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IL-1 signaling is critical for expansion but not generation of autoreactive GM-CSF$^+$ Th17 cells

Ilgiz A Mufazalov, Carsten Schelmbauer, Tommy Regen, Janina Kuschmann, Florian Wanke, Laureen A Gabriel, Judith Hauptmann, Werner Müller, Emmanuel Pinteaux, Florian C Kurschus, Ari Waisman

Corresponding author: Ari Waisman & Ilgiz Mufazalov, Mainz University

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(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 30 May 2016

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

As you can see, the referees find the analysis interesting and are supportive of publication here. They raise a number of different issues that shouldn't involve too much work to sort out. In particular, referee #3 would like to see some additional insight into how IL-1 promotes expansion of pathogenic Th17 cells. Given the positive feedback, I would like to invite you to submit a suitably revised manuscript for our consideration.

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:

This elegant and interesting study by the Waisman laboratory clarifies the role of IL1 signaling in organ-specific autoimmunity. The authors show that autoreactive and pathogenic IL-17A+ GM-CSF+ CD4 T cells are expanded in vivo in response to pertussis toxin in an IL-1R-dependent process. Using a novel mouse model allowing T cell-specific deletion of IL1-R1, they further show that pathogenic autoreactive Th17 cells, and notably the IL-17A+ GM-CSF+ component, need T cell-intrinsic IL1 signaling to accumulate following immunization. This had a clear impact on EAE severity and on the accumulation of autoreactive Th17 cells within the central nervous system (CNS). Importantly, some autoreactive Th17 cells still make it to the CNS but promote modest disease owing to an elevated Treg/Th17 ratio locally. Finally, indirect data suggest that the reduced accumulation of Th17 cells in mice deficient for IL1-R1 in T cells results from impaired expansion/survival rather than from impaired generation.

This study is well performed and the manuscript logically presented. The results are important, particularly in light of the recent emphasis on IL-1 in autoimmune and autoinflammatory diseases.

The following minor concerns should be addressed in order to further strengthen the paper or improve clarity:
1- The authors should indicate whether the number of CD11b+ myeloid cells in secondary lymphoid organs is altered in mice that globally lack IL1-R1 at the steady state (i.e., without immunization).
2- Figure 2C & D and subsequent figures, the label on some figures is not clear. Specifically IFNγ+/- is ambiguous and could be changed or even removed.
3- Figure 3B: is there a decrease in the total number of MOG-specific CD4 T cells as assessed by CD40L up-regulation in IL1-R1-deficient mice (global or T cell-specific) or is the decrease selective for Th17 MOG-specific T cells? This is important to help decipher whether antigenic priming or T cell differentiation or expansion is affected in these mice.
4- Figure 4A: do the statistical analyses take into consideration repeated measures. A more global approach to EAE severity, rather than day-by-day comparisons, would seem more appropriate.
5- Figure 5C & D: is there any significant decrease in the frequency or absolute numbers of IL-17A+ CD4 T cells in IL-1R1ΔT as compared to WT mice?
6- The authors state that IL1β acts on proliferation of GM-CSF-producing Th17 cells. Could the results be instead explained by differential migration and/or survival of these cells?
7- In the last point of their discussion, the authors may want to refer and briefly discuss the data from Steve Lacroix et al. that were just published in JEM.

Referee #2:

This study shows that PTx treatment in mice immunized with MOG/CFA induces IL-1β signaling. Myeloid cells from WT mice treated with PTx show an increased IL-1β expression, while those from IL-1R1 KO mice showed only low amounts of IL-1β expression and PTx treatment had no effect in altering these levels in the KO mice.
Next, the authors focused on Th17 cells. Mice treated with PTx had higher MOG-reactive Th17 cell frequencies. In IL-1R1 KO mice, there was a major decrease in the number of these cells and PTx had no effect. Moreover, PTx treatment in WT enhances the GM-CSF co-expression by MOG-reactive Th17 cells, while this effect was absent in KO mice. Also, in a model where IL-1R1 is specifically knocked out in TCRβ+ cells, GM-CSF+ Th17 cells were reduced, but still detectable. Therefore, the authors state that the lack of IL-1β signaling is rather due to impaired proliferation instead of priming of these cells.
In complete IL-1R1 KO, EAE cannot be induced. However, for IL-1R1 KO in only TCRβ+ cells, mice showed a mild disease with a lower amount of CNS infiltrating Th17 and GM-CSF+Th17 cells compared to WT. This indicates that the presence of other cells able to react to IL-1β can still induce EAE. Finally, they show that following in vitro restimulation in the presence of IL-23, T cells from WT and IL-1R1 KO (complete and TCRβ+ cells) can induce EAE after adoptive transfer into RAG-/- mice.

Overall opinion:
This is an interesting and well-written paper. This novelty of this study is mainly that KO of IL-1R1 in TCRβ+ cells is not mandatory for EAE induction, even though this signaling pathway seems to be important for (pathogenic) Th17 development The only negative aspect is that the study has been performed solely in mice; human data would be an important addition for making the link to

pathological conditions.

A few suggestions which could improve the manuscript:
1) There is still a small number of IL-1R1+ cells present in the Kos. Therefore could this contribute to the low numbers of pathogenic Th17 cells in these mice? Thus, the conclusion that IL-1 is only important for proliferation and expansion of Th17 cells and not for their generation seems a bit strong.
2) Since MOG-Specific Th17 cells numbers are largely reduced in IL-1R1 KO mice, how many events are acquired for the MOG-specific Th17 cells
3) It seems that the effect observed is primarily seen in the MOG-specific Th17 cell population. Since the percentage in IL-1R1 KO is so low, is it still reliable to look into subset within this cell population?
4) Codarri et al. (2011) show cells co-expressing IFN-γ and GM-CSF. These cells seem also to be pathogenic. Although the IL-1R1 expression on these cells seems to be lower compared to Th17 cells, does PTx have an effect on these cells and what about the IL-1R1KO?
5) Is there a difference in total cell numbers in the spleen after EAE induction in WT and IL-1R1 KO (complete or specifically in TCRβ+ cells)?
6) Since in vitro restimulation in the presence of IL-23 seems to induce pathogenic Th17 cells, what would happen if IL-23 would be injected in MOG/CFA immunized IL-1R1 KO mice? Would this restore the pathogenic capacity of the Th cells?
7) What about IL-1β in humans? Does it induce pathogenic Th17 cells? Can in vitro blockade of IL-1β in human cells reduce the numbers of these pathogenic cells?

Referee #3:

The paper by Mufazalov et al., " IL-1 signaling is critical for expansion but not generation of autoreactive GM-CSF+ Th17 cells" reports the interleukin-1β (IL-1β) is critical for pathogenic Th17 (IL-17+GM-CSF+) cell expansion but not initiation during EAE. The authors also showed that the pathogenicity of IL-1R1 deficient Th17 cells can be fully restored by IL-23 stimulation during EAE transfer experiment. Altogether, this manuscript describes that the role of IL-1 in enhancing pathogenic Th17 cells rather than their generation.

Major critique:
This is a wonderful paper that further strengthens the role of IL-1 signaling in Th17 cells, the paper demonstrates that this is due to role of IL-1 in expanding Th17 cells. Although the experiments in this manuscript are well performed, the concept of IL-1 signaling inducing Th17 cell pathogenicity has been previously described (Caroline Sutton et al, JEM, 2006; Yeonseok Chung et al, Immunity, 2009), nevertheless the paper adds more to the existing information on the role of IL-1 in development of Th17 cells. However, further mechanistic insight into how IL-1 functions in expanding Th17 should be addressed. The question really is: what does IL-1 signaling do to induce expansion of Th17 cell but not other T cell subsets? Overall paper is well done, data presented is of good quality and strengthens the insight in the role of IL-1 in inducing Th17 cells.

1. The authors showed that IL-1R1 deficient myeloid cells don't response to PTx. It is not clear whether PTx administration induces higher frequency of myeloid cells in WT mice since the author described that impaired IL-1 signaling results in reduced myeloid cells In (Fig. 1A). If it is not, then why the deficient mice exhibit lower cell frequency per se at the basal level.

2. The authors should also determine the effect of IL-1 on other Th subsets as controls (Fig. 2A), so that they can conclude the specific role of IL-1 on Th17 cells. In addition, if the authors want to prove the antigen specificity of pathogenic Th17 cells, they should use MOG tetramers for antigen specificity.

3. The authors claim that IL-1 is critical for Th17 cell expansion but not initiation. However, it is possible that IL-1 is equally important for stability or maintenance (Fig. 5). This could be easily addressed by using an IL-17a fate reporter.

5. The authors should compare the IL-17A+GM-CSF+IFNγ+ percentage in WT, IL-1R1ΔT and IL-1R1-/- T cells after in vitro stimulation with IL-23, to determine whether IL-23 can restore the IL-

1R deficient signaling (Fig. 5).

6. The expansion of pathogenic Th17 cells should also be reflected on their increased expression of IL-23R, which the author didn't show in this manuscript. To conclude pathogenicity of IL-1R1 deficient T cells was fully restored by IL-23 polarization and expansion, the authors should show the requirement of IL-23 signaling by adoptively transferring activated T cells into Rag-/- and IL-23R-/- Rag-/- mice.

In summary, this is an interesting paper showing that IL-1 signaling induces pathogenic GM-CSF+ Th17 cell expansion but not initiation, adding one more piece to the puzzle of the role of IL-1 in differentiation of Th17 cells. Adding a mechanistic molecular insight by which IL-1 induces expansion of pathogenic Th17 cells will strengthen the paper.

1st Revision - authors' response 05 September 2016

Referee #1:

This elegant and interesting study by the Waisman laboratory clarifies the role of IL1 signaling in organ-specific autoimmunity. The authors show that autoreactive and pathogenic IL-17A+ GM-CSF+ CD4 T cells are expanded in vivo in response to pertussis toxin in an IL-1R-dependent process. Using a novel mouse model allowing T cell-specific deletion of IL1-R1, they further show that pathogenic autoreactive Th17 cells, and notably the IL-17A+ GM-CSF+ component, need T cell-intrinsic IL1 signaling to accumulate following immunization. This had a clear impact on EAE severity and on the accumulation of autoreactive Th17 cells within the central nervous system (CNS). Importantly, some autoreactive Th17 cells still make it to the CNS but promote modest disease owing to an elevated Treg/Th17 ratio locally. Finally, indirect data suggest that the reduced accumulation of Th17 cells in mice deficient for IL1-R1 in T cells results from impaired expansion/survival rather than from impaired generation.

This study is well performed and the manuscript logically presented. The results are important, particularly in light of the recent emphasis on IL-1 in autoimmune and autoinflammatory diseases.
We thank the reviewer for his/her positive response and the enthusiasm.

The following minor concerns should be addressed in order to further strengthen the paper or improve clarity:
1- The authors should indicate whether the number of CD11b+ myeloid cells in secondary lymphoid organs is altered in mice that globally lack IL1-R1 at the steady state (i.e., without immunization).
Indeed, global deficiency in IL-1 signaling resulted in lower numbers of $CD11b^{+}$ myeloid cells and in reduced IL-1β production already under steady state conditions. We have added these data now in Fig 1 new panels A-C.

2- Figure 2C & D and subsequent figures, the label on some figures is not clear. Specifically IFNγ+/- is ambiguous and could be changed or even removed.
We have now double-checked the labels of the figures and changed them according to the suggestion of the reviewer.

3- Figure 3B: is there a decrease in the total number of MOG-specific CD4 T cells as assessed by CD40L up-regulation in IL1-R1-deficient mice (global or T cell-specific) or is the decrease selective for Th17 MOG-specific T cells? This is important to help decipher whether antigenic priming or T cell differentiation or expansion is affected in these mice.
IL-1R1 deficiency resulted in reduced frequencies and numbers of total MOG-specific CD4 T cells equally in $IL\text{-}1R1^{\Delta T}$ and $IL\text{-}1R1^{-/-}$ mice (new Appendix Fig S3). This reduction was not restricted only to conventional MOG-specific Th17 cells, but also included cells expressing IFNγ and/or GM-CSF (new Fig EV4). Importantly, the majority of WT IFNγ and/or GM-CSF positive cells express either the characteristic of Th17 lineage cytokine IL-17 or represent ex-Th17 cell populations, as was shown previously by our group and others (Eur J Immunol. 2010 Dec;40(12):3336-46, Nat Immunol. 2011 Mar;12(3):255-63, J Immunol. 2016 Jun 15;196(12):4893-904). Moreover analysis of IL-1R1 expression indicated that Th17 cells express the highest levels of IL-1R1 (new Fig 2A-D).

We concluded that overall reduced numbers of MOG-specific CD4 T cells stems from impaired cell expansion during their stage of IL-1 responders (i.e. Th17 cells) and concentrated our study on Th17 cells.
Antigenic priming was not directly addressed in the current study, however the same degree of reduction of MOG-specific CD4 T cells detected in IL-1R1$^{\Delta T}$ and IL-1R1$^{-/-}$ mice indicate that this is a T cell intrinsic phenomenon, since antigen-presenting cells should not be targeted by CD4-Cre mediated recombination.

4- Figure 4A: do the statistical analyses take into consideration repeated measures. A more global approach to EAE severity, rather than day-by-day comparisons, would seem more appropriate.
We improved statistical analysis by including new data and also providing analysis of the area under the curve (new Fig 5A-C).

5- Figure 5C & D: is there any significant decrease in the frequency or absolute numbers of IL-17A+ CD4 T cells in IL-1R1ΔT as compared to WT mice?
We detected no significant differences in numbers of Th17 cells harvested from EAE diseased mice regardless of the genotypes of transferred cells. Statistical analysis is now added to the modified Fig 6K (former Fig 5D). Differences in frequencies of IL-17A^{+} CD4 T cells in IL-1R1$^{\Delta T}$ group compare to WT group were also not significant when repeated experiments were taken into consideration.

6- The authors state that IL1β acts on proliferation of GM-CSF-producing Th17 cells. Could the results be instead explained by differential migration and/or survival of these cells?
We would like to thank the reviewer for this suggestion. We addressed CCR6 expression by mutant Th17 cells and found no differences compare to WT cells (new Fig 5L-N). Together with data obtained in adoptive transfer system we speculate that IL-1 signaling is not critically involved in Th17 cells migration. Although we did not directly address effects of IL-1 signaling on survival of Th17 cells we showed an increased yield of Th17 cells when IL-1β was added *in vitro* (new Fig 6A-D). Also in another study we show that addition of IL-1 increased proliferation index of WT CD4 T cells, while cells isolated from IL-1R1$^{\Delta T}$ did not respond to IL-1β administration (PLoS One. 2016 Aug 23;11(8):e0161505). Together we concluded that IL-1 is primarily needed for the expansion (and, most likely, survival) of pathogenic CD4 T cells.

7- In the last point of their discussion, the authors may want to refer and briefly discuss the data from Steve Lacroix et al. that were just published in JEM
We are thankful for this suggestion. Now we discuss Steve Lacroix's findings within the manuscript.

Referee #2:

This study shows that PTx treatment in mice immunized with MOG/CFA induces IL-1β signaling. Myeloid cells from WT mice treated with PTx show an increased IL-1β expression, while those from IL-1R1 KO mice showed only low amounts of IL-1β expression and PTx treatment had no effect in altering these levels in the KO mice.
Next, the authors focused on Th17 cells. Mice treated with PTx had higher MOG-reactive Th17 cell frequencies. In IL-1R1 KO mice, there was a major decrease in the number of these cells and PTx had no effect. Moreover, PTx treatment in WT enhances the GM-CSF co-expression by MOG-reactive Th17 cells, while this effect was absent in KO mice. Also, in a model where IL-1R1 is specifically knocked out in TCRβ+ cells, GM-CSF+ Th17 cells were reduced, but still detectable. Therefore, the authors state that the lack of IL-1β signaling is rather due to impaired proliferation instead of priming of these cells.
In complete IL-1R1 KO, EAE cannot be induced. However, for IL-1R1 KO in only TCRβ+ cells, mice showed a mild disease with a lower amount of CNS infiltrating Th17 and GM-CSF+Th17 cells compared to WT. This indicates that the presence of other cells able to react to IL-1β can still induce EAE. Finally, they show that following in vitro restimulation in the presence of IL-23, T cells from WT and IL-1R1 KO (complete and TCRβ+ cells) can induce EAE after adoptive transfer into RAG-/- mice.

Overall opinion:

This is an interesting and well-written paper. This novelty of this study is mainly that KO of IL-1R1 in TCRβ+ cells is not mandatory for EAE induction, even though this signaling pathway seems to be important for (pathogenic) Th17 development. The only negative aspect is that the study has been performed solely in mice; human data would be an important addition for making the link to pathological conditions.

We thank the reviewer for the interest in our study and the helpful criticism. To gain insight into the importance of IL-1 signaling for human CD4 T cells we addressed the expression of IL-1R1 by cells isolated from the peripheral blood of healthy donors. We found that human Th17 cells, like murine cells, also expressed the highest levels of IL-1R1 compare to other T helper subsets (New Fig 2). Moreover, different populations of human Th17 cells represented similar to murine cells pattern of IL-1R1 expression (new Fig 2E, F). We also discuss these findings in more details below.

A few suggestions which could improve the manuscript:

1) There is still a small number of IL-1R1+ cells present in the Kos. Therefore could this contribute to the low numbers of pathogenic Th17 cells in these mice? Thus, the conclusion that IL-1 is only important for proliferation and expansion of Th17 cells and not for their generation seems a bit strong.

Indeed current Fig 4A (the modified former Fig 3A) shows that 1.38% WT CD4 T cells express IL-1R1, while in both groups of mutant mice this value decreased by log scale and doesn't exceed 0.16% which is, we believe, a background level of IL-1R1 staining. To exclude that cells, which potentially escaped CD4-Cre mediated recombination in IL-1R1$^{\Delta T}$ mice, are responsible for EAE development we performed staining on cells recovered from the inflamed CNS. We could show that MOG responding Th17 cells isolated from IL-1R1$^{\Delta T}$ and IL-1R1$^{-/-}$ EAE diseased mice are indistinguishable in terms of IL-1R1 expression (Data now included in the new Fig 7 in panels D and E).

2) Since MOG-Specific Th17 cells numbers are largely reduced in IL-1R1 KO mice, how many events are acquired for the MOG-specific Th17 cells

The reviewer refers to the analysis of peripheral MOG-specific Th17 cells recovered from IL-1R1 deficient mice, which is now presented in Fig 3A and in Fig 4B (former Fig 2A, 3B). Numbers of events detected by FACS in CD40L^{+}IL17-A^{+} gate are outlined below:

Figure 3A: WT (PBS) 329 events; WT (PTx) 700 events; IL-1R1$^{-/-}$ (PBS) 50 events; IL-1R1$^{-/-}$ (PTx) 29 events.

Figure 4B: WT 583 events; IL-1R1$^{\Delta T}$ 77 events; IL-1R1$^{-/-}$ 85 events.

3) It seems that the effect observed is primarily seen in the MOG-specific Th17 cell population. Since the percentage in IL-1R1 KO is so low, is it still reliable to look into subset within this cell population?

Indeed, acquired numbers of MOG-specific Th17 cells in the group of IL-1R1 deficient mice are low as indicated above. However, the subsequent analysis of this population showed a similar pattern of the IFNγ and GM-CSF staining in the WT and knock out mice, with low statistical variability (Fig 3C, 3D, 4B, 4D). Another way of analyzing these data would be to merge individual FACS data within given group of animals, which will make, however impossible following statistical analysis. Therefore we propose to leave the present analysis.

4) Codarri et al. (2011) show cells co-expressing IFN-γ and GM-CSF. These cells seem also to be pathogenic. Although the IL-1R1 expression on these cells seems to be lower compared to Th17 cells, does PTx have an effect on these cells and what about the IL-1R1KO?

Indeed pathogenicity of CD4 T cells is strongly correlated with IFNγ and GM-CSF expression, and such cells were underrepresented when IL-1R1 was deleted or PTx was not included in the immunization protocol (new Fig EV4). By using cell-fate reporter systems our group and others have shown previously that in the context of EAE, IFNγ and GM-CSF positive cells are mainly (or even exclusively) originated from Th17 cells (Eur J Immunol. 2010 Dec;40(12):3336-46, Nat Immunol. 2011 Mar;12(3):255-63, J Immunol. 2016 Jun 15;196(12):4893-904). Moreover, IFNγ and GM-CSF producers negative for IL-17A show dramatically reduced IL-1R1 expression compare to Th17 cells (new Fig 2A, C). Therefore we focused our research on Th17 cells, the cell type, which is mainly respond to IL-1 and represent a progenitor subset of the pathogenic T cells.

5) Is there a difference in total cell numbers in the spleen after EAE induction in WT and IL-1R1 KO (complete or specifically in TCRβ+ cells)?

We did not detect significant diffirencies in the splenic cell numbers of EAE desisead mice deficient on IL-1 signaling (new Appendix Fig S4).

6) Since in vitro restimulation in the presence of IL-23 seems to induce pathogenic Th17 cells, what would happen if IL-23 would be injected in MOG/CFA immunized IL-1R1 KO mice? Would this restore the pathogenic capacity of the Th cells?
We are thankful for this suggestion. We performed treatment of mice with IL-23 and found a stimulatory effect on EAE severity of both WT and IL-1R1$^{\Delta T}$ mice (new Fig EV5).

7) What about IL-1β in humans? Does it induce pathogenic Th17 cells? Can in vitro blockade of IL-1β in human cells reduce the numbers of these pathogenic cells?
In *in vitro* systems IL-1β was reported to have a strong stimulatory effect on pathogenic human Th17 cells generation, which was suppressed by IL-1β neutralization (Nature. 2012 Apr 26;484(7395):514-8). Moreover drugs designed to suppress IL-1 signaling show beneficial effect in clinic, which is now briefly discussed within the revised manuscript. And, as it was outlined above, we addressed IL-1R1 expression on human Th17 cells and present data in new Fig 2 in panels E and F.

Referee #3:

The paper by Mufazalov et al., " IL-1 signaling is critical for expansion but not generation of autoreactive GM-CSF+ Th17 cells" reports the interleukin-1β (IL-1β) is critical for pathogenic Th17 (IL-17+GM-CSF+) cell expansion but not initiation during EAE. The authors also showed that the pathogenicity of IL-1R1 deficient Th17 cells can be fully restored by IL-23 stimulation during EAE transfer experiment. Altogether, this manuscript describes that the role of IL-1 in enhancing pathogenic Th17 cells rather than their generation.

Major critique:
This is a wonderful paper that further strengthens the role of IL-1 signaling in Th17 cells, the paper demonstrates that this is due to role of IL-1 in expanding Th17 cells. Although the experiments in this manuscript are well performed, the concept of IL-1 signaling inducing Th17 cell pathogenicity has been previously described (Caroline Sutton et al, JEM, 2006; Yeonseok Chung et al, Immunity, 2009), nevertheless the paper adds more to the existing information on the role of IL-1 in development of Th17 cells. However, further mechanistic insight into how IL-1 functions in expanding Th17 should be addressed. The question really is: what does IL-1 signaling do to induce expansion of Th17 cell but not other T cell subsets? Overall paper is well done, data presented is of good quality and strengthens the insight in the role of IL-1 in inducing Th17 cells.
We thank the reviewer for the encouraging comments and the enthusiasm for our paper. It is always nice to be acknowledged for your own work.
We show that Th17 cells are the main cell type among CD4 T cells that express IL-1R1, which makes them responsive to IL-1 (new Fig 2). Previous studies suggested that IL-1 signaling leads to the activation of the NF-κB pathway which regulate cell survival and proliferation (reviewed in Dev Comp Immunol. 2004 May 3;28(5):415-28). In the present work we did not address directly the downstream mechanism of IL-1R1 stimulation but rather described in details the physiological outcome. Such investigations are of great interest, which we believe should result in an independent future reports.

1. The authors showed that IL-1R1 deficient myeloid cells don't response to PTx. It is not clear whether PTx administration induces higher frequency of myeloid cells in WT mice since the author described that impaired IL-1 signaling results in reduced myeloid cells In (Fig. 1A). If it is not, then why the deficient mice exhibit lower cell frequency per se at the basal level.
Non-immunized IL-1R1$^{-/-}$ mice displayed impaired development of CD11b^{+} myeloid cells (new Fig 1A-C), pointing to the crucial role of IL-1 signaling. After immunization with MOG/CFA such mice persisted to presented low numbers of myeloid cells, including those that produce IL-1β, in secondary lymphoid organs (Fig 1D-H). We concluded that IL-1 signaling is important for the development and function of CD11b^{+} myeloid cells. We apologies for not stating it clearly at the time of initial submission.

2. The authors should also determine the effect of IL-1 on other Th subsets as controls (Fig. 2A), so that they can conclude the specific role of IL-1 on Th17 cells. In addition, if the authors want to prove the antigen specificity of pathogenic Th17 cells, they should use MOG tetramers for antigen specificity.
We thank the reviewer for this suggestion. The effects of IL-1 signaling on GM-CSF and IFNγ expression are now presented in new Fig EV4. The specificity of such stimulatory effect stems from the fact that Th17 cells are the progenitor cell type for GM-CSF and IFNγ producers in the context of EAE (Eur J Immunol. 2010 Dec;40(12):3336-46, Nat Immunol. 2011 Mar;12(3):255-63, J Immunol. 2016 Jun 15;196(12):4893-904) and express the highest levels of the IL-1R1 (new Fig 2A, C).
To exclude the non-specific CD4 T cells activation during restimulation of cells *ex vivo* we performed a control experiment with the non-relevant peptide OVA added to the restimulation medium. As expected, under such conditions MOG-specific Th17 cells were not detectable (new Fig EV3).

3. The authors claim that IL-1 is critical for Th17 cell expansion but not initiation. However, it is possible that IL-1 is equally important for stability or maintenance (Fig. 5). This could be easily addressed by using an IL-17a fate reporter.
Previously we developed an alternative fate reporter system based on IL-17Fcre mediated recombination (J Immunol. 2009 Feb 1;182(3):1237-41). Culture of cells isolated from IL-17Fcre/eYFP mice did not reveal effect of IL-1 on the maintenance of Th17 cells identity in our preliminary experiments. Frequencies and numbers of MOG-specific fate mapped Th17 cells (eYFP+ cells) that lost Th17 cell signature cytokines IL-17A and IL-17F were equal in cultures supplemented with IL-1β or not (Fig R1). We are currently investigating mechanisms of Th17 cell plasticity, which is an essential part of an independent study and therefore we ask not to use this data in the current report. (Data not included in the Peer Review Process File)

5. The authors should compare the IL-17A+GM-CSF+IFNγ+ percentage in WT, IL-1R1ΔT and IL-1R1-/- T cells after in vitro stimulation with IL-23, to determine whether IL-23 can restore the IL-1R deficient signaling (Fig. 5).
We are thankful for this suggestion and we apologies for not providing these data at the time of submission. Addition of IL-23 to cell cultures increased frequencies and numbers of MOG-specific IL-1R1 deficient Th17 cells, including its GM-CSF$^+$ subset (new Fig 6D-F) and IFNγ$^+$ subset (new Fig 6G). The same culture conditions were later used in an adoptive transfer EAE.

6. The expansion of pathogenic Th17 cells should also be reflected on their increased expression of IL-23R, which the author didn't show in this manuscript. To conclude pathogenicity of IL-1R1 deficient T cells was fully restored by IL-23 polarization and expansion, the authors should show the requirement of IL-23 signaling by adoptively transferring activated T cells into Rag-/- and IL-23R-/- Rag-/- mice.
We thank the reviewer for this very interesting suggestion. However, we do not have at present the mice in our lab and could not perform the cross in a timely manner. We hope the reviewer understands the difficulties in performing this experiments and will not insist on including that in the revised manuscript.

In summary, this is an interesting paper showing that IL-1 signaling induces pathogenic GM-CSF+ Th17 cell expansion but not initiation, adding one more piece to the puzzle of the role of IL-1 in differentiation of Th17 cells. Adding a mechanistic molecular insight by which IL-1 induces expansion of pathogenic Th17 cells will strengthen the paper.
Once again, we thank the reviewer for the interest in our study.

2nd Editorial Decision 22 September 2016

Thanks for sending us your revised manuscript. The revision has now been reviewed by the three referees and their comments are provided below. As you can see, they appreciate the introduced revisions and support publication here.

I am therefore very pleased to accept the manuscript for publication here.

REFEREE REPORTS

Referee #1:

The authors have addressed satisfactorily my previous concerns by performing new experiments and via text/figure changes. This process has further increased the quality and clarity of the manuscript. I think that this paper represents an important and solid contribution in the field of T cell-mediated tissue inflammation and autoimmunity.

Referee #2:

The authors have answered all my queries. Excellent work.
Congratulation.

Referee #3:

The authors have done a wonderful job in responding all the concerns. There are no more concerns.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ari Waisman
Journal Submitted to: The EMBO J
Manuscript Number: EMBOJ-2016-94615

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA, see 1.b (below)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was chosen based on ethical limitations of unnecessary animals sacrifice. Usually experimental groups consisted of 3-5 mice, which in most cases was enough for proper statistical analysis. When necessary (and indicated) samples from different experiments were pooled together.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Animals from different experimental groups were randomly mixed in housing cages.
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	Scoring mice for EAE symptoms was performed in "blind" manner. The investigator did not know the genotype and the group (treated/untreated) of mice at a day of scoring.
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.	Sample distribution might be estimated based on plot diagrams with individual values and by indicated SD/SEM values.
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

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).	Information within supplementary files (Appendix, Table S1 and S2).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All mice were bred in-house under SPF conditions. Experiments were performed with 6-14 weeks old mice on C57BL/6 background (males and females). Detailed genetic modifications of the il1r1 locus described in a separated study (European journal of immunology, Vol. 46, pp. 912-918).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal Care and Use Committee (IACUC) from the Land of Rhineland Palatine (RLP) approved all experiments with Permit Number 23 177-07/G12-1-057.
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.	Compliance is confirmed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The study with human cells from buffy coats was performed in approval by the local ethics committee (Ethics Committee of the Landesärztekammer Rheinland-Pfalz).
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.	Donors were randomly selected healthy volunteers who participated in this study after written informed consent in accordance with the Helsinki Protocol and the Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	NA
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).	NA
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).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. 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.	NA

G- Dual use research of concern

23. 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.	NA