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Altered BCR signaling quality predisposes to autoimmune disease and a pre-diabetic state

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(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 02 March 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees appreciate the findings on the functions of the related kinases Syk and Zap-70 and find the paper suitable for publication in the EMBO Journal. They raise a number of specific concerns that should be addressed to strengthen the findings. Given the comments provided, I would like to ask you to submit a suitably revised manuscript that address the raised concerns in full. I should add that it is EMBO Journal policy to allow a single major round of revision only and that it is therefore important to address the raised concerns 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://www.nature.com/emboj/about/process.html

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

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

This is a very interesting and potentially important manuscript that analyzes the consequences of altering signaling quality of B cells utilizing a zap70 KI into the Syk locus. The experiments are well performed, the conclusions reasonably supported by the experiments, but there are a handful of experimental issues that should be addressed to strengthen the authors' conclusions.

Major issues:
1. Figure 3- In figures 3B-D, the authors have utilized total splenic B cells in their analysis. Given the differences in B cell subsets between KI and wt mice (figure 2A- where T2 cells appear to be over-represented in the KI vs WT mice), the correct analysis is with purified B cell subsets and not total B cells. Since T2 B cell signaling is different from FO or MZ B cells, these differences may give rise to the differences observed in figures 3B-D rather than actual differences in signaling.
2. Figure 3B- Authors should comment on differences in total Ig-beta expression in the control mice.
3. Figure 4- the authors should address whether there are any physiological consequences of these biochemical signaling defects- e.g., are bleeding times different between wt and KI mice?
4. Figure 6- There are two different phenotypes here that each require additional analysis.
a. Glomerulonephritis- the nature of the GN needs additional analysis. As there is no anti-dsDNA Abs, though there are immune complexes as evidence by Ig staining and autoantibodies that react with yet-to-be determined cytoplasmic antigens, is there complement deposition in the glomeruli? Is there systemic complement activation as measured by decreases in serum C3 and C4 levels? Is there proteinuria in these mice and is there elevations in serum creatinine and blood urea nitrogen levels?
b. Diabetes mellitus- Again, the nature of this needs additional analysis. What is the histology early and late in the pancreas- is there a T cell infiltrate or insulitis? With respect to the glucose challenge in figure 6D, the authors should actually be measuring serum C-peptide levels, not just blood glucose levels.

Minor issues:
1. Figure 2F. Accumulation of the various B cell subsets as mice age should be quantified compared to wt control as the authors did in figure 2B.
2. Supplemental figure 3C- the authors should provide a statistical analysis over several mice with respect to the differences observed with CD4+Foxp3+ T cells.

Referee #2

The protein tyrosine kinases Syk and ZAP70 are key signal transducer elements of the B cell antigen receptor (BCR). Several studies suggest that the ratio at which Syk and ZAP70 are coexpressed is critical for the proper development and selection of B cells. Imbalanced coexpression is associated with various diseases such as autoimmune phenomena and B cell malignancies. Using a 'knock in' strategy, which drives expression of ZAP70 under the control of Syk promotor elements, Kiefer and colleagues now demonstrate that the two kinases differently affect the quality of BCR signal output which in turn predisposes for various autoimmune reactions. The provided data are convincing and support the authors' conclusions. The manuscript is well written. Altogether this is a timely and state-of-the-art analysis of 'pathogenic' BCR signal transduction. I have only some minor comments and suggestions.

1) I am puzzled by the term 'hypomorphic' Syk to describe the genetic make-up of the cells (see abstract and text). The 'knock in' strategy basically replaced the expression of Syk by that of ZAP70. This is something different. Hence I strongly recommend to avoid the term 'hypomorphic' Syk.

2) Authors claim that the altered cellular responses and the biased B cell development in the mutant mice are caused by compromised Erk1/2 activation. Indeed, this might be possible. However, Erk1/2 signaling was the only pathway the authors have investigated. When making such a firm statement authors should either analyze additional survival pathways or weaken that argument.

3) Figure 3. Authors describe in the text that kinases were produced in CHO cells (page 8). By contrast, the figure legend says that they were produced in COS cells.

4) In figure 4 authors have investigated ITAM-mediated signaling in wild-type and mutant platelets. In this case the observed differences can not immediately be ascribed to the kinase replacement because these cells lack de novo protein synthesis, and hence, different turn over rates of the two kinases may play a major role. Do platelets express ZAP70 at all? It should be fairly easy to analyze ZAP70 expression in platelets by western blotting.

5) Given that the replacement of Syk by ZAP70 affects not only B lymphocytes but also other cell types, I wonder whether this important message should not be included into the title/abstract in order to make the paper even more attractive for a wide readership.

Referee #3

The manuscript by Konigsberger et al. describes a new knockin mouse model where the Zap70 cDNA is placed into the Syk gene. This mouse line expresses Zap-70 in all haematopoietic cell types, which normally express Syk. The question addressed by this mouse line is whether Zap-70 can replace Syk in its function, two tyrosine kinases which show some redundancy, especially during B- and T-cell development. The authors analyse B- and T-cell development, as well as specific functions of macrophages and neutrophils and platelets, which all normally express Syk. Interesting differences are detected in these cell types: B cells are generally affected in their development and also show impaired signalling, while T cell development is normal. B cells accumulate as MZ B cells and the knockin mice develop autoimmune phenotypes. Macrophages and granulocytes are analysed in their phagocytosis potential that is normal, while platelets have impaired ITAM-mediated signalling known to depend on Syk. This is an interesting and quite comprehensive study in many cell types.
I have some minor points which should be addressed:
- In the results (page 5, 6) and discussion (page 12) the authors write about a positive selection checkpoint. Although some people call it like this, this is not a generally used term, I would replace it by pre-BCR checkpoint.
- On page 6 bottom the changes in T cells are discussed (Fig.2C and 2D). This discussion is unclear to me. I see no big changes in T cell populations, anyway.
- on page 7 middle the increased MZ B cell phenotype is discussed in terms of a non-obese diabetic mouse line. I do not understand the relevance of this mouse model here. Later the authors discuss the MZ B cell increase in terms of BCR signalling quality, which determines between MZ and FO B cells. Another argument could be B cell lymphopenia, which leads to the fill up of a MZ B cell compartment with rather normal cell numbers. This has been observed in a lot of BCR transgenic lines (see e.g. review Martin and Kearney Nat Rev Immunol 2002).
- Fig.1 H. How were the cells gated for this cell cycle analysis?
- Fig.2: Here it is crucial to give absolute cell numbers for all the populations (A-F). Otherwise it is hard to judge whether and how much the B cell populations are decreased. Are MZ B cells just relatively increased, is the total MZ cell number normal? The same question exists for the T cell changes. To give the changes in percentage is not enough. One would like to know whether the total T cell numbers are increased or normal.
- Fig.6 C Please show some quantification of the increased glomeruli. How often observed? Statistics? Please state whether proteinurea was observed and whether life span of the mice was affected.

1st Revision - authors' response 20 April 2012

Referee #1

... Major issues:
1. *Figure 3: In figures 3 B-D, the authors have utilized total splenic B cells in their analysis. Given the differences in B cell subsets between KI and wt mice (figure 2A- where T2 cells appear to be over-represented in the KI vs WT mice), the correct analysis is with purified B cell subsets and not total B cells. Since T2 B cell signaling is different from FO or MZ B cells, these differences may give rise to the differences observed in figures 3B-D rather than actual differences in signaling.*

We agree with the referee's comment on differential responses of mature versus transitional B cell subsets. To account for this issue, we sorted wildtype T2/MZ splenic B cells using a previously published (Su et al., 2002, *JI*) set of surface markers ($B220^{+}CD21^{hi}CD24^{hi}$). We included the anti-IgM-mediated phospho-tyrosine pattern of this population in Figure 3 C. Given the strong reduction of total B cells in the Syk^{ki} mouse, it was technically impossible to sort sufficient purified knock-in B cells to also include a specific subpopulation for biochemical comparison. We propose, based on the composition of the Syk^{ki} spleen, that wildtype $B220^{+}CD21^{hi}CD24^{hi}$ (T2/MZ) and Syk^{ki} $B220^{+}CD19^{+}$ B cells, which accumulate preferentially at the T2/MZ stage, are valid populations for the comparison of *ex vivo* kinase activity. We are pleased to report that the conclusions drawn from this experiment remain unaltered. It is indeed the kinase exchange that leads to the attenuated BCR-mediated signalling observed in Figure 3 B-D.

2. *Figure 3B: Authors should comment on differences in total Ig-beta expression in the control mice.*

We want to comment the reviewer's valuable point as follows: Material in the control lane was precipitated with an isotype matched hamster antibody of irrelevant specificity, which generated a non-specific band at a higher molecular weight, but most importantly no signal at the molecular weight of Ig-beta. Precipitation of lysates from ki mice with anti Ig-beta specific antibodies, in addition to Ig-beta (which we have indicated with red arrows), brought down lower molecular weight material. We think this lower molecular weight material is non-specific and results from the presence of inappropriately self restricted autoimmune Ig in ki mice. 1^{st} If it truly was Ig-beta it should become phosphorylated and show up in the pTyr blot, which is not the case. 2^{nd} 0 and 2 min time points were measured by splitting the same lysate and is appears unlikely that within 2 mins Ig-beta concentration is massively upregulated.
We rather think that some cellular material was preferentially bound by autoreactive Ig. After crosslinking of surface Ig by anti-IgM, the avidity of the complex for this material was largely increased. Some of these complexes may have stayed intact during our gentle Triton X-100 lysis and thereby the material may have become co-precipitated.

3. *Figure 4: The authors should address whether there are any physiological consequences of these biochemical signaling defects- e.g., are bleeding times different between wt and KI mice?*

We addressed the reviewer's comment and measured bleeding times by tail-tip amputation of wildtype C57Bl/6, heterozygous and homozygous Zap-70 knock-in mice. In the cohort of animals available for these experiments, we did not detect a significant difference between heterozygous and homozygous knock-in mice. However, we prefer not to include these data in the manuscript, for the following reasons:
1. Bleeding analysis by tail-tip amputation should be done exclusively with animals that have not previously experienced tip amputations. Presence of scar tissue from an earlier amputation leads to large variations in the results obtained. Because the operational routine of our animal facility involves mandatory genotyping by tail resection of all mice at weaning, we did not have appropriate cohorts of untouched animals available during this revision. Accordingly, the preliminary data generated displayed inappropriately large variations within the heterozygous and knock in groups.
2. We are generating non-tail-tip genotyped cohorts of animals, however, breeding of sufficiently large numbers of mice will take more time than appropriate during this review process.
3. The detailed analysis of the hemodynamic parameters and behaviour of Zap-70 knock in mice is the subject of an ongoing collaborative study. We will make exactly this issue into the central point of a future manuscript, therefore we prefer to not include the preliminary data in the context of the present study.

4. *Figure 6: There are two different phenotypes here that each require additional analysis.*
a. Glomerulonephritis- the nature of the GN needs additional analysis. As there is no anti-dsDNA Abs, though there are immune complexes as evidence by Ig staining and autoantibodies that react with yet-to-be determined cytoplasmic antigens, is there complement deposition in the glomeruli? Is there systemic complement activation as measured by decreases in serum C3 and C4 levels? Is there proteinuria in these mice and is there elevations in serum creatinine and blood urea nitrogen levels?

Following the reviewer's suggestions, we further characterized the developing glomerulonephritis and analyzed C3 deposition in ctrl and knock-in glomeruli. Syk^{ki} glomeruli stained weakly positive for C3 and the resulting data are now included in Figure 6. Furthermore, we measured systemic activation of complement by serologically assessing levels of C3 and C4, which is now included in Supplementary Figure 6. Knock-in mice showed a mild but significant proteinuria and these data have also been included in Figure 6. Finally, we have included creatinine and blood urea nitrogen levels in the supplementary data set (Supplementary Figure 6).

b. Diabetes mellitus- Again, the nature of this needs additional analysis. What is the histology early and late in the pancreas- is there a T cell infiltrate or insulitis? With respect to the glucose challenge in figure 6D, the authors should actually be measuring serum C-peptide levels, not just blood glucose levels.

Here, we histologically assessed early and late pancreatic sections for cellular infiltrate and included the data in Supplementary Figure 6. In addition, C-peptide levels were analyzed and are included in Figure 6. The implications of the additional analysis have been discusses in the text (page 11).

Minor issues:
1. *Figure 2F: Accumulation of the various B cell subsets as mice age should be quantified compared to wt control as the authors did in figure 2B.*

We changed Figure 2F and now quantify the accumulation of marginal zone B cells as requested.

2. *Supplemental figure 3C: The authors should provide a statistical analysis over several mice with respect to the differences observed with CD4+Foxp3+ T cells.*

We provide the statistical analysis (n=4 animals/group) of nTreg cell numbers (Supplementary Figure 3).

Referee #2

..... I have only some minor comments and suggestions.
1) *I am puzzled by the term 'hypomorphic' Syk to describe the genetic make-up of the cells (see abstract and text). The 'knock in' strategy basically replaced the expression of Syk by that of ZAP70. This is something different. Hence I strongly recommend to avoid the term 'hypomorphic' Syk.*

We concur with referee #2, the terminology ‚hypomorphic' should be avoided in the present context. We therefore removed the term 'hypomorphic' from the manuscript and have either replaced it by a description of the genetic alterations introduced or changed it to 'hypoactive' solely were kinase activity was assessed.

2) *Authors claim that the altered cellular responses and the biased B cell development in the mutant mice are caused by compromised Erk1/2 activation. Indeed, this might be possible. However, Erk1/2 signaling was the only pathway the authors have investigated. When making such a firm statement authors should either analyze additional survival pathways or weaken that argument.*

Following the reviewer's suggestion, we have tried to investigate other pathways. However, due to the severe reduction in B cell numbers, we were unable to biochemically analyze additional survival pathways. We have therefore weakened the argument in the discussion section (page 13, line 6), as suggested by the referee.

3) *Figure 3. Authors describe in the text that kinases were produced in CHO cells (page 8). By contrast, the figure legend says that they were produced in COS cells.*

Kinases were expressed in CHO cells. We apologize for the mistake and changed the figure legend and the materials and methods part accordingly.

4) *In figure 4 authors have investigated ITAM-mediated signaling in wild-type and mutant platelets. In this case the observed differences can not immediately be ascribed to the kinase replacement*

because these cells lack de novo protein synthesis, and hence, different turn over rates of the two kinases may play a major role. Do platelets express ZAP70 at all? It should be fairly easy to analyze ZAP70 expression in platelets by western blotting.

We agree with the referees' comment, prepared washed platelets and analyzed Syk and Zap-70 protein expression in wildtype, heterozygous and homozygous knock-in platelets. The robust expression of Zap-70 in knock-in platelets, in the absence of increased degradation, strongly resembled our analysis of bone marrow cells. This analysis, which has been included in Supplementary Figure 5, argues against different turnover rates as the reason for the observed differences in platelets.

5) *Given that the replacement of Syk by ZAP70 affects not only B lymphocytes but also other cell types, I wonder whether this important message should not be included into the title/abstract in order to make the paper even more attractive for a wide readership.*

As the larger focus of the manuscript lies on B cells and we argue that B cells are the causative part for the autoimmune phenotype observed, we would like to stick to the original title which should nevertheless be an attractive header for a broad readership. We have included additional information on myeloid cells in the abstract.

Referee #3

….. This is an interesting and quite comprehensive study in many cell types.

I have some minor points which should be addressed:
1. *In the results (page 5, 6) and discussion (page 12) the authors write about a positive selection checkpoint. Although some people call it like this, this is not a generally used term, I would replace it by pre-BCR checkpoint.*

We changed the term 'positive selection' into 'pre-BCR checkpoint'.

2. *On page 6 bottom the changes in T cells are discussed (Fig.2C and 2D). This discussion is unclear to me. I see no big changes in T cell populations, anyway.*

There is roughly a doubling of CD4+ and CD8+ T cells in knock-in spleens shown in Figure 2C. This is also the case for the respective CD4+ and CD8+ eff/mem populations. The later is now far more clearly depicted, as we have changed Fig 2D and the text accordingly from relative representation to absolute numbers. We believe that now the differences pertaining to the absolute increase in T cells are far clearer. Also the fact that this expansion happened in a completely balanced fashion, resulting only in negligible changes of the T cellular subsets, should be more obvious.

3. *On page 7 middle the increased MZ B cell phenotype is discussed in terms of a non-obese diabetic mouse line. I do not understand the relevance of this mouse model here. Later the authors discuss the MZ B cell increase in terms of BCR signalling quality, which determines between MZ and FO B cells. Another argument could be B cell lymphopenia, which leads to the fill up of a MZ B cell compartment with rather normal cell numbers. This has been observed in a lot of BCR transgenic lines (see e.g. review Martin and Kearney Nat Rev Immunol 2002).*

We discussed the finding of Marino et al. because in NOD mice marginal zone B cells not only accumulate with age and onset of frank diabetes, but they were shown to be essentially capable of presenting the autoantigen insulin to pancreatic T cells. As Zap-70 knock-in B cells showed anti-insulin reactivity and anti-insulin titers can be increased by stimulating the cells with LPS, to which MZ B cells vigorously react, we found it worth mentioning. We appreciate that our model is essentially different from BCR transgenic model systems like the anti-HEL or the anti-phosphorylcholine models as the kinase exchange likely generates a polyclonal B cell repertoire (only 0.08% of total B cells react to insulin). We fully agree to the argument that lymphopenia might be another reason influencing peripheral B cell selection and have therefore, following the reviewer's notion, included this argument in the text (page 14, line 21).

4. *Fig.1 H. How were the cells gated for this cell cycle analysis?*

Cell cycle analysis was done on fraction C ($B220^+CD43^+CD24^+BP1^+$) B cells. We included an according statement in the legend to Figure 1.

5. *Fig.2: Here it is crucial to give absolute cell numbers for all the populations (A-F). Otherwise it is hard to judge whether and how much the B cell populations are decreased. Are MZ B cells just relatively increased, is the total MZ cell number normal? The same question exists for the T cell changes. To give the changes in percentage is not enough. One would like to know whether the total T cell numbers are increased or normal.*

We have fully included the reviewer's comments. Instead of giving percentages in Figure 2B, C, D, and F, we now calculated absolute numbers in order to make e.g. the accumulation of marginal B cells more comprehensive. The representative dot plots in Figure 2A were left unchanged to better visualize the B cell arrest at the T2 stage (total cell number statistics are depicted in Figure 2B). We have intentionally kept Figure 2E unchanged, because the ratio is identical, irrespective if it is calculated from percentages or absolute numbers.

6. *Fig.6 C Please show some quantification of the increased glomeruli. How often observed? Statistics?*

We now show a quantification of glomerular size and the requested statistics in Figure 6.

Please state whether proteinurea was observed and whether life span of the mice was affected.

We included the aforementioned (referee 1) data on proteinuria in Figure 6 and state on the life span in the text. Lifespan was apparently normal up to the time points, where animals were sacrificed for experimental analysis. This ranged up to 60 weeks as in the case of glomerulonephritis scoring.

Additional Correspondence 18 May 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has know been seen by referee #1 who appreciate the introduced changes and supports publication in the EMBO Journal. I am therefore pleased to proceed with the acceptance of the paper for publication here. You will receive the formal acceptance letter shortly.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Referee #1:

The authors nicely address most of the questions I raised in the previous review.

