Enhanced BCR signaling inflicts early plasmablast and germinal center B cell death

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HIGHLIGHTS
- BCR signaling without adequate further stimulation can inflict cell death
- Inappropriate BCR signaling can inhibit extra-follicular PC differentiation
- Increased BCR signaling induces apoptosis in early GC B cells
- BCR signaling intensity affects affinity-dependent B cell selection

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Enhanced BCR signaling inflicts early plasmablast and germinal center B cell death

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SUMMARY
It is still not clear how B cell receptor (BCR) signaling intensity affects plasma cell (PC) and germinal center (GC) B cell differentiation. We generated Cγ1Cre/w1Ptpn6fl/fl mice where SHP-1, a negative regulator of BCR signaling, is deleted rapidly after B cell activation. Although immunization with T-dependent antigens increased BCR signaling, it led to PC reduction and increased apoptosis. Dependent on the antigen, the early GC B cell response was equally reduced and apoptosis increased. At the same time, a higher proportion of GC B cells expressed cMYC, suggesting GC B cell-Tfh cell interactions may be increased. GC B cell numbers returned to normal at later stages, whereas affinity maturation was suppressed in the long term. This confirms that BCR signaling not only directs affinity-dependent B cell selection but also, without adequate further stimulation, can inflict cell death, which may be important for the maintenance of B cell tolerance.

INTRODUCTION
Specific interaction between antigen and the B cell receptor (BCR) is the key signal for B cell selection and activation (Niiro and Clark, 2002; Yam-Puc et al., 2018). After initial activation in vivo, B cells may differentiate into plasma cells (PCs) through rapid extra-follicular expansion or become germinal center (GC) cells that will undergo BCR affinity maturation for antigen (MacLennan, 1994; MacLennan et al., 2003; Victora and Nussenzweig, 2012). GCs contribute to long-lived humoral responses by producing high-affinity antibody-forming PCs and memory B cells (MacLennan, 1994; Victora and Nussenzweig, 2012; Weisel et al., 2016). High-affinity neutralizing antibodies represent a crucial mechanism by which vaccines or natural infections confer sterilizing immunity protecting against re-exposure to the same pathogen (Bachmann et al., 1994; Steinhoff et al., 1995). Two major signals regulate B cell activation leading to antibody production: signals from T helper cells have been studied intensely in recent years (Oropallo and Cerutti, 2014; Shulman et al., 2013; Victora et al., 2010), whereas less attention has been given to the impact of BCR signaling during selection of B cells by antigen (Khalil et al., 2012; Mueller et al., 2015). Although signals from pathogen recognition receptors may participate in B cell activation (Li et al., 2013; Pone et al., 2015), the interaction between antigen and BCR has been described as crucial to control whether activated B cells enter the GC or undergo rapid PC differentiation in extra-follicular proliferative foci. B cell clones undergoing a strong initial interaction with antigen can efficiently differentiate into extra-follicular PCs contributing to the rapid early phase of the antibody production (Paus et al., 2006). B cells expressing a wide range of BCR affinities become pre-GC B cells after T-B interaction (Dal Porto et al., 2002; Schwickert et al., 2011; Victora et al., 2010). Higher affinity BCRs can induce stronger signal transduction than lower affinity ones (Kouskoff et al., 1998). BCR occupancy is a product of BCR affinity and antigen concentration, and concentration of free antigen can be limited by antibody feedback (Toellner et al., 2018).

B cell activation upon BCR ligation can be amplified by Toll-like receptor (TLR) signaling (Castro-Dopico and Clatworthy, 2019; Pone et al., 2012), and this may act at very early stages before T cell help is available. However, these innate immune receptors may also have an inhibitory effect on B cell activation, such as TLR-9, which can inhibit antigen processing and presentation by B cells, inhibiting acquisition of T cell help (Akkaya et al., 2018a, 2018b). The effect of all this on cell fate decisions during B cell differentiation merits more attention.
The Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase (PTP)-1 (SHP-1), encoded by the \textit{Ptpn6} gene, negatively regulates BCR signaling primarily via its binding to the immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors CD72, CD22, Fc\textit{y}RIIB, paired Ig-like receptor (PIR)-B, and FCRL3 (Adachi et al., 2001; D’Ambrosio et al., 1995; Kochi et al., 2009; Maeda et al., 1998; Nitschke and Tsubata, 2004). Although FCRL3 can mediate B cell activation through TLR-9 stimulation, this seems to be independent of SHP-1 (Li et al., 2013). SHP-1 is expressed and constitutively activated in all B cells, and its specific deletion on B cells results in systemic autoimmunity (Pao et al., 2007). SHP-1 is highly expressed and activated in GC B cells, suggesting that BCR signaling is negatively regulated during differentiation of GC B cells (Khalil et al., 2012). BCR signaling has been shown to be absent in dark zone (DZ) GC B cells (Stewart et al., 2018), whereas there is more signal transduction in light zone (LZ) B cells competing for selection signals through affinity-dependent activation of their BCR (Mueller et al., 2015).

To test how BCR signaling inhibition by SHP-1 affects antigen-induced B cell differentiation, we generated \textit{Cg1}^{\textit{Cre/wt}}\textit{Ptpn6}^{fl/fl} mice, in which the T-dependent B cell activation induces SHP-1 deletion in most B cells (Roco et al., 2019). Most induction of immunoglobulin class switch recombination (CSR) happens during the initial phase of cognate T cell-B cell interaction before GCs are formed, which is accompanied by rapid strong induction of IgG1 germline transcripts (Marshall et al., 2011; Roco et al., 2019; Toellner et al., 1998). Although CD40 ligation and interleukin (IL)-4 are strong inducers of IgG1 germline transcripts (Stavnezer et al., 2008), their expression is not necessarily followed by CSR. Here we use Cre recombinase located inside the IgG1 heavy chain locus as a reporter for successful T-dependent B cell activation (Casola et al., 2006; Roco et al., 2019). Using \textit{Cg1}^{\textit{Cre}} mice that contain a Cre-deletable version of SHP-1 (\textit{Ptpn6}), we show that \textit{Cg1}^{\textit{Cre/wt}}\textit{Ptpn6}^{fl/fl} B cells exhibit stronger BCR signaling. Paradoxically this leads to a smaller extra-follicular IgG1+ PC response and to death of GC B cells, resulting in reduced affinity maturation in the GC.

**RESULTS**

**Increased apoptosis in extra-follicular plasma cells of \textit{Cg1}^{\textit{Cre/wt}}\textit{Ptpn6}^{fl/fl} mice**

B cells binding antigen with higher affinity are more likely to differentiate into extra-follicular PCs (O’Connor et al., 2006; Paus et al., 2004). To test whether deletion of the negative regulator of BCR signaling, SHP-1, affects the early extra-follicular PC response to immunization, \textit{Cg1}^{\textit{Cre/wt}}\textit{Ptpn6}^{fl/fl} and \textit{Cg1}^{\textit{Cre/wt}}\textit{Ptpn6}^{fl/fl}, in the following abbreviated as Shp1\textit{fl/wt} and Shp1\textit{fl/fl} mice, were immunized with sheep red blood cells (SRBCs) intravenously. The \textit{Cg1}^{\textit{Cre}} allele reports expression of IgG1 germline transcripts (Casola et al., 2006), which are strongly induced after the initial interaction of B cells with T helper cells before PCs or GCs appear (Marshall et al., 2011; Roco et al., 2019; Zhang et al., 2018). This should lead to efficient deletion of SHP-1 in extra-follicular PCs and GC founder B cells. Spleens were analyzed 5 days post immunization, when the extra-follicular PC response peaks and early GCs have formed (Zhang et al., 2018).

Against expectation, flow cytometry showed that Shp1\textit{fl/fl} PC numbers were reduced by 50% (Figure 1A). This primarily affected IgG1-switched PCs, whereas non-switched IgM PCs developed in similar numbers as in Shp1\textit{fl/wt} control animals (Figure 1B). Testing deletion of SHP-1 in PCs by flow cytometry showed that Shp1\textit{fl/wt} and Shp1\textit{fl/fl} PC expressed similar amounts of SHP-1 (Figures S1A and S1B), suggesting that the surviving PCs had not deleted SHP-1. Immunohistology, using IRF4 as a marker for PCs, confirmed reduced PC foci in the splenic red pulp, primarily in the IgG1-switched PCs of Shp1\textit{fl/fl} mice (Figure 1C). PCs emerging from GCs at the GC-T zone interface (GTI) (Zhang et al., 2018) were unaffected at this point (Figure S1C). These data indicate that increased BCR signaling after initial B cell activation inhibits extra-follicular PC differentiation.

Hyper-activation of B cells through BCR signaling can lead to programmed cell death (Akkaya et al., 2018b; Parry et al., 1994; Tsubata et al., 1994b; Watanabe et al., 1998). To test whether cell death was responsible for the smaller extra-follicular PC response, apoptotic cells were detected using Annexin V and 7-AAD staining. This showed a minor increase in the proportion of apoptotic PCs (Annexin V+ and 7-AAD+) in Shp1\textit{fl/fl} mice (Figure 2A). Also, the expression of active caspase-3 on different isotypes of PCs showed that IgG1+ PCs of Shp1\textit{fl/fl} animals were more likely to express active caspase-3 (Figure 2B). Immunohistology confirmed an increase in active caspase-3+ cells in the IRF-4+ extra-follicular splenic foci of Shp1\textit{fl/fl} mice (Figure 2C). Interestingly, apoptosis was increased despite the similar SHP-1 protein expression in Shp1\textit{fl/fl} PCs that survived to this stage (Figure S1A). Therefore, it is likely that at earlier stages the differences in apoptosis rates were even more pronounced. Taken together, this indicates that an inappropriate increase in BCR signaling can negatively affect extra-follicular PC generation through increased cell death.
SRBC-induced GC formation of Cγ1Cre/wtPtpn6fl/fl mice is unaffected

In established GCs, BCR signaling is limited by SHP-1 hyper-phosphorylation, and this is important for GC maintenance (Khalil et al., 2012). To test how SHP-1 deletion, starting from the earliest stages of GC development, affects the GC response we followed GC B cell differentiation in Cγ1Cre/wtPtpn6fl/fl mice 5 days after SRBC immunization. Surprisingly, at this early stage there was no significant change in the number of GC B cells in Shp1fl/fl mice (Figure 3A). Flow cytometry confirmed a reduction of SHP-1 staining intensity in all GC B cells (Figure S2A), indicating that most GC B cells had deleted the gene. The increase in SYK phosphorlyations seen in GC B cells in Shp1fl/fl mice confirmed that SHP-1 deletion does increase BCR signaling in this system (Figure 3B). In contrast to what was seen in extra-follicular PCs, cell death in GC B cells, evaluated by flow cytometric analysis of Annexin V/7-AAD and active caspase-3 staining, was not increased at this stage (Figure 3C). Immunohistology confirmed that there were no obvious changes in GCs in Shp1fl/fl compared with Shp1fl/wt mice (Figure 3D). These data indicate that increased BCR signaling after initial B cell activation does not affect the formation of GCs.

Germinal center B cell responses and affinity maturation to hapten protein are impaired in Cγ1Cre/wtPtpn6fl/fl mice

While SRBC immunization rapidly induces B cell activation and differentiation (Zhang et al., 2018), it also has a T-independent component. Primary foot immunization with 4-hydroxy-3-nitrophenyl acetyl coupled to chicken γ-globulin (NP-CGG) in alum induces strong IL-4 expression in T cells, Th2 type B cell activation, and rapid differentiation of extra-follicular PCs as well as GC in the draining popliteal lymph nodes (Toellner et al., 1998). Furthermore, this antigen allows the identification of antigen-specific antibodies.

To better estimate the response in B cells that had an actual history of Cre-recombinase expression, we generated Cγ1Cre/wtPtpn6fl/wt and Cγ1Cre/wtPtpn6fl/fl on the ROSA26tm1G background, which contain a Cre-inducible membrane-tagged version of eGFP (Muzumdar et al., 2007). Eight days post subcutaneous immunization with NP-CGG, Shp1fl/fl draining lymph nodes showed a reduced numbers of PCs (Figures 4A and 4B), similar to what was seen in Shp1fl/fl spleens after SRBC immunization. Again, PCs surviving to this
Stage contained normal amounts of SHP-1 (Figures S2B and S2C). GCs developing in Shp1\textsuperscript{fl/fl} mice were smaller (Figure S2D) and numbers of GC B cells were reduced (Figures 4A and 4B). This went along with increased GC B cell apoptosis (Figure 4C). Again, SHP-1 deletion (Figure S2E) resulted in increased BCR signaling, as detected by increased SYK phosphorylation (Figure 4D).

To test the hypothesis that B cell are dying by apoptosis because they are not receiving sufficient T cell help, we evaluated the expression of cMYC, which is induced after GC B cell stimulation by Tfh cells (Calado et al., 2012; Dominguez-Sola et al., 2012; Luo et al., 2018). Despite expectation, cMYC was found in a larger number of Shp1\textsuperscript{fl/fl} GC B cells (Figure 4E), suggesting more efficient Tfh help. These results suggest that, similar to what is seen in extra-follicular PCs, overstimulation of GC B cells leads to increased cell death. Deletion of C-terminal Src kinase (Csk), another downstream inhibitor of BCR signaling, led to a similar reduction of the GC B cell response in C\textsubscript{g1}C\textsubscript{re}Ptpn6\textsuperscript{fl/fl} mice after 8 and 14 days of NP-CGG immunization (Figures S3A and S3B).

**Figure 2. Plasma cell apoptosis is increased in SRBC immunized C\textsubscript{g1}C\textsubscript{re}Ptpn6\textsuperscript{fl/wt} mice**

Apoptosis rate on PCs was analyzed 5 days post SRBCs immunization in C\textsubscript{g1}C\textsubscript{re}Ptpn6\textsuperscript{fl/wt} and C\textsubscript{g1}C\textsubscript{re}Ptpn6\textsuperscript{fl/fl} mice. (A) Representative dot plots show apoptosis rate based on the binding of Annexin V and the dead cell dye 7-AAD (pregated on PCs; top panel). Annexin V\textsuperscript{+} 7-AAD\textsuperscript{-} cells were considered as early apoptotic cells, and Annexin V\textsuperscript{+} 7-AAD\textsuperscript{+}, cells as late apoptotic cells. Summary data (bottom panel; % of plasma cells; results are combined from two independent experiments).

(B) Active caspase-3 expression on IgG1\textsuperscript{+} or IgM\textsuperscript{+} PCs. Graphs on the right show the percentage of active caspase-3\textsuperscript{+} cells within IgG1\textsuperscript{+} or IgM\textsuperscript{+} plasma cells. Results are combined from three independent experiments.

(C) Spleen sections from Shp1\textsuperscript{fl/wt} and Shp1\textsuperscript{fl/fl} mice staining for B cell follicles (IgD, green), T cell zone (CD4, blue), and PCs (IRF4 in red) in the top, or active caspase-3\textsuperscript{+} cells (Caspase-3 in red) in the bottom. Ratio of active caspase-3\textsuperscript{+} pixel/IRF4\textsuperscript{+} pixel, representative of one of two independent experiments. Each symbol corresponds to one animal; horizontal lines indicate the mean. n.s. not significant, *p < 0.05 (two-tailed t test).
To test the effects of SHP-1 deletion on later stages of the response to NP-CGG, splenic GC response and affinity maturation were monitored after intraperitoneal primary immunization of Shp1\(^{fl/wt}\) and Shp1\(^{fl/fl}\) mice. Similar to what was seen in lymph nodes, increased BCR signaling led to a reduced early GC response 8 days after immunization, but this effect was lost at later stages of the response (Figures 4F and S4A). NP-specific IgG1 was marginally reduced, whereas NP-specific antibody affinity in Shp1\(^{fl/fl}\) mice did not increase at late stages after immunization (Figure 4G), showing that despite the normalization in GC B cell numbers, there is a long-term effect on the efficiency of affinity-dependent B cell selection. A similar reduction of NP-specific IgG and IgG1 titers and antibody affinity were seen in C\(^{g1}\)\(^{Cre/wt}\) Csk\(^{fl/fl}\) animals (Figures S3C and S3D).

**DISCUSSION**

Enhanced BCR signaling due to specific deletion of SHP-1 in all B cells during development leads to B1a B cell subset expansion and results in autoimmunity (Pao et al., 2007). Few studies have tested the effects of
artificially enhanced BCR signaling in mature B cells that had undergone normal B cell development (Davidzohn et al., 2020; Li et al., 2014). The model presented here allows normal B cell development and increased BCR signaling by deletion of SHP-1 only after mature and naive B cells are activated by signals that may induce class-switching to IgG1.

Figure 4. Germinal center B cell responses and affinity maturation to NP-CGG are impaired in Cγ1Cre/wtPtpn6fl/fl mice

(A) Germinal center B cell responses in popliteal lymph nodes (PLN) 8 days post NP-CGG immunization of Cγ1Cre/wtPtpn6fl/wt ROSAmTmG and Cγ1Cre/fl/flPtpn6fl/wt ROSAmTmG mice. Sequential gating strategy for the identification of PCs (lymphocytes/singlets/live/tomato+C0GFP+B220+C0CD138) and GC B cells (lymphocytes/singlets/live/tomato+C0GFP+CD138−CD220+CD38−Fas+).

(B) Summary of PC and GC B cell numbers (% of live cells and total numbers per LN); three independent experiments.

(C) Active caspase-3 on GC B cells from PLNs 8 days post NP-CGG immunization (% of GC B cells; results are representative of one experiment).

(D) SYK phosphorylation in GC B cells (MFI on GC B cells).

(E) Relative percentage of GC area containing cMYC-expressing cells. Each symbol represents a different GC. Data combined from two independent experiments.

(F) Splenic NP-specific GC B cells at different time points after immunization (total cell numbers per spleen; results are from one to two independent experiments).

(G) Serum antibody titers for NP-specific IgG1 (left panel) and relative affinity of NP-specific IgG1 (right panel). Results are from two to three independent experiments; horizontal lines indicate the mean. n.s. not significant, *p < 0.05, **p < 0.01, ***p < 0.001 (B–E two-tailed t test and F–G two-way ANOVA).
SHP-1 inhibits BCR signaling through SYK (Adachi et al., 2001), and activated B cells in the current model show clear signs of pSYK overexpression after B cell activation.

After antigen-mediated BCR stimulation and T cell help, B cells may differentiate into extra-follicular PCs (MacLennan et al., 2003) or become GC precursor cells to start GC reactions (Victora and Nussenzweig, 2012). The exact mechanisms that control this fate decision is still controversial and under scrutiny. It has been shown that B cells experiencing a strong initial interaction with antigen more efficiently differentiate into extra-follicular PCs (Paus et al., 2006). Here, we show that higher signaling through the BCR affects PC differentiation in unexpected ways. 

Although the frequency of apoptosis in the PC compartment seems high, this appears to fit with the substantial PC death during the contraction of the immune response (McCarron et al., 2017; Smith et al., 1996; Sze et al., 2000). Kinetics of cell death in extra-follicular foci in the primary response are scarce, but it has been estimated that the daily cell loss should be over 20%, enough to account for their massive decline (Smith et al., 1996). PC death in the extra-follicular response is sharp and short (Sze et al., 2000), whereas GC responses and apoptosis therein are happening over longer periods, and apoptotic GC B cells are efficiently removed by tingible body macrophages. Therefore, it seems quite possible that removal of apoptotic cells in the extra-follicular response is less efficient.

Although the effect on the size of the GC compartment in NP-CGG immunized mice was only transient, the higher pSYK levels clearly indicate considerably increased signal transduction in GC B cells. pSYK levels were also increased in GC B cells induced by SRBC immunization, although there was less obvious effect on GC size. This may be explained by the fact that the response to SRBC immunization is less dependent on T cell help, and that GC B cells are able to survive and expand for a limited time without T cell help (de Vinuesa et al., 2000). A recent study testing the inhibition of pSYK degradation in GC B cells using mixed bone marrow chimeras (Davidzohn et al., 2020) showed that increased SYK signaling led to an increase in the GC LZ compartment. Further differentiation of these LZ B cells depended on Tfh cell help (Davidzohn et al., 2020; de Vinuesa et al., 2000; Gitlin et al., 2015; Shulman et al., 2013). We show here that SHP-1 deletion in the GC leads to higher levels of pSYK. Many of these GC B cells are able to recruit efficient Tfh cell help, indicated by the increased expression of cMYC (Calado et al., 2012; Dominguez-Sola et al., 2012). However, many GC B cells undergo apoptosis. BCR overstimulation and inadequate expression of
cMYC can induce cell death (Akkaya et al., 2018b; Meyer and Penn, 2008; Watanabe et al., 1998), and this may be responsible for the reduced viability of B cells at the early stages of the GC response.

GCs are not only sites of affinity maturation. B cell selection in the GC also guarantees peripheral tolerance (Goodnow et al., 1989; Russell et al., 1991). The data shown here could reflect the deletion of autoreactive GC B cells that encounter inadequate BCR signaling and are not able to recruit adequate Tfh cell help in time. In the same way, higher affinity SHP-1-deficient GC B cells may be deleted because they are not recruiting sufficient Tfh cell help. This would indicate that affinity-dependent BCR signaling not only is important for affinity-dependent B cell selection but also that the balance of BCR signaling and Tfh cell-mediated rescue may regulate tolerance during GC B cell responses.

Limitations of the study
Although CSR occurs during the initial T cell:B cell interaction before GC formation or extra-follicular plasmablasts differentiation, very early signals influencing B cell fate decisions might have been overlooked. Furthermore, it must be considered that SHP-1 gene deletion may not necessarily translate into immediate reduction of SHP-1 levels due to the half-life of existing kinases inside the cell, making some delays in our observations. On the other hand, our interpretations are limited to Th2-type responses, further evaluation of BCR signals to other type of responses would be needed for more general conclusions.

Resource availability
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Kai-Michael Toellner (k.m.toellner@bham.ac.uk).

Materials availability
Materials generated in this study are available upon request. Materials and resources are summarized in Table S1.

Data and code availability
No data sets or code were generated or analyzed in this study. All software is commercially available.

METHODS
All methods can be found in the accompanying Transparent methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102038.

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AUTHOR CONTRIBUTIONS
Conceptualization, J.C.Y.-P. and K.-M.T. Investigation, J.C.Y.P., L.Z., R.A.M.-A., L.G.-I., and Y.Z. Formal analysis J.C.Y.P. Resources, Y.A.S., M.S., and K.-M.T. Writing – Original Draft. J.C.Y.-P. Writing – Review & Editing, J.C.Y.-P. and K.-M.T. All authors reviewed and edited the final version of the manuscript. Supervision and Funding Acquisition, K.-M.T.

DECLARATION OF INTERESTS
The authors declare no personal, professional, or financial conflict of interest.
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Supplemental Information

Enhanced BCR signaling
inflicts early plasmablast
and germinal center B cell death

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Supplementary Figure 1. SHP-1 expression on PCs and GC-associated PCs during the response to SRBCs, related to Figure 1. Cy1Cre/wtPtpn6fl/wt (fl/wt) and Cy1Cre/wtPtpn6fl/fl (fl/fl) mice were immunised with SRBCs. A. SHP-1 expression in PCs was determined 5 days post immunisation. Representative FACS plot (left) and summary data (right) of SHP-1 expression (MFI) in all PCs. B. SHP-1 expression in IgG1+ PCs. Results are representative of two independent experiments. Grey histograms in A and B show the fluorescence minus one (FMO) control for SHP-1 staining. C. Quantification of IRF4+ PCs from Cy1Cre/wtPtpn6fl/wt (fl/wt) and Cy1Cre/wtPtpn6fl/fl (fl/fl) mice 5 days post SRBC immunisation in the GC-T zone interface (Zhang et al., 2018). Spleens were stained with IRF4 (red), IgD (green) and CD4 (blue). T, T zone; GC, germinal centre. Bar, 100 µm. (% of GC area; results are representative of one of two independent experiments). Horizontal lines indicate the mean.
Supp. Fig. 2

A. From GC B cells:

MFI of SHP-1

fl/wt fl/fl

0 200 400 600 800 1000 1200

GC B cells

B. From Plasma cells:

MFI of SHP-1

fl/wt fl/fl

0 500 1000 1500

Plasma Cells

C. From IgG1+ PCs:

MFI of SHP-1

fl/wt fl/fl

0 500 1000 1500

IgG1+ Plasma Cells

D. Shp1

fl/wt fl/fl

IgD CD4 PNA

E. From GC B cells:

MFI of SHP-1

fl/wt fl/fl

0 5 10 15 20

GC area

% of LN section

SHP-1

\*
Supplementary Figure 2. SHP-1 expression is decreased in GC B cells from Cγ1Cre/wtPtpn6fl/fl mice, related to Figure 3 and Figure 4. A. Cγ1Cre/wtPtpn6fl/wt (fl/wt) and Cγ1Cre/wtPtpn6fl/fl (fl/fl) mice were immunised with SRBCs and SHP-1 expression was determined 5 days post immunisation. Representative FACS plot (left) and summary data (right) of SHP-1 expression (MFI of GC B cells; results are representative of one of two independent experiments). B-C. SHP-1 expression was determined 8 days post NP-CGG immunisation. Representative FACS plot (left) and summary data (right) of SHP-1 expression (MFI) on PCs (B), IgG1+ PCs (C); results are representative of one of two independent experiments. D. Lymph node sections from Shp1fl/wt (fl/wt) and Shp1fl/fl (fl/fl) mice staining for B cell follicles (IgD, green), T cell zone (CD4, blue) and the GC marker PNA (red), scale bar 500 μm. PNA+IgD- areas were calculated as percentage of total LN area. E. Representative FACS plot (left) and summary data (right) of SHP-1 expression (MFI) on GC B cells. Grey histograms in FACS plots show the fluorescence minus one (FMO) control for SHP-1 staining. Each symbol represents one animal. *P < 0.05, **P < 0.01 (two-tailed t-test).
Supplementary Figure 3. B cell responses in the absence of CSK, related to Figure 4. A. Cy1\textsuperscript{Cre/wt}\textit{CSK}\textsubscript{fl/wt} (black circles) and Cy1\textsuperscript{Cre/wt}\textit{CSK}\textsubscript{fl/fl} (white circles) mice were immunised with NP-CGG and the PC and the GC responses were analysed 8 and 14 days post immunisation. (% of live cells; results are combined from two independent experiments). B. Spleen sections from \textit{CSK}\textsubscript{fl/wt} (fl/wt) and \textit{CSK}\textsubscript{fl/fl} (fl/fl) mice staining for B cell follicles (IgD, brown) and T cell zone (CD3, blue). GCs are identified as IgD-CD4\textsuperscript{+} (black arrows), scale bar 200 \textmu m. C. Serum antibody titres for NP-specific IgM, IgG and IgG1 8 and 14 days post-immunisation and the relative affinity of IgG1 to NP (D), 14 days post-immunisation. (Results are from one to two independent experiments at each time-point). Horizontal lines indicate the mean. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (A and D two-tailed t-test and C two-way ANOVA).
Supplementary Figure 4. In situ splenic GC response after NP-CGG immunisation of $\gamma_1^{\text{Cre/wt}}\text{Ptpn6}^{\text{fl/wt}}$ and $\gamma_1^{\text{Cre/wt}}\text{Ptpn6}^{\text{fl/fl}}$ mice, related to Figure 4. $\gamma_1^{\text{Cre/wt}}\text{Ptpn6}^{\text{fl/wt}}$ and $\gamma_1^{\text{Cre/wt}}\text{Ptpn6}^{\text{fl/fl}}$ mice were immunised with NP-CGG and spleen sections were stained for B cell follicles (IgD, blue), T cell zone (CD4, green) and Bcl6+ germinal centres (red) 8, 14, and 21 days post-immunisation, scale bar 200 μm. Bcl6+IgD- areas were calculated as percentage of total spleen area. Each symbol corresponds to one animal.
### REAGENT or RESOURCE | SOURCE | IDENTIFIER
--- | --- | ---
**Antibodies**
Rat anti-CD4 (GK1.5), allophycocyanin | eBiosciences | Cat # 17-0041-82
Rat anti-IgD (11-26c.2a), BrilliantViolet421 | BioLegend | Cat # 405725
Goat anti-IgG1, Alexa Fluor 633 | Invitrogen, UK | Cat # A21126
Goat anti-mouse IRF4 (M-17), purified | Santa Cruz Biotech | Cat # sc-6059
Rabbit anti-mouse active Caspase 3 (C92-605), purified | BD Biosciences | Cat # 55956
Sheep anti-IgD, polyclonal | Abcam | N/A
Donkey anti-rabbit, Cy3 | Jackson ImmunoResearch Laboratories | Cat # 711-165-152
Donkey anti-sheep, Alexa Fluor 488 | Jackson ImmunoResearch Laboratories | Cat # 713-545-147
Donkey anti-goat, Alexa Fluor 555 | Invitrogen, UK | Cat # A21432
Rat anti-CD16/32 (93), purified | BD Biosciences | Cat # 01241A
Rat anti-B220 (RA3-6B2), BrilliantViolet510 | BioLegend | Cat # 103248
Rat anti-CD138 (281-2), BrilliantViolet711 | BioLegend | Cat # 142519
NP - Phycoerythrin | In house | N/A
Rat anti-CD38 (90), Fluorescein isothiocyanate | eBiosciences | Cat # 11-0381-82
Rat anti-Fas (Jo2), BrilliantViolet605 | BD Biosciences | Cat # 740367
Rat anti-IgG1 (X56), Allophycocyanin | BD Biosciences | Cat # 550874
Mouse anti-IgM (Igh-6b), Phycoerythrin | BD Biosciences | Cat # 553521
Mouse anti-pSYK (I120-722), Phycoerythrin | BD Phosflow | Cat # 558529
Monoclonal-rabbit anti-SHP-1 (C14H6), purified | Cell Signalling Technology | Cat # 3759S
Swine anti-rabbit, biotin | DAKO | Cat # E043101-2
Goat anti-IgM, AP | Southern Biotech | Cat # 1021-04
Goat anti-IgG1, AP | Southern Biotech | Cat # 1070-04
NP18-CGG | In house | N/A
ACK lysing buffer | Gibco | Cat # A1049201
Annexin V apoptosis detection kit | BD Biosciences, USA | Cat # 556547
Foxp3/Transcription Factor Fixation/Permeabilization Foxp3 kit | eBioscience | Cat # 00-5523-00

**Bacterial and Virus Strains**
Inactivated *Bordetella pertussis* | LEE laboratories, BC, USA | N/A

**Biological Samples**
Sheep red blood cells (SRBCs) | TCS Biosciences, UK | Cat # SB069

(Table S1 continued on next page)
# Supplementary Table S1. Materials and Resources, related to Figures 1-4

## Chemicals, Peptides, and Recombinant Proteins

| Chemical/Protein | Supplier | Catalog Number |
|------------------|----------|----------------|
| BSA              | Sigma-Aldrich | Cat # A9418  |
| Streptavidin-Alexa Fluor 555 | Life Technologies, UK | Cat # S32355 |
| Streptavidin-Texas Red Phycoerythrin | BD Biosciences | Cat # 551487 |
| ProLong Gold antifade | Invitrogen, UK | Cat # P36930 |
| NP₁₅-BSA         | In house  | N/A            |
| NP₂-BSA          | In house  | N/A            |
| p-nitrophenyl phosphate in Tris buffer (SIGMAFAST) | Sigma-Aldrich | Cat # N2770 |

## Experimental Models: Organisms/Strains

| Organism | Supplier | Catalog Number |
|----------|----------|----------------|
| Mouse: Cγ¹Cre/+ | Casola S, et al, 2006. | N/A |
| Mouse: Ptpn6fl/wt | Pao Li, et al, 2007 | N/A |
| Mouse: ROsa¹mT/mG | The Jackson Laboratory | JAX: 007576 |
| Mouse: Cskfl/wt | Schmedt et al., 1998 | N/A |

## Software and Algorithms

| Software | Supplier | Catalog Number |
|----------|----------|----------------|
| FIJI Win64 (ImageJ d 1.47) | Schindelin et al., 2012 | N/A |
| ZEN      | Carl Zeiss Germany | N/A |
| BD FACSDiva | BD Biosciences | N/A |
| FlowJo v10 | FlowJo LLC, USA | N/A |
| GraphPad Prism 7 | GraphPad | N/A |
Transparent Methods

Mice

Eight to twelve-week-old female and male mice were used for all procedures. \( \text{Cy}^1_{\text{Cre/wt}} \text{Ptpn6}^{\text{fl/wt}} \) (Shp1\(^{\text{fl/wt}} \)) and \( \text{Cy}^1_{\text{Cre/wt}} \text{Ptpn6}^{\text{fl/fl}} \) (Shp1\(^{\text{fl/fl}} \)) mice were generated by the mating of \( \text{Cy}^1_{\text{Cre/wt}} \) (kindly donated by S Casola, IFOM, Milan, Italy) (Casola et al., 2006) and Ptpn6\(^{\text{fl/wt}} \) animals (Pao et al., 2007). Ptpn6\(^{\text{fl/wt}} \) animals had been backcrossed extensively onto C57BL6. For some experiments, \( \text{Cy}^1_{\text{Cre/wt}} \text{Ptpn6}^{\text{fl/wt}} \) mice were crossed with ROSA\(^{mT/mG} \) animals (007576; Jackson Laboratory), which express a membrane tagged version of dTomato inserted into the ROAS26 locus. Cre-expression replaces this by expression of membrane-tagged eGFP (Muzumdar et al., 2007). Animal experiments were licensed by the UK Home Office according to the Animals Scientific Procedures Act 1986 and approved by local ethics committee, University of Birmingham, UK.

METHOD DETAILS

Immunisation

\( 2 \times 10^8 \) sheep red blood cells (SRBCs) (TCS Biosciences, UK) in PBS were injected intravenously in the lateral tail vein. NP (4-hydroxy-3-nitrophenyl acetyl) was conjugated to CGG (Chicken \( \gamma \)-globulin) at a ratio of NP\(_{18}\)-CGG. Mice were immunised intraperitoneally (i.p.) with 50\( \mu \)g NP\(_{18}\)-CGG precipitated in alum plus \( 10^5 \) chemically inactivated \textit{Bordetella pertussis} (LEE laboratories, BC, USA) or subcutaneously on the plantar surface of the foot with 20\( \mu \)g NP\(_{18}\)-CGG precipitated in alum plus \( 10^5 \) chemically inactivated \textit{Bordetella pertussis}.

Immunofluorescence

Spleens and popliteal lymph nodes obtained at different time-points post-immunisation were frozen and cryosectioned. Slides were rehydrated in PBS and blocked using 1\% BSA (Sigma-Aldrich) in PBS for 30 min. Antibodies were diluted at the optimal dilution in PBS, 1\% BSA and incubated in a humid dark chamber for 1 h. Allophycocyanin-CD4 (GK1.5), BrilliantViolet421-IgD (11-26c.2a), Alexa633-goat anti-IgG1, goat anti-mouse IRF4 (M-17), rabbit anti-mouse active Caspase 3 (C92-605), and sheep anti-IgD (Abcam) were used. Secondary antibodies were Cy3-conjugated donkey anti-rabbit and Alexa488-conjugated donkey anti-sheep, Alexa555-conjugated donkey anti-goat and streptavidin Alexa555-conjugated. Slides were mounted in ProLong Gold antifade reagent (Invitrogen, UK) and left to dry in a dark chamber for 24 h. Images were taken on an Axio Scan Z1 microscope (Zeiss). Image data were processed using FIJI (Schindelin et al., 2012) or ZEN (Carl Zeiss Germany).
Flow Cytometry

Cell suspensions were prepared from spleens and popliteal lymph nodes obtained at different time-points post-immunisation. For intracellular staining, red blood cells were lysed by ACK lysing buffer (Gibco). Cell suspensions were blocked by CD16/32 (93) diluted in FACS buffer (PBS supplemented with 0.5% BSA plus 2mM EDTA), and then followed with staining cocktail: BrilliantViolet510 B220 (RA3-6B2), BrilliantViolet711 CD138 (281-2), NP- Phycoerythrin for detecting antigen specific B cells (in house), Fluorescein isothiocyanate CD38 (90), BrilliantViolet605 Fas (Jo2), Allophycocyanin IgG1 (X56), Phycoerythrin IgM (Igh-6b), rabbit anti-mouse active caspase 3 (C92-605), streptavidin-Texas Red Phycoerythrin, Phycoerythrin pSYK (120-722), monoclonal-rabbit anti-SHP-1 (C14H6). Swine anti-rabbit biotin to detect rabbit anti-mouse active caspase 3. Annexin V apoptosis detection kit was used to stain apoptotic and dead cells. For intracellular/intranuclear staining, cell suspensions were treated after surface staining with the Foxp3/Transcription Factor Fixation/Permeabilization Foxp3 kit (eBioscience, Carlsbad, CA), according to manufacturer specification. Samples were acquired using a LSRFortessa Analyzer (BD Biosciences, USA). Data were analysed with FlowJo v10 (FlowJo LLC, USA).

ELISA

Serial dilutions of serum samples were analysed by ELISA on NP_{15}-BSA (5 μg/ml)–coupled microtiter plates to detect NP-specific IgG1, or NP_{2}-BSA (5 μg/ml)–coupled microtiter plates to measure the high-affinity fraction of IgG1. Alkaline phosphatase (AP)-conjugated primary antibodies anti-IgG1 (Southern Biotech) were developed with p-nitrophenyl phosphate dissolved in Tris buffer (SIGMAFAST, Sigma-Aldrich). The absorbance was measured at 405 nm, plotted against dilution and relative antibody titres were read as the dilution where absorbance reached an arbitrary threshold. Relative affinity was calculated by dividing ELISA titres derived from NP_{2}-BSA–coupled plates by ELISA titres derived from NP_{15}-BSA-coupled plates.

Statistical analysis

All analysis was performed using GraphPad Prism 7 software. To calculate significance two-tailed Student’s t-test or two-way ANOVA were used. Statistics throughout were performed by comparing pooled data obtained from all independent experiments. Statistical analysis of data plotted on a log scale were done from log transformed data. P values <0.05 were considered significant (*). *p<0.05, ** p< 0.01, *** p<0.001, ****p<0.0001.
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