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Malt1 protease inactivation efficiently dampens immune responses but causes spontaneous autoimmunity

Maike Jaworski, Benjamin Marsland, Jasmine Gehrig, Werner Held, Stéphanie Favre, Sanjiv A Luther, Mai Perroud, Déla Golshayan, Olivier Gaide and Margot Thome

Corresponding author: Margot Thome, University of Lausanne

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1st Editorial Decision 05 June 2014

Thank you for submitting your manuscript to The EMBO Journal. Two referees have now evaluated your study and their comments are provided below.

As you can see both referees find the analysis interesting and suitable for publication in The EMBO Journal. They raise a number of specific concerns that I suspect that you should be able to resolve. Given the positive feedback from the referees I would like to invite you to submit a suitably revised manuscript for our consideration. I should add that it is EMBO Journal policy to allow only a single major round of revision only and it is therefore important to address the concerns raised at this stages.

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

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

REFEREE REPORTS

Referee #1:

MALT1 is a protease that functions in several immunoreceptor signaling pathways by acting as a scaffold protein as well as by proteolytically processing a number of substrates. However, the relative contribution of scaffold versus catalytic MALT1 activities is still unknown. Jaworski et al have generated MALT1 catalytic inactive (protease dead) knockin mice and compared these mice with full MALT1 knockout and wild type mice.

This is a straightforward approach that provides some highly interesting and sometimes surprising results. First, this study shows that several of the innate and adaptive immune responses that were previously shown to be disrupted in MALT1 knockout mice are also affected in knockin mice expressing a catalytic inactive MALT1 mutant. Secondly, this study shows that MALT1 catalytic inactive knockin mice are protected in mouse models of multiple sclerosis and colitis, further illustrating the important role of MALT1 proteolytic activity and its potential for therapeutic targeting. Third and very surprising, whereas MALT1 knockout mice do not develop any inflammatory phenotype, MALT1 protease dead mice do show spontaneous inflammation in the stomach. It is further shown that this phenotype is T cell intrinsic and that MALT1 knockout and knockin mice have a severe reduction in regulatory T cells. Based on Treg transfer experiments the authors conclude that this defect in Tregs is responsible for the inflammatory phenotype in the protease dead MALT1 knockin mice.
Altogether, this is an interesting study that is of high importance in the context of the proposed role of MALT1 as a therapeutic target in autoimmune disease and lymphoma. The experiments have been well performed and the manuscript is clearly written.

Major comments that should be addressed:

1. Although the Treg transfer experiments that were performed may suggest that a defect in Tregs is at the origin of the inflammatory phenotype in MALT1 knockin mice, this is not fully proven. It could still be that transfer of Tregs (or increasing the absolute number of Tregs) is also protective in wild type mice subjected to an inflammatory disease model. The authors should at least discuss possible alternative explanations. For example, it is unclear if the active site mutation in MALT1 may also exert some dominant negative effect. Normally substrates binding to MALT1 are released upon cleavage. This means that the MALT1 protease dead mutant is potentially still able to bind substrates, but these substrates are no longer properly released, because they cannot be cleaved. In this way substrates may be taken out of their normal cellular context and an accumulation of MALT1-substrate complexes could have effects that are different from mere MALT1 protease inactivation or the complete loss of MALT1. A potential dominant effect of MALT1(C/A) calls for a more thorough analyses of heterozygous MALT1 +/ki mice.

2. The inflammatory phenotype of the MALT1 knockin mice is restricted to gastritis. What could be the reason for this specificity? Why does the absence of Tregs not result in inflammation in other tissues? The phenotype in other tissues could be very mild and manifested only when mice are getting older. Did the authors also analyze older mice? To investigate if the inflammation is systemic it would also be of interest to analyze the presence of proinflammatory cytokines in the serum of mice (TNF, IL1, IL17, ...).

3. It is known that infection with Helicobacter species may frequently occur and spread easily in mice reared under SPF conditions. Moreover, there is a high prevalence of rather uncommon Helicobacter species that may be a consequence of the current routine procedures used for health screening of SPF mice (Bohr et al., J. Clin. Microbiol. March 2006 vol. 44 no. 3 738-742). Because Helicobacter pylori infections have been associated with gastritis and MALT lymphoma, it is tempting to speculate that the observed gastritis in MALT1 knockin mice reflects Helicobacter infection. The authors should check this. Also, analyzing the effect of antibiotics treatment on the gastritis phenotype could learn a lot.

4. I do have some problems with the number of mice used in several experiments. Most experiments have only been done on very small groups of mice (n=3). Although differences are shown to be statistically significant, it is questionable if results on such small groups are fully reliable (especially

in these cases where differences between setups seem to be rather small). Also, in many cases the figure legend mentions a group size of greater than or equal to 3. The authors should be more specific here. Also for figure 4 F, the text mentions that 2 out of 8 mice tested showed T cell infiltration in the colon, but only data for 4 mice (or 3 in the WT mice) are shown in the figure itself.

5. It is not clear from the data that are shown if MALT1 KO or KI mice are fully or only partially protected from colitis. This should at least be mentioned in the text.

6. The authors suggest that increased RelB expression, which is associated with RelB mediated inhibition of NF-kB RelA, is responsible for decreased expression of FoxP3. However, there may be alternative explanations. For example, decreased FoxP3 expression may also reflect decreased FoxP3 mRNA stability as a consequence of the absence of MALT1 mediated Regnase 1 cleavage. The authors should therefore analyze if decreased FoxP3 expression in ki/ki mice reflects decreased transcription or decreased stability.

7. the authors claim that impaired FoxP3 expression is at the origin of reduced Treg numbers in MALT1 knockout and knockin mice. However, fig. 7C mainly shows a delay in FoxP3 expression with still significant FoxP3 expression in ki/ki mice. It seems unlikely that this is sufficient to explain the almost complete absence of Tregs. In this context, it would also be interesting to measure the amount of Treg cells (CD25+ Foxp3+) that is obtained after stimulation with anti-CD3/CD28 and TGF-beta in vitro.

8. Fig. 8: the authors should illustrate the efficiency of Treg reconstitution after adoptive Treg transfer.

9. mechanistically, it is still not very clear why the MALT1 knockout mice, which also lack Tregs and show reduced anti-CD3 induced T cell activation, do not show accumulation of activated T cells and gastritis. This should be discussed in more detail.

Minor comments that should be addressed:

1. Expression data for MALT1 and protease dead MALT1 is only shown for total splenocytes (Fig. 1A and B). Equal expression should also be shown for purified T and B cells (fig. 1 C and D).

2. on page 7, Jaworski et al. refer to two papers (Ruland et al. and Ruefli-Brasse et al.) to claim that IkBa degradation is impaired in antigen stimulated T and B cells. However for B cells, Ruland et al. only reports a minor influence of MALT1 deficiency on IkBa degradation. This should be corrected in the text.

3. Also concerning JNK activation, Jaworski et al. show that the MALT1 KO mice have a partial or strong reduction in JNK activation in T and B cells, respectively. This is in line with the published data of Ruland et al., but is in contrast with the data mentioned in the paper of Ruefli-Brasse, showing that JNK activation is not impaired in T and B cells. What is the reason for the discrepancy between the published data from the study of Ruefli-Brasse and the newly generated data in the present manuscript, using the same mice provided by the lab of Ruefli-Brasse?

4. fig 1B: the reference for the antibody of the neo-epitope of bcl10 should be Rebeaud, 2008

5. Legend titles of fig7 and fig8 are switched

6. Figure 6: histological analysis and serum IgE/IgG1 levels (Fig. 6B and C) are only shown for knockin mice. Similar information related to full knockout mice should also be provided.

Non-essential suggestions:

The authors show that substrate cleavage is absent in in vitro stimulated T or B cells from protease dead MALT1 knockin mice. Given the unexpected phenotype of MALT1 protease dead mice, it may be of interest to demonstrate that MALT1 catalytic activity is also absent in vivo. One could compare for example substrate cleavage in splenocytes of wild type, MALT1 KO, and MALT1 protease dead mice treated with anti-CD3.

Referee #2:

An essential role for MALT1 paracaspase for the activation of an immune response has been provided by the generation of MALT1 ko mice. MALT1 adaptor functions and proteolytic activity have been suggested to play key roles, but so far the contribution of proteolytic activity has not been addressed in a mouse model. For this purpose the authors generated knock-in mice expressing exclusively the catalytically inactive C472A mutant in mice (MALT1 ki/ki). To assign adaptor and protease function, they directly compared the phenotypes of MALT1 ki and ko animals. Indeed, most of the defects in immune cell development and function of MALT1 ko are also found in the MALT1 ki mice and both mice are protected from induction of EAE. Thus, the data reveal the importance of the protease for triggering an appropriate immune response, but the immune response is not as severely affected as in MAKT1 deficient mice. Unexpectedly and in contrast to MALT1 ko mice, the MALT1 ki mice also display an activated T cell phenotype and develop autoimmune gastritis. Indeed, Treg development is severely impaired in MALT1 ko and ki mice and by Treg transfer experiments the author show that the autoimmune phenotype is rescued by functional Treg cells. The authors suggest a model that despite dampened immune cell activation, protease inactive MALT1 affects the balance of the immune response and that the loss of Treg cells induces autoimmunity. This phenotype is not evident from MALT1 ko animals, because here the complete absence of an immune cell activation prevents the necessity of negatively acting Treg cells.

This is a highly interesting and very well performed study. The data are clear and the manuscript is overall written very well. There is good evidence for the suggested immune balancing function of the MALT1 protease, even though it is not finally proven, which is in fact very difficult. Some points should be addressed or taken into consideration.

The authors interpret the phenotype by a loss of function of protease activity. However, MALT1 C472A does not necessarily only reflect a loss of function mutation, because even though substrates cannot be cleaved, they are still potentially recognized and bound by MALT1. This is especially an issue for proteases and could even lead to dominant effects by dragging out either MALT1 itself or substrate proteins from their original cellular complexes. Thus, the situation of this genetic inactivation may be quite different from a pharmacological inhibition, where the enzyme never sees the substrate. Actually, other mouse models would be required to address in how far effects are solely due to defective stimulus dependent MALT1 activation. This could be done either by mutating the catalytic cysteine plus the substrate binding pocket or by mutating lysine 644 that is essential for MALT1 activation by ubiquitination. I think this issue should at least be discussed in the context of the therapeutic impact.

All analyses have been carried out until the age of 15 weeks. What is happening to the mice beyond this age? Are they recovering or is the disease progressing so that they need to be sacrificed? Is the autoimmune phenotype restricted to gastritis?
The complete protection of MALT1 ki mice from EAE is indeed somewhat surprising given the activated T cell phenotype and the severe reduction of Treg cells. How is this explained and what age where the mice for EAE induction? May the age of the mice affect the outcome of EAE?

Figure 2B: Differences in CFSE dilution especially between ki and ko are hard to see. Maybe an overlay or quantification would be better.

Figure 3D: Is cleavage of other MALT1 substrates like RelB or Regnase-1 affected upon zymosan stimulation in ki and ko mice?

Figure 6B, C and Discussion: Is only RelB increased in the thymocytes? How about Regnase-1 and CYLD also in comparison to MALT1 ko thymocytes? Are RelB and other substrates more binding to MALT1 in ki cells (see above)? The interpretation also on page 17 is focused too much on the negative function of RelB. Actually, FoxP3 mRNA is not so severely reduced in ki cells, which could argue that mRNA regulation e.g. by Regnase-1 is quite important. A combined effect of multiple substrates is more likely.

On page 11 at the bottom the author write MALT1 C474A instead of C472A.

1st Revision - authors' response 08 August 2014

Referee #1:

Major comments that should be addressed:

1. Although the Treg transfer experiments that were performed may suggest that a defect in Tregs is at the origin of the inflammatory phenotype in MALT1 knockin mice, this is not fully proven. It could still be that transfer of Tregs (or increasing the absolute number of Tregs) is also protective in wild type mice subjected to an inflammatory disease model. The authors should at least discuss possible alternative explanations. For example, it is unclear if the active site mutation in MALT1 may also exert some dominant negative effect. Normally substrates binding to MALT1 are released upon cleavage. This means that the MALT1 protease dead mutant is potentially still able to bind substrates, but these substrates are no longer properly released, because they cannot be cleaved. In this way substrates may be taken out of their normal cellular context and an accumulation of MALT1-substrate complexes could have effects that are different from mere MALT1 protease inactivation or the complete loss of MALT1. A potential dominant effect of MALT1(C/A) calls for a more thorough analyses of heterozygous MALT1 +/ki mice.

We agree that the defect in Tregs is not the only explanation for the inflammatory phenotype of the mice. An alternative explanation that we have discussed (see discussion top of p.19) is that additional defects (such as a potential generation of autoreactive T cells due to potential defects in thymic selection) may contribute.

We have analyzed heterozygous MALT1 +/ki mice in several experiments that are included in the manuscript (Fig. 1C, 6A and expanded Fig. E6 A) as well as in additional experiments that are not included in the paper. In the experiments included in the manuscript, heterozygous mice behaved like wt mice and distinct from ki/ki mice with respect to substrate cleavage (Fig. 1C), autoimmunity-induced weight loss (Fig. 6A), spleen and lymph node cellularity (ext. Fig. 6A). In additional data that are not included in the paper, we also saw no differences between heterozygotes and wildtype mice with respect to percentages and total numbers of B- and T cells in spleens and lymph nodes, basal serum IgE levels and EAE induction. Therefore, we can reasonably exclude a dominant negative effect of the catalytically inactive mutant.

2. The inflammatory phenotype of the MALT1 knockin mice is restricted to gastritis. What could be the reason for this specificity? Why does the absence of Tregs not result in inflammation in other tissues? The phenotype in other tissues could be very mild and manifested only when mice are getting older. Did the authors also analyze older mice? To investigate if the inflammation is systemic it would also be of interest to analyze the presence of proinflammatory cytokines in the serum of mice (TNF, IL1, IL17, ...).

Presently, we have no explanation for the preferentially gastric autoimmune phenotype, but it is interesting to note that certain strains of mice also develop a preferentially gastric autoimmune phenotype upon thymectomy. This was first reported in 1980 by Nishizuka and collaborators (Lab Invest. 1980, 42:387-95) and to the best of our knowledge the exact reasons for this still remain unknown. It is possible (and actually likely) that our mice would also develop additional, broader autoimmune features upon ageing (this is now briefly discussed on page 18), but we have been unable to assess this thoroughly because the disease is progressing and in accordance with our institutional animal welfare regulations we have to sacrifice the mice as soon as they lose more than 15% of their weight (which usually happens within the first 15 weeks of life of the mice).

We have tried to assess the levels of the proinflammatory cytokines TNF, IL1 and IL17 in the serum of unmanipulated wildtype and knock-in mice but there were no significant changes in IL-1a levels and signals for IL-17A and TNFa were below detection levels, which would argue against a systemic inflammation (we have tested serum of mice up to the age of 15 weeks).

3. It is known that infection with Helicobacter species may frequently occur and spread easily in mice reared under SPF conditions. Moreover, there is a high prevalence of rather uncommon Helicobacter species that may be a consequence of the current routine procedures used for health screening of SPF mice (Bohr et al., J. Clin. Microbiol. March 2006 vol. 44 no. 3 738-742). Because Helicobacter pylori infections have been associated with gastritis and MALT lymphoma, it is tempting to speculate that the observed gastritis in MALT1 knockin mice reflects Helicobacter infection. The authors should check this. Also, analyzing the effect of antibiotics treatment on the gastritis phenotype could learn a lot.

In the past we have treated the mice repeatedly with antibiotics (since we originally attributed the swollen lymph nodes to a potential presence of infections) but did not notice an improvement of the condition. We then transferred the mice through embryo transfer into a specific pathogen-free environment, and the symptoms still persisted. Our mice were routinely (every 3 months) tested and found to be negative for the presence of helicobacter species using a PCR-based, pan-helicobacter specific test of feces and liver that includes (but is not restricted to) detection of H. pylori, H. bilis, H. hepaticus, H. rodentium and H. tiphlonius. Therefore, it is unlikely that H. pylori (nor other helicobacter species) are driving the phenotype, but we can obviously not completely exclude that other, presently undetectable gastric infections may contribute to the phenotype.

4. I do have some problems with the number of mice used in several experiments. Most experiments have only been done on very small groups of mice (n=3). Although differences are shown to be statistically significant, it is questionable if results on such small groups are fully reliable (especially in these cases where differences between setups seem to be rather small). Also, in many cases the figure legend mentions a group size of greater than or equal to 3. The authors should be more specific here. Also for figure 4 F, the text mentions that 2 out of 8 mice tested showed T cell infiltration in the colon, but only data for 4 mice (or 3 in the WT mice) are shown in the figure itself.

The small number of mice used in individual experiments is due to the fact that we cannot breed the knock-in mice homozygously, ki/ki mice thus always have to be derived from heterozygous breedings, with consequently limited numbers of ki/ki mice and control littermates. For all main figures, exact numbers of mice per group have now been indicated. For Fig. 4F, we performed 2 independent experiments, in each of which only 1 out of 4 mice showed T-cell infiltration in the colon. This is now explicitly mentioned in the text referring to Fig. 4F.

5. It is not clear from the data that are shown if MALT1 KO or KI mice are fully or only partially protected from colitis. This should at least be mentioned in the text.

In each of the two independent sets of experiments performed, 3 out of 4 knock-in mice tested were resistant to colitis, and knock-out mice were completely protected, our interpretation is thus that the ki mice develop colitis "with lower penetrance". We have now indicated the numbers of mice in each of the two experiments in the text to make this clear (see point 4 above).

6. The authors suggest that increased RelB expression, which is associated with RelB mediated inhibition of NF-kB RelA, is responsible for decreased expression of FoxP3. However, there may be alternative explanations. For example, decreased FoxP3 expression may also reflect decreased FoxP3 mRNA stability as a consequence of the absence of MALT1 mediated Regnase 1 cleavage. The authors should therefore analyze if decreased FoxP3 expression in ki/ki mice reflects decreased transcription or decreased stability.

We agree that the increased RelB level is only one out of several possible explanations for the defect in nTreg development, and that a combined lack of cleavage of various substrates is more likely to provide the full explanation, which is now explicitly mentioned in the discussion on page 18.

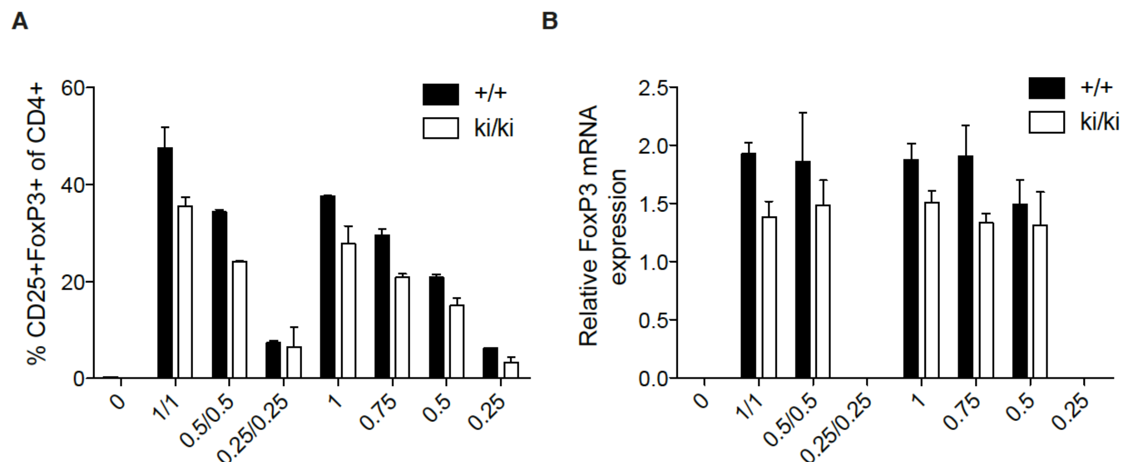
We have assessed FoxP3 mRNA stability in the in vitro system used to assess inducible Tregs (iTregs) generation (unfortunately FoxP3 mRNA stability could not be assessed in the thymus because of the minimal number of Tregs remaining in the ki/ki mice). In the in vitro system, we saw no significant effect of a MALT1 inhibitor (new expanded data Fig. E7 A) or of MALT1 deficiency

or catalytic inactivation (new expanded data Fig. E7 B) on FoxP3 mRNA stability, suggesting that MALT1 activity (and MALT1-dependent Regnase-1 cleavage) may not be required to stabilize FoxP3 mRNA in this in vitro system. Nevertheless, we systematically saw a delay in the inducible upregulation of FoxP3 mRNA levels in the in vitro system (Fig. 7C). These findings give some interesting insight into the role of MALT1 in iTreg generation that are now described in more detail in the results and discussion section of the revised manuscript. However, the exact reasons for the defect in FoxP3+ Treg cells in vivo are most likely complex and require further investigations that are beyond the scope of this manuscript.

7. the authors claim that impaired FoxP3 expression is at the origin of reduced Treg numbers in MALT1 knockout and knockin mice. However, fig. 7C mainly shows a delay in FoxP3 expression with still significant FoxP3 expression in ki/ki mice. It seems unlikely that this is sufficient to explain the almost complete absence of Tregs. In this context, it would also be interesting to measure the amount of Treg cells (CD25+ Foxp3+) that is obtained after stimulation with anti-CD3/CD28 and TGF-beta in vitro.

We believe that the strongly impaired presence of CD25+ FoxP3+ Treg cells in the mice most likely reflects a severe defect in nTreg cell development, combined with a partial defect in iTreg induction.

We have assessed the numbers of iTreg (CD25+ FoxP3+) cells that can be generated in vitro upon treatment with various concentrations of CD3/CD28 and with TGF-beta, and found correlating mild differences in iTreg proportions and FoxP3 mRNA levels (figure below is for referee's information only, naïve T cells were stimulated for 48h with anti-CD3, anti-CD28, IL-2 and TGF-beta as indicated in the methods section). However, we cannot exclude that these in vitro stimulation conditions do not fully reflect the conditions required to induce iTreg cell development in vivo, which may be more strongly affected.



8. Fig. 8: the authors should illustrate the efficiency of Treg reconstitution after adoptive Treg transfer.

In the reconstituted mice, the percentage of Treg cells remained lower than in unreconstituted mice (less than 1% of CD4+ T cells were CD25- and FoxP3-positive; of those > 40 % were GFP-positive), nevertheless this was sufficient to efficiently rescue the autoimmune phenotype, suggesting that the endogenous Treg cells in the knock-in mice were compromised in their function. These data are now illustrated in the new expanded Fig. E 8.

9. mechanistically, it is still not very clear why the MALT1 knockout mice, which also lack Tregs and show reduced anti-CD3 induced T cell activation, do not show accumulation of activated T cells and gastritis. This should be discussed in more detail.

We believe that the main reason for the absence of autoimmunity features in MALT1 knock-out mice is the efficient blunting of their T- and B-cell responses, while MALT1 ki mice still show minimal B- and T-cell responses (see for example Fig. 2 A, B, F and H). We have now discussed this in more detail in the manuscript (see page 18).

Minor comments that should be addressed:

1. Expression data for MALT1 and protease dead MALT1 is only shown for total splenocytes (Fig. 1A and B). Equal expression should also be shown for purified T and B cells (fig. 1 C and D).

We have included these expression data in the new expanded Fig. E1 C.

2. on page 7, Jaworski et al. refer to two papers (Ruland et al. and Ruefli-Brasse et al.) to claim that IkBa degradation is impaired in antigen stimulated T and B cells. However for B cells, Ruland et al. only reports a minor influence of MALT1 deficiency on IkBa degradation. This should be corrected in the text.

We apologize for this error and have corrected the text accordingly.

3. Also concerning JNK activation, Jaworski et al. show that the MALT1 KO mice have a partial or strong reduction in JNK activation in T and B cells, respectively. This is in line with the published data of Ruland et al., but is in contrast with the data mentioned in the paper of Ruefli-Brasse, showing that JNK activation is not impaired in T and B cells. What is the reason for the discrepancy between the published data from the study of Ruefli-Brasse and the newly generated data in the present manuscript, using the same mice provided by the lab of Ruefli-Brasse?

We can only speculate about the reasons underlying this discrepancy, which is now explicitly mentioned in the results section on page 7. It may include the use of distinct anti-P-JNK antibodies, differences in protein loading (loading of higher amounts of lysates will make differences less apparent) or subtle differences in the stimulation protocols. Since JNK activation is not the main focus of our manuscript, we have retained from discussing this further.

4. fig 1B: the reference for the antibody of the neo-epitope of bcl10 should be Rebeaud, 2008

We apologize for the error. The reference has been removed from the legend. The correct reference is indicated in the materials and methods section.

5. Legend titles of fig7 and fig8 are switched

We apologize for this error, which has now been corrected.

6. Figure 6: histological analysis and serum IgE/IgG1 levels (Fig. 6B and C) are only shown for knockin mice. Similar information related to full knockout mice should also be provided.

The corresponding information for knockout mice has now been included in Fig. 6B and 6C and the corresponding description of the results.

Non-essential suggestions:

The authors show that substrate cleavage is absent in in vitro stimulated T or B cells from protease dead MALT1 knockin mice. Given the unexpected phenotype of MALT1 protease dead mice, it may be of interest to demonstrate that MALT1 catalytic activity is also absent in vivo. One could compare for example substrate cleavage in splenocytes of wild type, MALT1 KO, and MALT1

protease dead mice treated with anti-CD3.

We agree with the reviewer that this would be potentially interesting, but think that these experiments exceed the scope of the present manuscript.

Referee #2:

This is a highly interesting and very well performed study. The data are clear and the manuscript is overall written very well. There is good evidence for the suggested immune balancing function of the MALT1 protease, even though it is not finally proven, which is in fact very difficult. Some points should be addressed or taken into consideration.

The authors interpret the phenotype by a loss of function of protease activity. However, MALT1 C472A does not necessarily only reflect a loss of function mutation, because even though substrates cannot be cleaved, they are still potentially recognized and bound by MALT1. This is especially an issue for proteases and could even lead to dominant effects by dragging out either MALT1 itself or substrate proteins from their original cellular complexes. Thus, the situation of this genetic inactivation may be quite different from a pharmacological inhibition, where the enzyme never sees the substrate. Actually, other mouse models would be required to address in how far effects are solely due to defective stimulus dependent MALT1 activation. This could be done either by mutating the catalytic cysteine plus the substrate binding pocket or by mutating lysine 644 that is essential for MALT1 activation by ubiquitination. I think this issue should at least be discussed in the context of the therapeutic impact.

We agree with the reviewer that mutating the catalytic site Cys may have additional effects on MALT1 that we cannot exclude with this model. In the past we have assessed RelB binding to human wt and C464A MALT1, and found no differences in substrate binding (our unpublished findings).

We also do not believe that we have a dominant negative effect. As mentioned above in response to a similar concern raised by referee 1, we have analyzed heterozygous MALT1 +/ki mice in several experiments that are included in the manuscript (Fig. 1C, 6A and ext Fig E6 A) as well as in additional experiments that are not included in the paper. In the experiments included in the manuscript, heterozygous mice behaved like wt mice and distinct from ki/ki mice with respect to substrate cleavage (Fig. 1C), autoimmunity-induced weight loss (Fig. 6A), spleen and lymph node cellularity (expanded Fig. E6 A). In additional data that were not included in the paper, we also saw no differences between heterozygotes and wildtype mice with respect to percentages and total numbers of B- and T cells in spleens and lymph nodes, basal serum IgE levels and EAE induction. Therefore, we can reasonably exclude a dominant negative effect of the catalytically inactive mutant.

Since similar concerns about possible dominant negative effects were raised by both referees, we have now briefly addressed this topic in the discussion on page 16.

All analyses have been carried out until the age of 15 weeks. What is happening to the mice beyond this age? Are they recovering or is the disease progressing so that they need to be sacrificed? Is the autoimmune phenotype restricted to gastritis?

As already mentioned above in response to a similar question raised by referee 1, we have no explanation for the preferentially gastric autoimmune phenotype, but it is interesting to note that certain strains of mice also develop a preferentially gastric autoimmune phenotype upon thymectomy. This was first reported in 1980 by Nishizuka and collaborators (Lab Invest. 1980, 42:387-95) and to the best of our knowledge the exact reasons for this still remain unknown. It is possible (and actually likely) that our mice would also develop additional, broader autoimmune features upon ageing (this is now briefly mentioned in the text), but we have been unable to assess this thoroughly because the disease is progressing and in accordance with our institutional animal welfare regulations we have to sacrifice the mice as soon as they lose more than 15% of their weight (which usually happens within the first 15 weeks of life of the mice).

The complete protection of MALT1 ki mice from EAE is indeed somewhat surprising given the activated T cell phenotype and the severe reduction of Treg cells. How is this explained and what age where the mice for EAE induction? May the age of the mice affect the outcome of EAE?

We used the mice at the youngest possible age for reliable EAE induction (usually starting MOG immunizations at the age of 10 weeks) to minimize the impact of the spontaneous autoimmune gastritis on the general health condition of the mice. The age of the mice may indeed affect the outcome of EAE, and it is possible that mice would be less well protected if they were used at later ages (which was not possible because mice had to be sacrificed around the age of 15 weeks). We would like to stress though that the mice were not only resistant to induction of EAE symptoms, but also showed a complete absence of T-cell priming (since no Th1 and Th17 polarization was observed). We speculate that complete protection from EAE may be due to the fact that this is a Th1/Th17 model, whereas the autoimmune gastritis shows features of a Th2 response that may be less efficiently blunted.

Figure 2B: Differences in CFSE dilution especially between ki and ko are hard to see. Maybe an overlay or quantification would be better.

We have improved this by showing overlays of the histograms.

Figure 3D: Is cleavage of other MALT1 substrates like RelB or Regnase-1 affected upon zymosan stimulation in ki and ko mice?

It was technically more difficult to assess MALT1 substrate cleavage in zymosan-stimulated monocytes than in activated lymphocytes, possibly because zymosan triggers MALT1 activation with lower efficiency and/or because of higher background of monocyte lysates. In addition to Bcl-10, we could also detect impaired cleavage of RelB and CylD in zymosan-stimulated cells of ki or ko mice (this is now included in expanded Fig. E5 B). Because of high background, cleavage of the MALT1 substrate Regnase1 was not well detectable in monocyte lysates.

Figure 6B, C and Discussion: Is only RelB increased in the thymocytes? How about Regnase-1 and CYLD also in comparison to MALT1 ko thymocytes? Are RelB and other substrates more binding to MALT1 in ki cells (see above)? The interpretation also on page 17 is focused too much on the negative function of RelB. Actually, FoxP3 mRNA is not so severely reduced in ki cells, which could argue that mRNA regulation e.g. by Regnase-1 is quite important. A combined effect of multiple substrates is more likely.

As also mentioned above in response to Referee 1, we agree that the increased RelB level is only one out of several possible explanations for the defect in nTreg development, and that a combined lack of cleavage of various substrates is more likely to provide the full explanation, which is now explicitly mentioned in the discussion.

In the past we have assessed RelB binding to human wt and C464A MALT1, and found no differences in substrate binding (our unpublished findings). Except for Bcl10, binding of other substrates to MALT1 was not detectable by co-IP.

We have assessed FoxP3 mRNA stability in the in vitro system used to assess inducible Tregs (iTregs) generation (unfortunately FoxP3 mRNA stability could not be assessed in the thymus because of the minimal number of Tregs remaining in the ki/ki mice). In the in vitro system, we saw no significant effect of a MALT1 inhibitor (new expanded data Fig. E7 A) or of MALT1 deficiency or catalytic inactivation (new expanded data Fig. E7 B) on FoxP3 mRNA stability, suggesting that MALT1 activity (and MALT1-dependent Regnase-1 cleavage) may not be required to stabilize FoxP3 mRNA in this in vitro system. Nevertheless, we systematically saw a delay in the inducible upregulation of FoxP3 mRNA levels in the in vitro system (Fig. 7C). These findings give some interesting insight into the role of MALT1 in iTreg generation that are now described in more detail in the results and discussion section of the revised manuscript. However, the exact reasons for the defect in FoxP3+ Treg cells in vivo are most likely complex and require further investigations that

are beyond the scope of this manuscript.

We did not see reproducible changes in the levels of total A20 and CylD levels, but these substrates anyway show only partial cleavage by MALT1 under strong conditions of stimulation. We therefore cannot conclude whether A20 or CylD levels (or their cleavage products) might affect thymic Treg development.

On page 11 at the bottom the author write MALT1 C474A instead of C472A.

We apologize for this error that has now been corrected.

2nd Editorial Decision 14 September 2014

Thank you for submitting your revised manuscript to The EMBO Journal. The original referees have now seen your study and their comments are provided below.

As you can see below, both referees appreciate the introduced changes and support publication here. Referee #1 suggests adding some clarifications to the Treg reconstitution experiments - only text changes are needed. Once we get the revised version back in then we will accept the paper for publication here.

Congratulations on a very nice paper!

REFEREE REPORTS

Referee #1:

The authors have addressed all my concerns and changed the manuscript accordingly.
In my opinion the manuscript is ready to be published but may benefit of still adding some extra clarification related to the Treg reconstitution experiments. More specifically, the authors show that the number of Tregs in Treg reconstituted MALT1 C/A mice is even lower than in the non reconstituted mice (suppl fig E8) (1% versus 2%). The authors show, however, that of this 1%, 40% are GFP positive. Do the authors mean that all remaining Tregs in the MALT1 C/A mice are inactive, while the GFP positive cells are active and although still very low in numbers this is sufficient to completely protect against disease development. Maybe a short note on this in the manuscript would be helpful to clarify it for the readers. Also, they should indicate at what time after reconstitution efficiency was measured.

Referee #2:

This is a highly interesting manuscript. The data are very convincing and technical quality is high. All my questions have been addressed adequately. Some questions remain: Is the autoimmune gastritis driven by absence of Tregs? Are the effects on FOXP3 protein only a result of reduced transcription? In how far is enhanced IFNy involved in the pathogenesis. However, I think it is important to publish these results to stimulate further scientific discussion and research on MALT1 protease activity.

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