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reports

MALT1 targeting suppresses CARD14-induced psoriatic dermatitis in mice

Van Nuffel, E., Staal, J., Baudelet, G., Haegman, M., Driege, Y., Hochepied, T., Afonina, I. S., & Beyaert, R.

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   21 October 2019

Thank you for the submission of your research manuscript to our journal. I apologize for the delayed handling of your manuscript, but we have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, referees 1 and 2 also point out several concerns and have a number of suggestions for how the study should be strengthened. Both referees indicate that it will be essential to document the phenotype and the disease scoring of CARD14E138A mice better and to investigate the cause of weight loss and death, since it is unlikely to be caused by health problems in the skin only but might be due to additional expression of the transgene in the gastrointestinal tract.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) 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 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 the revisions further.

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. Please reformat your references according to the numbered style of EMBO Reports.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) 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.

4) 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.

5) 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>)

6) You have already correctly formatted the Supplementary information as Expanded View (EV). Please note that we can only typeset 5 EV figures.

Should the revision result in more Supplementary figures, then these 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.

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
<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

8) Our journal 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>.

9) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical

methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.

- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

10) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (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."

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

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.

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REFEREE REPORTS

Referee #1:

Mutations in the gene encoding for CARD14 have been linked to the development of psoriasis. Previous work by the authors in vitro showed that gain-of-function CARD14 mutants recruit and activate the paracaspase MALT1 to drive NF-kB/JNK signaling and the expression of proinflammatory genes. In this manuscript, Van Nuffel et al investigate the impact of targeting MALT1 in vivo by using new mouse models of psoriatic dermatitis. Although mice engineered to overexpress a CARD14 gain-of-function mutant (CARD14E138A) in their keratinocytes did not display signs of skin abnormalities, they failed to survive after birth unless MALT1 is absent. By contrast, the inducible induction of CARD14E138A in keratinocytes was not lethal and led to a psoriatic phenotype. Signs of skin disease were significantly ameliorated by keratinocyte-specific deletion of MALT1 or by the inhibition of MALT1 protease activity.

Major points
1. In addition to the epidermis, K5 and K14 are also expressed in the stratified epithelium of salivary glands oesophagus and in the stomach. Because K5CreTg/+CARD14E138A/+ or ieCARD14E138A mice do not show any overt/macroscopical signs of psoriatic disease (besides the ears), it would be crucial to investigate whether these organs express CARD14E138A/+, and whether they are affected, as this could play a role on the quick onset of death as well as on some aspect of the disease. For instance, an issue with the salivary glands or the gastrointestinal tract might prevent these mice from eating normally.

2. Why was the ratio calculated after one week (Fig.1)? The authors should provide more information on when exactly the K5CreTg/+CARD14E138A/+ mice die. Also, are these animals born dead or do they die in the days following natural birth? A survival curve for MALT1+/+ WT, MALT1EKO WT, MALT1+/+ ieCARD14E138A and MALT1EKOieCARDE138A would be helpful. In the case pups are alive, is the weaning occurring normally, do they die of dehydration given that no overt phenotype of psoriasis is detected.

3. The anti-CARD14 used did not detect endogenous CARD14 in normal skin samples. How do the authors explain this apparent discrepancy? The specificity of the antibody should be tested on

human and mouse extracts, as well as the expression CARD14 and CARD14E138A in other tissues.

4. The administration of MLT-827 potently prevents psoriasis in mice with keratinocyte-specific CARD14E138A gain-of-function, suggesting that MALT1 scaffold function is dispensable. It would be important to assess NF-kB activation in these samples and to define the impact of MLT-827. What is the impact of NF-kB in the disease?

Additional points
1. In general, the figure legends need to be more elaborate; i.e. which IHC staining was used? In Fig. 2C, are mice treated with tamoxifen? Please, also indicate what arrows are pointing to, refer to the size of the scale bar as it is hard to read on the figure itself, etc.

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5. In the methods section, how the draining of the lymph nodes is done should be explained.

6. The Reference for Demeyer A et al 2019 is incomplete.

Referee #2:

The manuscript by Van Nuffel et al. addresses the role of MALT1 in psoriasis induction by keratinocyte-specific expression of a constitutively active mutant of CARD14 (E138A). A role for this mutant in the MALT1-dependent activation of keratinocytes and induction of psoriasis-like features has already been supported by previous studies identifying activating CARD14 mutations in psoriatic patients, by the generation of heterozygous mice expressing CARD14 mutants (which develop psoriasis-like lesions), and by studies showing that expression of CARD14 mutants induce inflammatory gene expression in keratinocytes in vitro. The authors now newly demonstrate a keratinocyte-intrinsic role for CARD14 in the development of psoriasis. Moreover, they show that genetic or pharmacological inhibition of MALT1 function alleviates CARD14-E138A-induced psoriasis-like symptoms. The data are mostly of high technical quality, but some figure panels need improvement and some findings seem contradictory or preliminary.

Major concerns with Figures:

1) Fig. 1E: the text states that "mice did not show signs of disease until at least 6 months of age", but this is not appropriately illustrated by the photo of a single mouse of each genotype at month 6. It would seem more appropriate to specify which signs of disease were scored and to illustrate them appropriately.

2) Fig. 2B: the observed weight loss of up to 18% over 4 days of gene induction can hardly be attributed to health problems in the skin only. The authors should investigate the underlying reasons by performing a more thorough analysis of the health status of the mice.

3) Fig. EV2B: these findings suggest that the proposed leaky expression of CARD-E138A induces skin thickening in an entirely MALT1-independent manner, which is counterintuitive and contradictory to the rest of the findings reported in the paper. This clearly needs to be discussed.

4) Fig. 3C/D/E: the inhibitor seems to have a dramatic effect on ear thickening but only a minor effect on the thickness of the epidermis, which may suggest that it acts more on the skin-infiltrating immune cells than on the keratinocytes themselves. Please comment.

5) Fig. 3F: the observed increase in the in vitro responses of T-cells isolated from the ear-draining lymph node of ieCARD14-E138A animals is intriguing, but it is not clear whether this results from the tissue context or whether this represents a general change in T-cell responses in these mice. The

authors should perform an additional control and analyze T-cell responses from a lymph node that is not draining the skin.

Minor points:

1) Fig. EV2C: the morphological differences are difficult to see in the photos, it would be better to enlarge the photos and show the ears only

2) Fig. 3G: inducible expression of CARD14 E138A and treatment of the inhibitor has a pronounced effect on pro-inflammatory cytokines. Does this simply reflect the numbers of the respective cytokine-producing cells in the tissue?

3) It is not always clear how many mice were used and how representative the data are (concerns Fig. 2B, 2C, 3C)

1st Revision - authors' response   9 February 2020
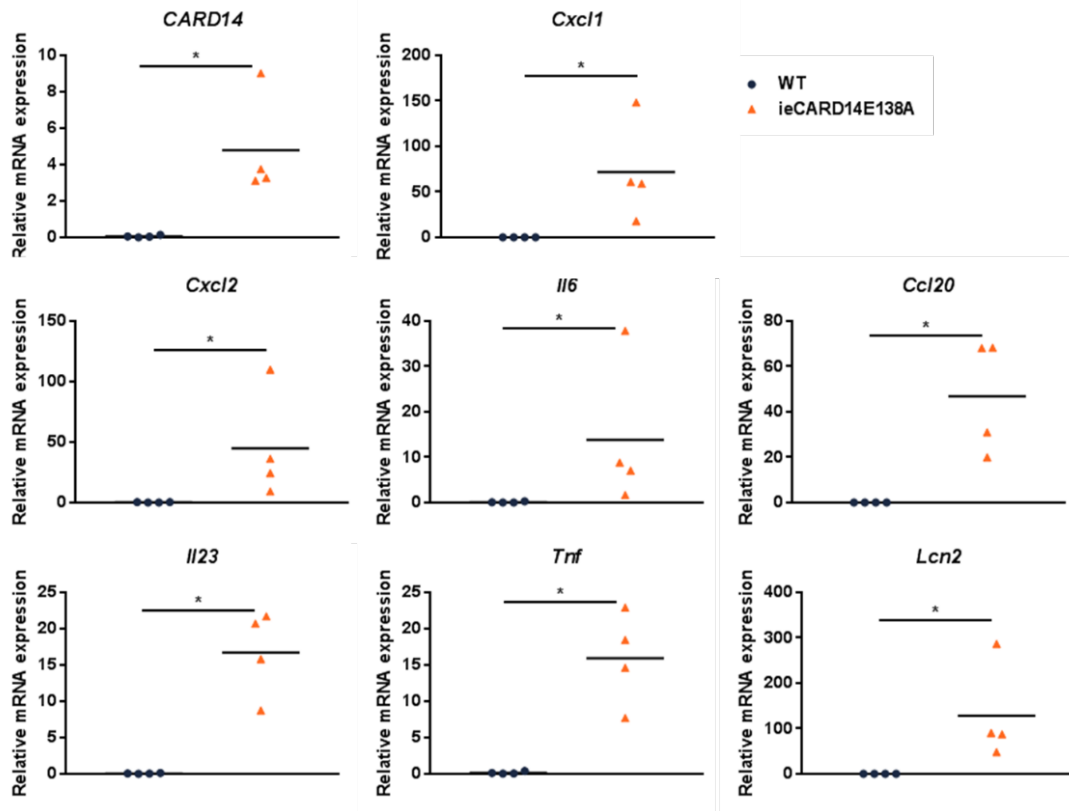
Referee #1:

Mutations in the gene encoding for CARD14 have been linked to the development of psoriasis. Previous work by the authors in vitro showed that gain-of-function CARD14 mutants recruit and activate the paracaspase MALT1 to drive NF-kB/JNK signaling and the expression of proinflammatory genes. In this manuscript, Van Nuffel et al investigate the impact of targeting MALT1 in vivo by using new mouse models of psoriatic dermatitis. Although mice engineered to overexpress a CARD14 gain-of-function mutant (CARD14E138A) in their keratinocytes did not display signs of skin abnormalities, they failed to survive after birth unless MALT1 is absent. By contrast, the inducible induction of CARD14E138A in keratinocytes was not lethal and led to a psoriatic phenotype. Signs of skin disease were significantly ameliorated by keratinocyte-specific deletion of MALT1 or by the inhibition of MALT1 protease activity.
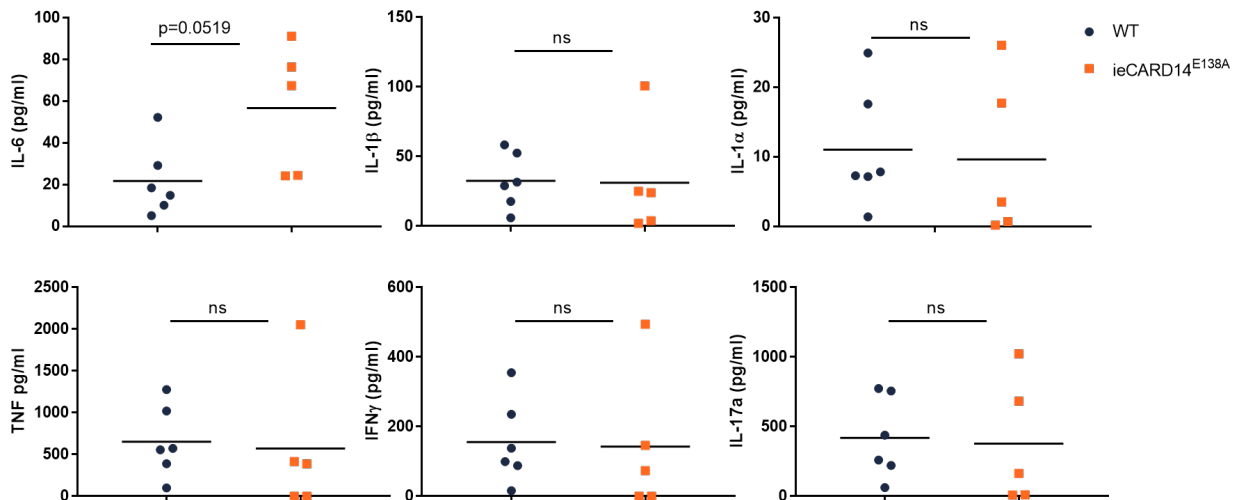
Major points

1. In addition to the epidermis, K5 and K14 are also expressed in the stratified epithelium of salivary glands oesophagus and in the stomach. Because K5CreTg/+CARD14E138A/+ or ieCARD14E138A mice do not show any overt/macroscopical signs of psoriatic disease (besides the ears), it would be crucial to investigate whether these organs express CARD14E138A/+, and whether they are affected, as this could play a role on the quick onset of death as well as on some aspect of the disease. For instance, an issue with the salivary glands or the gastrointestinal tract might prevent these mice from eating normally.

Response: Thank you for this valid suggestion. To investigate the expression of the CARD14^{E138A} transgene we have made use of the fact that CARD14^{E138A} is expressed as a bicistronic transgene with GFP (see Figure 1A), and performed immunohistochemistry for GFP on various tissues that might show K14 driven Cre activity upon tamoxifen treatment (now included in the manuscript as figure EV3E). This indicated that next to the ears, the CARD14^{E138A}/GFP transgene is also expressed in stratified epithelium of the tongue and the epithelium of salivary glands of mice treated for 4 days with tamoxifen. The GFP signal in the esophagus was very weak, indicating minimal expression of the transgene in this organ. To further investigate the impact of CARD14^{E138A} expression in these tissues, we also checked for inflammatory cell infiltration by performing immunohistochemistry for CD45, which was clearly detectable at the base of the stratified epithelium of the tongue and more weakly in the salivary gland. Moreover, increased expression of several inflammatory cytokines and chemokines was detected in the tongue of ieCARD14^{E138A} mice (see graph below for the information of the reviewer). Finally, we noticed that the stomach of ieCARD14^{E138A} mice was empty (quantified by stomach weight in figure EV3A of the revised manuscript) and that blood glucose levels were lower compared to WT controls (figure EV3B of the revised manuscript) 4 days after tamoxifen treatment. Together these results let us speculate that inflammation in the oral cavity prevents ieCARD14^{E138A} mice from eating normally, leading to the observed loss in body weight. These results are now also mentioned in the revised manuscript.
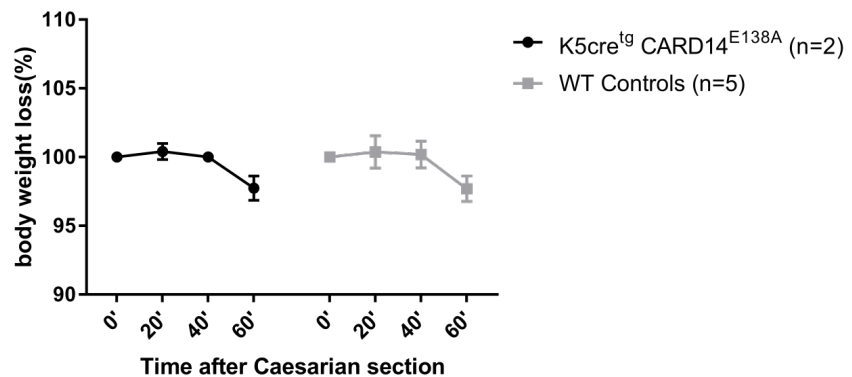
We could not detect a significant difference in transepidermal water loss through the skin (figure EV3D in revised paper) or a significant difference in body temperature (figure EV3C in revised paper). In addition, analysis of the serum of ieCARD14^{E138A} mice did not show a consistent increase of pro-inflammatory cytokines such as IL-1α, IL-1β, TNF, IFNγ, IL-17 (see figure below for the information of the referee). IL-6 levels were slightly increased but not consistently in all ieCARD14^{E138A} mice. Together these results indicate that the mice do not suffer from weight loss due to fever or cachexia caused by excessive production of cytokines.

2. Why was the ratio calculated after one week (Fig.1)? The authors should provide more information on when exactly the K5CreTg/+CARD14E138A/+ mice die. Also, are these animals born dead or do they die in the days following natural birth? A survival curve for MALT1+/+ WT, MALT1EKO WT, MALT1+/+ ieCARD14E138A and MALT1EKOieCARDE138A would be helpful. In the case pups are alive, is the weaning occurring normally, do they die of dehydration given that no overt phenotype of psoriasis is detected.

Response: We receive material for genotyping from our mouse breeding facility when pups are approximately 1 week old and therefore we used these results to calculate the ratio after 1 week. We have set up breedings to follow the pups closely after birth, showing that pups expressing CARD14^{E138A} are born normally but die within 24 hours after birth (as mentioned in the manuscript). Because of this small time window, it is difficult to determine a survival curve with an exact timing.
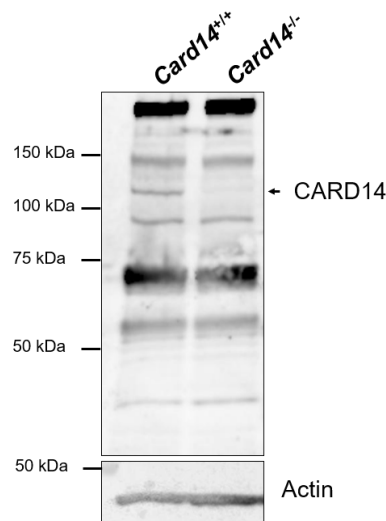
To assess if these mice die because of serious dehydration, we have closely followed their body weight loss after caesarian section (see graph below). These results indicate that pups expressing CARD14^{E138A} do not show severe dehydration, which is in line with the fact that also ieCARD14^{E138A} mice do not show increased transepidermal water loss after four days tamoxifen treatment (now added as Figure EV3D). Based on the observed CARD14^{E138A} expression and inflammation in the tongue and salivary glands, leading to weight loss in tamoxifen treated ieCARD14^{E138A} mice, we speculate that mortality in pups is due to similar events.
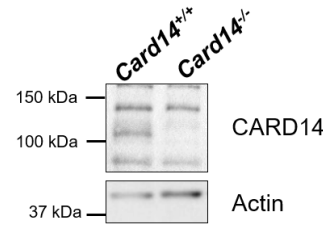

3. The anti-CARD14 used did not detect endogenous CARD14 in normal skin samples. How do the authors explain this apparent discrepancy? The specificity of the antibody should be tested on human and mouse extracts, as well as the expression CARD14 and CARD14E138A in other tissues.

Response: Because the quality of available CARD14 antibodies is rather weak, both in terms of sensitivity and specificity, it is very hard to indicate the correct band corresponding to endogenous CARD14 in total cell lysates (figure 3B). We have therefore validated the CARD14 antibody in total ear tissue and primary keratinocytes derived from CARD14 wild type and knockout mice that we have in house (Card14$^{tm1b(EUCOMM)Hmgu}$). With this antibody we can detect an immunoreactive band with a size between 100 and 150 kDa, corresponding to the predicted size of full length CARD14 (113 kDa), which is absent in lysates derived from Card14 knockout mice (see graph below for the information of the reviewer). In addition, we have validated this antibody on the human CARD14 expressing cell line LNCaP by RNA interference (72 hours after transfection with CARD14 siRNA or scrambled siRNA). Based on this information, it might be that in figure 3B the band between the 100 and 150 kDa molecular weight markers corresponds to endogenous mouse CARD14. In the transgenic ieCARD14^{E138A} mice, the clear detection of human CARD14^{E138A} might reflect its higher expression from the Rosa26 promoter and/or a possible higher sensitivity of the antibody for human CARD14 versus mouse CARD14.
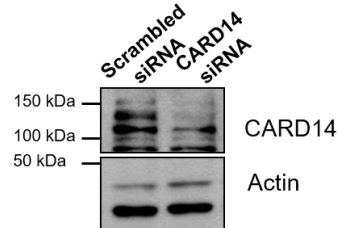
Lysate of total ears of *Card14^+/+* and *Card14^-/-* mice

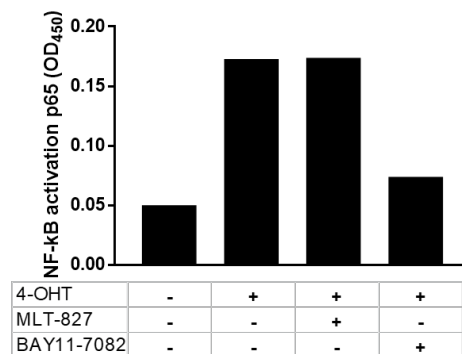**Lysates of mouse primary keratinocytes**

Lysates of LNCaP cells 72 hours after transfection with scrambled or CARD14 siRNA

4. The administration of MLT-827 potently prevents psoriasis in mice with keratinocyte-specific CARD14E138A gain-of-function, suggesting that MALT1 scaffold function is dispensable. It would be important to assess NF-kB activation in these samples and to define the impact of MLT-827. What is the impact of NF-kB in the disease?

Response: It has previously already been shown that MALT1 protease inhibition by MLT-827 does not block MALT1 scaffolding function required to engage T cell receptor-induced IKK signaling in T cells (Bardet et al., Immunol Cell Biol. 96, 81-99, 2018). To address the reviewer's comment, we have isolated primary keratinocytes from ieCARD14^{E138A} mice and treated them with 1 µM 4-hydroxytamoxifen (4-OHT) for 24 hours to induce CARD14^{E138A} expression in the absence or presence of the MALT1 inhibitor MLT-827 or the IKK inhibitor BAY11-7082. NF-κB activation was measured by analyzing nuclear cell extracts for the presence of p65 with the TransAM NF-κB p65 transcription factor assay (Active motif). Stimulation of cells with 4-OHT (leading to CARD14^{E138A} expression) increased nuclear p65 compared to unstimulated cells, which was reduced upon treatment with the IKK inhibitor BAY11-7082 (graph below for the information of the reviewer). In contrast, treatment with the MALT1 inhibitor MLT-827 did not have an impact on the nuclear localization of p65.
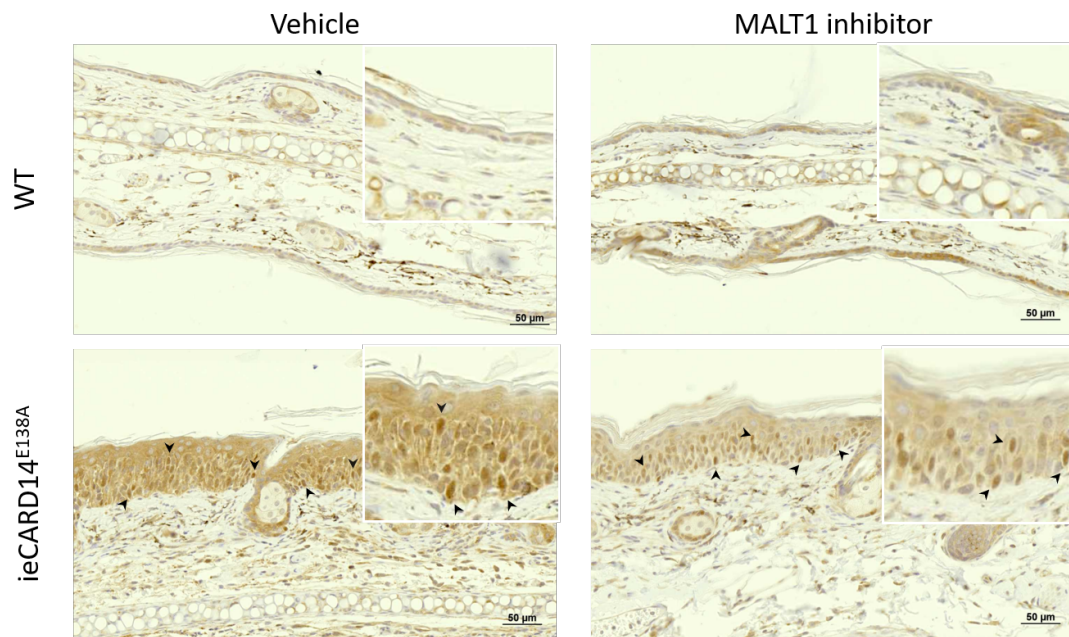
Nuclear lysates of ieCARD14^{E138A} primary keratinocytes

4-OHT	-	+	+	+
MLT-827	-	-	+	-
BAY11-7082	-	-	-	+

To further investigate the effect of MLT-827 on CARD14^{E138A}-induced NF-κB activation in vivo, we analyzed the localization of p65 in the ears of ieCARD14^{E138A} mice treated with tamoxifen in the absence or presence of MLT-827. Immunohistochemistry showed nuclear p65 staining in epidermal cells (arrows) of CARD14^{E138A} expressing mice, which was not affected by MLT-827 (see figure below for the information of the reviewer).

Wang and collegues already showed before that the IKK inhibitor BAY11-7082 can reduce ear thickening and epidermal thickening in CARD14$^{E138A/+}$ mice (Wang et al., Immunity 49:66-79, 2018). Together, these published data and our own in vitro and in vivo data show that NF-κB has an impact on disease development, but that the protective effect of MLT-827 on disease development is specifically mediated by its inhibition of MALT1 proteolytic activity, without affecting MALT1 scaffold function and IKK signaling. Most likely, MLT-827 prevents psoriasis-associated cytokine production and inflammation by affecting the cleavage of MALT1 substrates (e.g. regnase-1 and roquin-1/-2) that are known to regulate the expression of specific proinflammatory cytokines at the posttranscriptional level. It should also be noted that inhibition of disease by MLT-827 might not only reflect inhibition of MALT1 proteolytic activity in keratinocytes, but also effects on other cell types (e.g. endothelial cells and T cells) in which MALT1 might be indirectly activated and contribute to psoriasis pathology.

As the specific effects of MLT-827 on MALT1 proteolytic activity, as well as the effect of IKK inhibition on CARD14-induced psoriasis, have been described before, we did not include the above mentioned data in the revised paper.

Additional points
1. In general, the figure legends need to be more elaborate; i.e. which IHC staining was used? In Fig. 2C, are mice treated with tamoxifen? Please, also indicate what arrows are pointing to, refer to the size of the scale bar as it is hard to read on the figure itself, etc.

Response: We have carefully checked the manuscript and have described the experiments in the figure legends in more detail.

2. Page 7, second paragraph, Fig. 2E should be Fig. 2F

Response: We have adapted the text accordingly.

3. On Fig. 3C, it seems that a mouse group is missing.

Response: The two curves of the control groups were overlapping. We have now adapted the figure so that both groups are more visible.

4. In the introduction, it is not clear as why the authors quote some and not other substrates for MALT1.

Response: We have adapted the introduction and now refer to a recent review describing MALT1 substrates.

5. In the methods section, how the draining of the lymph nodes is done should be explained.

Response: We did not drain lymph nodes but isolated lymph nodes that drain the ears and isolated the lymphocytes from these lymph nodes. We have adapted the materials and methods section as follows to explain more clearly how these lymphocytes were obtained:

'Single cell suspensions from ear-draining lymph nodes were obtained by homogenizing the organ through a 70 µm cell sieve. The isolated lymphocytes were counted and 200 000 lymphocytes were seeded in the CD3/CD28-coated plates for in vitro restimulation.'

6. The Reference for Demeyer A et al 2019 is incomplete.
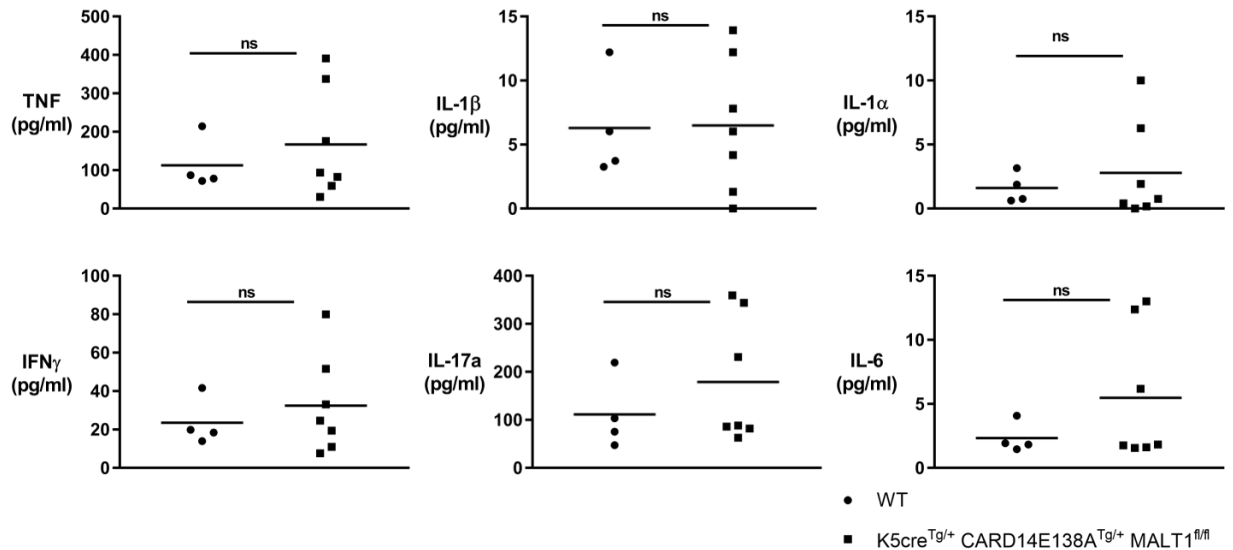
We have now updated this reference.

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Major concerns with Figures:

1) Fig. 1E: the text states that "mice did not show signs of disease until at least 6 months of age", but this is not appropriately illustrated by the photo of a single mouse of each genotype at month 6. It would seem more appropriate to specify which signs of disease were scored and to illustrate them appropriately.

Response: To check if epidermal CARD14^{E138A} expression in MALT1 deficient mice had an effect on the skin, we scored ear thickness of mice between 4 and 7 months old, which was found to be comparable to littermate controls. Histological analysis of the epidermis of the skin and the ears did not show signs of epidermal inflammation such as epidermal thickening or inflammatory cell infiltration (Figure 1G and H of the revised manuscript). Strong GFP expression in the epidermis indicated expression of the CARD14^{E138A}/GFP transgene (Figure EV1C of the revised paper). Also the body weight was not affected (Figure 1G). We also analyzed the serum for the presence of several inflammatory cytokines such as IL-6, TNF, IFNγ, IL-17, IL-1β and IL-1α, but their levels were mostly close to the detection limit of the ELISA and did not change significantly between WT and CARD14^{E138A} expressing MALT1 deficient mice (figure shown below for the information of the reviewer).

Cytokine levels in serum of WT and K5cre$^{Tg/+}$ CARD14E138A$^{tg/+}$ MALT1$^{fl/fl}$ mice aged between 4 and 7 months (ns non-significant, determined by Mann-Whitney U test).
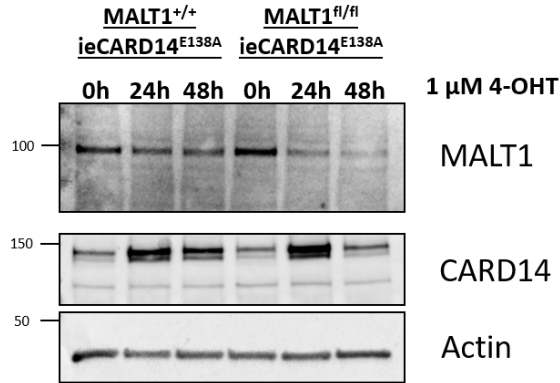

2) Fig. 2B: the observed weight loss of up to 18% over 4 days of gene induction can hardly be attributed to health problems in the skin only. The authors should investigate the underlying reasons by performing a more thorough analysis of the health status of the mice.

Response: We have performed several experiments to address this question and we refer to our response to reviewer 1 (question 1) for more details.

3) Fig. EV2B: these findings suggest that the proposed leaky expression of CARD-E138A induces skin thickening in an entirely MALT1-independent manner, which is counterintuitive and contradictory to the rest of the findings reported in the paper. This clearly needs to be discussed.

Response: We indeed think that marginal spontaneous K14creER activity might induce leaky CARD14^{E138A} expression in the absence of tamoxifen treatment, leading to basal thickening of the ears in both MALT1$^{+/+}$ and MALT1$^{fl/fl}$ ieCARD14^{E138A} mice compared to wild type mice. This might be explained by the fact that the CARD14^{E138A} transgene is already induced by a single recombination event, while two recombination events need to be induced to obtain MALT1 deficiency in keratinocytes. Therefore, it might be that the minor leaky K14creER activity is not sufficient to induce complete MALT1 deletion. In addition, it might also be that due to MALT1 protein stability, MALT1 protein expression levels do not diminish as fast as CARD14^{E138A} protein expression levels rise. This is now briefly discussed in the revised paper.

To test this hypothesis, we have cultured primary keratinocytes derived from MALT1$^{+/+}$ and MALT1$^{fl/fl}$ ieCARD14^{E138A} mice and stimulated them with 1 μM 4-hydroxytamoxifen (4-OHT) to induce K14creER-mediated recombination. Western blotting for CARD14^{E138A} and MALT1 showed that we can already detect leaky CARD14 expression without 4-OHT stimulation (0h timepoint), while MALT1 is still strongly expressed in both MALT1$^{+/+}$ and MALT1$^{fl/fl}$ keratinocytes (see figure below for the information of the reviewer). CARD14^{E138A} expression is significantly increased after 24h 4-OHT treatment, while MALT1 protein expression in MALT1$^{fl/fl}$ mice is only partially reduced at that time point and is even still slightly expressed at 48 hours. Together, these results explain why both MALT1$^{+/+}$ and MALT1$^{fl/fl}$ ieCARD14^{E138A} mice show similar basal ear thickening.

MALT1⁺/⁺ ieCARD14^E138A   MALT1^fl/fl ieCARD14^E138A

| 0h | 24h | 48h | 0h | 24h | 48h | 1 μM 4-OHT |

100 — MALT1
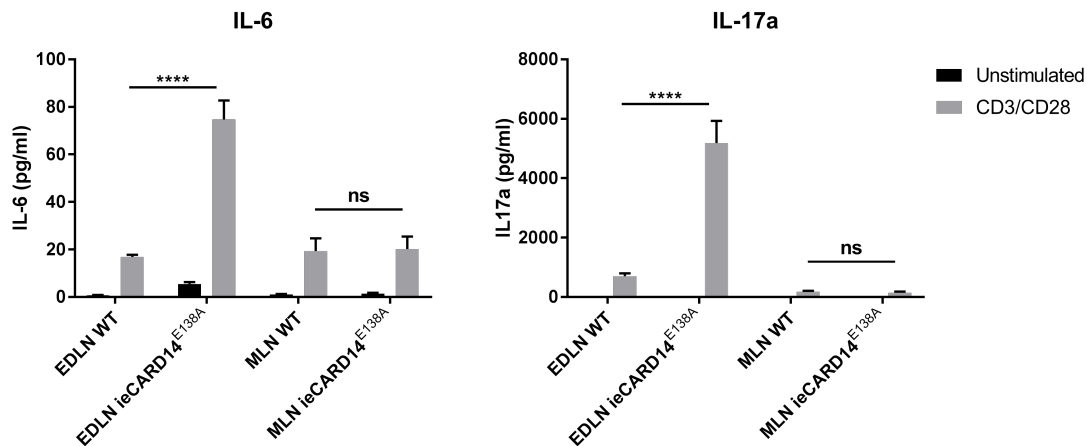
150 — CARD14

50 — Actin

4) Fig. 3C/D/E: the inhibitor seems to have a dramatic effect on ear thickening but only a minor effect on the thickness of the epidermis, which may suggest that it acts more on the skin-infiltrating immune cells than on the keratinocytes themselves. Please comment.

Response: Indeed, the effect of the inhibitor on the epidermal thickness is rather limited compared to its effect on the total ear thickness. The total ear thickness does not only result from epidermal thickening but also from inflammation and edema of the tissue. Reduced ear thickening upon oral treatment with the MALT1 inhibitor MLT-827 might thus also reflect these events. Moreover, MLT-827 might also affect the function of MALT1 in other cell types present in the skin, such as T cells and endothelial cells, which may contribute to inflammation and ear thickening. This is now briefly mentioned in the revised manuscript.

5) Fig. 3F: the observed increase in the in vitro responses of T-cells isolated from the ear-draining lymph node of ieCARD14-E138A animals is intriguing, but it is not clear whether this results from the tissue context or whether this represents a general change in T-cell responses in these mice. The authors should perform an additional control and analyze T-cell responses from a lymph node that is not draining the skin.

Response: As suggested, we have repeated the experiment and have taken along lymphocytes of mesenteric lymph nodes, next to lymphocytes of the ear-draining lymph nodes. In contrast to the observed increase in the in vitro response of T cells (increased IL-6 or IL-17 production after 72 hours stimulation with anti-CD3/CD28) from ear-draining lymph nodes isolated from ieCARD14^E138A mice, we could not detect a similar effect in the case of lymphocytes from mesenteric lymph nodes. This indicates that the general T-cell responses in ieCARD14^E138A mice are not affected. (see figure below for the information of the reviewer).
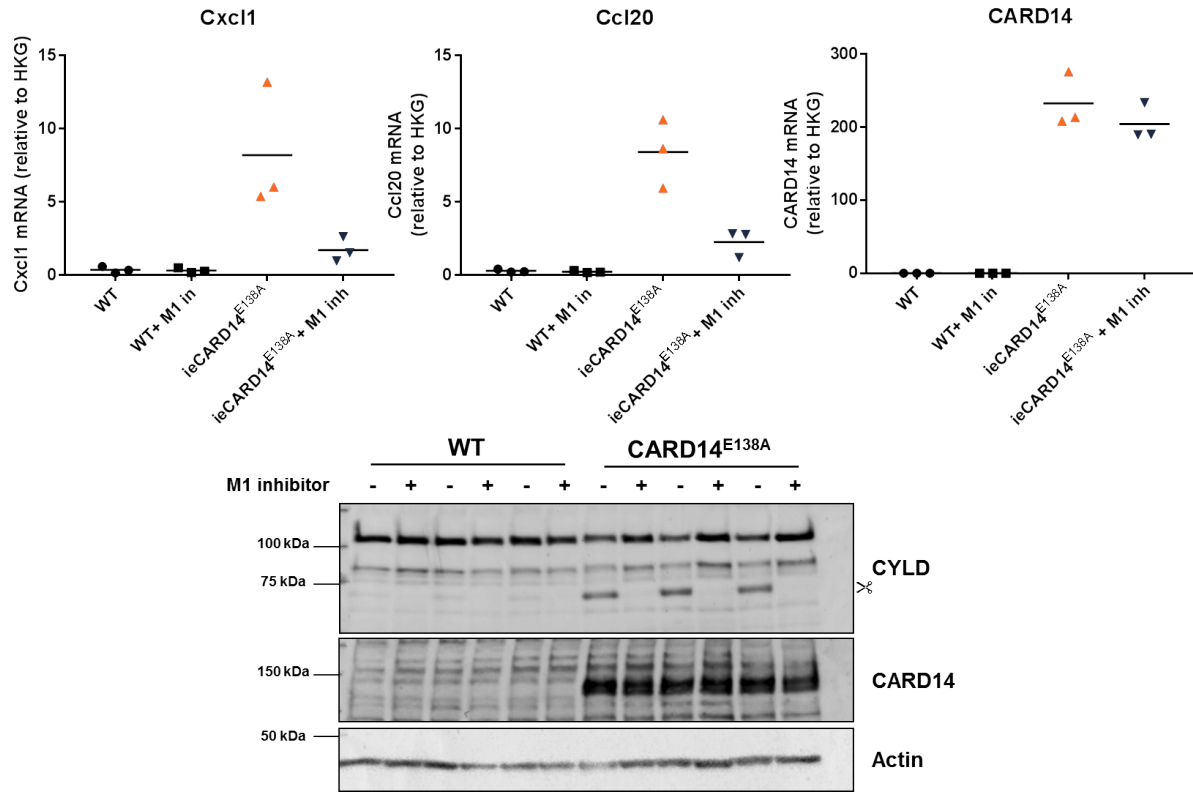
IL-6

IL-17a

Unstimulated
CD3/CD28

Minor points:

1) Fig. EV2C: the morphological differences are difficult to see in the photos, it would be better to enlarge the photos and show the ears only

Response: We have now adapted and enlarged the figures so that the changes are more visible.

2) Fig. 3G: inducible expression of CARD14 E138A and treatment of the inhibitor has a pronounced effect on pro-inflammatory cytokines. Does this simply reflect the numbers of the respective cytokine-producing cells in the tissue?

Response: As we investigated gene expression on lysates of total ears, the observed differences in gene expression will reflect gene expression coming from all cells in the tissue, including infiltrating immune cells next to keratinocytes. To investigate the effect of MALT1 protease inhibition on gene expression in keratinocytes, we treated primary keratinocytes isolated from ieCARD14^{E138A} mice with 1 μM 4-OHT in the absence or presence of a MALT1 protease inhibitor (1 μM) and analyzed MALT1-mediated cleavage of CYLD as well as gene expression of Cxcl1 and Ccl20 by Western blot and qPCR, respectively. This also showed that inhibition of MALT1 proteolytic activity in keratinocytes (reflected by reduced CYLD cleavage) is associated with a reduction in expression of Cxcl1 and Ccl20 (see figure below for the information of the reviewer), suggesting that the in vivo effects at least partially reflect gene expression in keratinocytes.

3) It is not always clear how many mice were used and how representative the data are (concerns Fig. 2B, 2C, 3C)

Response: We apologize for this. We have carefully checked the manuscript and added the necessary information.

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

As you will see, all referees are very positive about the study and request only minor changes to clarify text and figures.

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

****
Abstract

CARD14 gain-of-function mutations cause psoriasis in humans and mice. Together with BCL10 and the protease MALT1, mutant CARD14 forms a signaling node that mediates increased NF-κB signaling and proinflammatory gene expression in keratinocytes. However, it remains unclear if psoriasis in response to CARD14 hyperactivation is keratinocyte-intrinsic or requires CARD14 signaling in other cells. Moreover, the in vivo effect of MALT1 targeting on mutant CARD14-induced psoriasis has not yet been documented. Here we show that inducible keratinocyte-specific expression of CARD14E138A in mice rapidly induces epidermal thickening and inflammation as well as increased expression of several genes associated with psoriasis in humans. Keratinocyte-specific MALT1 deletion as well as oral treatment of mice with a specific MALT1 protease inhibitor strongly reduce psoriatic skin disease in CARD14E138A mice. Together, these data illustrate a keratinocyte-intrinsic causal role of enhanced CARD14/MALT1 signaling in the pathogenesis of psoriasis and show the potential of MALT1 inhibition for the treatment of psoriasis.

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REFEREE REPORTS

Referee #1:

Overall, the authors satisfactorily addressed my concerns with this revised manuscript. Nevertheless, I recommend the following amendments to the text:

- The authors should make clear that CARD14E138A expression is driven by K5/K14 promoters and that this is not restricted to the epidermis, as indicated page 6 (lines 1 and 3). This is particularly important given that mice more likely die because an expression of CARD14 E138A and inflammation in tongue and salivary glands.
- On Fig. EV2, females appear heavier than males. Can the authors double check that symbols were not inverted?
- Molecular weight markers are missing on Fig. 3B.

Referee #2:

The authors have adequately addressed all issues that I had raised.

Reviewer 1:

-The authors should make clear that CARD14E138A expression is driven by K5/K14 promoters and that this is not restricted to the epidermis, as indicated page 6 (lines 1 and 3). This is particularly

important given that mice more likely die because an expression of CARD14 E138A and inflammation in tongue and salivary glands.
Re: This was already clarified and discussed in detail in the last paragraph of the corresponding section, but we have now also made some changes in the text of the previous paragraphs where we introduce the specific K5/K14 cre mouse lines.

-On Fig. EV2, females appear heavier than males. Can the authors double check that symbols were not inverted?
Re: we apologize for this mistake. Symbols have been corrected.

-Molecular weight markers are missing on Fig. 3B.
Re: now added

Accepted   31 March 2020

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.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Prof. Dr. Rudi Beyaert
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2019-49237-T

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 justified
→ 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 χ2 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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to predetermine sample size. Based on our previous experience, we aimed to analyse 4-10 mice per group in each experiment, but the exact numbers of animals used in experiments were also determined by breeding outcomes.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical method was used to predetermine sample size. Based on our previous experience, we aimed to analyse 4-10 mice per group in each experiment, but the exact numbers of animals used in experiments were also determined by breeding outcomes.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the experiments.
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.	No randomization has been used to allocate animals to treatment. We did make sure that the average ear thickness and body weight at the start of the experiment was similar between the groups that received MALT1 inhibitor or vehicle.
For animal studies, include a statement about randomization even if no randomization was used.	No specific method of randomization had been used to allocate animals to a treatment.
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.	Measurements of epidermal thickness was performed blinded and measuring ear thickness and body weight in mice was done blinded for the genotype and/or treatment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Measurements in mice such as body weight and ear thickness were performed blinded for the genotype and/or treatment
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For tests that require normal distribution, normal distribution was confirmed. Non-parametric tests were used when data were not normally distributed or if the sample size was to small to determine normal distribution.
Is there an estimate of variation within each group of data?	The results are expressed as mean ± SEM or individual datapoints are displayed.

Is the variance similar between the groups that are being statistically compared?	Yes

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).	The following antibodies have been used: anti-CARD14 (10400-1-AP, Proteintech), anti-CYLD (sc-74435, Santa Cruz), anti-BCL10 cleavage-specific (gift from Thijs Baens, Cistim Leuven vzw, Leuven , Belgium), anti-BCL10 (sc-5273, Santa Cruz), anti-MALT1 (32494, Cell Signaling Technology) and anti-β-actin-HRP (sc-47778, Santa Cruz). Secondary HRP-conjugated anti-mouse or anti-rabbit IgG antibody were purchased from Thermo Fisher scientific (31432 and 31464). The following antibodies were used for flow cytometry: CD16/CD32 (553142 , BD), MHCII-eFluor 450 (# 48-5321-80, eBioscience), CD64-BV711 (Biolegend, 139311), Siglec F-PE (BD, 552126), CD45-APC-eFluor780 (47-0451-82, eBioscience), CD8-PerCP-Cy5.5 (45-0081, eBioscience), CD3-PE-Cy5 (55-0031, Tonbo Biosciences), CD19-PE-Cy5 (15-0193, eBioscience), CD11c-PE-Cy7 (117317, Biolegend), CD11b-BV605 (563015, BD), γδ-TCR-APC (17-5711, eBioscience), Ly6G-AF700 (561236, BD), CD4-APC-eFluor780 (47-0042, eBioscience). All commercial antibody validations are available on manufacturers' websites
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not applicable

* 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.	Species, strain, gender, age, genetic modification status and housing and husbandery conditions have been described in the materials and methods.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal protocols were approved by the ethics committee of Ghent University
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 have consulted the ARRIVE guidelines to make sure that we reported all relevant aspects of the animal studies.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
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.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
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.	Not applicable
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.	Not applicable

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	Plasmids were deposited in the BCCM/GeneCorner plasmid collection along with detailed descriptions of cloning strategy and plasmid sequence (http://bccm.belspo.be/about-us/bccm-genecorner). All other data are available from the authors upon reasonable requests.
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).	Source data for the western blots is submitted.
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).	Not applicable
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.	Not applicable

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.	Not applicable