European Journal of Immunology

Supporting Information

for

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Tobias Haas, Simon Heidegger, Alexander Wintges, Michael Bscheider, Sarah Bek, Julius C. Fischer, Gabriel Eisenkolb, Martina Schmickl, Silvia Spoerl, Christian Peschel, Hendrik Poeck and Jürgen Ruland

Card9 controls Dectin-1-induced T-cell cytotoxicity and tumor growth in mice

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Haas Tobias, Heidegger Simon, Wintges Alexander, Bscheider Michael, Bek Sarah, Fischer Julius C., Eisenkolb Gabriel, Schmickl Martina, Spoerl Silvia, Peschel Christian, Poeck Hendrik and Ruland Jürgen

Correspondence: Prof. Dr. Jürgen Ruland, Institut für Klinische Chemie und Pathobiochemie, Klinikum rechts der Isar, 81675 München, Germany

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Review Timeline:
Submission date: 24-Oct-2016
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Handling Executive Committee member: Reis e Sousa

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- 19-Dec-2016

Dear Dr. Haas,

Please accept my sincere apologies for the prolonged delay in processing the review of your Manuscript ID eji.201646775 entitled "Card9 signaling controls Dectin-1-mediated cytotoxic T-cell priming that can protect from tumor challenge" which you submitted to the European Journal of Immunology. One of the referee reports was severely delayed and there was a difference in opinion for which we sought additional advice.

The comments of the referees are included at the bottom of this letter. Even though ref#1 suggested rejection based on the low novelty, the other referee and the Executive Editor would like to see a revised version of your manuscript that takes into account all the comments of the referees (including ref#1 comments). This edited version 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. 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 referee(s) 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,
Laura Soto Vazquez

On behalf of Prof. Caetano Reis e Sousa

Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

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Reviewer: 1

Comments to the Author

This short report by Haas et al. confirms the critical role of Card9 for Dectin-1 signaling and for linking innate recognition of Dectin-1 agonists (such as fungal stimuli, beta-glucan-1,3) to adaptive immunity. This well accepted function of Card9 has been demonstrated in particular for the priming of CD4+ T cells. Haas et al. follow a previously published study (LeibundGut-Landmann et al. 2008), which linked Dectin-1 signaling to CTL priming (without specifically assessing Card9-dependence of the response). Using the very same experimental setups, the authors show here that Card9 is required for the responses described back-then, including the antigen-specific proliferation and cytokine production by CD8+ T cells in response to OVA protein + curdlan in vitro, CTL priming in vivo in OVA+curdlan vaccinated mice and the protection from tumor challenge in these vaccinated mice. The authors further show that Dectin-1-dependent CTL

priming is independent of the inflammasome (despite the strong activation of the NLRP3 inflammasome by Dectin-1/Card9-signaling), and that tumor immunity in response to OVA+curdlan vaccination is dependent on CD8+ T cells, but independent of NK cells. While the study is well performed it does not add much new to the field.

Specific comments:

1. In Fig. 1A-C, the authors use CpG1826 and PAM3CSK in addition to Curdlan for comparison of Dectin-1, Card9 and MyD88-dependent responses without commenting on it in the text. While the response to CpG1826 and PAM3CSK is clearly MyD88-dependent, it is also at least partially Card9-dependent. Moreover, it appears that CpG1826-stimulated conditions induce IFN-g production despite very limited induction of proliferation, i.e. proliferation is even reduced compared to the “no adjuvant” condition. Moreover, the Curdlan induced response is only partially Dectin-1 dependent (although Curdlan is used as a specific Dectin-1 agonist), while at the same time it displays a partial MyD88-dependence (13.5% and 17.1% IFNg+ cells in Dectin-1 and MyD88-/- conditions compared to 24.3% for WT). In Fig. 1B, it would be helpful to see the quantification of proliferation separately from IFN-g production and also to see a summary graph for n independent samples rather than just representative plots.
2. In the legend to Fig. 1C, the authors write “Significance levels refer to the “no adjuvant group” of each respective genotype.” Is this correct?
3. Fig. 2A: Statistical significance for the Cp1826-treated Wild-type group in comparison to what?
4. Curdlan-boosted vaccination leads to dramatic reduction in tumor growth with only very small tumor volumes measured on day 20 post-challenge (Fig. 3B). Still, 50% of the animals dye within 60 days. How did tumor growth progress in OVA+Curdlan-vaccinated WT animals? Why do the authors not show tumor volumes until later time points? The conclusion provided on page 7 “We observed that Curdlan-boosted vaccination resulted in significant tumor growth delay with complete rejection of OVA-expressing tumor cells in 50% of the treated animals” is not accurate.
5. In Fig. 3D, the authors should show the depletion efficiency for the anti-CD8 and anti-NK1.1 treated animals.
6. Again in Fig. 3D, the figure legend “Curdlan + OVA + anti-CD8 + NK1.1” should be changed to “Curdlan + OVA + anti-CD8 + anti-NK1.1”.
7. DC-T cell cultures: there is no indication about the duration of the DC-T cell co-cultures.

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Reviewer: 2

Comments to the Author

In this study Haas et al demonstrate that Curdlan-induced CTL cross-priming is mediated by CARD9 and it is independent of inflammasome activation. Vaccination with OVA and Curdlan resulted in efficient cross-priming that was dependent on CARD9. This resulted in clearance of primary tumors dependent on CD8 cells, a block in formation of metastasis and increased survival. All of these effects were dependent on CARD9. It is well established that Curdlan signals through Dectin-1 and CARD9, however this has not previously been shown for Curdlan-induced cross-priming of CTLs. This study is potentially interesting; however, there are several concerns that should be addressed by the authors.

Specific comments:

Fig. 2B - The following statement describes the effect in 2B-C - â€œBoth CTL proliferation and IFN-gï€ release were mediated by DC-intrinsic Dectin-1 and downstream Card9 activation but did not require MyD88, a central adapter molecule for Toll-like receptor signaling.â€• However, from the flow plots the effect on proliferation is not clear. Please provide the data in another manner to clarify the effect on proliferation.

Fig. 2B - Please provide a graph with the replicates of the % IFN-g producing cells or number of IFN-g producing CD8 cells. There seems to be less IFN-g in the CARD9-/- and MyD88-/- cells with no adjuvant. If this is real when the replicates are graphed together then this basal reduction in the KO cells needs to be mentioned/discussed. Potentially related to this CpG stimulation resulted in reduced IFN-g production (Fig. 2B & C). Do the authors think that CpG signaling is partially dependent on CARD9 or that the cells have an intrinsic defect. This issue needs to be addressed/discussed.

Figs 2 & 3 - The number of mice are stated for these experiments in the figure legends, however they don”t state how many experiments and whether the data is representative of one experiment or cumulate from several experiments.

Fig. 3C - What statistical test was used for the survival curves - it is not clear from the statistics section or the figure legend.

There is a serious lack of a discussion in this paper. I recognize that it is a short communication but the data still needs to be discussed in relation to related papers.

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First Revision – authors' response 25-Jan-2017

Reviewer #1

This short report by Haas et al. confirms the critical role of Card9 for Dectin-1 signaling and for linking innate recognition of Dectin-1 agonists (such as fungal stimuli, beta-glucan-1,3) to adaptive immunity. This well accepted function of Card9 has been demonstrated in particular for the priming of CD4+ T cells. Haas et al. follow a previously published study (LeibundGut-Landmann et al. 2008), which linked Dectin-1 signaling to CTL priming (without specifically assessing Card9-dependence of the response). Using the very same experimental setups, the authors show here that Card9 is required for the responses described back-then, including the antigen-specific proliferation and cytokine production by CD8+ T cells in response to OVA protein + curdlan in vitro, CTL priming in vivo in OVA+curdlan vaccinated mice and the protection from tumor challenge in these vaccinated mice. The authors further show that Dectin-1-dependent CTL priming is independent of the inflammasome (despite the strong activation of the NLRP3 inflammasome by Dectin-1/Card9-signaling), and that tumor immunity in response to OVA+curdlan vaccination is dependent on CD8+ T cells, but independent of NK cells. While the study is well performed, it does not add much new to the field.

We thank the reviewer for pointing out that our study is well performed and also acknowledge that Dectin-1 signaling has previously been linked to CTL cross-priming, as mentioned in detail in our manuscript's introduction. However, we strongly disagree with the reviewer's opinion that our study does not add much new to the field, as we clearly identify the Card9 branch as the master regulator of CTL priming after Dectin-1 activation. Furthermore, to our knowledge, our study is the first to demonstrate the importance of the Dectin-1/Sky pathway for CTL priming *in vivo* using genetic models. Finally, we demonstrate that the efficacy of Curdlan-based tumor vaccines is indeed based on CTL function and independent of the NLRP3 inflammasome.

Specific comments:

1. In Fig. 1A-C, the authors use CpG1826 and PAM3CSK in addition to Curdlan for comparison of Dectin-1, Card9 and MyD88-dependent responses without commenting on it in the text. While the response to CpG1826 and PAM3CSK is clearly MyD88-dependent, it is also at least partially Card9-dependent. Moreover, it appears that CpG1826-stimulated conditions induce IFN-g production despite very limited induction of proliferation, i.e. proliferation is even reduced compared to the 'no adjuvant' condition. Moreover, the Curdlan induced response is only partially Dectin-1 dependent (although Curdlan is used as a specific Dectin-1 agonist), while at the same time it displays a partial MyD88-dependence (13.5% and 17.1% IFNg+ cells in Dectin-1 and MyD88-/- conditions compared to 24.3% for WT). In Fig. 1B, it would be helpful to see the quantification of proliferation separately from IFN-g production and also to see a summary graph for n independent samples rather than just representative plots.

We thank the reviewer for pointing out the problems with the representation of our data in Fig. 1B and 1C. Similar concerns were raised by Reviewer 2. We followed the reviewers´ suggestion and have now

analyzed intracellular FACS data as a summary graph for several independent samples and present these data now in the new Supporting Information Figure 1B (the number of independent samples is now indicated in the legend). This new representation not only underscores the central role of Card9 for Curdlan-induced CTL cross-priming *in vitro,* but also clarifies that CpG-induced CTL cross-priming, used as a control here, does not require Card9. Addition of further experimental replicates to the ELISA analysis of IFN-γ in CTL supernatants confirmed that CpG-induced CTL IFN-γ production was independent of Card9 (as depicted in new Figure 1B). Our original data presentation ("representative histogram") had raised doubts whether Card9 may have a role in TLR9 dependent CTL cross-priming, which was why we had then decided to analyze PAM3CSK as an alternative, well-established MyD88-dependent TLR agonist. Including those TLR1/2 data into the manuscript introduced unnecessary confusion, since analyzing all available data now settled this issue. We therefore decided to remove the TLR1/2 data.

According to the reviewer´s request, we now also analyzed Curdlan-induced CTL proliferation separately, which depended on Card9 and is now represented in the new Supporting Information Figure 1A.

Our current analysis further clarifies that Curdlan-induced CTL-priming in fact depends on Dectin-1 but not on MyD88, whereas CpG-induced CTL-priming is independent of Dectin-1 (IFN-γ from CTL supernatants as analyzed by ELISA in new Fig. 1B). Similar trends, albeit not always statistically significant, can be seen for these control groups in the intracellular IFN-γ stains depicted in the new Supporting Information Figure 1B.

Although analysis of antigen specific CTLs, specific lytic capacity of CTLs and *in vivo* analyses of Curdlan- vs CpG-induced CTL responses (as represented in Figures 2, 3 and Supporting Information Figure 2) are certainly most powerful to clarify any doubts from *in vitro* CTL analyses in Figure 1 which may remain, we do believe that reconsidering our data according to the reviewer´s concerns helped to improve the representation of our findings and strengthen the conclusions drawn therefrom.

2. In the legend to Fig. 1C, the authors write 'Significance levels refer to the 'no adjuvant group' of each respective genotype.' Is this correct?

We thank the reviewer for this comment. Indeed, the figure legend in its current form was wrong. Significance levels refer to the respective wild-type group. This has been clarified by brackets in the revised figure. The figure legend has been corrected accordingly.

3. Fig. 2A: Statistical significance for the Cp1826-treated Wild-type group in comparison to what?

An asterisk without brackets indicates comparison to the WT 'No adj.' group. This has been clarified in the figure legend 2A and equivalent figure legends.

4. Curdlan-boosted vaccination leads to dramatic reduction in tumor growth with only very small tumor volumes measured on day 20 post-challenge (Fig. 3B). Still, 50% of the animals dye within 60 days. How did tumor growth progress in OVA+Curdlan-vaccinated WT animals? Why do the authors not show tumor volumes until later time points? The conclusion provided on page 7 'We observed that Curdlan-boosted vaccination resulted in significant tumor growth delay with complete rejection of OVA-expressing tumor cells in 50% of the treated animals' is not accurate.

Figure 3B has been revised: The mean tumor growth is now shown until the time point when the first animal per group succumbed to tumor progression. This has been clarified in the revised figure legend. The complete tumor growth curves of each individual mouse are now shown in the new Supporting Information Figure 2A, demonstrating that indeed 6 out of 12 OVA + Curdlan-vaccinated WT mice were free of visible or palpable tumors. The overall survival in Figure 3C now shows a follow-up of 80 days.

Although our data now allow to maintain the essence of our finding, we would however rephrase the original sentence in the manuscript "*Curdlan-boosted vaccination resulted in significant tumor growth delay with complete rejection of OVA-expressing tumor cells in 50% of the treated animals*" by replacing *tumor cells* with *tumors,* since we refer to visible or palpable tumors and not to a histologic analysis.

Accordingly, we now show longer follow-ups with the CTL and NK cell depletion experiments and represented these data in the new Figure 3D as overall survival. The mean tumor growth is now shown in the new Supporting Information Figure 3A. This extended analysis did not change interpretation of the original data.

5. In Fig. 3D, the authors should show the depletion efficiency for the anti-CD8 and anti-NK1.1 treated animals.

Data showing the depletion efficiency of the anti-CD8 and anti-NK1.1 antibodies are now included as new Supporting Information Fig. 3B.

6. Again in Fig. 3D, the figure legend 'Curdlan + OVA + anti-CD8 + NK1.1' should be changed to 'Curdlan + OVA + anti-CD8 + anti-NK1.1'.

The figure legend has been corrected as mentioned by the reviewer.

7. DC-T cell cultures: there is no indication about the duration of the DC-T cell co-cultures.

The DC / T cell co-cultures were analyzed after 48 hours. This has been clarified in the revised methods section.

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Reviewer #2:

In this study Haas et al demonstrate that Curdlan-induced CTL cross-priming is mediated by CARD9 and it is independent of inflammasome activation. Vaccination with OVA and Curdlan resulted in efficient cross-priming that was dependent on CARD9. This resulted in clearance of primary tumors dependent on CD8 cells, a block in formation of metastasis and increased survival. All of these effects were dependent on CARD9. It is well established that Curdlan signals through Dectin-1 and CARD9, however this has not previously been shown for Curdlan-induced cross-priming of CTLs. This study is potentially interesting; however, there are several concerns that should be addressed by the authors.

We thank the reviewer for his positive and supportive comments and his suggestions that helped to further improve our manuscript.

Specific comments:

Fig. 2B – The following statement describes the effect in 2B-C – "Both CTL proliferation and IFN-g release were mediated by DC-intrinsic Dectin-1 and downstream Card9 activation but did not require MyD88, a central adapter molecule for Toll-like receptor signaling." However, from the flow plots the effect on proliferation is not clear. Please provide the data in another manner to clarify the effect on proliferation.

Please also see our response to reviewer #1 comment #1.

We thank the reviewer for this remark and acknowledge that the statement that "CTL proliferation and IFN-g release were mediated by DC-intrinsic Dectin-1 and downstream Card9 activation but did not require MyD88" was not an entirely accurate description of the original representation of our data. The new Supporting Information Figure 1A now depicts Card9-dependency of Curdlan-induced CTL proliferation separately. The new Figure 1B now depicts that Curdlan-induced CTL IFN-γ production depends on Dectin-1 and Card9 but not on MyD88.

However, since we do not formally show that CTL proliferation depends on Dectin-1**,** we changed the wording on page 5 of our revised manuscript to "CTL priming with robust IFN-γ release was mediated by DC-intrinsic Dectin-1 and downstream Card9 activation but did not require MyD88, a central adapter molecule for Toll-like receptor signaling".

Fig. 2B – Please provide a graph with the replicates of the % IFN-g producing cells or number of IFN-g producing CD8 cells.

Similar concerns were raised by Reviewer 1. We followed the reviewers´ suggestion and now analyzed intracellular FACS data as a summary graph for several independent samples and present these data in the new Supporting Information Figure 1B (number of independent samples now indicated in the legend). This new representation not only underscores the central role of Card9 for Curdlan-induced CTL cross-priming *in vitro,* but also clarifies that CpG-induced CTL cross-priming, used as a control here, does not require Card9.

There seems to be less IFN-g in the CARD9-/- and MyD88-/- cells with no adjuvant. If this is real when the replicates are graphed together then this basal reduction in the KO cells needs to be mentioned/discussed. Potentially related to this CpG stimulation resulted in reduced IFN-g production (Fig. 2B & C). Do the authors think that CpG signaling is partially dependent on CARD9 or that the cells have an intrinsic defect. This issue needs to be addressed/discussed.

Analyzing pooled intracellular IFN-γ stains from several experiments (new Supporting Information Figure 1B) instead of a representative histogram, proliferation of cross-primed CTLs (new Supporting Information Figure 1A) and more experimental replicates of the ELISA supernatant IFN-γ analysis of cross-primed CTLs (new Figure 1B), we can now say that there are no significant differences between activation levels of different genotypes analyzed in this study. Furthermore, according to the data in Figure 1B and Supporting Information Figure 1B, CTL cross-priming by CpG does not depend on Card9.

Figs 2 & 3 – The number of mice are stated for these experiments in the figure legends, however they don't state how many experiments and whether the data is representative of one experiment or cumulate from several experiments.

All data are either representative of at least two experiments or have been pooled of two independent experiments. This has been clarified in the revised figure legends.

Fig. 3C – What statistical test was used for the survival curves – it is not clear from the statistics section or the figure legend.

Survival was analyzed using the Log-rank test. This has been clarified in the statistics section.

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There is a serious lack of a discussion in this paper. I recognize that it is a short communication but the data still needs to be discussed in relation to related papers.

As suggested by the reviewer, we now discuss our findings in greater detail, thus adding a more refined perspective to the interpretation of our results.

Second Editorial Decision - 09-Feb-2017

Dear Dr. Haas,

It is a pleasure to provisionally accept your manuscript entitled "Card9 controls Dectin-1-induced T-cell cytotoxicity and prevention of tumor growth" 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: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). 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,
Laura Soto Vazquez

on behalf of Prof. Caetano Reis e Sousa

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European Journal of Immunology
e-mail: ejied@wiley.com
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