EMBO reports

SHLD2 promotes class switch recombination by preventing inactivating deletions within the Ig locus

Alexanda Ling, Meagan Munro, Natasha Chaudhary, Conglei Li, Maribel Berru, Brendan Wu, Daniel Durocher, and Alberto Martin
DOI: 10.15252/embr.201949823

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Review Timeline:

Submission Date:	5th Dec 19
Editorial Decision:	15th Jan 20
Revision Received:	23rd Apr 20
Editorial Decision:	12th May 20
Revision Received:	19th May 20
Accepted:	25th May 20

Editor: Esther Schnapp

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 15th Jan 2020

Dear Dr. Martin

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, while referee 2 is more negative, both referees 1 and 3 support the publication of your work following careful revisions. I would therefore like to give you the opportunity to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter blots in these cases.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be

cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

8) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In this manuscript, Martin and colleagues examine CSR in Shieldin 2 k/o (Shld2-/-) mice and demonstrate that loss of Shld2 impairs CSR and can suppress embryonic lethality of a Brac1 exon 11 deleted mice. The authors demonstrate that loss of Shld2 does not impair V(D)J recombination or lymphocyte development. However, Shld2-/- B cells and Shld2-deficient CH12 cells are impaired in undergoing CSR. They also observe a novel phenotype in the mutant B cells in that they permanently lose expression of Ig upon induction of CSR likely due to recombination-mediated loss of constant region exons. Given that the shieldin complex acts downstream of 53BP1 and restricts end-resection, the experiments strongly suggest that Shld2 is a key effector of 53BP1-mediated regulator of end-processing during CSR.

The roles of the shieldin complex in CSR and in end-protection have been demonstrated earlier by others and one could question the novelty of this work. However, given that the authors have generated a mouse model and have mechanistically resolved why the BCR negative cells exist in CH12 cells mutated for NHEJ genes, the work will be of much relevance and interest to the field in general. The manuscript is very well-written, the experiments are solid and there are only a few technical issues outlined below that the authors should resolve. Otherwise, this is a solid study that should be published.

1. The authors should provide a better description of the Brac1□11 mutation.
2. Can the authors do a western blot for Shld2 in wt and k/o B cells? And it is not clear what RNA is generated from the targeted alleles.
3. Fig 2. The authors should provide representative flow plots showing the switching defects to each isotype would be useful.
4. Fig. 2C. An AID ko control would have been useful to assess the lower limit of the assay.
5. What is the frequency of GC B cells in the shld2-/- at homeostasis and after NP-CGG immunization?

Referee #2:

This manuscript examines the phenotype of Shld2-deficient mice and B cells. The findings reveal no major defect in V(D)J recombination and a substantial defect in CSR. Defects in CSR have been reported previously by others through analysis of cell lines deficient in components of the shieldin complex, including Shld2. The authors also find that deficiency in Shld2 compensates genetically for mutation of Brca1, that Shld2-deficient B cells (ex vivo stimulated) and Shld2-deficient CH12 cells show an increase in Ig-lo/neg cells, that deficiency in many NHEJ factors in CH12 cells also results in an increase in Ig-lo/neg cells, and finally, that in 53BP1-deficient CH12 cells, that some Igh alleles contain aberrant deletions.

The work reported here is generally well done though as noted below, some of the PCR analyses lack important controls. The main issue regarding this paper is scope/novelty of the findings. As noted above, defective CSR in shieldin-deficient and Shld2-deficient B cells has already been reported, and extending this to mice is not a substantial advance. The finding regarding Brca1 is a nice refinement of what was known from previous genetic analyses, e.g., with 53BP1. The heart of the claim for novelty rests on the finding of genomic deletions during CSR in 53BP1-deficient B cells (with only a very little bit of data gathered in Shld2-deficient cells). Presumably, this is a feature of CSR in many NHEJ-deficient backgrounds. It's an interesting observation. It was known that the DSBs associated with CSR would still be generated in such backgrounds, and it was known that they could participate in translocations, and they would have to be repaired somehow for the cells to survive. But their fate had not been carefully examined. This work is a start in this direction. Substantial deletions are a pretty obvious candidate for the outcome, but worth documenting. Overall, I find the work below what would be expected for novelty/significance for publication in EMBO Reports. I offer suggestions for experiments below in hopes they will be helpful, not because I think they will necessarily make the paper suitable for EMBO Reports.

Specific comments:

1. Last sentence of abstract wasn't properly edited.
2. Figure 1F: the authors should show percent GFP + cells before and after imatinib treatment so that readers can better appreciate the effect of the CRISPR treatments. These cells frequently have substantial GFP+ cells before treatment.
3. Figure S5B needs a legend on the figure.
4. Figure 5A is confusing. First, what is "TA" and "T1"? They are mentioned but not defined in the figure legend. Second, the lack of a full length WT control sample for IgM and IgA on the gels is confusing and makes interpretation of the data very difficult. Maybe all of these transcripts contain deletions. The same problem affects Fig. 5C and Fig. S7A. I really can't make out what is going on.
5. Controls are also missing in Figs 5C and S7C and D: amplification from DNA prepared from Ig-hi clones where deletions are not expected. Without this, some or much of the long range PCR data in these figures could simply be due to deletions that occur during PCR amplification. The lack of careful analysis of control DNA makes it difficult to assess some of the central conclusions of the paper.
6. It is peculiar that so little of the analyses in Fig. 5 and S7 were done with Shld2-deficient cells. Shld2 is the focus of the paper and Shld2-/- cells should be analyzed carefully and extensively. Along the same lines, the sequence analysis of Fig. 5D is inadequate. Two clones is a very small amount of data and no sequencing has been performed for Shld2-deficient products, nor for deletions that I expect will show up when amplifying from Ig-hi clones where deletions are not expected.
7. It is surprising that no attempt was made to identify and characterize deletion products from ex

vivo stimulated B cells from Shld2-deficient mice.

Referee #3:

"SHLD2 promotes class switch recombination by preventing inactivating deletions within the Ig locus" by Ling et al is an elegant study with straight-forward results, demonstrating that SHLD2 is crucial not only for antibody class switch recombination (CSR) but also for genomic stability in B cells.

A role for the shieldin complex (SHLD1, 2, 3 and Rev7) in CSR was previously demonstrated in cell lines, where it was proposed to act downstream of 53BP1. 53BP1 is known to protect DNA double-strand break (DSB) ends from resection, thus promoting non-homologous end-joining (NHEJ) over homologous recombination (HR). This study generated novel shld2-deficient mice by CRISPR/Cas9 and undertook a very thorough examination of CSR by analyzing isotype switching in serum, in vitro stimulated spleen B cells, and in immunized mice analyzed by ELISA and Elispot. Defective CSR was observed in all cases, while B cell and thymic development and VDJ recombination were completely normal. Furthermore, the authors found that shld2-deficiency can suppress the lethality of brca1-deficiency, supporting its role in suppression of HR.

The phenotype of SHLD2-deficiency mirrors that of 53BP1-deficiency, which is known to have drastically reduced CSR efficiency. The authors show that, rather than reduced CSR, 53BP1- and SHLD2-deficiency both result in non-productive CSR, associated with large deletions in the Ig locus. Between 20 and 40% of cells become permanently IgLO - initially defined as IgM-neg and IgG1-neg (the two most common sIg). While this could indicate switching occurred to other isotypes, the authors confirmed in two ways that this does in fact result from loss of Ig expression. The cell line CH12 switches almost exclusively to IgA, and 20 to 40% of SHLD2- CH12 cells became IgLO upon CSR induction (IgM-neg/IgA-neg). More importantly, loss of Ig was confirmed molecularly at both the RNA and DNA level.

Altogether, this study convincingly shows that SHLD2-deficiency has the same phenotype as 53BP1-deficiency, and that these proteins act to protect DSB DNA ends from end resection so that they can be used by the NHEJ pathway for CSR. It was not formally shown that SHLD2 acts downstream of 53BP1, but this is strongly suggested by the similar phenotypes. This point is suggested, but not overstated by the authors, and is not essential to these very important findings. The extent of the DNA degradation in the absence of SHLD2 is a novel finding that shows how important this factor is not only for CSR, but for activated B cells to maintain production of any isotype of Ig.

The data is well displayed with appropriate statistical analysis. There are several typos/grammatical errors throughout the text that should be corrected prior to publication.

Cross-comments by referee 1:

I would strongly maintain that the manuscript should be published once (and if) the authors address the comments raised by the reviewers. The presence of the Ig negative cells in DNA repair mutants have confounded the field for a while and a mechanistic explanation provided in this manuscript is significant enough for publication. True the Shieldin k/o mouse has been published before but here

the authors have generated their own model, which will be useful to the field. And even confirmatory data (this paper goes way beyond that) is useful for a complex set of proteins like the shieldins. So, I would strongly encourage you to give the authors a chance to resubmit a revised manuscript.

Preamble: We thank our Reviewers and Editor for their suggestions and encouraging comments. We have amended our manuscript in response to all these suggestions/comments. The responses and changes (red font in the manuscript) are detailed below:

Referee #1 (Remarks to the Author):

In this manuscript, Martin and colleagues examine CSR in Shieldin 2 k/o (Shld2-/-) mice and demonstrate that loss of Shld2 impairs CSR and can suppress embryonic lethality of a Brac1 exon 11 deleted mice. The authors demonstrate that loss of Shld2 does not impair V(D)J recombination or lymphocyte development. However, Shld2-/- B cells and Shld2-deficient CH12 cells are impaired in undergoing CSR. They also observe a novel phenotype in the mutant B cells in that they permanently lose expression of Ig upon induction of CSR likely due to recombination-mediated loss of constant region exons. Given that the shieldin complex acts downstream of 53BP1 and restricts end-resection, the experiments strongly suggest that Shld2 is a key effector of 53BP1-mediated regulator of end-processing during CSR.

The roles of the shieldin complex in CSR and in end-protection have been demonstrated earlier by others and one could question the novelty of this work. However, given that the authors have generated a mouse model and have mechanistically resolved why the BCR negative cells exist in CH12 cells mutated for NHEJ genes, the work will be of much relevance and interest to the field in general. The manuscript is very well-written, the experiments are solid and there are only a few technical issues outlined below that the authors should resolve. Otherwise, this is a solid study that should be published.

1. The authors should provide a better description of the Brac1Δ11 mutation.

Response: We have modified the text to better describe the BrcaΔ11 mutation.

2. Can the authors do a western blot for Shld2 in wt and k/o B cells? And it is not clear what RNA is generated from the targeted alleles.

Response: Unfortunately, there is not a good commercial antibody to detect SHLD2 to our knowledge.

3. Fig 2. The authors should provide representative flow plots showing the switching defects to each isotype would be useful.

Response: We have embedded representative flow plots in Figure EV2A.

4. Fig. 2C. An AID ko control would have been useful to assess the lower limit of the assay.

Response: In hindsight, we agree that an AID-KO control would demonstrate the lower limit of the assay. As this control would have needed to be done alongside the analysis of the other data, which would have required a repetition of all the other analyses, and because the experiments we

can do are limited during the current crisis, we cannot complete these experiments. Nevertheless, the data show that *Shld2*$^{-/-}$ CSR is impaired in response to NP-CGG immunization and similar to the CSR defect demonstrated by *53bp1*$^{-/-}$ mice.

5. What is the frequency of GC B cells in the shld2-/- at homeostasis and after NP-CGG immunization?

Response: We have assessed the frequency of GC B (B220^{+} GL-7^{+} FAS^{+}) cells in WT and *Shld2*$^{-/-}$ mice at homeostasis and after NP-CGG immunization and embedded the data in Figure EV3. The data does not show a difference in GC B cell frequency between WT and *Shld2*$^{-/-}$ mice at homeostasis or after immunization.

Referee #2 (Remarks to Author):

The work reported here is generally well done though as noted below, some of the PCR analyses lack important controls. The main issue regarding this paper is scope/novelty of the findings. As noted above, defective CSR in shieldin-deficient and Shld2-deficient B cells has already been reported, and extending this to mice is not a substantial advance. The finding regarding Brca1 is a nice refinement of what was known from previous genetic analyses, e.g., with 53BP1. The heart of the claim for novelty rests on the finding of genomic deletions during CSR in 53BP1-deficient B cells (with only a very little bit of data gathered in Shld2-deficient cells). Presumably, this is a feature of CSR in many NHEJ-deficient backgrounds. It's an interesting observation. It was known that the DSBs associated with CSR would still be generated in such backgrounds, and it was known that they could participate in translocations, and they would have to be repaired somehow for the cells to survive. But their fate had not been carefully examined. This work is a start in this direction. Substantial deletions are a pretty obvious candidate for the outcome, but worth documenting.

Response: We agree with the reviewer that the observation of increased deletion within the Ig locus in 53BP1 and Shld2-deficient B cells that leads to loss of Ig expression is an interesting observation. In response to the point that only a few SHLD2-deficient clones were analyzed (also point 6 below), we have now increased this analysis substantially and is now shown in Fig 5C, and 6C,D.

Specific comments:
1. Last sentence of abstract wasn't properly edited.

Response: Thank you for pointing this error out. We have now corrected this.

2. Figure 1F: the authors should show percent GFP + cells before and after imatinib treatment so that readers can better appreciate the effect of the CRISPR treatments. These cells frequently have substantial GFP+ cells before treatment.

Response: We have embedded a supplemental figure showing the basal frequency of GFP+ cells prior to imatinib treatment in Figure EV1F; all populations had <8% GFP+ cells prior to treatment, and the normalized frequency is shown in Figure 1F.

3. Figure S5B needs a legend on the figure.

Response: Thank you for the correction, we have amended the figure legend (now Fig EV4B).

4. Figure 5A is confusing. First, what is "TA" and "T1"? They are mentioned but not defined in the figure legend. Second, the lack of a full length WT control sample for IgM and IgA on the gels is confusing and makes interpretation of the data very difficult. Maybe all of these transcripts contain deletions. The same problem affects Fig. 5C and Fig. S7A. I really can't make out what is going on.

Response: We apologize for the lack of clarity. “TA” and “T1” are *53bp1*$^{-/-}$ CH12 clones independently Cas9-edited with different sgRNA; we have amended the figure caption to clarify this fact. We have also amended the figure layout and descriptions to hopefully provide more clarity. We have also included positive control samples for IgM and IgA reverse-transcription PCR experiments, as well as data from *Shld2*$^{-/-/-}$ clones both Ig^{lo} and IgA^{+} subclones.

5. Controls are also missing in Figs 5C and S7C and D: amplification from DNA prepared from Ig-hi clones where deletions are not expected. Without this, some or much of the long range PCR data in these figures could simply be due to deletions that occur during PCR amplification. The lack of careful analysis of control DNA makes it difficult to assess some of the central conclusions of the paper.

Response: Thank you for the suggestion. We have repeated the long-range PCR experiment with some *Shld2*$^{-/-/-}$ IgA^{+} and additional *Shld2*$^{-/-/-}$ Ig^{lo} subclones, and also modified the PCR protocol (mainly by increasing extension time); with respect to the latter modification, most Ig^{lo} clones now show a LR-PCR product(s) and supports the conclusion that Ig^{lo} cells are a subset of switched cells.

6. It is peculiar that so little of the analyses in Fig. 5 and S7 were done with Shld2-deficient cells. Shld2 is the focus of the paper and Shld2-/- cells should be analyzed carefully and extensively. Along the same lines, the sequence analysis of Fig. 5D is inadequate. Two clones is a very small amount of data and no sequencing has been performed for Shld2-deficient products, nor for deletions that I expect will show up when amplifying from Ig-hi clones where deletions are not expected.

Response: We agree that more data is necessary. We have sequenced additional long-range-PCR products from WT, *53bp1*$^{-/-/-}$, and *Shld2*$^{-/-/-}$ Ig^{lo} clones and presented them in Figure 6D. We have also carried out more RT-PCR analysis on additional *Shld2*$^{-/-/-}$ Ig^{lo} clones shown in Figure 5C. The long-range-PCR data captures both switching on the productive allele (with the VDJ exon) as well as the non-productive allele; nevertheless, the data show that most of the LR-PCR products have extensive deletion into the IgA constant region exons and along with RT-PCR data

in Figure 5C supports the conclusion that Ig^{lo} cells are a subset of switched cells, albeit with overactive resection and deletion into the IgA constant region.

7. It is surprising that no attempt was made to identify and characterize deletion products from ex vivo stimulated B cells from Shld2-deficient mice.

Response: It is difficult to clone *ex vivo* B cells and thus a more technically sophisticated single-cell long-range-PCR approach would be necessary to address this question. Nevertheless, we believe that the improved and additional RT-PCR, long-range-PCR, and sequencing data on WT, *53bp1*$^{-/-}$, *Shld2*$^{-/-/-}$ Ig^{lo} and IgA^{+} CH12 clones robustly demonstrate that Ig^{lo} cells are the product of deletions into the acceptor constant region during the course of CSR.

Referee #3 (Remarks to Author):

"SHLD2 promotes class switch recombination by preventing inactivating deletions within the Ig locus" by Ling et al is an elegant study with straight-forward results, demonstrating that SHLD2 is crucial not only for antibody class switch recombination (CSR) but also for genomic stability in B cells.

Response: We thank this reviewer for this comment.

The data is well displayed with appropriate statistical analysis. There are several typos/grammatical errors throughout the text that should be corrected prior to publication.

Response: Thank you for pointing these out, which were also noticed by the other reviewers. We have now gone through the manuscript to ensure that such typos are correct.

1st Revision - Editorial Decision 12th May 2020

Dear Dr. Martin,

Thank you for the submission of your revised manuscript. We have now received the comments from the referees and both support its publication now. We can therefore in principle accept your manuscript.

Only a few more minor changes will be required:

Please complete the section E in the author checklist.

Fig 1E is called out after 1B and Fig 5C is called out after 5A, please correct.

The 2 EV tables are in one file, they need to be one file per table, please upload them as Table EV1 and Table EV2.

The APPENDIX FILE is missing a table of content with page numbers, please add.

The blots in figures 5 and 6 could be better quality.

The EMBO reports reference style is numbered, however, this style will change on the 1st of July to the Harvard style. I assume that you will be able to resubmit your final manuscript within a few weeks, and in this case, please correct the current reference style to the numbered EMBO reports style (a link can be found in our guide to authors).

I attach to this email a related manuscript file with comments from the data editors. Please address all comments in the final manuscript file.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Authors have addressed all comments. Manuscript is ready for publication.

Referee #2:

The authors have addressed my concerns with changes to the text and figures and with new experiments that substantially increase the amount of molecular data provided on Shld2-deficient cells. This is a solid and useful study. Regarding scope and novelty, I defer to the judgement of the other two reviewers and the editor. There are certainly novel and interesting findings in this manuscript.

Immunology
UNIVERSITY OF TORONTO

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http://www.albertomartinlab.ca/

19 May 2020

Dear Esther,

Thank you for your time and manuscript corrections:

- We have corrected the figure panel order in Figures 1 and 5 to match the appearance in text.
- We have separated the EV tables into separate files.
- We have added a table of contents with page numbers to the Appendix.
- We have increased the resolution of the images in Figures 5 and 6.
- We have added information regarding replicates and statistics in the figure legend text.

These changes have been colored in red. Let me know if we have addressed all of your concerns.

Sincerely,

Alberto Martin

2nd Revision - Editorial Decision 25th May 2020

Dr. Alberto Martin
University of Toronto
Immunology
1 King's College Circle 7302
Toronto, ON M5S 1A8
Canada

Dear Dr. Martin,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: ALBERTO MARTIN
Journal Submitted to: EMBO REPORTS
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were not pre-determined. We typically select 4-10 mice per group (experimental and control groups)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample sizes were not pre-determined and no statistical methods was done to determine sample size. We typically select 4-10 mice per group (experimental and control groups).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from the analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	I looked over the data myself to ensure that subjective bias was reduced
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was carried out.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	This was not carried out or applicable to this study
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done in this study
5. For every figure, are statistical tests justified as appropriate?	We carried out Student's t tests and two-way analysis of variance (ANOVA) for all experiments. We consulted a statistician for these analyses
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We did not test for normal distribution, but the differences between the groups was substantial enough that none was required.
Is there an estimate of variation within each group of data?	not determined

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Although this was not determined, there is no reason to believe that the variance would differ between the two groups compared

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	These were provided in the Methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines are authenitc, and are mycoplasma free

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	all this information was provided in the Methods
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The experimental procedures were approved by the Animal Care Committee of University of Toronto. This was provided in the Methods section
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We are in compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable (N/A). No human subjects in this manuscript.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A