重链基因的 mice 进行交配。在这些 mice 中，表达 Igλ 轻链（~15% 的总 B 细胞）的 B 细胞形成针对 NP 抗原的 BCR（Shih et al., 2002a）。细胞系 DsRed+ SykY317F B1-8hi 和控制 PA-GFP+ B1-8hi B 细胞被转导到宿主 mice，随后用 NP-OVA 免疫到后肢。为了增加转移细胞在 GC 中的比例并减少实验中的变异性，将细胞移入到 host mice 中。

图 2. SykY317F mice 呈现高水平的 PC 和血清免疫球蛋白的增加。(A) 脾中 WT 和 Syk(317F) mice 中的淋巴细胞频率（n = 4 或 6，两个独立实验，两尾 Student’s t 检验）。(B 和 C) GC B 细胞（B220+ FAS+ CD38low）和 PCs（CD138+）在脾中未免疫 WT 和 Syk(317F) mice（n = 5，两个独立实验的 B 和 n = 13，四个独立实验的 C，两尾 Student’s t 检验）。(D) 未免疫 WT 和 Syk(317F) mice（n = 4 或 5，两个独立实验，两尾 Student’s t 检验）中 IgM、IgA 和 IgG1 的血清水平（n = 13，四个独立实验）。(E) 流式细胞术图和在脾和 BM 中的 CD138+ PC 频率及 PC 对 B 细胞比率的量化（n = 4，两个独立实验的脾和 n = 7，四个独立实验的 BM，两尾 Student’s t 检验）。(F) 在 BM 中的 CD38+ IgG1+ 记忆 B 细胞（n = 8，两个独立实验，两尾 Student’s t 检验）。每个点代表单个 mouse；条形图代表平均值。*, P < 0.05; **, P < 0.01; ****, P < 0.0001; ns, 不显著; SSC-A, 侧散度面积。

目的，SykY317F mice 被与表达 B1-8hi 重链基因的 mice 进行交配。在这些 mice 中，B 细胞表达 Igλ 轻链 (~15% 的总 B 细胞) 形成针对 NP 抗原的 BCR（Shih et al., 2002a）。细胞系 DsRed+ SykY317F B1-8hi 和控制 PA-GFP+ B1-8hi B 细胞被转移至 host mice，随后用 NP-OVA 免疫到后肢。为了增加转移细胞在 GC 中的比例并减少实验中的变异性，将细胞移入到 host mice 中。
setting, we used host mice in which most of the B cells express a BCR that binds hen egg lysozyme (MD4 mice) and cannot respond to the NP antigen. Flow-cytometric analysis of the different GC subpopulations, 7 d after immunization, revealed that both SykY317F B1-8hi and WT B cells entered the GC response to a roughly similar extent (Fig. S3 A). However, after an additional 7 d, the SykY317F B1-8hi were not detected in the GC cell population (Fig. S3 A). As observed in the polyclonal experiment (Fig. 3 G), we did not detect a significant difference in proliferation of transferred WT and SykY317F B1-8hi B cells by EdU staining (Fig. S3 B). Thus, we conclude that Syk degradation regulates GC persistence independently of variation in BCR specificity and relative affinity during GC seeding.

We further examined whether the reduction in SykY317F B cells within GCs is a result of enhanced apoptosis. To this end, WT and SykY317F B1-8hi B cells were transferred into host mice,
and 7 d after immunization, B cells were stained with fluorescent Annexin V, which binds phosphatidylserine on membranes of apoptotic cells. Follicular B cells did not show significant binding of Annexin V, whereas control B cells that were subjected to apoptosis by heat shock showed high levels of Annexin V binding (Fig. S3 C). Analysis of GC cells did not reveal a significant difference in Annexin V staining between WT and SykY317F B cells (Fig. S3 C). Thus, the reduction in the proportion of SykY317F B cells cannot be explained by a significant increase in apoptosis within the GC compartment.

**PC generation in GCs but not memory cell formation is attenuated by Syk degradation**

Our initial analysis of SykY317F mice revealed that they host high levels of PCs in their spleen and BM (Fig. 2 C). Furthermore, the increase in serum IgG1 levels indicated that antibody generation through a T cell–dependent response is enhanced in these mice (Fig. 2 D). Based on these observations, as well as the lack of measurable defects in other GC functions, we speculated that the reduced proportion of SykY317F B cells in the GC compartment may be a result of enhanced differentiation of GC cells into PCs that contribute to the excess levels of T cell–dependent IgG1 antibodies in the serum. To interrogate this possibility, we examined the GC and PC compartments in lymph nodes of chimeric mice that were coreconstituted with WT and SykY317F BM cells. Since the BM reconstitution efficiency varied between mice and experiments, we examined and present the frequency of follicular B cells, GC B cells, and PC of eight individual chimeric mice. 14 d after immunization with NP-KLH, we found that the proportion of WT B cells in the GC was higher compared with SykY317F GC B cells; however, in all of the individual chimeric mice, regardless of BM reconstitution efficiency, the frequency of SykY317F PCs was disproportionally higher compared with their percentage in the GC (Fig. 4 A). Normalization of the PC compartment to their corresponding subpopulations in the GC on day 14 after immunization revealed an increase in the proportions of SykY317F PCs relative to their proportions in the GC compartment (Fig. 4 B). A similar analysis of CD38+ IgG1+ did not reveal an increase in the proportion of SykY317F memory B cells in immunized chimeric mice (Fig. 4 C). These findings suggest that SykY317F GC B cells are more effective in generating PCs than their WT counterparts.

To evaluate whether these PCs depend on T cell functions for their formation at the specific time point of the analysis, we ablated the CD4+ T cells during the peak of the GC response by injection of anti-CD4–depleting antibody to chimeric mice. This treatment resulted in nearly complete ablation of CD4 and GC cells in immunized mice (Fig. S3, D and E). Most importantly, depletion of CD4 T cells resulted in a considerable reduction in the number of PCs in the LN, demonstrating that these cells were recently formed in a T cell–dependent manner (Fig. 4 D and Fig. S3 F). These findings indicate that Syk degradation in B cells attenuates generation of PCs during the T cell–dependent immune response.

To examine specifically whether the CD138+ population of SykY317F B cells that was detected in lymph nodes by flow cytometry was indeed composed of GC-derived antibody forming cells, we used a pulse-chase approach that allows tracking of previously proliferating B cells in the GC reaction (Weisel et al., 2016; Li et al., 2018). Immunized chimeric mice were injected intravenously with EdU to label proliferating cells, and after 24 h, the proportions of EdU-positive cells within the PC compartment were examined by flow cytometry. We found that both WT and SykY317F PCs were fluorescently labeled, suggesting that these cells divided within the GC compartment during the 24-h pulse period before the analysis (Fig. 4 E). Analysis of a shorter pulse of EdU (2.5 h) revealed that PCs rarely proliferate outside the GCs, and no enhanced EdU incorporation was observed in SykY317F PCs (Fig. S4, A and B). Thus, these experiments confirm that WT and SykY317F PCs of GC origin are formed in our experimental analysis. Collectively, we conclude that Syk degradation in GC B cells impedes PC formation.

We found that acquisition of mutations that enhance immunoglobulin affinity was not defective in the Syk GC B cells. Nonetheless, the enhanced generation of PCs during the GC response in SykY317F mice predicts that serum antibody affinity would be reduced. To examine specifically serum antibody affinity, we immunized WT and SykY317F mice and measured the amount of antigen-specific antibodies over time. ELISA for total NP-specific antibodies (NP20) as well as for high-affinity antibodies (NP4) revealed a small defect in antibody generation at a later time point during the immune response when the GC size is reduced (Fig. 4 F). Calculation of high-affinity versus total antibody ratio revealed that the affinity of the serum antibodies of the SykY317F mice was constantly lower compared with the WT mice (Fig. 4 G). Together, we conclude that the increase in serum antibody affinity is less effective in SykY317F mice.

**Proper GC compartmentalization depends on Syk degradation**

During the GC response, B cells iteratively migrate between the DZ, where they mutate their immunoglobulins and proliferate, and the LZ, where they interact with antigens and receive T cell–derived selection signals that further promote reentry into the DZ (Allen et al., 2007b; Victora and Nussenzweig, 2012). DZ B cells express high levels of CXCR4 and low levels of CD86, whereas LZ B cells show an inverse expression pattern (Victora et al., 2010; Allen et al., 2004). DZ to LZ ratio analysis revealed that these compartments were occupied almost normally by SykY317F B cells in chimeric mice 7 d after immunization (Fig. 5 A); however, on day 14 and 21 after antigen challenge, when the proportion of the SykY317F B cells in the GC was reduced, the DZ to LZ ratio of SykY317F GC B cells was repeatedly skewed toward the LZ compartment (Fig. 5 B). These findings demonstrate that Syk degradation is required for effective persistence of B cells in the GC over time and acquisition of a DZ phenotype.

To further substantiate our conclusions, we took an alternative approach to study Syk functions by conditionally ablating Syk expression during the GC response. To this end, we crossed SykY317F mice with mice expressing ERT2-Cre under the ubiquitin C promoter to produce a mouse strain wherein we can conditionally ablate Syk expression after GC formation. To specifically examine if Syk levels in B cells affect the DZ/LZ distribution in both WT and SykY317F GC B cells, we generated mixed BM chimeric mice in which ~50% of the B cells were derived from
Figure 4. PC formation in GCs is attenuated through Syk degradation. (A) Representative flow cytometry plots of GC (B220+ FAS+ CD38low) and PC (CD138+) frequencies in popliteal LNs of chimeric hosts 14 d after immunization with NP-KLH. (B) Quantifications of follicular, GC, and PC compositions in individual WT and SykY317F chimeric mice 14 d after immunization are shown. The bar graph shows analysis of GC to PC ratios in individual chimeric mice (n = 9, two independent experiments, two-tailed Student’s t test). (C) Representative flow cytometry plots and quantification of memory (CD38+ IgG1+), GC, and PC ratios in immunized mice (n = 5, two independent experiments, two-tailed Student’s t test). (D) Quantification of CD4+ T cells, GC, and PC percentages following injection of CD4-depleting antibody or PBS to immunized mice. Gating strategy is shown in Fig. S3 (n = 6, two independent experiments, two-tailed Student’s t test). (E) Representative flow cytometry plots and quantification of EdU+ PCs 24 h after injection of EdU into mice 14 d after immunization (n = 0.5, two independent experiments, two-tailed Student’s t test). Each dot in the graphs represents a single mouse; bars represent the mean. (F) Titers of total (NP20) and high-affinity (NP4) anti-NP IgG1 in the serum of NP-OVA immunized mice. (G) Ratios of high-affinity/total anti-NP antibodies. Lines represent the mean; error bars represent SEM (in F and G, n = 5–7, three independent experiments, two-tailed Student’s t test). *, P = 0.05; ***, P < 0.001; ****, P < 0.0001; ns, not significant.
CD45.1\(^+\) WT and ~50% were CD45.2\(^+\) Syk\(^{fl/fl}\) ERT2-Cre. Chimeric mice were treated with tamoxifen on day 7 after immunization with NP-KLH, when GCs were fully established, and the B cell subpopulations were examined 11 d after immunization. Flow-cytometric analysis of GCs in LNs of immunized mice revealed that the Syk\(^{fl/fl}\) ERT2-Cre B cells were under-represented in the GC compartment (Fig. 5 C). Most importantly, analysis of the LZ and DZ compartments in the GC revealed that Syk-deficient B cells were enriched in the GC DZ compartment (Fig. 5 D). No difference in GC size and zonal distribution were observed between Syk\(^{fl/fl}\) and Syk\(^{Y317F}\) ERT2-Cre-expressing mice that did not receive tamoxifen (Fig. S4, C and D). Together, these results complement our findings using Syk\(^{Y317F}\) mice and demonstrate that transition from DZ to LZ cellular state also depends on the expression of Syk.

**Syk degradation regulates gene expression in LZ B cells and attenuates the Kras/Erk pathway**

Our findings indicate that Syk plays a major role in B cell functions specifically in the LZ, where antigens are presented as immune complexes on FDCs (Heesters et al., 2014). To gain an additional insight regarding Syk activity in the different GC zones and in propagating downstream signals, LZ and DZ B cells were sorted from immunized chimeric mice, and their global gene expression was examined by mRNA sequencing. When comparing the transcriptome of DZ-derived WT versus Syk\(^{Y317F}\) B cells, we found that very few genes were differentially expressed (24 genes were significantly up-regulated, and 2 genes were down-regulated; Fig. 6, A–C), suggesting that Syk degradation plays only a minor role in regulating the transcriptome of the DZ cells. In sharp contrast, mRNA analysis of LZ cells revealed a massive change in gene expression of the Syk\(^{Y317F}\) B cells compared with WT B cells; 84 genes were significantly up-regulated and 363 genes were down-regulated in Syk\(^{Y317F}\) LZ B cells (Fig. 6, B and C). To identify signaling pathways that were changed in Syk\(^{Y317F}\) B cells, the gene signatures of LZ B cells were examined by gene set enrichment analysis (GSEA). This analysis revealed a significant change in the expression of genes that are associated with the Kras pathway (Fig. S5 A; Ob-hora et al., 2003; Deininger et al., 2005).
Figure 6. Gene expression profiles in the LZ and the Kras/Erk pathway in GC B cells are regulated by Syk degradation. (A and B) Volcano plots showing gene expression in DZ (CXCR4hi CD86lo; A) and LZ (CXCR4lo CD86hi; B) B cells, 14 d after immunization with NP-KLH. The x axis specifies the log2 of the fold-changes (FC), and the y axis shows the log10 of the p-adj values. Dotted horizontal and vertical lines indicate the filtering criteria (FC = ±1 and p adj = 0.05). Blue dots represent differentially expressed genes. (C) Quantification of the differential significantly expressed genes in the LZ and DZ compartments of SykY317F mice. The down-regulated genes in the LZ and the up-regulated genes in the DZ of SykY317F mice were statistically significant, P < 0.05 (chi-square). (D) Flow-cytometric histograms and quantification of pErk in WT and SykY317F B cells that were left unstimulated or stimulated with anti-IgM at different time points. Each graph represents the mean ± SEM (n = 3, three independent experiments, two-tailed Student’s t test). (E) Representative flow cytometry plots of GC, PC, and follicular B cell composition in chimeric mice (~50% AIDCre/+ GFP and ~50% AIDCre/+ Rosa26stop-fl KrasG12D B cells) 21 d after immunization with NP-KLH. The graph shows the percentage of B cells in the GC (B220+FAS+ CD38low) normalized to the follicular B cell compartment (B220+FAS−CD38hi; n = 10, three independent experiments for GC and n = 7, two independent experiments for PC, two-tailed Student’s t test). (F) Flow cytometry plots and graphs of DZ and LZ distribution in individual chimeric mice as in E. Each line represents a single chimeric mouse (n = 7, two independent experiments, two-tailed Student’s t test). In E and F, each dot represents a single mouse; bars represent mean. *, P = 0.05; ****, P < 0.0001. gMFI, geometric mean fluorescence intensity; p-adj, adjusted P value.
To verify if the Kras pathway is enhanced in SykY317F B cells, we examined Erk phosphorylation in response to immunoglobulin stimulation in vitro. B cells were stimulated with anti-IgM for different time periods, followed by fixation and permeabilization for pErk intracellular staining. Flow-cytometric analysis revealed that in analogy to the prolonged calcium signaling in SykY317F B cells (Fig. 1 E), the magnitude of Erk phosphorylation was higher in these cells compared with WT B cells (Fig. 6 D). These findings indicate that prolonged activation of Erk through the Kras pathway may promote some of the phenotypes observed in SykY317F mice.

To determine whether the Kras/Erk pathway plays a role in maintaining the LZ state in GC in vivo, we crossed mice that express Cre under the activation-induced cytidine deaminase (AID) promoter to a mouse strain that carries a knock-in gene of a constitutively activated form of Kras in its Rosa26 locus (KrasG12D; Jackson et al., 2001). In this model, continuous Kras activation occurs in GC B cells as well as in B cells that previously expressed AID, but not in the immature or naive B cell compartments. To examine how Kras activation affects B cell compartmentalization in the GC, we generated mixed BM chimeric mice from BM cells of control mice (AIDCre/+ GFP), and mice expressing activated Kras in their BM cells (AIDCre/− KrasG12D). Chimeric mice were immunized with NP-KLH, and the proportion of the two B cell types in the GC was examined after 21 d. Flow-cytometric analysis of GCs in the chimeric mice revealed that B cells expressing activated Kras were over-represented in the GC compartment compared with WT B cells (Fig. 6 E). Thus, constitutive Kras activation is sufficient to enhance GC occupancy. This outcome was different from the one observed in SykY317F B cells, wherein BCR signaling intensity was increased only upon ligand-induced Syk activation. In contrast, in KrasG12D mice, signal propagation occurs constitutively regardless of immune receptor activation. Furthermore, in the chimeric mice, more PCs were produced from the cells that expressed activated Kras; however, it was impossible to assess if this observation was a result of enhanced signaling or enhanced GC occupancy (Fig. 6 E). Most importantly, analysis of DZ and LZ distribution in individual chimeric mice revealed that enhanced Kras activation promoted the LZ phenotype in GC B cells, as was observed in SykY317F B cells (Fig. 6 F). Thus, these experiments show that activation of the Kras pathway downstream of Syk partially phenocopies the skewed DZ/LZ segregation observed in the SykY317F mice.

Syk degradation attenuates the PI3K/Foxo1 pathways and expression of PC transcription factors in follicular and GC B cells

Our gene expression analysis revealed that inhibition of Syk degradation affected the PI3K pathway (Fig. S5 A). Foxo1 is a master regulator of the DZ, and in its absence or when the PI3K pathway is over-activated, the GC contains only LZ B cells (Sander et al., 2015; Dominguez-Sola et al., 2015). BCR triggering leads to activation of the PI3K pathway and downstream activation of Akt as well as to deactivation of Foxo1 by phosphorylation (Kurosaki et al., 2010). To evaluate whether Syk degradation regulates the PI3K pathway, we stimulated B cells derived from WT and SykY317F B cells with anti-IgM in vitro and examined Akt and Foxo1 phosphorylation by Western blot assay. This analysis revealed that the magnitude of phosphorylated Akt and Foxo1 was higher in SykY317F B cells compared with WT B cells (Fig. 7, A and B). The steady-state levels of total Foxo1 were similar in WT and SykY317F B cells (Fig. S5 B). We conclude that Syk degradation is required for attenuation of the PI3K pathway, and promotes Foxo1 activation by attenuating its phosphorylation.

To examine BCR signaling specifically in GC B cells, lymph nodes were removed from immunized mice, and cell suspensions were treated with anti-IgM and anti-IgG antibodies for 20 min followed by analysis of ribosomal protein S6 phosphorylation (p-S6), a target that is downstream of the BCR and PI3K/Akt pathway. We found that p-S6 was higher in SykY317F B cells compared with WT B cells (Fig. 7 C). These results demonstrate that Syk degradation is required for attenuation of BCR signaling in GC B cells.

Previous studies established that the activation of Erks and PI3K enhances the generation of PCs by promoting expression of the PC transcription factors Irf4 and Blimp-1 (Yasuda et al., 2011; Shi et al., 2015; Setz et al., 2018). In addition, it was found that artificial activation of Syk promotes Blimp-1 expression in B cells; however, the role of Syk degradation in this process was not examined (Herzog et al., 2009). Furthermore, Blimp-1 repression activity antagonizes Bcl6 and extinguishes the GC program and in combination with high levels of Irf4 promotes PC differentiation (Öchiai et al., 2013; Nutt et al., 2015; Minnich et al., 2016). Based on these observations, we examined whether the prolonged BCR signaling and Erk activation in SykY317F B cells lead to enhanced expression of PC transcription factors in vitro. Fresh follicular SykY317F B cells did not show expression of Blimp-1 and Irf4 without stimulation (Fig. S5, C and D). In addition, both naive SykY317F and WT B cells that were isolated from mouse spleens did not express Irf4 and Blimp-1 after 3 d in culture without activation signals (Fig. 7, D and E), demonstrating that SykY317F B cells do not differentiate into PCs spontaneously. However, in response to anti-IgM stimulation, SykY317F B cells showed enhanced Blimp-1 and Irf4 expression compared with WT cells (Fig. 7, D and E). To examine whether BCR triggering of SykY317F B cells promotes expression of PC transcription factors more effectively in the GC compartment as well, we purified GC B cells from WT and SykY317F mice that were immunized with NP-KLH and stimulated the cells with anti-IgM and anti-IgG for 16 h. We found that SykY317F GC B cells expressed high levels of Blimp-1 and Irf4 in response to BCR stimulation compared with their WT counterparts (Fig. 7, F and G). Collectively, we conclude that Syk degradation in response to BCR triggering attenuates the Kras and PI3K pathways, and prevents excessive expression of PC transcription factors in antigen-engaged follicular and GC B cells.
The major purpose of the GC reaction is to generate memory B cells and PCs that secrete high-affinity antibodies and provide long-lasting protection from previously encountered pathogens. Here, we describe a novel molecular mechanism of BCR signal transduction that controls the balance between PC formation and GC retention. We find that extended BCR signaling through Syk supports the formation of PCs, whereas progressive Syk degradation attenuates plasma cell formation.
degradation attenuates and limits the duration of the signal transduction and thereby promotes B cell transition into a DZ cell state.

Based on measurements of calcium signaling induced in response to BCR ligation in Cbl-deficient B cells, previous studies concluded that Cbl plays a role as a negative regulator of BCR signaling (Sohn et al., 2003); however, so far, a direct connection between Cbl and BCR signaling was not established. Thus, it seems that the BCR regulates the degradation events of specific downstream factors rather than directly controlling Cbl functions (Ise and Kurosaki, 2019). We find that inhibition of Cbl-mediated Syk degradation is sufficient to replicate the negative outcome of Cbl deletion in B cells (Lupher et al., 1998; Rao et al., 2002), suggesting that the Cbl-dependent attenuation of BCR activation functions specifically through this molecular mechanism. A recent study demonstrated that Cbl prevents premature PC differentiation by ubiquitylation and degradation of Irf4 and Cbl itself (Li et al., 2018). Our study adds an additional and specific Cbl-mediated mechanism in proximity to the BCR that suppresses the expression of PC transcription factors. Thus, post-translational mechanisms that regulate degradation events of proximal and distal BCR downstream signals, as well as negative feedback loops by phosphorylation and dephosphorylation events, attenuate BCR signaling and suppress PC generation (Li et al., 2018; Shlomchik et al., 2019; Guo et al., 2019).

During the GC response, B cells proliferate and insert mutations into their immunoglobulin genes followed by antigen engagements and interactions with T cells in the LZ (Victoria and Nussenzweig, 2012). Whereas BCR signaling plays a key role throughout the B cell response, our findings demonstrate that BCR-induced Syk degradation in vivo primarily regulates B cell functions after GC formation. This suggests that regulation of BCR signal transduction through Syk decay plays a more significant role when the B cells are subjected to a selective pressure, i.e., when the antigen is a limiting factor, and some of the cells acquire affinity-enhancing mutations. B cell transition from the LZ to the DZ requires T cell help and depends on the expression of Myc and Foxo1, the transcription factor that regulates the DZ program (Sander et al., 2012; Dominguez-Sola et al., 2012, 2015; Sander et al., 2015). Transient inhibition of LZ to DZ transition through BCR signaling and Foxo1 inactivation is expected to increase B cell encounters with T cells that promote the expression of Myc (Biram et al., 2019; Luo et al., 2018). A concomitant decline in Foxo1 constraining signals through BCR signaling attenuation, coupled to Foxo1 and Myc transcription, may optimally promote B cell transition to the DZ (Luo et al., 2019). Thus, we suggest that initial positive BCR signals followed by long lasting signal transduction desensitization through degradation events and negative feedback loops promote T cell–dependent entry to the DZ. Nonetheless, since the BCR signal is expected to become weaker over time, and since enhanced B cell signaling through Syk did not endow GC B cells with preferential advantage in clonal expansion, it seems that selection of the high-affinity variants for enhanced proliferation in the DZ is mediated by antigen uptake and presentation to T cells (Gitlin et al., 2014, 2015; Kwak et al., 2018). In contrast, enhanced BCR signaling through Syk favors B cell differentiation into PCs.

In the DZ, Foxo1 is highly expressed (Sander et al., 2015; Dominguez-Sola et al., 2015), and since most of the antigen is not retained in this compartment, BCR signaling is not expected to induce Foxo1 inactivation by phosphorylation in this zone. Therefore, there is no need for negative regulation by degradation of proximal BCR signals in the DZ. This conclusion is further supported by the finding that Cbl is expressed primarily in the GC LZ (Li et al., 2018) and by our transcriptomic analyses that revealed only minor differences in gene expression between WT and SykkY317F B cells in the DZ. Thus, we conclude that the regulation of BCR signal transduction by Syk degradation is a specialized mechanism associated with the GC LZ, where interactions with antigens and affinity-based selection by T cells take place.

BCR triggering induces numerous phosphorylation events that propagate downstream signals, followed by rapid desensitization of the signaling pathways through phosphatase activity (Geahlen, 2009; Luo et al., 2019). As opposed to phosphorylation and dephosphorylation events that are transient and can be rapidly reversed, replenishment of Syk protein levels after its degradation in the LZ depends on gene expression. Thus, the major difference between these two desensitization mechanisms is the time scale of the signal transduction recovery. It is possible that the phosphorylation of proximal BCR signals followed by dephosphorylating events allow the cells to serially engage with antigen over time, while Syk decay is a process with a long-lasting effect on cell fate. During the GC reaction, B cells interact with a limiting amount of antigen that is deposited as immune complexes on FDCs, found in the GC LZ (Heesters et al., 2014). As opposed to initial priming of B cells, most of the cognate interactions in the GC are very short-lived (∼3 min), suggesting that the BCR of GC B cells is triggered sequentially as the cells migrate over the FDC network (Schwickert et al., 2007; Suzuki et al., 2009). Based on these early observations and our new findings, we suggest that Syk is constantly subjected to transient phosphorylation events in parallel to a progressive decrease in Syk protein levels by degradation. The level of Syk might serve as a decay timer that initially favors LZ retention, when the amount of Syk is high within the cells, while the progressive reduction in Syk levels as a result of its gradual degradation may favor transition from the LZ to the DZ state. Furthermore, based on Syk depletion experiments performed by us and others (Luo et al., 2018), it seems that Syk reexpression in the DZ, among other factors, is required for B cell transition into an LZ cell state, possibly through replenishment of BCR tonic signaling. Collectively, we conclude that Syk is an important regulator of the cyclic reentry process in GCs.

It was previously shown that activation of the Kras downstream effectors, Erk1/2, leads to phosphorylation and degradation of the GC master regulator, Bcl6 (Nie et al., 1998), and indeed it was demonstrated that a transitional B cell population in the GC expresses less Bcl6 at the protein level (Basso and Dalla-Favera, 2015; De Silva et al., 2016; Laidlaw et al., 2017; Ise et al., 2018). Erk proteins are essential for PC differentiation by regulating the expression of Irf4 and Blimp-1 (Yasuda et al., 2011) through phosphorylation of Pax5, a repressor of Blimp-1.
SykY317F mice were generated as described below. WT mice were purchased from the Jackson Laboratory. NP31-KLH (Invitrogen), respectively. After purification, 2 µg/ml anti-CD43 magnetic beads or CD4+ T cell-negative isolation kits (Miltenyi Biotec), respectively. After purification, 2–4 × 10^6 B1-8hi B cells were transferred intravenously into host mice before immunization. In experiments wherein B1-8hi B cells were transferred into WT mice, 0.5 × 10^6 OT-II T cells were cotransferred. In competition experiments, the ratio of adoptively transferred cells was 50:50.

**Chimeric mice**

For generation of chimeric mice, WT hosts were irradiated with 950 rad and injected intravenously with BM cells derived from various donor mice. In Fig. 2, Fig. 3 A, Fig. 4, and Fig. 5, mice were reconstituted with 30–50% SykY317F and 20–50% CD45.1 or GFP WT BM cells. In Fig. 5, C and D, mice were reconstituted with 50% Syk^fl/fl^ ERT2-Cre and 50% CD45.1 WT BM cells. In Fig. 6, E and F, mice were reconstituted with 50% AIDCre/+ Rosa26^fl-stop-fl^ KrasG12D and 50% SJL or AIDCre/+ GFP BM cells. Mice were used 8–16 wk after BM reconstitution.

**Immunization and treatments**

Mice were injected with 25 µl PBS containing 10 µg NP31-KLH in alum into the hind footpads. In experiments involving cell transfer, mice were injected with 10 µg NP16-OVA precipitated in alum into the hind footpads. Cre-mediated deletion of Syk was triggered by tamoxifen administration (Sigma-Aldrich, 15 mg/mouse) by oral gavage on day 7 after immunization. For CD4 blocking after primary immunization, mice were injected with CD4 blocking antibody (GK1.5, Bio-X-Cell) or PBS administered both intravenously (200 µg) and into hind footpads (10 µg) 10 d after immunization.

**Flow cytometry**

Single-cell suspensions were obtained by forcing popliteal lymph nodes through mesh into PBS or RPMI containing 2% serum and 1 mM EDTA. Cells were incubated with 2 µg/ml anti-CD16/32 (clone 93) for blockage of Fc receptors for 5–10 min. Cell suspensions were washed and incubated with fluorescein isothiocyanate-labeled antibodies (Table S1) for 20–40 min. GC cells were gated as live/single, B220^CD38^ FAS+. Early activated B cells in chimeric mice, cell suspensions were stained for GCs (CD38^low^ FAS^+^), CD45.1, and CD45.2. For intracellular staining, cells were permeabilized using the Foxp3/Transcription factor-staining buffer set (eBioscience). Apoptosis was quantified by staining the cells with Alexa Fluor 647-conjugated Annexin V. For in vivo proliferation analysis, mice were injected intravenously and in the hind footpad with 2 mg and 0.25 mg EdU (Invitrogen), respectively, in PBS. Cells were then stained for surface antigens as described above and processed using Click-it EdU-488 or 647 (Molecular Probes) according to the manufacturer’s protocols. For intracellular calcium analysis, splenocytes were loaded with 2 µM Indo-1 acetoxymethyl (Indo-1 AM, Invitrogen) in PBS+/− containing 0.5% BSA at 37°C for 30 min. After loading, cells were washed and stained with B220-A780 (eBioscience). Cells were washed and resuspended in PBS+/−/0.5% BSA. After a baseline was established, cells were stimulated with 1 or 10 µg/ml F(ab’)_2 anti-mouse IgM (Jackson ImmunoResearch) and immediately analyzed for 10 min by flow cytometry using an LSRII flow cytometer. Intracellular calcium levels were determined by measurement of the fluorescence ratio of 440/40 nm to 530/30 nm emissions (violet to
Experiment, WT and SykY317F mice were intradermally immunized (Jackson ImmunoResearch) at 37°C for 16 h. For the p-S6 expression, 10 µg/ml biotinylated anti-IgG, and 1 µg streptavidin incubated in B cell medium and stimulated with 10 µg/ml anti-CD45.2+ B cells were stimulated with anti-IgM (10 µg/ml) for 3 d and for Blimp-1 and Irf4 expression, purified B cells were incubated with anti-IgM (10 µg/ml) for the indicated time periods. The reaction was stopped by adding paraformaldehyde to a 1.5% final concentration followed by incubation for 10 min at room temperature. Fixed cells were then permeabilized with ice-cold methanol and incubated for 10 min at 4°C. Cells were washed twice with PBS 1% BSA to remove residual methanol. Samples were then incubated with anti-B220 and pErk or pSyk antibodies for 30 min at 4°C. FACS analysis was performed as described below.

**Stimulation of naive B cells in vitro**

Spleens from WT and SykY317F mice were processed by forcing spleen tissue through mesh into PBS. Cells were seeded at 10⁶/ml and then incubated at 37°C in B cell medium (RPMI-1640 medium supplemented with 10% FBS, penicillin/streptomycin, gentamycin, glutamine, pyruvate, nonessential amino acids, and 50 µM β-mercaptoethanol) with or without stimulation for various times as indicated in the figure legends. For CD86 up-regulation, spleen cells were stimulated with anti-IgM (10 µg/ml) for 16 h, and for Blimp-1 and Irf4 expression, purified CD45.2+ B cells were stimulated with anti-IgM (10 µg/ml) for 3 d in the presence of CD45.1+-supporting spleen cells. B cells were stained with the indicated antibodies and analyzed by flow cytometry, and CD45.1+ cells were gated out from the analysis. For pErk and pSyk analysis, cells were incubated with anti-IgM (10 µg/ml) for the indicated time periods. The reaction was stopped by adding paraformaldehyde to a 1.5% final concentration followed by incubation for 10 min at room temperature. Fixed cells were then permeabilized with ice-cold methanol and incubated for 10 min at 4°C. Cells were washed twice with PBS 1% BSA to remove residual methanol. Samples were then incubated with anti-B220 and pErk or pSyk antibodies for 30 min at 4°C. FACS analysis was performed as described below.

**Stimulation of GC-derived B cells**

Spleenic B cells were purified from WT and SykY317F mice 14 d after NP-KLH intraperitoneal immunization using CD43 magnetic beads. Purified B cells were incubated with PE-conjugated IgD and CD38 antibodies followed by magnetic bead depletion of labeled cells with anti-PE beads (Miltenyi Biotec). The enriched cell population contained ~80% GC B cells. These cells were incubated in B cell medium and stimulated with 10 µg/ml anti-IgM, 10 µg/ml biotinylated anti-IgG, and 1 µg streptavidin (Jackson ImmunoResearch) at 37°C for 16 h. For the p-S6 experiment, WT and SykY317F mice were intradermally immunized with NP-KLH in CFA for 12 d. Spleen-derived cells were warmed to 37°C with 5% CO₂ in medium for 30 min. After the incubation, 20 µg/ml biotinylated anti-IgG was added for 5 min followed by streptavidin cross-linking and anti-IgM stimulation (10 µg/ml) for an additional 20 min.

**Western blot analysis**

Naïve splenic B cells were isolated using anti-CD43 magnetic beads (Miltenyi Biotec). Purified B cells were kept unstimulated, or stimulated with anti-IgM (10 µg/ml) for the indicated time periods at 37°C. The cells were then lysed in radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, protease inhibitors, and phosphatase inhibitors). Lysates were centrifuged for 15 min at 20,000 g at 4°C. Cleared lysates were boiled with SDS reducing sample buffer for 5 min, separated by SDS-PAGE (Bio-Rad), and transferred to nitrocellulose membranes. Blots were blocked with 5% skim milk in Tris-buffered saline + 0.1% Tween for 1 h at room temperature and incubated with specific primary antibodies overnight at 4°C with gentle agitation. The following antibodies were used for biochemical studies: anti-Syk (clone: 5F5, Biolegend); anti-pFoxo1 (polyclonal, Cell Signaling Technology), anti-Foxo1 (clone: C29H4, Cell Signaling Technology), anti-pAkt (clone D9E, Cell Signaling Technology), anti-β-actin (clone: BA3R, Thermo Fisher Scientific) and anti-GAPDH (clone: 6C5, Merck Millipore). Horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch) and ECL Reagent (Biological Industries) were used for detection.

**Analysis of BCR internalization by flow cytometry**

B cells were incubated with biotin-anti-mouse IgM (10 µg/ml, Biolegend) at 4°C for initial BCR binding and then incubated at 37°C for 0, 5, 10, and 30 min. Biotin-F(ab’)₂-anti-IgM remaining on the cell surface after the incubation was stained with APC-streptavidin and quantified using flow cytometry. The data were expressed as percentages of the cell surface-associated biotin-anti-IgM antibody at time 0 (Sharma et al., 2009).

**ELISA**

Serum was collected from unimmunized mice, and IgM, IgA, and IgG1 antibodies in the serum were detected by ELISA using anti-mouse IgM, IgA, and IgG1–horseradish peroxidase, respectively. For antibody affinity maturation analysis, WT and SykY317F mice were immunized intraperitoneally with 100 µg NP-OVA in alum. Serum was collected from mice every 7 d. NP-specific antibodies in serum were quantified by ELISA with NP-BSA or NP20-BSA that was coated on 96-well plates. NP-specific IgG1 in serum of immunized mice was detected using anti-mouse IgG1–horseradish peroxidase.

**Single-cell immunoglobulin analysis**

Popultal lymph nodes from immunized mice were harvested and processed for flow cytometry analysis. Cells were sorted using a FACS Aria cell sorter (BD Bioscience) into 96-well plates containing lysis buffer (PBS containing 3 U/µl RNAsin and 10 mM dithiothreitol). cDNA was purified using random primers (NEB) as previously described (von Boehmer et al., 2016).

For chimeric mice (50% SykY317F and 50% GFP) analysis, cell suspensions were stained for dump (CD4, CD8, GR-1, F4/80) and gated as dump⁻ B220⁺ Fas⁺GL-7⁺IgG1⁺GFP⁻ or dump⁺ B220⁺ Fas⁺ GL-7⁺IgG1⁻GFP⁺ representing WT and SykY317F GC B cells, respectively. IgG1 heavy-chain sequence was amplified twice using primers for the IgG1 constant region (5’-GAAGGGTGGCACAC CGCTGGAC-3’ in the first reaction and 5’-GCTAGGGAAATAGC CCTTGCAC-3’ in the second reaction) together with a mix of primers for the variable regions (Table S2; Ho et al., 2016).

For WT and SykY317F VH186.2 sequence analysis, cell suspensions were gated as dump⁻ B220⁺CD138⁻ GL-7⁻FAS⁻ IgG1⁺ IgG1⁺. Nested PCR was used to amplify VH186.2 segment of IgG1 heavy chains. NP-specific VH186.2 IgG1 clones were amplified using the primers: VH186.2 (5’-CTAGTAGCAACTGCAACCGGT GACATTCTCAGGTGCAGCTGCAGGAGTC-3’) and IgG1 outer (5’-GGAAGGTGGCACACCGGCGTGGAC-3’) followed by a second reaction with the VH186.2 and IgG1 inner primers (5’-GCTCAG GGAATAGCCTTGAC-3’). For total V(D)J sequencing of IgG1...
used as described (Jaitin et al., 2014), developed for single-cell RNA sequencing and transcriptome analysis (GSL Biotech). mRNA was captured with 12 μl of oligo(dT) primers for the variable regions. For gene expression analysis, 1×10^5 cells from each population were sorted into 50 μl of lysis/binding buffer (Life Technologies). RNA sequencing to produce expression libraries with a minimum of two replicates per population.

MARS-seq analysis was done using the UTAP transcriptome analysis pipeline (Kohen et al., 2019) of the Weizmann Institute Bioinformatics Unit. Reads were trimmed using Cutadapt and mapped to the Mus_musculus genome (UCSC mm10) using STAR (Dobin et al., 2013) v2.4.2a with default parameters. The pipeline quantifies the genes annotated in RefSeq (extended by 1,000 bases toward the 5’ edge and 100 bases in the 3’ direction). Counting of sequenced reads was done using htsseq-count (Anders et al., 2015; union mode). Genes having minimum of five UMI-corrected reads in at least one sample were considered. Normalization of the counts and differential expression analysis was performed using DESeq2 (Love et al., 2014) with the following parameters: betaPrior = True, cooksCutoff = FALSE, independentFiltering = FALSE. Raw P values were adjusted for multiple testing using the procedure of Benjamini and Hochberg. The threshold for significant differential expression was: padj ≤ 0.05, |log2FoldChange| ≥ 1 and baseMean ≥ 5.

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Figure S1. IgM and IgG1 expression and Syk phosphorylation at position Y348 in WT and SykY317F mice. (A and B) Flow cytometry analysis and quantification of IgM expression on WT and SykY317F follicular B cells (A) and IgG1 expression on WT and SykY317F GC B cells (B220+ FAS+ CD38low;B; n = 4 in A and n = 5 in B, two independent experiments, two-tailed Student’s t test). Each dot in the graphs represents a single mouse; bars represent the mean. (C) Flow cytometry histograms and quantification of pSyk in WT and SykY317F B cells that were left unstimulated or stimulated with anti-IgM at different time points. The gMFI of pSyk (Tyr 348) after 2 and 10 min of stimulation was normalized to the gMFI at time 0. Each graph represents the mean of ± SEM (n = 3, three independent experiments, two-tailed Student’s t test). **, P ≤ 0.01; ns, not significant.
Figure S2. Syk^{Y317F} mice exhibit normal B cell development. (A–C) Representative flow-cytometric analysis and quantification of Syk^{Y317F} and WT B cells in different developmental stages in the BM (A), peripheral blood (B), and spleen (C). Each dot represents a single mouse; bars represent the mean ($n=4–7$, two independent experiments, two-tailed Student’s t test). ns, not significant.
Syk degradation is required for B cell persistence in GCs independently of their relative BCR affinity in a T cell–dependent manner. (A) Analysis of transferred B1-8hi and B1-8hi SykY317F B cells in GCs (B220+ FAS+CD38low) in MD4 recipient mice 7 and 14 d after immunization with NP-OVA. Each dot in the graphs represents a single mouse; bars represent the mean (n = 5, two independent experiments, two-tailed Student’s t test). (B and C) Representative flow-cytometric analysis and quantification of EdU+ (B) and Annexin V+ (C) in B1-8hi and B1-8hi SykY317F B cells that were cotransferred into WT hosts. Mice were immunized with NP-OVA, and GCs were analyzed 7 d later. Each dot in the graphs represents a single mouse; bars represent the mean (n = 6–8, two independent experiments, two-tailed Student’s t test). (D–F) Gating strategy for CD4+ T cells (D), GC B cells (E), and PCs (F) following CD4-depleting antibody or PBS injection into NP-KLH–immunized chimeric mice (~50% GFP+, ~50% SykY317F). Quantification of the data are shown in Fig. 4 C. ****, P < 0.0001; ns, not significant.
Figure S4. Short-term EdU incorporation in PCs and ERT2-Cre expression effect on the GC reaction. (A and B) Representative flow-cytometric plots and quantification of EdU⁺ PCs (CD138⁺) in popliteal lymph nodes (A) of NP-KLH immunized mice (day 14) and in the BM (B) 2.5 h after injection of EdU into immunized mice (n = 4, two independent experiments, two-tailed Student’s t test). (C and D) Representative flow-cytometric plots and quantification of GC size (C), DZ, and LZ (D) GC B cell distribution in Sykfl/fl and Sykfl/fl ERT2-Cre mice 7 d after immunization without tamoxifen treatment. Each dot in the graphs represents a single mouse; bars represent the mean (n = 3, one experiment, two-tailed Student’s t test). ns, not significant; SSC-A, side scatter-area.
Figure S5. Changes in gene expression that are related to the Kras and PI3K pathways in WT and SykY317F mice. (A) GSEA plot showing enrichment of the Kras and PI3K/Akt/MTOR signaling pathways in LZ GC B cells. False discovery rate q value = 0.023 and false discovery rate q value = 1, respectively. Analysis is based on the gene expression data shown in Fig. 6. (B) Foxo1 protein levels were determined by Western blot analysis of B cells derived from WT and SykY317F mice that were stimulated with anti-IgM for the indicated time. Signals were normalized to β-actin (n = 1, one experiment). (C and D) Representative histograms and quantification of Blimp-1 (C) and Irf4 (D) expression in follicular B cells (B220+FAS+CD38hi) and PCs (CD138+) of WT and SykY317F mice. Each dot in the graphs represents a single mouse; bars represent the mean (n = 3, one experiment, two-tailed Student’s t test). ns, not significant; *, P < 0.05; **, P ≤ 0.01.
Table S1. List of antibodies used for flow cytometry

| Antigen   | Fluorophore       | Clone       | Manufacturer   | Concentration, µg/ml |
|-----------|-------------------|-------------|----------------|----------------------|
| B220      | V500              | RA3-6B2     | BD             | 1                    |
| B220      | APC-Alexa Fluor 780/e450 | RA3-6B2 | eBioscience    | 1                    |
| CD138     | BV605             | 281-2       | Biolegend      | 0.5                  |
| CD38      | FITC              | 90          | Biolegend      | 1                    |
| CD38      | Alexa Fluor 700   | 90          | eBioscience    | 1                    |
| F4/80     | APC-Alexa Fluor 780 | BM8        | eBioscience    | 1                    |
| FAS       | PE-Cy7            | Jo2         | BD             | 0.33                 |
| GL-7      | Alexa Fluor 647/PerCP-Cy5.5 | GL7 | Biolegend | 2.5                 |
| Gr-1      | APC-Alexa Fluor 780 | RB6-8C5 | eBioscience | 1                    |
| Igλ       | PE                | RML-42      | Biolegend      | 1                    |
| Streptavidin | Alexa Fluor 647 |            |                | 1                    |
| IgM       | Biotin            | RMM-1       | Biolegend      | 1                    |
| IgM       | PerCP-CeFluor710  | II/41       | eBioscience    | 1                    |
| IgG1      | BV421/FITC        | RMG1-1      | Biolegend      | 1                    |
| IgD       | Alexa Fluor 647   | 11-26c.2a   | Biolegend      | 1                    |
| CD4       | Alexa Fluor 488/Alexa Fluor 780 | GK1.5 | Biolegend | 1                    |
| CD8       | Pacific Blue      | 53-6.7      | Biolegend      | 1                    |
| CD8       | Alexa Fluor 700   | 53-6.7      | eBioscience    | 1                    |
| CD19      | Pacific Blue      | 6D5         | Biolegend      | 1                    |
| CD19      | FITC              | M819-1      | Biolegend      | 1                    |
| CD3       | PE                | I45-2C11    | Biolegend      | 1                    |
| CD21      | APC-Cy7           | 7E9         | Biolegend      | 1                    |
| CD24      | PeCy7             | M1/69       | Biolegend      | 1                    |
| CD23      | FITC              | B3B4        | Biolegend      | 1                    |
| CXCR4     | BV421             | L276F12     | Biolegend      | 1                    |
| CD86      | PE/FITC           | GL-1        | Biolegend      | 0.08                 |
| pERK      | Alexa Fluor 488   | 4B11B69     | Biolegend      | 2.5                  |
| CD45.1    | BV421             | A20         | Biolegend      | 1                    |
| CD45.1    | Alexa Fluor 780   | A20         | eBioscience    | 1                    |
| CD45.2    | PE                | 104         | Biolegend      | 1                    |
| Blimp-1   | Alexa Fluor 647   | 5E7         | Biolegend      | 1                    |
| IrF4      | Alexa Fluor 488/PerCP-Cy5.5 | IRF4.3E4 | Biolegend | 1                    |
| p-S6 (Ser235/236) | Alexa Fluor 647 | D57.2.2E | Cell Signaling Technology | 1 |
| Primers                              | Primer sequence 5’-3’                                   |
|--------------------------------------|--------------------------------------------------------|
| Variable region primers              |                                                        |
| HP1F-mLEADER-1                       | GTAACACTTTTAAATGGGTATCCAGTGT                           |
| HP1F-mLEADER-2                       | GTCTAATTTTAAAGGTGTCAGTGTC                           |
| HP1F-mLEADER-3                       | AGCAACAGCTACAGGTCAGGTCCACTCC                         |
| HP1F-mLEADER-4                       | TCTTCTCTGTGTCAGTAACTRCAGG                            |
| HP1F-mLEADER-5                       | TTGCTATTCTGTGAGGTCAGCAGCTGCCC                         |
| HP1F-mLEADER-6                       | GTGACATTCTCTAGCTGTCCTRCC                              |
| HP1F-mLEADER-7                       | TGATACGTGTGCACTGTGTCCTGG                              |
| HP1F-mLEADER-8                       | GTTTTTTATCAAGGTGCATTGT                              |
| HP1F-mLEADER-9                       | CTATTCTGTGACGGCAGCTGCCCAAAG                         |
| VH186.2 variable region primer       | CTAGTACACTGCAACCGGTGATTACTCTGAGGTCAGGAGGTC          |
| Constant region primers              |                                                        |
| Cy1-outer                            | GGAAGGTGTGCACACCGGCTGGAC                              |
| Cy1-inner                            | GCTCAAGGAAATAGCCCTTTGAC                               |