European Journal of Immunology

Supporting Information
for
DOI 10.1002/eji.201545800

Heather M. Grifka-Walk, David A. Giles and Benjamin M. Segal

IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

European Journal of Immunology

IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23

Grifka-Walk, Heather; Giles, David; Segal, Benjamin

Correspondence: Mr. Giles, David, BSRB 4238, 109 Zina Pitcher Place, Ann Arbor, Michigan, United States, 48109

Review Timeline:	Submission date:	14-May-2015
	First Editorial decision:	11-Jun-2015
	Revision/s received:	17-Jun-2015
	Second Editorial decision:	09-Jul-2015
	Accepted:	22-Jul-2015

Handling Executive Committee member: Prof. Britta Engelhardt

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 - 11-Jun-2015

Dear Mr. Giles,

Manuscript ID eji.201545800 entitled "IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

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

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.**

European Journal of Immunology

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

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

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

Yours sincerely,
Katharina Schmidt

On behalf of
Prof. Britta Engelhardt

Dr. Katharina Schmidt
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com

www.eji-journal.eu

Get the latest articles delivered directly to your desktop ! Register now for the free Wiley Alerting Service at
http://onlinelibrary.wiley.com/myprofile/alertManager

****

Reviewer: 1

Comments to the Author
The main finding described in this manuscript is that IL-23 is not required for EAE development in the adoptive EAE model. In this model, myelin-specific Th cells were re-activated in vitro in the presence of IL-12; and were thus polarized in Th1 lineage. The authors do not reference a paper by Thakker et al. (J. Immunol. 2007 Feb 15;178(4):2589-98.) that addresses questions similar to those in the current manuscript. Thakker et al. report that Th cells that developed in IL-23p19-/- mice, then were re-activated in

European Journal of Immunology

vitro in the presence of IL-12 had markedly reduced encephalitogenicity upon adoptive transfer into WT recipients, compared with Th cells that originate from WT donors. It appears that the authors of the current manuscript did not observe reduced encephalitogenicity of Th cells that developed in the absence of IL-23 signaling. The manuscript would be improved by mentioning relevant findings from other groups. Even though the novelty and significance of the findings described in the manuscript are somewhat limited, they can nevertheless be a useful addition to the field. The manuscript is well written and the findings clearly presented.

Reviewer: 2

Comments to the Author

1. In this study, CFA was used during the immunization. In contrast, previous studies from this lab with similar ex vivo differentiation have used IFA. TLR stimulation undoubtedly contributes to the differentiation of the T cells via APC activation. Was the data different with CFA versus IFA in regards to ex vivo cytokine production following activation with IL-12 or IL-23 in the IL-12p40 T cells?
2. For figure 1A, the text states that the lymph nodes cells were stimulated â€˜ex vivo with antigen plus either recombinant IL-12 or IL-23â€™. However, the figure legend indicates that the cells were stimulated with IL-12 and IFNg (Th1) or IL23, IL-1a and anti-IFNg (Th17). The sentence in the text is misleading because the ex vivo manipulation is more extensive than indicated. What time post-stimulation was flow cytometry performed in Fig. 1A?
3. Why is the term â€˜bona fideâ€™ Th1 cells used?
4. At what time point post-stimulation was tbet and rorc mRNA levels measured in Figure 1B?
5. In the rest and rechallenge experiments, splenocytes from what mice were used for the APCs?
6. It would be beneficial to see the EAE courses for longer than 15 days to see if there are differences in course, not just disease onset.
7. It is unclear how cytokine levels were measured in the CNS. What is exactly meant by â€˜supernatants of CNS homogenatesâ€™?
8. There seem to be several controls missing from these experiments. For example, in Figure 2A, there should have been a condition in which lymph node cells did not receive IL-12 or IL-23. These controls should have been used for all the experiments, including the flow cytometry, real-time PCR and cytokine multiplex bead assay.
9. There are numerous study not cited that are relevant to this study. The low number of references may be due to limitations of a short communication.
10. The authors should discuss in more detail why the data in other papers is potentially flawed, given the contradictory results.
11. This is an interesting study. However, more controls are necessary to draw firm conclusions, particularly given the number previous studies that have contradictory data.

European Journal of
Immunology

Reviewer: 3

Comments to the Author
Grifka-Walk et al investigated the need of IL-23 for pathogenicity of T cells in a murine model of multiple sclerosis, EAE. As the title of their article states, they could show that Th1 cells generated under conditions where IL-23 signaling was missing were able to induce EAE in mice, thus showing that it is possible to induce this disease without IL-23 signaling.
There are some other points in the paper, but I think the authors will agree with me that the disease in the absence of IL-23 signaling is really the major one.
Now, we can ask if this point by itself is sufficient to make this manuscript important enough for publication in the EJI. I am afraid I cannot agree to that, since the author do not really show much more than that. They do claim that the IL-23-less EAE is characterized by monocyte-rich CNS infiltrates and has more proinflammatory cytokines, but in my opinion this is only the consequence of stronger EAE, meaning quantitative difference and not qualitative one.
What would make this work more important? I will list here some points, but the major one, or the critical one in my mind is #1.

1. What about plasticity? The authors refer to the paper of Hirota et al from 2009 (similar paper appeared also in the EJI in 2008), but do not contribute much beyond it. Do T cells need to be first TH-17 to become the â€œrealâ€• pathogenic Th1 cells? The authors here show that one can bypass the need for IL-23 if use passive disease, but what about TH-17 cells? The only way to show that is to use the technique used by Hirota or Kurschus and cross an IL-17-Cre mouse with a reporter mouse and the IL-23 receptor deficient animals, and observe if the highly pathogenic Th1 cells express the reporter gene, namely they were at an earlier stage IL-17 producers or not. The original papers on TH-17 did not include IL-23, but only TGF beta and IL-6. Maybe these cytokines are sufficient to drive the generation of TH-17 that then become the really pathogenic Th1 cells?
2. I am not sure what exactly figure 3A shows. Is that really spinal cord? What is the green area around the spine? Is that bone? Why is it green? In my opinion the MBP staining just did not work.
3. In figure 3B, what is the rest of the CD45+CD11b+ cells? Microglia? Please show the FACS plots also, and clarify exactly which cells are present in the inflamed CNS, show separately brain and spinal cord.
4. Do TH-17 cells lacking IL-12 signaling able to induce disease upon passive transfer or not? According to figure 2D, yes, but 3D, no. Please clarify.

European Journal of Immunology

First Revision – authors' response – 17-Jun-2015

Dear Editors,

Enclosed please find a revised version of our paper entitled "IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23". We have considered the reviewers' valuable and constructive comments, and have made changes to the manuscript, based on their suggestions. Changes from the original version are underlined in the revised manuscript. Below is a point-by-point reply to each point that was raised in the critique:

Reviewer 1:
"… The authors do not reference a paper by Thakker et al. (J. Immunol. 2007 Feb 15;178(4):2589-98.) that addresses questions similar to those in the current manuscript. Thakker et al. report that Th cells that developed in IL-23p19-/- mice, then were re-activated in vitro in the presence of IL-12 had markedly reduced encephalitogenicity upon adoptive transfer into WT recipients, compared with Th cells that originate from WT donors. It appears that the authors of the current manuscript did not observe reduced encephalitogenicity of Th cells that developed in the absence of IL-23 signaling. The manuscript would be improved by mentioning relevant findings from other groups. Even though the novelty and significance of the findings described in the manuscript are somewhat limited, they can nevertheless be a useful addition to the field. The manuscript is well written and the findings clearly presented."

RESPONSE: We appreciate Reviewer 1's comments that our study is "well-written" and would be a "useful addition to the field." The reviewer noted that we did not cite a pertinent study by Thakker et al. (2007). We thank Reviewer 1 for bringing this reference to our attention. We have cited that paper in the revised manuscript (reference #18) and discussed its findings in the Results section under the heading "Th1 cells can induce EAE independent of IL-23" (page 6, second paragraph).

Reviewer 2:

The comments of Reviewer 2 were numerated and will be addressed as such below:
1) "In this study, CFA was used during the immunization. In contrast, previous studies from this lab with similar ex vivo differentiation have used IFA. TLR stimulation undoubtedly contributes to the differentiation of the T cells via APC activation. Was the data different with CFA versus IFA in regards to ex vivo cytokine production following activation with IL-12 or IL-23 in the IL-12p40 T cells?"

RESPONSE: Previous studies from our lab that used IFA to immunize donors in adoptive transfer models were done with SJL mice. In contrast, induction of clinical EAE in C57BL/6 mice via adoptive transfer requires that donors be immunized with CFA for the generation of encephalitogenic effector cells. IFA primed, MOG-specific T cells from C57BL/6 donors are innocuous, even after polarization with IL-12 or IL-23 ex vivo (unpublished data). We recently demonstrated that C57BL/6 mice primed with MOG35-55 emulsified in IFA do not properly activate and mobilize myeloid cells, a critical step in EAE induction (Rumble et al., J Exp Med 2015). Since our KO mice and littermate controls are on a C57BL/6 background, we focused the experiments in this study on CFA primed effector T cells.

2) "For figure 1A, the text states that the lymph nodes cells were stimulated 'ex vivo with antigen plus either recombinant IL-12 or IL-23'. However, the figure legend indicates that the cells were stimulated with IL-12 and IFNg (Th1) or IL23, IL-1a and anti-IFNg (Th17). The sentence in the text is misleading because the ex vivo manipulation is more extensive than indicated. What time post-stimulation was flow cytometry performed in Fig. 1A?"

RESPONSE: The main text has been revised to indicate the cocktail of cytokines that were used for polarization (page 5). The amount of time that cells had been cultured at the time of harvest and the amount of time that they were subsequently stimulated with PMA/ionomycin ex vivo is now specified in the figure legend.

3) "Why is the term 'bona fide' Th1 cells used?"

RESPONSE: The term "bona fide" was used to indicate Th1 cells that are never exposed to IL-23 signaling. We recognize the ambiguity of this term and have replaced it throughout the manuscript with "IL-23-

European Journal of Immunology

independent Th1 cells".

4) "At what time point post-stimulation was tbet and rorc mRNA levels measured in Figure 1B?"

RESPONSE: The time point of transcription factor analysis is now specified in the figure legend.

5) "In the rest and rechallenge experiments, splenocytes from what mice were used for the APCs?"

RESPONSE: We have expanded the legend for Figure 1 to clarify this point. IL-12p40-/- cells were re-activated with IL-12p40-/- T-depleted splenocytes. IL-23R-/- and IL-12Rb2-/- T cells were re-activated with WT T-depleted splenocytes.

6) "It would be beneficial to see the EAE courses for longer than 15 days to see if there are differences in course, not just disease onset."

RESPONSE: We understand the benefit of extended disease courses; however, given the severity of paralysis induced by IL-23-independent Th1 cells, we were required to end our disease courses after 15 days in accordance with vivarium regulations.

7) "It is unclear how cytokine levels were measured in the CNS. What is exactly meant by 'supernatants of CNS homogenates'?"

RESPONSE: The Materials and Methods have been clarified to describe the "supernatants" used in CNS multiplex analysis.

8) "There seem to be several controls missing from these experiments. For example, in Figure 2A, there should have been a condition in which lymph node cells did not receive IL-12 or IL-23. These controls should have been used for all the experiments, including the flow cytometry, real-time PCR and cytokine multiplex bead assay."

RESPONSE: We and others have previously found that myelin-primed T cells reactivated under neutral conditions are incapable of inducing EAE (Shevach et al, 1999; Kroenke et al, 2008). We confirmed this in our IL-12p40-/- system (Supp Fig 2). The non-polarized T cells do not trigger inflammatory infiltration of the CNS, so it is not possible to repeat the flow cytometry studies shown in Figure 2 with that control. For this short communication, we chose to use IL-12-independent Th17 cells as a foil to the IL-23-independent Th1 cells.

9&10) "There are numerous study not cited that are relevant to this study. The low number of references may be due to limitations of a short communication." "The authors should discuss in more detail why the data in other papers is potentially flawed, given the contradictory results."

RESPONSE: We are limited by the space restrictions; however in light of reviewer comments, we have included a discussion of Thakker et al. (2007) and comments regarding the novelty of our data in light of previous reports (pages 4 and 6).

11) "This is an interesting study. However, more controls are necessary to draw firm conclusions, particularly given the number previous studies that have contradictory data."

RESPONSE: We appreciate the reviewer's observation that this is an "interesting study"; however, we contend the point that our results directly contradict those of previous studies. We acknowledge that, in contrast to our Th1 adoptive transfer paradigm, IL-23 is required for induction of EAE in C57BL/6 mice via active immunization. However, there are many factors that distinguish active immunization from adoptive transfer, including the administration of Bordetella pertussis toxin in the former. It is possible that insufficient quantities of IL-12 are generated in the microenvironment where myelin-reactive T cells undergo priming in actively immunized C57BL/6 mice to efficiently drive the differentiation of encephalitogenic effectors when IL-23 is absent. If so, ex vivo expansion in the presence of IL-12 may rescue the deficit. Irrespective of that possibility, a major point made in this study is that IL-23 is not universally required for induction of EAE, as illustrated using our particular experimental protocol. We do not claim that it is dispensible in every EAE model.
To our knowledge, this study is the first to use a Th1 adoptive transfer protocol in which IL-23 signaling in absent at every stage of disease. As mentioned above, we have now included a control in Supplementary

Figure S1, showing that MOG-primed cells derived from IL-12p40-/- system fail to induce EAE following ex vivo antigenic challenge under neutral conditions. Hence, in that paradigm polarization with either IL-12 or IL-23 is critical for the acquisition of encephalitogenicity.

Reviewer 3:

The (authors) claim that the IL-23-less EAE is characterized by monocyte-rich CNS infiltrates and has more proinflammatory cytokines, but in my opinion this is only the consequence of stronger EAE, meaning quantitative difference and not qualitative one.

It is true that we found EAE induced by IL-23 independent Th1 cells it be more severe than EAE induced by IL-12 independent Th17 cells in general. However, we deliberately matched mice between the 2 groups based on clinical scores for the experiments in Figure 3 A-C. This is specified in the Figure legend. Therefore we do not believe that the relatively high percentage of monocytes in CNS infiltrates and higher levels of certain cytokines in mice with Th1 mediated EAE is simply the consequence of more severe disease.

1) "What about plasticity? The authors refer to the paper of Hirota et al from 2009 (similar paper appeared also in the EJI in 2008), but do not contribute much beyond it. Do T cells need to be first TH-17 to become the "real" pathogenic Th1 cells? The authors here show that one can bypass the need for IL-23 if use passive disease, but what about TH-17 cells? The only way to show that is to use the technique used by Hirota or Kurschus and cross an IL-17-Cre mouse with a reporter mouse and the IL-23 receptor deficient animals, and observe if the highly pathogenic Th1 cells express the reporter gene, namely they were at an earlier stage IL-17 producers or not. The original papers on TH-17 did not include IL-23, but only TGF beta and IL-6. Maybe these cytokines are sufficient to drive the generation of TH-17 that then become the really pathogenic Th1 cells? "

RESPONSE: In the paper by Hirota et al. mentioned above, the authors crossed their IL-17-cre fate mapping reporter mice to an IL-23p19 deficient background, and the progeny were actively immunized with MOG in CFA. They found that very few cells in the draining lymph nodes of MOG-primed IL-23p19 deficient mice (as opposed to IL-23 sufficient mice) expressed IL-17 at any point. They concluded that the development of exTh17 cells is IL-23 dependent (Hirota, et al. Nature Immunology, 2011, 12:255-263). Therefore, we think it is unlikely that the IL-23 independent, IL-12 polarized T cells used in our experiments are exTh17 cells. Consistent with this contention, we now an include additional experiment showing that MOG-primed CD4+ T cells recovered from IL-23-deficient mice fail to express rorc, a factor critical for early Th17 cell development, at any point during polarization ex vivo (Supplementary Figure 1). We feel that it is beyond the scope of this study to cross the their IL-17-cre fate mapping reporter mice to our different knock-out mice .

2) "I am not sure what exactly figure 3A shows. Is that really spinal cord? What is the green area around the spine? Is that bone? Why is it green? In my opinion the MBP staining just did not work."

RESPONSE: Figure 3A shows a spinal cord within the intact spinal column. This preparation is critical to preserve the integrity of the perivascular and meningeal immune infiltrates. The stained regions pointed out by the reviewer are descending nerves that are myelinated before exiting the spinal column. The bone is the unstained region between the nerves and DAPI-intense bone marrow on the edges of the section.

3) "In figure 3B, what is the rest of the CD45+CD11b+ cells? Microglia? Please show the FACS plots also, and clarify exactly which cells are present in the inflamed CNS, show separately brain and spinal cord."

RESPONSE: Our flow gating strategy for each figure has been added to the supplement. The remainder of the CD45+ CD11b+ cells are Ly6G+ neutrophils and MHC-II(lo) monocytes and dendritic cells. Due to space restrictions and given the spinal cord-centric nature of the animal model, we have chosen to represent only the spinal cord.

4) "Do TH-17 cells lacking IL-12 signaling able to induce disease upon passive transfer or not? According to figure 2D, yes, but 3D, no. Please clarify."

RESPONSE: While IL-12-independent Th17 cells are encepahlitogenic they are not as potent as their IL-23 indpendent Th1 counterparts, as reflected in both reduced incidence and reduced severity. This is consistent across both figures.

We are hopeful that the revised manuscript is now acceptable for publication in the European Journal of Immunology.

Thank you for your consideration.

Sincerely,

Benjamin M. Segal, MD
Holtom-Garrett Professor of Neurology
Director, UM Multiple Sclerosis Center

Second Editorial Decision - 09-Jul-2015

Dear Mr. Giles,

It is a pleasure to provisionally accept your manuscript entitled "IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23" 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,
Katharina Schmidt

on behalf of
Prof. Britta Engelhardt

Dr. Katharina Schmidt
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com

European Journal of
Immunology

www.eji-journal.eu