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

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 26 September 2014

Thank you for the phone conversation today discussing what experiments can be done in a revised version. Given that significant revisions can be undertaken to strengthen the findings, I would like to invite you to submit a revised manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision only and that it is therefore important to resolve the major issues raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In this manuscript entitled "CD19 and BAFF-R can Signal B cell survival in the absence of Syk" Hobeika et al. describe the consequences of B cell-specific, inducible ablation of the non receptor tyrosine kinase Syk in adult mice. The study is based on a novel mb1-CreERT2 driver allele

generated by the Reth group, which induces rapid and efficient deletion starting from the pro/pre B cell stage. While bone marrow B cells are largely not affected, consequences of Syk deletion show in the spleen, where follicular B cells are approx. 3-fold and MZ B cells approx. 10-fold reduced. While transitional B cells of the spleen are not altered, the authors argue that this may be due to rapid replenishment, peritoneal B1 B cells show a clear reduction. The unexpected large number of surviving follicular B cells allowed the authors to characterize this population in more detail. They show that tyrosine phosphorylation in response to non-specific phosphatase inhibition by pervanadate was largely reduced in Syk-deleted B cells and these cells completely failed to mobilize Ca2+ in response to BCR stimulation. Similarly, a number of BCR coupled signaling responses was blocked and proliferative responses were reduced. Adoptive transfer experiments in Rag2;yc-deficient mice suggested that the loss of B cells after Syk depletion resulted from cell death rather than a failure of replenishment from the bone marrow.

Syk-deficient B cells were still responsive to BAFF in culture and injection of an antibody blocking BAFF-receptor binding resulted in a near complete loss of the Syk-deficient B cell population in vivo, which together suggested that Syk-deficient B cells, in particular mature follicular B cells, still depend on BAFF-R-mediated survival signals. To analyze this phenomenon in more detail, the authors generated mice with a combined constitutive loss of CD19 and a B cell-specific inducible loss of Syk. Combined Syk/CD19 deficiency resulted in aggravated loss of mature B cells, which could be reverted to the level of single gene deletion, by simultaneous triple ablation of Syk, CD19 and the transcription factor FoxO1.

This study by the Reth group revisits the question of B cell fate in the absence of the essential BCR signaling mediator Syk. This is of fundamental importance as deletion of the BCR or Iga on mature B cells leads to rapid cell death. The nature of the underlying BCR-mediated survival signal, which could be continuous stimulation by low affinity autoantigen or a low-level tonic signal, remains unclear. The results reported here differ in a number of central points from a similar study published last year by the Tybulewicz group. Hobeika et al. report that B1 B cells are affected by inducible loss of Syk, they report only a mild upregulation of IgM on Syk-deficient B cells and most importantly, Syk-deficient B cells are still responsive to BAFF in vitro and in vivo. These findings then lead Hobeika et al. to speculate that survival of Syk-deficient B cells still depends on essential survival signals via the PI3-K pathway. They support this notion by the combination of inducible Syk-deletion with CD19 and FoxO1 KO'S. Here a grain of salt is the detail, that the CD19 deficiency results from a in locus knock in of CreERT2, which at least should result in a different CreERT2 gene dosage and hence deletion kinetics in the compound knock out. This point could be discussed in the manuscript.

The essential difference between the two Syk-deletion studies is that the mb1-CreERT2 allele is exclusively B cells specific and therefore preferable, while the R26-MCM driver used in the previous study results in a global deletion of Syk. In addition the floxed alleles used in these two studies are different. While Tybulewicz and coworkers argue for a joint function of Syk between the BCR and BAFFR to provide PI3-K/NFkB mediated survival signals and thereby explain the rapid B cell loss after Syk deletion, Hobeika et al. arrive at a fundamentally different interpretation, suggesting that also in the absence of Syk, PI3-K mediated signals are essential for the survival of about 30% of mature follicular B cells.

The manuscript by Hobeika et al., does however not provide experimental insights how BAFF-R signals PI3-K activation in Syk-deficient B cells, nor why Syk-deficient B cells die in the first place and what distinguished the surviving fraction from the cells dying. Here migration defects and defective signaling to mTOR are discussed. Could these possibilities be experimentally addressed in freshly Syk-deleted B cell populations?

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Page 18, line3: correct Schweighoffer et al. have shown

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Referee #2:

This manuscript addresses an important issue in Immunology, mainly what mechanisms are responsible for the long half-life of mature lymphocytes and how do they achieve the normal sized populations of these cells. In this regard, B cells and T cells seem to be under different control mechanisms, and this study addresses the problem for B cells. What is known is that B cell survival requires two distinct signals: a soluble cytokine called BAFF (the levels of which rise when the B cell population is decreased and vice versa), and expression of the B cell antigen receptor (BCR), for which the tyrosine kinase Syk is a major signaling component. The BCR survival signal requires Syk and signaling to PIP3 and Akt.

A paper published last year by Schweighoffer et al (referenced in the manuscript), took almost the identical approach used here to conditionally delete Syk in mature B cells and examine the effect on survival of mature B cells. Both groups used a floxed allele of Syk and an estrogen receptor-regulated Cre (activated by tamoxifen treatment of the mice) to delete Syk and then follow the survival/death of B cells and examine their properties (phenotype, responsiveness, etc.). In some ways, the results are similar, but in other ways they are in conflict. Schweighoffer et al. found that when Syk is deleted, most B cells (80% in their hands for mature follicular B cells) die over the course of several weeks. When they analyzed the B cells with deleted Syk before they died, they concluded that these cells responded poorly to BAFF in vitro (Figure 2A of their paper). This suggested to the authors that BAFF receptor may somehow connect to the BCR and induce it to signal, and they found that stimulation of normal B cells with BAFF induced some signs of BCR signaling (tyrosine phosphorylation of BCR components, etc.). Therefore, they concluded that the two survival signals of B cells actually converge in that BAFF receptor triggers some signaling from the BCR via Syk, which in turn provides a critical part of the BAFF survival signal. This conclusion is disputed by the current manuscript.

The Hobeika et al. manuscript has deleted Syk and finds again death of the majority of follicular B cells (2/3 in their hands rather than 4/5), but have concluded that the B cells lacking Syk still respond to BAFF as a survival signal. For example, Fig. 6A looks at the response to BAFF in vitro and find a smaller difference, 50-60% of the wild type B cells survived at day 6-8, whereas for Syk-deficient B cells it was about 30%. The results are somewhat different from the other paper (smaller effect in this manuscript, but an effect in the same direction). Conceivably the differences are minimized or enhanced (depending on which groups' data) by in vitro conditions such as BAFF concentration, culture medium (including FCS and anti-oxidants), cell concentration, and/or O2 concentrations in the incubator. To the credit of the authors of this manuscript, they have done the more important experiment, which is to assess the issue in vivo by putting in a monoclonal antibody that blocks the BAFF receptor and looking at the effect on B cell survival in vivo (Figure 6C). They find that the Syk-deficient B cells are substantially reduced in numbers by this treatment, which disagrees with the conclusions of Schweighoffer et al. Given the importance of this experiment to addressing the different conclusions of the prior paper, this experiment should be examined more carefully by showing treatment of wild type control mice as well as mice with Syk-deleted B cells, and showing some sort of kinetic analysis of wild type mice vs. mice with Syk-deleted B cells. A more detailed analysis may further contradict the main conclusion of the Schweighoffer paper, but it is also possible that a more detailed analysis will give an intermediate result and provide some support for the prior conclusion.

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Point-by-point answers to the referee's comments:

Referee #1

In this manuscript entitled "CD19 and BAFF-R can Signal B cell survival in the absence of Syk" Hobeika et al. describe the consequences of B cell-specific, inducible ablation of the non receptor tyrosine kinase Syk in adult mice. The study is based on a novel mb1-CreERT2 driver allele generated by the Reth group, which induces rapid and efficient deletion starting from the pro/pre B cell stage.

This is indeed the first report showing the efficiency of our B cell specific mb1-CreER^{T2} knock-in allele in deleting Syk and FoxO1 after a few applications of Tam. The mb1-CreER^{T2} strain is already being distributed to other groups throughout the world.

While bone marrow B cells are largely not affected, consequences of Syk deletion show in the spleen, where follicular B cells are approx. 3-fold and MZ B cells approx. 10-fold reduced. While transitional B cells of the spleen are not altered, the authors argue that this may be due to rapid replenishment, peritoneal B1 B cells show a clear reduction. The unexpected large number of surviving follicular B cells allowed the authors to characterize this population in more detail. They show that tyrosine phosphorylation in response to non-specific phosphatase inhibition by pervanadate was largely reduced in Syk-deleted B cells and these cells completely failed to mobilize Ca2+ in response to BCR stimulation. Similarly, a number of BCR coupled signaling responses was blocked and proliferative responses were reduced. Adoptive transfer experiments in Rag2;yc-deficient mice suggested that the loss of B cells after Syk depletion resulted from cell death rather than a failure of replenishment from the bone marrow.

Syk-deficient B cells were still responsive to BAFF in culture and injection of an antibody blocking BAFF-receptor binding resulted in a near complete loss of the Syk-deficient B cell population in vivo, which together suggested that Syk-deficient B cells, in particular mature follicular B cells, still depend on BAFF-R-mediated survival signals. To analyze this phenomenon in more detail, the authors generated mice with a combined constitutive loss of CD19 and a B cell-specific inducible loss of Syk. Combined Syk/CD19 deficiency resulted in aggravated loss of mature B cells, which could be reverted to the level of single gene deletion, by simultaneous triple ablation of Syk, CD19 and the transcription factor FoxO1.

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As rightly pointed out by the reviewer the presence of multiple CreER^{T2} copies in the mb1-CreER^{T2};Syk$^{fl/fl}$;CD19$^{-/-}$ compound mice may lead to accelerated recombination of the Syk locus at day 6 and 8 (see new Fig E11). However at day 10 the mb1-CreER^{T2} alone is able to delete the floxed Syk gene in all mature B cells to almost 100%. Accordingly, for the experiments depicted in Fig 7 we analyzed the murine B cells after 30 days of Tam application (see Method parts) and not after 15 days to rule out that the effect in the decrease of the cellularity in the compound strain is

due to a difference in the deletion kinetics. This point is described on page 13 of our revised manuscript.

The essential difference between the two Syk-deletion studies is that the mb1-CreERT2 allele is exclusively B cells specific and therefore preferable, while the R26-MCM driver used in the previous study results in a global deletion of Syk. In addition the floxed alleles used in these two studies are different. While Tybulewicz and coworkers argue for a joint function of Syk between the BCR and BAFFR to provide PI3-K/NFkB mediated survival signals and thereby explain the rapid B cell loss after Syk deletion, Hobeika et al. arrive at a fundamentally different interpretation, suggesting that also in the absence of Syk, PI3-K mediated signals are essential for the survival of about 30% of mature follicular B cells.

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As suggested by the reviewer, we have performed additional experiments to compare cell migration and mTOR signaling in wild type and Syk-deleted B cells (day 10 and day 20 respectively). The transwell migration experiments with the chemokines CCL21, CXCL12, and CXCL13 showed that Syk-deficient B indeed have a migration defect in particular in the migration towards the chemokines CXCL12 and CCL21 (see new Fig E5).

To analyse mTOR signaling, we used intracellular FACScan analysis to monitor a downstream target of mTOR signaling, the Ser240/244 phosphorylation at the activation loop of the ribosomal protein S6. We found that upon anti-Kappa F(ab')$_2$ and to lesser extent pervanadate stimulation Syk-deficient mature B cells do not phosphorylate S6 (see new Fig E4).This supports the notion of a reduced metabolic fitness of Syk-deficient mature B cells. Concerning the comment that we do "not provide experimental insights how BAFF-R signals PI3-K activation in Syk-deficient B cells" *we want to point out that we show in our manuscript for the first time, that CD19, one of the major activators of the PI3-K signaling pathway in mature B cells, is required for the survival of Syk-deficient B cells. This is in line with the recent publication of Jellusova et al. that BAFF-R employs CD19 for PI3-K signaling as cited in our manuscript.*

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We have now corrected these mistakes in the new manuscript and in the legend of Fig 1 as well as in the Fig 4B and we have edited the three references in the discussion.

Referee #2

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As a point of caution, we want to mention here that what so far has been clearly shown experimentally is that when B cells loose their BCR mIgM heavy chain, they die and that this death can be rescued by constitutive signaling through PIP3 and Akt. Whether or not the resting BCR is processing a survival signal via Syk is not at all clear and rather unlikely as discussed in our manuscript. At least in our nanoscale proximity studies we have not seen Syk close to the BCR in resting B cells (see Fig. 3 of Infantino et al J Exp Med. 2010). In all of our experiments over the last 15 years we always find Syk associated with the activated BCR where it plays an essential role in BCR opening and signal amplification.

A paper published last year by Schweighoffer et al (referenced in the manuscript), took almost the identical approach used here to conditionally delete Syk in mature B cells and examine the effect on survival of mature B cells. Both groups used a floxed allele of Syk and an estrogen receptor-regulated Cre (activated by tamoxifen treatment of the mice) to delete Syk and then follow the survival/death of B cells and examine their properties (phenotype, responsiveness, etc.). In some ways, the results are similar, but in other ways they are in conflict. Schweighoffer et al. found that when Syk is deleted, most B cells (80% in their hands for mature follicular B cells) die over the course of several weeks. When they analyzed the B cells with deleted Syk before they died, they concluded that these cells responded poorly to BAFF in vitro (Figure 2A of their paper). This suggested to the authors that BAFF receptor may somehow connect to the BCR and induce it to signal, and they found
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We agree with this reviewer that the in vivo studies of BAFF-R function in Syk-deficient B cells are more relevant than the in vitro studies that only poorly mimic the situation in lymphatic tissues. As asked by the reviewer we performed a kinetics analysis of the survival of resting follicular B cell subset in the blood of Syk-deficient and WT mice over a period of 15 days after treatment with anti BAFF-R. This analysis over 15 days showed no significant difference in the loss of Syk-deleted B cells compared to the control B cells (see new Fig E9)

While I think it is important for these authors to do a more thorough job of analyzing blockade of BAFF in vivo in any case, simply doing that and nothing else would leave this manuscript mostly repeating the Schweighoffer et al study and disproving their main conclusion as the main advance. While this is obviously significant for the field, it would, if that is what the results support, pretty much leave us where we were before the Schweighoffer paper. We have two survival signals (BCR and BAFF receptor), but how they interact and talk to each other would remain unclear. The studies

presented in Figure 7 do address some additional issues, but they are conceptually related to previously published work such as the Srinivasan et al Cell, 2009 paper.

Our data shown in Fig. 7 are quite in line with the recent publication of Jellusova et al. showing that BAFF-R employs CD19 for PI3-K signaling as discussed in our manuscript. We do not think that our discovery that Syk-deficient B cells require CD19 for survival is a minor point as it puts the important issue of B cell survival in the right context.

2nd Editorial Decision 22 December 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #1 and the comments are provided below. As you can see the referee appreciates the introduced changes and support publication here. There are a few minor issues to resolve before acceptance here.

Congratulations on a very nice paper

REFEREE REPORTS

Referee #1:

The revised version of the manuscript by Hobeika et al. includes valuable additional data, that contribute to the understanding of the loss of mature B cells in mice after induced deletion of the kinase Syk. Particularly the inclusion of data demonstrating a metabolic as well as a migrational impairment of Syk-deficient mature B cells provides important hints, towards the underlying mechanisms resulting in the loss of mature deficient B cells. Both possibilities had previously been suggested but not experimentally backed up.

This reviewer also appreciates the kinetic analysis of Sykfl/fl recombination in the presence of different numbers of CreERT2 alleles, which is now shown in Fig. E11. It would have been informative to also include an additional panel in the suppl. Fig.11. showing the day 10 data. These, according to the text (on page 14), show that the kinetic differences that clearly exist at day 6 and 8 have resolved at later time points. I would be happy to take the authors word for it, but why not include this panel, when the data exist.

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Page 36, legend to Fig. 2: The text has been changed to match the labeling of the panels, however, the figure legend still needs to be corrected.
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2nd Revision - authors' response 23 December 2014

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We included now a panel B in Fig E11 showing the deletion of Syk in the majority of mature B cells derived from the mb1-CreERT2; Syk$^{fl/fl}$ at d10 and a complete Syk-deletion at d20 after continuous Tam treatmemt.

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We corrected all these issues in the main text as well as in the figures and figure legends.

