CD19 and BAFF-R can signal to promote B-cell survival in the absence of Syk

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Abstract

The development and function of B lymphocytes is regulated by numerous signaling pathways, some emanating from the B-cell antigen receptor (BCR). The spleen tyrosine kinase (Syk) plays a central role in the activation of the BCR, but less is known about its contribution to the survival and maintenance of mature B cells. We generated mice with an inducible and B-cell-specific deletion of the Syk gene and found that a considerable fraction of mature Syk-negative B cells can survive in the periphery for an extended time. Syk-negative B cells are defective in BCR, RP105 and CD38 signaling but still respond to an IL-4, anti-CD40, CpG or LPS stimulus. Our in vivo experiments show that Syk-deficient B cells require BAFF receptor and CD19/Pi3K signaling for their long-term survival. These studies also shed a new light on the signals regulating the maintenance of the normal mature murine B-cell pool.

Keywords  BAFF receptor; B-cell antigen receptor; CD19; mb1-CreERT², Syk

Subject Categories  Immunology; Signal Transduction

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Introduction

B lymphocytes play an essential role in the establishment of humoral immunity. Their production and maintenance requires the expression of a functional BCR, as well as signals from the B-cell environment (Dorschkind, 1990; Lam et al., 1997). In the adult mouse, B-cell development occurs first in the bone marrow (BM) in the presence of the chemokine CXCL12 and interleukin IL-7 (Grabstein et al., 1993; Nagasawa, 2007). The expression of a pre-BCR on the surface of B-cell precursors marks the transition from the pro- to the pre-B-cell stage (Grabstein et al., 1993; Pelanda et al., 2002a). The pre-BCR is an autonomously active receptor, signals of which drive clonal expansion and differentiation of pre-B cells into immature, BCR-expressing B cells (Kohler et al., 2008; Herzog et al., 2009). This signaling requires the interaction of the pre-BCR with the kinase Syk (Cheng et al., 1995; Reth & Nielsen, 2014). Therefore, B cells with defective genes for Syk or any of the four pre-BCR components have a complete or partial block in development (Turner et al., 1997; Otipoby et al., 2001; Yang & Reth, 2010).

Immature B cells migrate from the BM to the spleen, where they develop further through transitional B-cell stages into either marginal zone (MZ) or mature follicular B cells. In addition to mature B cells, the peritoneum of the mouse also harbors B1 B cells that are polyreactive and seem to require continuous BCR expression and stimulation for their survival (Hayakawa et al., 1985; Sindhava & Bondada, 2012). Therefore, the deletion of genes encoding components of the BCR signaling pathway frequently results in the loss of this B-cell type. In its monomeric form, the BCR is a 1:1 complex between the membrane-bound immunoglobulin molecule (mIg) and the Igα/Igβ heterodimer. On resting mature B cells, the BCR forms autoinhibited oligomers which prevent continuous signaling (Yang & Reth, 2010). Upon exposure to cognate antigens, some BCR oligomers are opened and phosphorylated on tyrosines in the immunoreceptor tyrosine-based activation motif (ITAM) of Igα and Igβ by the tyrosine kinases Lyn and Syk (Reth, 1989; Johnson et al., 1995). This results in the binding of Syk to the BCR via its tandem SH2 domains, the rapid phosphorylation of neighboring BCR components and the amplification of the BCR signal via a Syk-mediated inside-out signaling process (Klasener et al., 2014).

Once bound to the BCR, Syk becomes fully activated and phosphorylates several downstream signaling elements, including the adaptor protein SLP65/BLNK. This organizes the calcium (Ca²⁺)
survive better when they lack FoxO1. In summary, both the BAFF signaling component, most prominently the p85/p110 PI3K heterodimer. Therefore, together with the adaptor protein BCAP, CD19 is the dominant activator of the PI3K pathway leading to B-cell survival and proliferation (Brunet et al., 1999; Datta et al., 1999; Monroe, 2006; Aiba et al., 2008; Srinivasan et al., 2009).

The B-cell-activating factor belonging to the TNF family (BAFF) and its receptor BAFF-R provide another important survival signal in mature B cells (Mackay et al., 2010; Harless et al., 2001). Both PI3K and BAFF-R signals regulate the homeostasis of mature B cells. Mice deficient for BAFF or BAFF-R have reduced follicular and MZ B-cell numbers, and the same holds true for defects in PI3K (Sasaki et al., 2004; Durand et al., 2009). Another factor implicated in the survival of mature B cells is the BCR itself. Indeed, the lack of the mIgM heavy chain in mature B cells results in the loss of BCR expression and a strong reduction in mature B-cell numbers (Lam et al., 1997). Whether this is due to the absence of a tonic pro-survival signaling or the induction of a death signal in mIgM-negative B cells is not yet clear. A tonic BCR signal may employ different signaling components than those involved in B-cell activation or it may use the standard BCR signaling components at reduced levels (Monroe, 2006). Of interest in this context is the recent finding that the deletion of the Syk gene in all hematopoietic cells of the mouse also results in strongly reduced B-cell numbers (Schweighoffer et al., 2013). Based on the finding that the Syk-negative B cells respond less well to BAFF in culture, the authors suggest that Syk is an essential intermediate between the BAFF-R and the tonic signal of the BCR. We have revisited this issue in a mouse strain, which permits an inducible deletion of mature B cells, we crossed mb1-CreERT2 mice with Sykfl/fl mice, in which the exon I of the Syk gene is flanked by cis-oriented loxp sites (floxed) (Saijo et al., 2003). The resulting mb1-CreERT2-Syklo/n mice were treated 5× with Tam every third day and analyzed on day 5 after the last treatment, 20 days after the beginning of the treatment. Splenic B cells isolated from the Tam-treated mice lacked both Syk transcripts and protein as demonstrated by a RT-PCR and a Western blot analysis (Fig 1B and C). Additionally, flow cytometric analysis revealed that lymph node (LN)-derived mature B cells from Tam-treated mb1-CreERT2-Syklo/n mice were devoid of intracellular Syk expression, in contrast to LN-derived B cells from mb1-CreERT2 control mice which also received Tam (Fig 1D). The efficient deletion of the floxed Syk allele in Tam-treated mb1-CreERT2-Syklo/n mice was further confirmed by PCR analysis of genomic DNA (Fig 1E). Notably, the floxed Syk gene is the most efficient Cre target we have studied, indicating that in B cells this gene locus is highly accessible to the Cre recombinase.

**The B-cell populations of Tam-treated mb1-CreERT2-Syklo/n mice**

Five days after the last Tam treatment, the frequencies and absolute cell numbers of pro-/pre- and immature B cells in the BM did not differ significantly between mb1-CreERT2 control and mb1-CreERT2-Syklo/n mice while recirculating B cells were slightly reduced (Fig 2A and C). In the spleen (SP) of Tam-treated mb1-CreERT2-Syklo/n mice, however, the relative and absolute numbers of mature follicular (M) and marginal zone (MZ) B cells were reduced threefold and tenfold, respectively (Fig 2B and D). In other lymphatic organs such as the peritoneal cavity (PC), the B1 cell numbers were also reduced in the Tam-treated mb1-CreERT2-Syklo/n mice (Fig 2E). B1 B cells proliferate constitutively, and this might contribute to a selection against Syk-negative B cells. We tested this by intracellular Syk staining in flow cytometric analysis and found that all B1 B cells in the PC of mb1-CreERT2-Syklo/n mice were Syk negative (Supplementary Fig S1). Interestingly, the transitional B-cell numbers in the spleen of the Tam-treated mice were not altered (Fig 2B), suggesting that this transitional B-cell pool is rapidly replenished with Syk-sufficient transitional B cells after the discontinuation of the Tam treatment. This notion is supported by a intracellular flow cytometric analysis of transitional and immature B cells in the BM of Tam-treated mb1-CreERT2-Syklo/n mice.
Sykfl/fl mice showing the appearance of Syk-positive B cells at day 5 but not day 2 after induction (Supplementary Fig S2).

A partial signaling defect in Syk-deficient mature B cells

The presence of a pool of Syk-negative B cells in the spleen of Tam-treated mb1-CreERT2;Sykfl/fl mice allowed us to study the signaling behavior of mature B cell in the absence of Syk. We first analyzed the expression of the BCR and coreceptors on the surface of splenic B cells derived from Tam-treated mb1-CreERT2 control and mb1-CreERT2;Sykfl/fl mice. In comparison with the control B cells, the Syk-negative B cells displayed slightly increased amounts of IgM, CD19 and CD23 on their surface (Fig 3A) even though the Syk-negative B cells were smaller than control B cells (data not shown).

Exposure of mature B cells to the phosphatase inhibitor pervanadate results in increased tyrosine phosphorylation (pY) (Wienands et al., 1996). We treated splenic B cells with pervanadate for 5 min and determined the intracellular pY levels by flow cytometry. In comparison with control B cells, the Syk-deficient B cells showed eightfold lower pY (Fig 3B). To test whether Syk-deficient B cells could mobilize intracellular Ca2+ upon BCR engagement, we stimulated isolated splenic B cells from Tam-treated mb1-CreERT2 control and mb1-CreERT2;Sykfl/fl mice with an anti-kappa F(ab’2)2 antibody. Only the control B cells, but not the Syk-negative B cells, mobilized
Ca\textsuperscript{2+} upon treatment with this stimulus (Fig 3C). Both B-cell populations responded equally well to ionomycin in this assay, indicating that the absence of a Ca\textsuperscript{2+} response in Syk-negative B cells is due to a defect of BCR signaling and not a problem of Ca\textsuperscript{2+} release per se (Fig 3D). In addition, no Ca\textsuperscript{2+} release was detected in Syk-negative B cells upon stimulation with anti-IgM F(ab\textprime\textsuperscript{1})\textsubscript{2}, anti-IgD or latrunculin (Supplementary Fig S3). Together, these experiments confirm the requirement of Syk for the Ca\textsuperscript{2+} release observed upon BCR activation, in line with data obtained from the Syk-deficient chicken B-cell line DT40 (Takata et al, 1994).

As another readout for BCR-dependent or BCR-independent B-cell signaling, we analyzed mTOR activation and the chemotactic
behavior of B cells from Tam-treated mb1-CreERT2 control and mb1-CreERT2;Sykfl/fl mice. Using the phosphorylation of ribosomal protein S6, an established indicator for mTOR activity, control B cells but not Syk-deficient mature B cells activated mTORC1, when stimulated with anti-kappa F(ab')2 (Supplementary Fig S4).

In transwell migration experiments with the chemokines CCL21, CXCL12 and CXCL13, Syk-deficient B cells migrated less well than control B cells in response to the chemokine CXCL12 (Supplementary Fig S5A), although the surface expression of the corresponding CXCL12 receptor, namely CXCR4, is slightly higher compared to the control B cells (Supplementary Fig S5B). These results are in line with the finding that by phosphorylating SWAP-70, Syk increases the chemotaxis and polarization of B cells particularly to CXCL12 (Pearce et al, 2011).

B-cell activation increases the expression of CD86 on the surface of stimulated mature B cells (Lenschow et al, 1993). We exposed B cells from Tam-treated mb1-CreERT2 control or mb1-CreERT2;Sykfl/fl mice to different stimuli and monitored their CD86 expression by flow cytometry. Contrary to control B cells, the Syk-negative B cells failed to upregulate CD86 when stimulated with anti-IgM F(ab')2, anti-CD38 and anti-RP105, indicating that direct and indirect signaling via the BCR is defective in the absence of Syk (Fig 4A).

Increased CD86 expression was, however, detected when Syk-negative B cells were stimulated with anti-CD40, IL-4, CpG, and to a
Survival of mature murine B cells without Syk

The results of a B-cell proliferation assay mirrored those of the CD86 expression analyses. Syk-negative B cells underwent fewer cell division cycles when cultured in the presence of anti-IgM (ab)2 or anti-RP105 antibodies (Fig 4B). Simultaneous signaling via BCR and CD40 exacerbated the differences in proliferation between Syk-negative and Syk-expressing B cells, whereas treatment of B cells with TLR9-activating CpG led to a similar rate of proliferation in both types of B cells (Fig 4B). The absence of Syk in the proliferating mb1-CreERT2;Sykfl/fl-derived B-cell population was verified by flow cytometric analysis of intracellular Syk expression (Supplementary Fig S6).

A fraction of Syk-negative B cells can survive for extended times in vivo

The partial loss of mature B cells in Tam-treated mb1-CreERT2;Sykfl/fl mice could be due to increased death of Syk-negative mature B cells and/or to a reduced input into the mature B-cell compartment. To distinguish between these possibilities, we injected Rag2−/−γc−/− mice, which cannot produce B or T cells, with equal numbers of mature splenic T or B cells from either mb1-CreERT2 or mb1-CreERT2;Sykfl/fl mice. The recipient mice were then treated a total of 5 times with Tam at 3-day intervals, and T- or B-cell cellularity was monitored 20 days later by flow cytometry (Fig 5A). While the number of T cells from mb1-CreERT2;Sykfl/fl mice was not altered by the Tam treatment, compared to the control, the number of mature B cells derived from the mb1-CreERT2;Sykfl/fl mice was reduced by approximately threefold (Fig 5B). Similar results were obtained from mice, which were injected after the last Tam treatment with anti-IL-7 receptor antibodies to block the migration of newly generated B cells from the bone marrow. In these mice, more than 40% of the mature splenic B cells survived without Syk for more than 40 days (Supplementary Fig S7), showing again that a considerable fraction of mature B cells can survive without Syk.

To study the survival of Syk-negative B cells in the presence of wild-type (WT) B cells, we injected Rag2−/−γc−/− mice with equal numbers of mature B cells derived from the spleen of either mb1-CreERT2;Sykfl/fl mice or mice expressing EGFP specifically in B cells (Fig 5C). B cells in the injected mice were harvested 5 days after the
last Tam treatment and analyzed by flow cytometry (Fig 5C). We found that one-third of the surviving B cells were Syk deficient (Fig 5D). The results obtained from the competitive adoptive transfer experiment are in line with the analysis of Tam-treated mice left for 2 months without treatment. In these mice, 20% of the mature B cells were still Syk negative (Supplementary Fig S8). In summary, these experiments indicate that a portion (20–30%) of the Syk-negative B cells are able to survive for prolonged times in the periphery, even when competing with wild-type B cells and despite having apparent signaling defects restricting their “fitness”. 

Figure 5. Deletion of Syk in mature B cells after transfer into Rag2<sup>−/−</sup> µC<sup>−/−</sup> mice. 

A Flow cytometric analysis of splenic B cells from Rag2<sup>−/−</sup> µC<sup>−/−</sup> mice. CD19 versus CD3e dot plots are shown. The mice were injected i.v. with 5 × 10⁶ splenic (CD19<sup>+</sup> CD93<sup>+</sup>) B cells and (CD3e<sup>+</sup>) T cells from Tam-untreated mb1-CreERT<sup>2</sup>;Syk<sup>fl/fl</sup> control or mb1-CreERT<sup>2</sup>;Syk<sup>fl/fl</sup> mice. The recipient mice were treated with Tam beginning 1 day after transfer as described in the Materials and Methods section. 

B Quantitative analysis of B and T cells from Rag2<sup>−/−</sup> µC<sup>−/−</sup> mice repopulated with mb1-CreERT<sup>2</sup> or mb1-CreERT<sup>2</sup>;Syk<sup>fl/fl</sup> splenocytes. Each symbol represents an individual mouse. Filled circles and squares represent Syk<sup>+/+</sup> B and T cells, respectively; open circles and squares represent Syk<sup>fl/fl</sup> B and T cells. 

C Flow cytometric analysis of Rag2<sup>−/−</sup> µC<sup>−/−</sup> mice repopulated with mature splenic B cells from WT mice (1 × 10⁷) carrying a GFP reporter construct or mature splenic B cells from mb1-CreERT<sup>2</sup>;Syk<sup>fl/fl</sup> mice (1 × 10⁷) at a ratio of 1:1. The recipient mice were treated with Tam as described in the Materials and Methods section. Shown are GFP versus CD19 dot plots to distinguish GFP<sup>+</sup> WT and GFP<sup>/−</sup> Syk<sup>fl/fl</sup> B cells before transfer (upper left panel), in the blood of the recipient mice at day 3 after transfer (upper right panel) or in the spleen of the recipient mice at day 20 after transfer (lower left panel). The cells were stained for the intracellular expression of Syk (lower right panel). 

D Quantitative analysis of WT or Syk<sup>fl/fl</sup> B cells. Absolute number of cells is shown. Filled circles represent WT B cells and open circles the Syk<sup>fl/fl</sup> B cells. 

Data information: In (B) and (D), two asterisks (**) indicate P < 0.01; P-values were obtained using two-tailed Student’s t-test.
Syk-negative B cells require BAFF for their survival

To test whether Syk-negative B cells can respond to the pro-survival factor BAFF, we cultured B cells from Tam-induced mb1-CreERT² or mb1-CreERT²;Syk⁻/⁻ mice in the absence or presence of BAFF (Fig 6A). Without BAFF, most B cells die within 3 days, whereas in the presence of BAFF, the B-cell cultures can be maintained for longer times. After 8 days of culture, 60% of the control and only 30% of the Syk-negative B cells were still viable (Fig 6A). This difference in the response to BAFF could not be due to an altered BAFF-R expression as the B cells from both mouse strains expressed similar amounts of BAFF-R on their surface (Fig 6B).

We next studied the BAFF dependence of Syk-negative B cells in vivo by injecting Tam-treated mb1-CreERT²;Syk⁻/⁻ mice with either 5A12 or 9B9 anti-BAFF-R antibodies, only the latter of which blocks BAFF binding to the receptor, and thus BAFF-R-mediated survival signals (Fig 6C). A recent study revealed that treatment of WT mice with the 9B9 antibody resulted in the elimination of 80% of the mature B cells (Rauch et al., 2009). Similarly, injection of Tam-induced mb1-CreERT²;Syk⁻/⁻ mice with the 9B9 antibody resulted in a tenfold reduction in the absolute number of splenic B cells. The non-blocking 5A12 antibody injection has no effect on the B-cell numbers. The reduction in Syk-negative B cells in anti-9B9-treated mice was observed to a different extent in different lymphatic tissues, including the SP, LN and PC (Fig 6D). A study of the mature follicular B-cell frequencies in the blood from control and B-cell-specific Syk-deficient mice revealed no significant difference in the kinetics of B-cell elimination at several time points (day 3 to day 15) after anti-BAFF-R treatment (Supplementary Fig S9).

These data indicate that Syk-negative B cells are, to a certain extent, responsive to BAFF and depend on BAFF–BAFF-R interactions for their long-term in vivo survival. The Syk family kinase ZAP-70 can partially replace Syk function in Syk-deficient pre-B cells (Schweighoffer et al., 2003). Therefore, one possibility for the long-term survival of Syk-negative mature B cells could be the expression of ZAP-70. To test this, B cells from mb1-CreERT²;Syk⁻/⁻ mice were subjected to immunoblot and flow cytometric analyses. However, no ZAP-70 expression was detected in these cells (Supplementary Fig S10). In line with these data, the Syk-negative mature B-cell population is not affected in mice lacking ZAP-70 (data not shown).

The survival of Syk- and CD19-negative B cells is rescued by FoxO1 deletion

Recent experiments implicate the PI3K signaling pathway in the survival and maintenance of mature B cells in the periphery.
Sykfl/fl;CD19 mice showed significantly reduced B-cell numbers in peripheral lymphoid tissues and a BCR signaling defect (Del Nagro et al., 2005). In this respect, they resemble the mb1-CreERT2;Sykfl/fl mouse strain (Fig 7). After 30 days of Tam treatment of mb1-CreERT2;Sykfl/fl;CD19−/− mice, the survival of mature B cells was severely compromised in comparison with CD19−/− or mb1-CreERT2;Sykfl/fl animals. The relative and absolute B-cell numbers in these mice were strongly reduced in the BM, especially the recirculating B cells (Fig 7A and C). A drastic reduction in the mature B cells was also detected in the SP of mb1-CreERT2;Sykfl/fl;CD19−/− mice (Fig 7B and D). These results indicate that CD19-derived signals support the survival of Syk-negative B cells and that Syk promotes the survival of CD19-negative B cells. Multiple copies of CreERT2 in the mb1-CreERT2;Sykfl/fl;CD19−/− compound mice lead to accelerated recombination of the Syk locus compared with the control mice on day 6 or 8 after Tam exposure (Supplementary Fig S11). However, at day 10, Syk deletion is complete in the majority of mature B cells of mb1-CreERT2;Sykfl/fl mice, and at day 20, these B cells are entirely devoid of Syk (Supplementary Fig S11B). For this reason, we do not think that gene dosage plays a critical role in our analysis performed at day 35 after Tam treatment.

One mechanism by which the CD19/PI3K signals can increase the survival of mature B cells is through the phosphorylation and subsequent proteasomal degradation of the pro-apoptotic transcription factor FoxO1 (Limon & Fruman, 2012). This effect of PI3K signaling can be mimicked by the deletion of the FoxO1 gene. To test the effect of FoxO1 deletion in a CD19/Syk double-deficient background, we generated mb1-CreERT2;Sykfl/fl;CD19−/−;FoxO1fl/fl mice. The absence of Syk and FoxO1 proteins was confirmed by

**Figure 7. Induction of Syk deletion in the absence of CD19 and FoxO1.**

A, B B lymphocytes derived from mb1-CreERT2;Sykfl/fl (first panel), CD19−/− (second panel), mb1-CreERT2;Sykfl/fl;CD19−/− (third panel) and mb1-CreERT2;Sykfl/fl;CD19−/−;FoxO1fl/fl (fourth panel) mice were analyzed by flow cytometry after Tam treatment (see Materials and Methods). (A) Flow cytometric analysis of BM-derived [A] or splenic [B] B cells. IgM versus IgD dot plots representing B220^+CD43^− BM-derived B cells are depicted in (A). The Fr D, E, and F are distributed as indicated. A CD19 versus B220 dot plot is depicted in (B).

C, D Statistical analysis of absolute B-cell numbers derived from the (C) the BM, Fr F and (D) splenic M B cells of mb1-CreERT2;Sykfl/fl (filled circles), mb1-CreERT2;CD19−/− (open circles), mb1-CreERT2;Sykfl/fl;CD19−/− (open squares) and mb1-CreERT2;Sykfl/fl;CD19−/−;FoxO1fl/fl (open diamonds). An asterisk (*) marks statistically significant differences (P < 0.05); two asterisks (**) indicate a P < 0.01. P-values were obtained by a two-tailed Student’s t-test. Shown are data from three to four mice per group.

E, F Intracellular flow cytometric analysis of (E) Syk and (F) FoxO1 in mb1-CreERT2;Sykfl/fl;CD19−/− (dashed line) and mb1-CreERT2;Sykfl/fl;CD19−/−;FoxO1fl/fl (solid line) B cells. In (E), B cells from mb1-CreERT2 mice served as a positive control for the Syk staining (dotted line). In (F), T cells from mb1-CreERT2;Sykfl/fl;CD19−/−;FoxO1fl/fl mice served as a positive control for FoxO1 expression showing that FoxO1 deletion was B cell specific.
flow cytometric analysis (Fig 7E and F), indicating efficient Cre-mediated deletion of the floxed Syk and FoxO1 genes in these mice. Interestingly, the ablation of FoxO1 expression restored the numbers of mature B cells to the levels of Syk or CD19 single-deficient B cells in both the BM and the SP (Fig 7A and B right panel; Fig 7C and D). Thus, the PI3K signaling pathway is indeed likely to be induced by CD19, to support the survival of Syk-negative B cells.

Discussion

We show here that approximately 25% of the mature peripheral B cells can survive in the mouse for at least 2 months without Syk, in a manner that requires BAFF-R and CD19 signaling. In contrast, deletion of the Syk gene in early B cells results in the appearance of a small number of immature IgM^+ B cells, which, however, fail to give rise to any mature B cells in the periphery (Cheng et al, 1995). Thus, pre-B and mature B cells have different requirements for Syk. Indeed, the pre-BCR is an autonomously signaling receptor that continuously engages Syk, whereas the BCR forms an autoinhibited oligomer on mature B cells that is not in contact with Syk. This notion is supported by a proximity ligation analysis showing that Syk is localized near the BCR only after BCR activation (Infantino et al, 2010; Klasener et al, 2014).

The presence of large amounts of Syk-negative mature B cells in the induced mb1-CreERT^2;Syk^f/f mice allowed us to analyze the in vivo role of this kinase in the activation of mature B cells. Our finding, that in the absence of Syk, mature B cells have a defective Ca^{2+} response, is consistent with an earlier study in Syk-negative DT40 B cells (Takata et al, 1994). This signaling defect is explained by the finding that the phosphorylation of the adaptor protein SLP-65/BLNK by Syk is required for the organization of a functional Ca^{2+} signalingosome. Signals from membrane proteins that are functionally connected to the BCR, such as CD38 and RP105, are also defective in Syk-negative B cells (Lund et al, 1996; Chan et al, 1998; Yazawa et al, 2003). Other receptor systems, however, such as CD40 and the innate immunity receptors TLR9 are not affected by the loss of Syk.

While the number of mature B cells is reduced only threefold in the induced mb1-CreERT^2;Syk^f/f mice, other B-cell subpopulation such as MZ B cells are reduced more than tenfold in the absence of Syk. This is surprising as MZ B cells are regarded as a more innate B-cell population that requires exposure to innate receptor stimuli for their maintenance and expansion (Cerutti et al, 2013). However, it is feasible that Syk is not only involved in signaling from the activated BCR, but also in B-cell migration and/or adhesion. In neutrophils and macrophages, Syk is involved in signaling from integrin and chemokine receptors (Mocsai et al, 2010). Indeed, we found that Syk-negative B cells display a defective CXCL12-driven chemotactic behavior (Supplementary Fig S5). Recently, it was shown that Syk phosphorylates the F-actin-binding protein SWAP-70, resulting in altered chemotaxis and polarization of B cells, particularly to CXCL12 (Pearce et al, 2011). Interestingly, SWAP-70^−/− mice also show reduced numbers of MZ B cells (Chopin et al, 2010). A loss of Syk might lead to a failure of T2 B cells to migrate to the MZ and thus to lower amounts of MZ B cells (Miosge & Goodnow, 2005).

It is not clear at present why 70% of the mature B cells disappear in the induced mb1-CreERT^2;Syk^f/f mice. Our transfer experiments, and the deletion of Syk in combination with an anti-IL-7R treatment, show that this loss is not primarily due to reduced production of mature B cells but is a feature of mature B cells themselves. Syk is also needed for the migration of transitional T0 B cells into the white pulp region of the spleen, where the cells develop into T1 B cells and receive additional survival signals (Henderson et al, 2010). Thus, a migration and/or adhesion defect, as discussed above, could be the cause of the loss of the mature B2 B cells in the induced mb1-CreERT^2;Syk^f/f mice. For example, if locally defined survival niches exist in the periphery that mature B cells need to visit from time to time to ensure their long-term survival, a defective migration of Syk-negative B cells would result in reduced cell numbers. Alternatively, within the cell, Syk may send a tonic signal to the mTOR pathway that controls the “fitness” of mature B cells. At least in B-cell tumor cells, such a Syk-mTOR connection has been described (Leseux et al, 2006; Fruchon et al, 2012; Carnevale et al, 2013). In line with this, we found that Syk-deficient mature B cells do not activate mTORC1 when stimulated with anti-Kappa F(ab′)^2 (Supplementary Fig S4).

Based on the finding that Syk-negative B cells respond less well to BAFF in culture, Schweighoffer et al suggested that Syk is an essential common component of the BCR and the BAFF-R, both of which mediate B-cell survival signaling (Schweighoffer et al, 2013). Our study confirms that in culture, Syk-negative B cells respond less well to the pro-survival factor BAFF. However, we also found that Syk-negative B cells still respond to BAFF in vivo and require this factor for their long-term survival in the mouse. Thus, an unresponsiveness to BAFF is not the reason for the reduction of the Syk-negative B cells in induced mb1-CreERT^2;Syk^f/f mice. Another discrepancy between these two studies is that we, in contrast to Schweighoffer et al, find a significant reduction in the B1 B-cell population in the induced mb1-CreERT^2;Syk^f/f mice. B1 B cells are polyreactive, slowly renewing cells that are strongly affected by the loss of positive BCR signaling components (Berland & Wortis, 2002). The reduction in the B1 B-cell numbers in the absence of Syk is in line with these studies. We have found that under certain conditions, B1 B cells can escape Cre-mediated deletion of the floxed Syk gene (Alsadeq et al, 2014). An incomplete Syk gene deletion may be the reason for this discrepancy. We verified by intracellular staining that Syk was indeed deleted in the B1 B-cell population that was still present in the induced mb1-CreERT^2;Syk^f/f mice.

The PI3K pathway can promote the survival of WT and Syk-deficient B cells. The importance of this pathway for B-cell survival was recently demonstrated in mice with a B-cell-specific activation of PI3K signaling via a dominant-active form of the p110α subunit (Sriniivasan et al, 2009). In these mice, B cells survive, even in the absence of BCR expression. Schweighoffer et al have shown that the survival of Syk-deficient B cells can be rescued, at least in part by the activation of the PI3K pathway (by deleting PTEN). The BCR coreceptor CD19 is a well-known PI3K activator, and we find that its expression is required for the survival of Syk-deficient B cells. The cytoplasmic tail of CD19 is phosphorylated by the Src-family kinase Lyn, thus allowing CD19 to connect to the p85/p110 PI3K module. It is thus not a surprise that CD19 can still be phosphorylated and function in the absence of Syk, although it was shown that Syk can also function upstream of PI3K in BCR signaling (Beitz et al, 1999). We recently found that CD19 is in close association with the IgD-BCR on resting B cells (Klasener et al, 2014). However,
whether CD19 inducibly or constitutively signals in Syk-deficient B cells remains to be determined. One important downstream target of the PI3K pathway is the transcription factor FoxO1, whose phosphorylation by Akt1 targets it for destruction. PI3K signaling can thus be mimicked by a FoxO1 gene deletion, and we found that such a deletion rescues mature Syk;CD19 double-deficient B cells from elimination. This provides a direct proof that the survival of Syk-deficient B cells involves not only BAFF but also PI3K signaling. In conclusion, our study suggests that two different receptor systems, namely BAFF-R and CD19, synergize to signal the survival not only of Syk-deficient but also of wild-type B cells.

Materials and Methods

Syk knockout mice

To delete Syk in mature B cells, the Syk"/" mouse strain, kindly provided by Prof. A. Tarakhovsky, was mated with mice expressing CreERT2 in a B-cell-specific manner (mb1-CreERT2). The CreERT2 insert was kindly provided by Prof. P. Chambon (Feil et al., 1997). The resulting mb1-CreERT2,Syk"/" mice express a Tam-inducible form of the Cre recombinase under the control of the mb-1 promoter, the mb-1 gene (encoding the Ig-ç protein) being B-cell-specific and expressed throughout B-cell development. In these mice, Tam treatment induces Cre activity and deletion of Syk in all B-cell subsets that have matured beyond the early pro-B-cell stage.

The CD19"/" strain has been described previously (Yasuda et al., 2013) and was kindly provided by Dr. M. Schmidt-Supprian. The FoxO1"/" mouse strain was described in Paik et al. (2007). All animal studies were absolved randomly and blindly on mice aged between 8 and 12 months and were carried out in accordance with the German Animal Welfare Act, having been reviewed by the regional council and approved under the license # G-09/103.

Tam treatment

Mice were treated by oral gavage with 6 mg of tamoxifen (Tam) citrate (Ratiopharm) dissolved in 20% ClinOleic acid (Baxter). The animals were treated for a total of five times at 3-day intervals and were then sacrificed 5 days after the last treatment, at day 20.

Isolation of genomic DNA and PCR analysis

Genomic (g) DNA was obtained from purified mature splenic and LN-derived B cells or from BM B cells culture using the Quick-gDNA MicroPrep isolation kit (Zymo Research). Amplification of the gDNA was achieved by PCR using following oligonucleotides primers. Syk F (d-band): 5'-GCC GTG TCT GTG CCT ACT GG-3' and 5'-GCT GGT GAA AAG GAC CTC T-3'; Syk F2 (f-band): 5'-GCC GTG TCT GTG CCT ACT GG-3' and 5'-TAC CTA ACC AAA CCC ACG GC-3', TC21 (loading control): 5'-GGG TCA TGT GTG GAA AAG AGG GC-3'; 5'-GTC GTA GAC GAT GAG GGG GG-3'.

mRNA isolation and RT-PCR

Total mRNA was isolated from whole mature B cells using the TRI Reagent kit (Sigma). B cells (5 × 10⁶) were lysed in TRI Reagent, and mRNA was isolated by chloroform/isopropanol precipitation. After ethanol washing and centrifugation, the RNA pellets were air-dried and resuspended in DEPC-treated water. RNA content was measured, and aliquots of 0.1 µg mRNA were reverse-transcribed using the first-strand cDNA synthesis kit (Fermentas) according to the manufacturer’s instructions. Amplification of the Syk cDNA was achieved by PCR using the primers Syk-cDNA-for: 5'-TTA GGA CCT GAA GGA GAA CC-3’ and Syk-cDNA-rev: 5’-AGG CTT TGG GAA GGA GTA GG-3’ and running 30 cycles with an annealing step at 60°C. For the amplification of the Hprt-cDNA, the DNA primers HPRT-F: 5'-GCT GGT GAA AAG GAC CTC T-3’ and HPRT-R: 5’-CAC AGG ACT AGA ACA CCT GC-3’ were used. PCR products were then separated and analyzed by agarose gel electrophoresis.

Immunoblot analysis

For immunoblotting, the samples were separated by denaturing SDS-gel electrophoresis, transferred onto a nitrocellulose membrane and probed with anti-Syk (N19; rabbit anti-mouse; Santa Cruz) or anti-ZAP-70 (N15; goat anti-mouse; Santa Cruz). Detection of GAPDH (Calbiochem) was used to demonstrate the amount of total protein loaded. Prior to incubation with the primary antibody (Ab), the membrane was blocked with PBS containing 5% milk powder and subsequently washed. Incubation of the membrane with the primary Ab diluted in PBS supplemented with 0.5% BSA (PAA Laboratories) and 0.1% NaN3 (Sigma) was performed for 1 h at room temperature (RT), followed by washing steps with PBS. Incubation with the secondary Ab, diluted in PBS, was carried out for 1 h at RT. Excess Ab was washed off, and immuno-reactive proteins were detected by the ECL chemoluminescence detection system (Amersham).

Intracellular cell staining

Intracellular cell staining was performed using the ADG fix&perm kit (Dianova) according to the manufacturer’s instructions. After fixation and permeabilization, cell suspensions isolated from various lymphoid organs were washed in saponin-based buffer (PBS; 0.5% saponin (Sigma); 0.2% BSA; 0.02% NaN3) and subsequently washed. Incubation of the membrane with the primary Ab diluted in PBS supplemented with 0.5% BSA (PAA Laboratories) and 0.1% NaN3 (Sigma) was performed for 1 h at room temperature (RT), followed by washing steps with PBS. Incubation with the secondary Ab, diluted in PBS, was carried out for 1 h at RT. Excess Ab was washed off, and immuno-reactive proteins were detected by the ECL chemoluminescence detection system (Amersham).

MACS depletion and cell sorting

Splenic B cells were obtained by MACS-based negative selection using the magnetically labeled B-cell isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. The cells were separated by AutoMACS (Miltenyi Biotec).

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The Abs used in the flow cytometry studies were anti- IgM-PE, anti-IgM-Cy5, anti-IgM-DyLight649, anti-IgD-PE, anti-CD93-APC, anti-CD21-FTTC, anti-CD23-PE, anti-CD5-PerCP-Cy5.5, anti-B220-PE, anti-biotin-PE (Becton and Dickinson, eBioscience, or Jackson) and biotinylated anti-BAFF-R (R&D).

CD93\(^+\) (mature) and CD93\(^-\) (transitional) B cells were obtained by cell sorting using FACS Aria (Becton Dickinson) or Dako Cytometry MoFlo (Dako) cell sorters. After FACSort separation, the B cells were analyzed for purity and viability using an anti-CD19 Ab (BioLegend clone 6D5) and 7-AAD (eBioscience), respectively.

**Ca\(^{2+}\) influx measurement**

Ca\(^{2+}\) influx in mature CD93\(^+\) B cells was measured using the intracellular fluorescent dye Indo-1 (Invitrogen) and the LSRII flow cytometer (Becton Dickinson). Cells (0.5-1 x 10\(^5\)) were resuspended in 1 ml plain Iscove’s medium (Biochrom AG) supplemented with 1% FCS. The Indo-1 dye was prepared 5 min prior to incubation with the cell samples and consisted of 25 \(\mu\)l Indo-1 (dissolved in DMSO), 25 \(\mu\)l Pluronic F-127 (Invitrogen) and 113 \(\mu\)l FCS. Indo-1 aliquots (15 \(\mu\)l) were then added to each sample, and cell samples were incubated for 45 min at 37\(^\circ\)C protected from light. Cells were then centrifuged at 300 x g and 4\(^\circ\)C. For analysis, the cell pellets were resuspended in 500 \(\mu\)l of Iscove’s medium supplemented with 1% FCS. The stimuli used in the experiments included 10 \(\mu\)g/ml polyclonal anti-IgM F(ab\(^\prime\))\(_2\) (Jackson Lab), 10 \(\mu\)g/ml anti-IgD (eBioscience, clone 10.4.22), 1 \(\mu\)M Ionomycin (Enzo) and 1 \(\mu\)M latrunculin A (Cayman).

**Transfer of mature B cells into Rag2\(^{-/-}\) mice**

Mature splenic B cells (CD19\(^+\)CD93\(^-\) ) (1 x 10\(^5\)) from mb1-CreER\(^{T2}\) or mb1-CreER\(^{T2}\)-Syk\(^{0/}\)\(^n\) mice were isolated by negative selection using the B-cell isolation kit (Miltenyi) in combination with anti-CD3 Ab and transferred into Rag2\(^{-/-}\) mice intravenously (i.v.). Recipient mice were treated with Tam every third day and cell populations were analyzed by flow cytometry 20 days after the transfer. For WT versus Syk\(^{0/}\)\(^n\) competition experiments, mature splenic B cells (CD19\(^+\)CD93\(^-\) ) from WT mice (1 x 10\(^5\)) carrying a GFP reporter construct (Pelanda et al, 2002b) or mature splenic B cells mb1-CreER\(^{T2}\)-Syk\(^{0/}\)\(^n\) mice (1 x 10\(^5\)) were purified or were incubated in the presence of the following stimuli: 10 \(\mu\)g/ml polyclonal anti-IgM F(ab\(^\prime\))\(_2\) (Jackson immunoresearch, goat anti-mouse polyclonal, catalog #115-006-075), 10 \(\mu\)g/ml anti-RP105 (eBioscience, clone RP/14), 10 \(\mu\)g/ml anti-CD38 (eBioscience, clone 90), 10 \(\mu\)g/ml anti-CD40 (eBioscience, clone 1C10), and 10 ng/ml mIL-4 (Miltenye Biotech); 2.5 \(\mu\)g/ml unmethylated CpG oligonucleotides (Invivogen, ODN 1826); and 10 \(\mu\)g/ml LPS (Sigma). The cells were harvested after 24 h of stimulation, and expression of the activation marker CD69 was analyzed by flow cytometry using anti-CD69 staining (eBioscience, clone GL1). To discriminate viable and dead cells, 7-AAD was added to each sample.

**Proliferation of mature B cells**

Proliferation of mature B cells was measured using the eFluor 670 proliferation dye (eBioscience). Mature CD93\(^-\) B cells (2 x 10\(^5\)) were labeled with eFluor 670 prior to cultivation according to manufacturer’s instruction. Aliquots of 1 x 10\(^5\) mature B cells were then cultured in 0.2 ml complete Iscove’s medium with 10% FCS for 72 h and were either left unstimulated or stimulated with anti-RP105, anti-IgM alone, anti-IgM and anti-CD40, anti-CD40 alone or unmethylated CpG oligonucleotides at the concentrations described above. Proliferation was measured by flow cytometry. Once again, 7-AAD staining was included in the analysis to discriminate between viable and dead cells. Phosphorylation of total intracellular pY was measured after stimulation of purified B cells with 50 \(\mu\)M pervanadate/H\(_2\)O\(_2\) (Sigma/Merck) for 5 min at 37\(^\circ\)C. The pervanadate/H\(_2\)O\(_2\) solution was prepared as described (Wienands et al, 1996). Cells were fixed immediately after phosphorylation and subjected to intracellular staining.

**Ex vivo survival assays**

Purified splenic B cells were cultured in complete Iscove’s medium supplemented with 10% FCS in the absence or presence of 100 ng/ml human recombinant (hr) BAFF (R&D) over a period of 8 days. The medium, supplemented with fresh hrBAFF, was exchanged every second day. The cells were harvested and analyzed by flow cytometry. Dead cells were excluded using 7-AAD and Annexin V staining.

**Treatment of mice with anti-IL-7R Ab**

mb1-CreER\(^{T2}\)-Syk\(^{0/}\)\(^n\) were treated with Tam for 35 days every third day. After confirming the deletion of Syk by intracellular staining day 20 after Tam treatment, mice were injected i.v. with 1 mg of anti-IL-7R-blocking Ab (rat anti-mouse, clone A7R34, BxCell and homemade). Mice remained under anti-IL-7R treatment every third day until day 40 after anti-IL-7R treatment when lymphocyte subpopulations in the SP, BM, LN, PP and PC were analyzed by FACS.
To generate the mb1-CreERT2;Sykfl/fl;CD19−/− mouse strain, mb1-CreERT2;Sykfl/fl;CD19−/− mice were crossed to CD19-CreERT2 mice expressing CreERT2 from the CD19 locus and therefore lacking CD19 expression in the homozygous state (Mark Schmidt-Supprian, personal communication and Yasuda et al., 2013). To generate the mb1-CreERT2;Sykfl/fl;CD19−/−;FoxO1fl/fl mouse strain, mb1-CreERT2;Sykfl/fl;CD19−/− mice were crossed to FoxO1fl/fl mice (Paik et al., 2007). The mb1-CreERT2 Sykfl/fl inducible mice (control for the CD19;Syk double-deficient mice) have one copy of CD19-CreERT2. The CD19−/− controls harbor mb1-CreERT2. The animals were treated with Tam for a total of 10 times at 3-day intervals, and the cells were subsequently analyzed 5 days after the last induction by flow cytometry.

**Ex vivo stimulation for pS6 detection**

The phosphorylation of S6 was measured after stimulation of purified mature B cells (1 × 10^5 cells) from mb1-CreERT2 and mb1-CreERT2;Sykfl/fl mice at day 20 after Tam treatment. Purified mature Syk-deficient B cells and the control cells were cultured overnight (O/N) in complete Iscove’s medium with 3% FCS. The cells were then stimulated with 10 μg/ml anti-Kappa F(ab)2, for 10 min (goat anti-mouse Polyclonal von Southern Biotech catalog# 1052-14). To assess the specificity of the anti-pS6 Ab, the cells were pre-treated with 500 nM pervanadate/H_2O_2 (Sigma/Merck) for 5 min and were stimulated with anti-Kappa F(ab)2 for additional 10 min. Stimulation with 50 μM peryvansadate/H_2O_2 (Sigma/Merck) for 5 min at 37°C served as a positive control for the phosphorylation.

**In vitro migration assay**

Cell migration was measured using 8-μm Transwell inserts (Greiner) for 24-well plates (Greiner). Iscove’s medium/3% FCS alone or supplemented with either 1 μg/ml CCL21, 0.2 μg/ml CXCL12 or 1 μg/ml CXCL13 (PeproTech) was added to the wells, and purified mature B cells (2 × 10^5 cells) from mb1-CreERT2 and mb1-CreERT2;Sykfl/fl mice at day 10 after Tam treatment were added into the inserts. The cells were allowed to migrate for 2.5 h at 37°C. The number of migrated cells and the number of cells in the insert were measured by flow cytometry for 1 min. Dead cells were excluded using 7-AAD. The relative migration was determined, and specific migration was assessed by subtracting the relative unspecific migration from the relative migration in response to the chemokines. The surface expression of CXCR4 (receptor for CXCL12) and CXCR5 (receptor for CXCL13) was assessed by flow cytometry using anti-CXCR4-BrilliantViolet421 (BioLegend) and anti-CXCR5-PE (BD) Abs.

**Statistical analysis**

Unpaired two-tailed Student’s t-tests (with n between three and five mice per group) were carried out using Prism 4 software (GraphPad Software Inc) to determine the statistical relevance of differences between groups. In all the carried experiments, the results are representative of at least three independent experiments.

**Supplementary information** for this article is available online: http://embj.embopress.org

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**Author contributions**

EH, EL-Z, VA, AA, SA, and RPo conducted the experiments. MWD and RPe provided materials, EH and MR designed the experiments, and EH, EL-Z, RPo and MR wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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Survival of mature murine B cells without Syk

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