European Journal of Immunology

Malt1 self-cleavage is critical for regulatory T cell homeostasis and anti-tumor immunity in mice

Mathijs Baens,1,2,3* Rocco Stirparo,1,4 Youlia Lampi,5,6 Delphine Verbeke,1,4 Roel Vandepoel,1,4 Jan Cools,1,4 Peter Marynen,1 Charles E. de Bock1,4 and Simon Bornschein.1,4*

Correspondence: Dr. Simon Bornschein, KU Leuven Department of Human Genetics, Leuven, Belgium

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Handling Executive Committee member: Prof. Shimon Sakaguchi

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision - 17-Apr-2018

Dear Dr. Bornschein,

Please accept our sincere apologies for the delay in the decision.

Manuscript ID eji.201847597 entitled "Malt1 self-cleavage induced NF-ÎºB activation is critical for regulatory T cell homeostasis and anti-tumor immunity" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

Both referees have a good opinion on the manuscript and have some requests to further improve the study.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees™ concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. For all data, including the new data generated for the revision of the manuscript, please report the number of independent experiments and number of samples per experiment (or experimental replicates). For flow cytometry data please show the full gating strategy, including the percentage of cells in the region or gate or event count. In the histograms/dot plots shown please report which fluorochromes were used and the scaling in the axis (log/lin). Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Marta Vuerich

On behalf of
Prof. Shimon Sakaguchi

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Reviewer: 1

Comments to the Author

In this study, the authors demonstrated that Malt1 self-cleavage plays a role for optimal T cell activation and regulatory T cell development. First, the authors showed that gene expressions including Tnf and proliferative responses are compromised in Malt1-cleavage-resistant CD4+ T cells, as in Malt1 protease-dead counterparts. Next, the authors generated and analyzed mice harboring a point mutation of Malt1 that does not undergo self-cleavage. They showed that these mice have defect of regulatory T cell development to a lesser extent as in mice with protease-dead Malt1. However, the protease activity, but not self-cleavage, of Malt1 resulted in the cause of systemic inflammation in mice. They observed that this was due to the decreased IL-2 availability, not due to Treg-intrinsic hypo-responsiveness to IL-2. Finally, the authors examined anti-tumor responses and found that Malt1 self-cleavage as well as its protease activity negatively regulate tumor development via the control of Treg number.

This is the first study that analyzing the functional roles of Malt1 self-cleavage activity in vivo, although there are several studies showing that Malt1 protease activity plays a role in maintaining immune homeostasis through control of Treg development. Overall, the phenotype of Malt1 self-cleavage-resistant mice in controlling tumor immunity is intriguing, although following concerns should be addressed.

Major comments

1. In Figure 1, the authors introduced Malt1 self-cleavage-resistant mutant using a set of in-vitro experiments, but these data were already published by the authors (Matijs Baens et. al., 2014, PLOS ONE). Thus, these data could be retracted from the main figure. Related to this, Supplemental Figure 1D was also reported. The authors should clarify what is found in this study and reorganize their findings more properly.

2. The authors claim that the levels of Malt1 was not altered in the Malt1 SR/SR mice. However, it looks the levels of Malt1 is lower in Malt1 SR/SR splenocytes compared with wild-type, especially after PMA/Ionomycin stimulation (Figure 1D). Thus, the authors need to quantify the expression of Malt1 by the mutations. Furthermore, the authors can show the levels of Malt1 p76 and p16 in Malt1 SR/SR mice.

3. The authors claim that Malt1 self-cleavage is required for optimal NF-kB activation. However, in Figure 1B and 1D, they showed that NF-kB activation was not altered in Malt1 SR/SR cells, whereas expressions of Tnf and Pdgfb were reduced in Malt1 SR/SR as well as Malt1 PD/PD cells. The authors imply that cleaved Malt1 p76 harbors an activity to induce NF-kB-targeted genes independent of NF-kB. Does Malt1 p76 act as a transcriptional activator in the nucleus? The authors can also consider analyzing the stability

of Il2 mRNA in Malt1 SR/SR and PD/PD cells. Given that this manuscript does not show a direct evidence that Malt1 self-cleavage induces NF-kB activation, the current title is not appropriate.

3. In Figure 3, the authors showed that Malt1SR/SR mice also show impaired development of thymic Treg cells. They also showed that Malt1SR/SR mice do not develop colitis, but did not discuss colonic Treg population. One might argue that if this developmental defect is due to decreased IL-2 availability, development of iTreg cells in the colon might be impaired as well. In vitro Treg differentiation assay will help to clarify whether defect of Treg cell population is T cell-intrinsic or not.

4. Interestingly the authors showed that Malt1SR/SR as well as Malt1PD/PD mice could suppress tumor progression compared to WT mice. However, given that Malt1SR/SR mice show much milder impairment of Treg recruitment into the tumor tissue than Malt1PD/PD mice, and that these mice do not develop autoimmunity, it is too early to make a conclusion that mild decrease in Treg cells within the tumor tissue is important for control of tumor progression. To strengthen this point, the author can examine additional tumor implantation models like B16 melanoma.

Minor comments

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3. typos. page 12 Matl1, page 14 then Malt1PD/PD mice, page 20 Spleenocyte.

Reviewer: 2

Comments to the Author

In their submitted manuscript, Baens and colleagues describe the phenotype of a mouse which carries a MALT1 mutant that cannot be auto-processed. The MALT1 protease has gain interest as drug target against certain lymphomas and to treat autoimmune diseases in the last decade. Similar to initiator caspases, the paracaspase MALT1 have been reported to be auto-processed (Baens et al., 2014), but the in vivo function was unclear.

The experimental design and the presented results are mostly of good quality, the data are not overinterpreted. I therefore recommend acceptance of the manuscript with minor revisions.

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+ The mp-Malt1 (membrane-targeted) needs more explanation. How is it targeted to the membrane and why does this lead to MALT1 protease activity?
+ Figure labelling needs to be improved. See e.g. Figure 1 Mcpip vs MCPIP or Il-6 vs Il2. Please unify.
+ Fig.1d: MCPIP cleavage is not convincing; could the authors provide a better blot?
+ Fig. 2h: Please clarify what neg.CTL mean.
+ Suppl. Figure 1D: IL2 reporter activation by p76 not very convincing. Maybe it would be better to focus only on the NF-kB-Luc data.
+ There are two very similar sentences on page 13 (see below). Please remove one of them.

In line with these observations, patients with Malt1 loss-of-function mutations, also show signs of autoimmune disease, IL-2 deficiency and reduced Treg frequency (Punwani et al., 2015; McKinnon et al., 2014).

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First Revision – authors' response - 17-Apr-2018

Reviewer: 1

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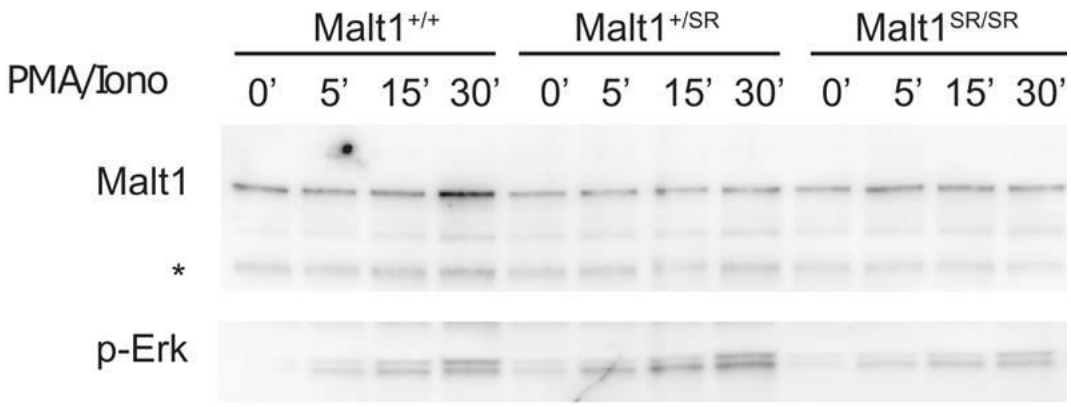
> **We appreciate the comment of the reviewer. Indeed, in our publication in PLOS ONE, we show a similar experiment. However, for the current report we used mouse Malt1 to demonstrate that the mutation in mouse has a similar effect to that we described earlier in human. Here, we show that mouse Malt1 and the PD and SR mutants behave identically. We have clarified this in the figure legend.**
>
> **Supplementary Figure 1D has not been reported previously. In the PLOS ONE paper we checked IL2 production in Jurkats and stable expressing mutants via ELISA upon PMA/ionomycin stimulation. Here we report transient expression in Jurkat (via electroporation) of p76 and the activation of both an NF-kB and IL2 Luciferase reporter, in contrast to full length Malt1 (or P/I stimulation).**

2. The authors claim that the levels of Malt1 was not altered in the Malt1 SR/SR mice. However, it looks the levels of Malt1 is lower in Malt1 SR/SR splenocytes compared with wild-type, especially after PMA/Ionomycin stimulation (Figure 1D). Thus, the authors need to quantify the expression of Malt1 by the mutations. Furthermore, the authors can show the levels of Malt1 p76 and p16 in Malt1 SR/SR mice.

We thank the reviewer for drawing attention to this point. These blots were carried out using whole spleen lysates and in general were challenging to get "clean" blots. We tried to visualize p16 or p76. p16 visualization failed as the antibody is human specific – does not react with mouse Malt1. Other Ab are not sensitive enough to detect p16 and the p76 neo-epitope Ab is also not sensitive enough (works only for overexpression experiments and in SSK41 lymphoma cells with a genomic MALT1 amplification and associated overexpression).

The Malt1-SR lanes were on the edge of the blot, and the perceived lower intensity towards the right end of the blot we speculate that the small intensity difference is more likely due to a technical artefact rather than a protein stability issue. We have included here for the reviewer to assess an independent blot which shows that Malt1 SR does not decrease at the protein level. We have clarified this observation in the text.



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We agree with the reviewer that in transient overexpression studies the Malt1-SR and PD mutants indeed show no effect on NF-kB reporter activation nor on phosphorylation of IkBa (proximal signaling events). The RNA-seq experiment however shows that there is a subset of genes that is affected by PD or SR, which was confirmed for selected genes as shown in supplementary figures 1A and B. This might indeed result (in part) from mRNA destabilization in case of the PD mice (MCPIP1 / Regnase1 is not inactivated), but not for the SR mice as Malt1 is still protease active, which indeed suggests that self-cleavage (p76) contributes to NF-kB signal strength.

In relation to the Malt1 p76 acting as a transcriptional activator within the nucleus, Ginster et al showed that N-terminal auto-proteolytic cleavage of MALT1 at R149 and generation of p76 that displays enhanced NF-kB signaling properties is regulated via its direct interaction with TRAF6, suggesting cytoplasmic control of NF-kB signal strength (PLoS One. 2017 Jan 4;12(1):e0169026). However, a nuclear role for MALT1 or p76 cannot be excluded. MALT1 contains nuclear export signals in its C terminus that serve to regulate

nuclear shuttling of BCL10 (Nakagawa et al. Blood. 2005 Dec 15;106(13):4210-6). As Bcl10 is a potential transcriptional activator that interacts with general transcription factor TFIIB (Liu et al., Biochem Biophys Res Commun. 2004 Jul 16;320(1):1-6), this suggests that MALT1 may modulate gene expression via Bcl10 nuclear expression. However, we believe that a more detailed and in-depth investigation of a nuclear role for Malt1 lies outside the scope of the current manuscript.

The authors can also consider analyzing the stability of Il2 mRNA in Malt1 SR/SR and PD/PD cells.

We agree this is an important point to consider and any increased IL2 mRNA stability in SR mice could explain higher IL2 production in Fig 3F. However, we believe these experiments albeit very interesting, do not fall within the scope of the current manuscript.

Given that this manuscript does not show a direct evidence that Malt1 self-cleavage induces NF-kB activation, the current title is not appropriate.

We thank the reviewer for this comment. Indeed, we adapted the title of the manuscript to better reflect our findings.

3. In Figure 3, the authors showed that Malt1SR/SR mice also show impaired development of thymic Treg cells. They also showed that Malt1SR/SR mice do not develop colitis but did not discuss colonic Treg population. One might argue that if this developmental defect is due to decreased IL-2 availability, development of iTreg cells in the colon might be impaired as well. In vitro Treg differentiation assay will help to clarify whether defect of Treg cell population is T cell-intrinsic or not.

We agree with the reviewer that it would be interesting to specifically follow-up on colonic Tregs in Malt1-impaired mice. Our preliminary data showed that, indeed, Treg in mesenteric lymph nodes were reduced as well. We performed the experiment proposed by the reviewer and were able to demonstrate that *in vitro* Treg differentiation is indeed defective in Malt1 PD/PD and to a lesser extent affected in Malt1 SR/SR mice, compared to Malt1 wt animals. Notably, the SR/SR is intermediate compared to Malt1 PD/PD which likely explains why the SR/SR mice do not suffer colitis. We amended the manuscript to include these data as a Supplementary Figure 3.

4. Interestingly the authors showed that Malt1SR/SR as well as Malt1PD/PD mice could suppress tumor progression compared to WT mice. However, given that Malt1SR/SR mice show much milder impairment of Treg recruitment into the tumor tissue than Malt1PD/PD mice, and that these mice do not develop autoimmunity, it is too early to make a conclusion that mild decrease in Treg cells within the tumor tissue is important for control of tumor progression. To strengthen this point, the author can examine additional tumor implantation models like B16 melanoma.

Indeed, we agree with the reviewer that this is an intriguing observation, especially from a clinical treatment point of view. We are currently trying to obtain clinical grade MALT1 inhibitors to follow up on this observation with MC38 and other tumor models such as B16 melanoma models in a follow-up study.

Minor comments

1. Full length Mcpip1 expression is reported to be decreased in response to PMA stimulation. However, the Figure 1D shows that the Mcpip1 levels are not altered following stimulation. The authors can examine the levels of Malt1 and Mcipi1 in later time points (1 or 2 hours after stimulation).

We thank the reviewer for commenting on this point. We agree that it would be interesting to follow up on the long-term stability of the different Malt1 substrates over time. However, we believe this is outside the scope of the current manuscript in which we wish to highlight that mutating the self-cleavage site does not alter that protease activity of Malt1.

2. Some of the Figure legends do not state how many times the authors did experiments for reproducibility.

We thank the reviewer for pointing out the lack of this information and we added all of these data in the manuscript and figure legends.

3. typos. page 12 “Matl1”, page 14 “then Malt1PD/PD mice”, page 20 “Spleenocyte”.

Thank you for highlighting these errors. These have now been corrected within the manuscript.

Reviewer: 2

Comments to the Author

In their submitted manuscript, Baens and colleagues describe the phenotype of a mouse which carries a MALT1 mutant that cannot be auto-processed. The MALT1 protease has gain interest as drug target against certain lymphomas and to treat autoimmune diseases in the last decade. Similar to initiator caspases, the paracaspase MALT1 have been reported to be auto-processed (Baens et al., 2014), but the in vivo function was unclear.

The experimental design and the presented results are mostly of good quality, the data are not overinterpreted. I therefore recommend acceptance of the manuscript with minor revisions.

Minor concerns:

+ The citations on page 4 is not formatted correctly (Hori, Shohei; Nomura, Takashi; Sakaguchi, 2002).

Thank you for highlighting this point. This has now been corrected and all references were adjusted.

+ kappas in NF-kB is not uniform (OK)

Thank you for highlighting this point. This has now been corrected.

+ The mp-Malt1 (membrane-targeted) needs more explanation. How is it targeted to the membrane and why does this lead to MALT1 protease activity?

We thank you the reviewer for asking us to clarify this point further in the manuscript. The myristoylation palmitoylation tag of the Lck Kinase targets the protein to the lipid raft membrane fraction which we have previously shown results the induction of MALT1 cleavage. This text has now been added and the appropriate reference (Baens et al, PLOS ONE, 2014)

+ Figure labelling needs to be improved. See e.g. Figure 1 “Mcpip” vs “MCPIP” or “Il-6” vs “Il2”. Please unify.

The protein labels have now all been edited to reflect mouse proteins. In Figure 2 we have removed the dash in the cytokine for consistency across the manuscript including the main text and the figures.

+ Fig.1d: MCPIP cleavage is not convincing; could the authors provide a better blot?

Thank you for this comment. These blots were carried out on whole spleen lysates pooled from mice within each genetic cohort. These blots were technically challenging and also similar comments were raised by Reviewer #1. We believe that although the blot currently in the manuscript is of high enough quality to highlight that the SR/SR mutant still retains the ability to cleave Mcpip compared to the proteolytically inactive PD/PD that cannot.

+ Fig. 2h: Please clarify what “neg.CTL“ mean.

We apologies for not making this clearer. Neg.CTL is an abbreviation for Negative Control. Assay buffer was used as background fluorescence negative control to assess relative serum concentrations (neg. CTL). We added this information to the figure legend of Figure 2.

+ Suppl. Figure 1D: IL2 reporter activation by p76 not very convincing. Maybe it would be better to focus only on the NF-kB-Luc data.

We agree with the reviewer that although the difference is statistically significant, the biological relevance is perhaps less compared to the NF-kB data. We have therefore removed the IL2 reporter chart and associated text within the manuscript.

+ There are two very similar sentences on page 13 (see below). Please remove one of them.

“In line with these observations, patients with Malt1 loss-of-function mutations, also show signs of autoimmune disease, IL-2 deficiency and reduced Treg frequency (Punwani et al., 2015; McKinnon et al., 2014).”

“In line with these observations, patients with Malt1 loss-of-function mutations, also show signs of autoimmune disease and Treg deficiency (Punwani et al., 2015; McKinnon et al., 2014).”

We thank the reviewer for highlighting this duplication in the text. We have deleted the first appearance of this sentence in the manuscript.

European Journal of Immunology

Second Editorial Decision - 29-Jun-2018

Dear Dr. Bornschein,

It is a pleasure to provisionally accept your manuscript entitled "Malt1 self-cleavage is critical for regulatory T cell homeostasis and anti-tumor immunity" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: https://onlinelibrary.wiley.com/toc/15214141/0/ja). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Shimon Sakaguchi

Dr. Nadja Bakocevic
Editorial Office
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