Peer review correspondence

European Journal of Immunology

Highly polarized Th17 cells induce EAE via a T-bet-independent mechanism

Heather M. Grifka-Walk, Stephen J. Lalor and Benjamin M. Segal

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Handling Executive Committee member: Prof. Rikard Holmdahl

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 – 4 June 2013

Dear Ben,

Manuscript ID eji.201343723 entitled "Stable Th17 cells induce EAE via a T-bet-independent mechanism" 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. You should also pay close attention to the editorial comments included below.

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.

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

Best wishes
Cate

On behalf of
Prof. Rikard Holmdahl

Dr. Cate Livingstone
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Reviewer: 1
Comments to the Author
Segal and colleagues describe a Tbet independent pathway to develop EAE. Tbet was long considered the 'TH1-master transcription factor'. Of course the term is misleading, but clearly Tbet was widely held responsible for the development of TH1 immune responses. Tbet was also previously claimed to be critical for the development of EAE and other auto-inflammatory diseases. Here they show that it is not absolutely essential and that T cells lacking Tbet are able to produce the pathogenic cytokine GM-CSF. This is an extension of the recent work by Duhen et al. which already demonstrated that Tbet is redundant in the development of EAE. The work here represents a clarification of some misconceptions about the necessity of transcription factors and polarization patterns for the development of a T cell driven autoimmune response. I am very much in favor of publishing this work, but I have some issues, which should be fixed before.

Major points:

1. Figure 2c is very unconvincing. I doubt that there is any GM-CSF producing cell detected here and the gate stats (0.75 vs 1.3) are not convincing at all. 2A shows that 10% of wt T cells make csf2, whereas this is reduced by 50% in tbetnull mice. This does not compare with the data in 2C. Why would it be the opposite after in vitro culture.? They go on and claim that the 'phenotype is maintained', but the data do not support this at all. The group has contributed to bring about the paradigm shift in understanding that GM-CSF rather than IL-17/IFNg promotes encephalitogenicity. So why are most figures dedicated to IL-17? In Fig 2A, they should also show a representative plot for GM-CSF rather than IFNg/IL-17.

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2. While primarily a matter of aesthetics, Fig1a is the one to convince readers of the redundancy of Tbet in the induction of EAE. It's hence a very important figure for their main message. The disease course must be run at least into some days after the peak to make this figure convincing.

Minor points:
1. Lastly, in the adoptive transfer experiments (Fig 3), they find that tbet-deficient T cells cause less severe disease than wt T cells. This should be discussed. Is that because there is a less GM-CSF (as shown in Fig 2?).
2. The title is misleading. The authors insinuate that TH17 cell stability has something to do with the encephalitogenicity of Tbet-deficient T cells. What they demonstrate is however that loss of Tbet somewhat promotes the TH17 phenotype. WT TH17 cells simply are unstable as demonstrated by fate mapping (they cite the correct references here). In order to demonstrate stability, genetic tagging and fate mapping is absolutely required. The use of a congenic marker will label the tbet gene, but not the fate of former IL-17 expressing cells. Their data do demonstrate that the loss of tbet leads to a stronger TH17 phenotype, which means that tbet is a negative regulator for TH17 polarization. TH17 stability is thus at best a consequence of the Tbet loss and not the other way around.

Reviewer: 2
Comments to the Author
Grifka-Walk et al report here that T-bet is not essential for EAE induced by Th17 cells. This is in contrast to previous studies (for example Bettelli, E et al 2004 JEM) which implicated an essential role of T-bet in EAE. In the absence of T-bet, Th17 cells are enriched both in the spleen and CNS of EAE mice without converting to IFN-g producing cells. These so called “stabilized” Th17 cells which were unable to convert to IFN-g producers mediated EAE equally.

New findings reported in this study indicate numerous mechanisms employed by the distinct T cell subsets to mediate autoimmune inflammation. This is an important study which has direct implications in using T-bet as a target to suppress brain autoimmunity.

I have following comments that should be addressed.
Comments:
• Although disease incidence was not affected, there was a huge difference in the disease scores of T-bet-/- T cell recipients (Figure 3B, 3D) suggesting a possible role for T-bet. Authors should properly address this issue and should discuss in their discussion.

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• From Figure 1D, T-bet deficient Th17 cells don't seem to produce GM-CSF. Medium value is similar to MOG peptide but in the results section reads as "… GM-CSF recall responses were comparable". This data doesn't add any significance to the story unless if it is proven that GM-CSF play or don't play any role in this experimental setting.

• Throughout the manuscript it is described as "stabilized Th17 cells", but the in vitro experiments in Figure 2 is too short (only 96 hours of primary stimulation and restimulation afterwards). Do Th17 cells maintain this phenotype even after extended culture period? Figure 2B can be removed.

• Figure 1d: T-bet-/- mice cells seems to produce lots of IFNg after MOG peptide stimulation. What is the explanation for this?

Reviewer: 3

Comments to the Author

In the manuscript by Grifka-Walk et al. "Stable Th17 cells induce EAE via a T-bet independent mechanism" the authors investigated the role of T-bet in the development of EAE. In particular, they addressed the question if Th17 cells once polarized toward the Th1 phenotytpe (ex-Th17 cells) acquire enhanced encephalalitogenic properties. The manuscript is of interest, I have few comments that need to be addressed:

Comments:

Fig.1: The frequency of CD4+IFN-g-producing cells in WT is 10 fold higher than that present in T-bet-/-, mice (panel C), while the amounts of IL-17 and IFN-g produced in response to anti-CD3 plus PMA are comparable between WT and T-bet-/- mice, do the authors have an explanation (panel D)? Is this the case also for CNS derived MNC? Did the authors evaluate the presence of CD8+IFN-g producing cells in CNS?

Fig. 2E: the plots related to the expression of activation markers need to be labelled (WT and T-bet-/-). Do the author have an explanation for the appreciable proliferation of the cells in response to medium alone? Moreover the CFSE dilution seems to be higher in WT cells when compared to T-bet-/- ones.

Fig. 3B: These are very impressive data that need to be better addressed in the manuscript

First Revision – authors' response – 2 July 2013

Reviewer: 1

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Major points:

„Figure 2c is very unconvincing. I doubt that there is any GM-CSF producing cell detected here and the gate stats (0.75 vs 1.3) are not convincing at all. 2A shows that 10% of wt T cells make csf2, whereas this is reduced by 50% in tbet null mice. This does not compare with the data in 2C. Why would it be the opposite after in vitro culture.? They go on and claim that the .phenotype is maintained., but the data do not support this at all. The group has contributed to bring about the paradigm shift in understanding that GM-CSF rather than IL-17/IFNg promotes encephalitogenicity. So why are most figures dedicated to IL-17? In Fig 2A, they should also show a representative plot for GM-CSF rather than IFNg/IL-17.

Response: The dot plot in Figure 2 A is gated on CD4+ T cells that were stimulated with PMA/ ionomycin in the presence of brefeldin for 6 hours prior to intracellular staining with cytokine-specific antibodies. In contrast, Figure 2C shows cytokine expression following 24 hours of stimulation with MOG peptide; brefeldin was added for the last 6 hours. This is now made clear in Figure legend 2 of the revised version. We find that the former protocol provides a stronger stimulus for upregulation of GM-CSF. In our experience, MOG-specific GM-CSF production is optimally detected when measured in supernatants by ELISA or Luminex assay after 48-72 hours of antigenic stimulation. Consequently, we have replaced the dot plots for GM-CSF and TNFƒÑ in Figure 2C with Luminex data (Supporting Information Fig. 1 in the revised manuscript). We believe that this change has improved the manuscript.
Based on the reviewer¡¦s recommendations we have added a representative plot for GM-CSF and TNFƒÑ in Figure 2A.

„While primarily a matter of aesthetics, Fig1a is the one to convince readers of the redundancy of Tbet in the induction of EAE. It's hence a very important figure for their main message. The disease course must be run at least into some days after the peak to make this figure convincing.

Response: We agree with the reviewer¡¦s comments. However, the majority of mice in our experiments are paralyzed by day 16 post-immunization and must be euthanized at that point according to the regulations of our animal care facility. Nonetheless, we believe that the data shown supports our conclusion that Tbet deficient mice remain susceptible to EAE following active immunization with myelin antigens.
Minor points:

„Lastly, in the adoptive transfer experiments (Fig 3), they find that tbet-deficient T cells cause less severe disease than wt T cells. This should be discussed. Is that because there is a less GM-CSF (as shown in Fig 2?).
Response: We have added a paragraph to the Discussion section that addresses possible mechanisms underlying diminished encephalitogenicity of Th17 polarized Tbetdeficient

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effector cells (including the role of GM-CSF).

„The title is misleading. The authors insinuate that TH17 cell stability has something to do with the encephalitogenicity of Tbet-deficient T cells. What they demonstrate is however that loss of Tbet somewhat promotes the TH17 phenotype. WT TH17 cells simply are unstable as demonstrated by fate mapping (they cite the correct references here). In order to demonstrate stability, genetic tagging and fate mapping is absolutely required. The use of a congenic marker will label the tbet gene, but not the fate of former IL-17 expressing cells. Their data do demonstrate that the loss of tbet leads to a stronger TH17 phenotype, which means that tbet is a negative regulator for TH17 polarization. TH17 stability is thus at best a consequence of the Tbet loss and not the other way around.

Response: We agree with the reviewer¡¦s comments and did not intend did not to imply that Tbet loss is the consequence of TH17 stability. Therefore we have removed the word ¡§stable¡¨ from the title and replaced it with ¡§highly polarized¡¨.

Reviewer: 2
Although disease incidence was not affected, there was a huge difference in the disease scores of T-bet-/- T cell recipients (Figure 3B, 3D) suggesting a possible role for T-bet. Authors should properly address this issue and should discuss in their discussion.

Response: We have added a paragraph to the Discussion section that addresses the role of T-bet in enhancing the pathogenicity of Th17 polarized myelin-reactive T cells.

From Figure 1D, T-bet deficient Th17 cells don.t seem to produce GM-CSF. Medium value is similar to MOG peptide but in the results section reads as ¡§¡K GM-CSF recall responses were comparable¡¨. This data doesn.t add any significance to the story unless if it is proven that GM-CSF play or don.t play any role in this experimental setting.

Response: We appreciate the reviewer bringing this point to our attention. We have reproducibly found high background levels of GM-CSF in 72 hour cultures of MOG primed Tbet-/- T cells, which obscure antigen-specific responses. Therefore, we have removed the panel showing GM-CSF expression from the Figure and references to it from the text. While experiments assessing the role of GM-CSF in EAE induced by WT and Tbet-/- Th17 cells are underway in our laboratory, we feel that they go beyond the scope of the current study.

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Throughout the manuscript it is described as ¡§stabilized Th17 cells¡¨, but the in vitro experiments in Figure 2 is too short (only 96 hours of primary stimulation and restimulation afterwards). Do Th17 cells maintain this phenotype even after extended culture period? Figure 2B can be removed.

Response: Of note, in Figure 2C the T cells were cultured with antigen and polarizing factors for 96 hours, rested for 96 hours and then restimulated with antigen for 24 hours prior to flow cytometry. We apologize that this was not clear in the Figure legend and the detailed protocol is now explicitly described in the revised manuscript. We also consider the Tbet-/- Th17 cells stable since, in comparison to WT cells, they retain high levels of IL-17 and low levels of IFNf× in the CNS 13-26 days post transfer into naive recipients (Fig. 3C and E).
We prefer to keep panel B in Figure 2 based on the remarks of the other reviewers.

Figure 1d: T-bet-/- mice cells seems to produce lots of IFNg after MOG peptide stimulation. What is the explanation for this?

Response: Previous studies have shown that Tbet-/- mice are capable of mounting substantive CD4+ T cell IFNf×$f$nresponses (ex. Way SS and Wilson CB. J Immunol. 2004 Nov 15;173(10):5918-22.) Th17 polarized Tbet-/- CD4+ T cells give rise to IFNg+IL-17+ double positive cells upon antigenic challenge ex vivo (Duhen R., et al., J Immunol. 2013 May 1;190(9):4478-82) which, along with NK cells, might be the source of IFNf× in our splenocyte cultures. Importantly, we consistently found that levels of IFNg were lower in Tbet-/- splenocyte cultures than in WT splenocyte cultures, as illustrated in Figure 1D.

Reviewer: 3

„Fig.1: The frequency of CD4+IFN-g-producing cells in WT is 10 fold higher than that present in T-bet-/-, mice (panel C), while the amounts of IL-17 and IFN-g produced in response to anti-CD3 plus PMA are comparable between WT and T-bet-/- mice, do the authors have an explanation (panel D)? Is this the case also for CNS derived MNC? Did the authors evaluate the presence of CD8+IFN-g producing cells in CNS?

Response: Figure 1 C shows intracellular cytokine expression in CD4+ T cells following 6 hours of stimulation with PMA/ ionomycin ex vivo. Figure 1 D shows levels of cytokines that accumulated in supernatants during four days of culture in the presence of PMA/fÑCD3. In the latter situation, cytokine levels represent a steady state that reflects the balance between rates of secretion and consumption /degradation over a several day timespan. We speculate that, since Tbet-/- cells express low levels of IFNf× receptor, they might not internalize IFNg to the same extent as WT cells. On the other hand, the

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flow cytometric data reflects intracellular stores at the time of harvest which, we believe, is more representative of Th phenotype in vivo.
We have not measured cytokine responses of CNS derived MNC following stimulation with anti-CD3 plus PMA. (For various experiments we have used PMA / ionomycin or MOG peptide). We only find a small percent of CD8+ T cells in the CNS of MOG-primed C57BL/6 mice with EAE.

„ Fig. 2E: the plots related to the expression of activation markers need to be labelled (WT and T-bet-/-). Do the author have an explanation for the appreciable proliferation of the cells in response to medium alone? Moreover the CFSE dilution seems to be higher in WT cells when compared to T-bet-/- ones.

Response: For the revised manuscript we have labeled the plots (WT and Tbet-/-) in the plots in Figure 2E.
The CFSE data in Fig. 2E represent T cell proliferation following priming in vivo, primary antigenic challenge ex vivo, rest and rechallenge. Although the cell lines are washed and reconstituted with naive T-depleted splenocytes prior to the resting phase, we suspect that small numbers of contaminating APC bearing MOG peptide/ Class II complexes might be carried over and stimulate background proliferation. In repeated experiments, there was no statistically significant difference in CFSE dilution between WT and Tbet-/- T cells.

„Fig. 3B: These are very impressive data that need to be better addressed in the manuscript

Response: As mentioned in our responses to reviewers 1 and 2, in the revised manuscript we address the observation that encephalitogenic Tbet-/- effector cells are less potent in disease induction than their WT counterparts.

Second Editorial Decision – 12 July 2013

Dear Dr. Segal,

It is a pleasure to provisionally accept your manuscript entitled "Highly polarized Th17 cells induce EAE via a T-bet-independent mechanism" 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 (copyright forms etc.) 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

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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,
Karen Chu

On behalf of Prof. Rikard Holmdahl

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