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Peer review correspondence

miR-148a is upregulated by Twist1 and T-bet and promotes Th1-cell survival by regulating the proapoptotic gene Bim

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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 – 16 April 2014

Dear Dr. Mashreghi,

Please accept my sincere apologies for the prolonged delay in processing the review of your Manuscript ID eji.201444633 entitled "miR-148a is upregulated by Twist1 and T-bet and promotes the survival of T helper 1 cells by regulating the proapoptotic gene Bim", which you submitted to the European Journal of Immunology has been reviewed. One of the referee reports was severely delayed and we therefore sought additional assessment. The comments of the referees are included at the bottom of this letter.

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European Journal of Immunology

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. 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. David Gray

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Reviewer: 1

Comments to the Author
The manuscript by Haftmann et al. demonstrated a role of miR-148a in promoting the survival of Th1 cells through targeting Bim. Moreover, the authors have shown that both Twist and T-bet are required for the induction of miR-148a in repeatedly stimulated Th1 cells. Specifically, by using ChIP approach, it was shown that Twist can directly bind to the upstream of the miR-148a gene, implying that Twist can directly promote the expression of miR-148a in Th1 cells. Overall, the study was performed in a straightforward

European Journal of Immunology

manner and the data present in the manuscript are mostly of good quality. However, there is an obvious disconnection between the known negative role of Twist in controlling Th1 responses and the observation of Twist-dependent miR-148a induction in promoting the persistence of repeatedly activated Th1 cells described in this current study. There was no explanation or even a discussion provided by the authors. Moreover, it has been recently shown that miR-148a could function as an oncomiR through targeting Bim in Glioblastoma (Kim et al., 2014 Cancer Res), so that aspect of the paper, while still important in the context of Th1 cells, is unlikely to add to the general appeal of the work. Therefore, the novelty of the paper relies very heavily on the observation of Twist- (or T-bet-) dependent miR-148a induction, for which there are also issues and concerns remained to be addressed in this current manuscript.

SPECIFIC EXPERIMENTAL POINTS

1. In Fig.4, the authors have shown that treatment of miR-148a antagomir could result in reduced survival in repeatedly stimulated Th1 cells. In addition to using scramble antagomir as controls, I think it is important to treat Th2 and Th17 cells with the same miR-148a antagomirs in these experiments as controls. Since miR-148a is not induced in repeatedly stimulated Th2 and Th17 cells as the authors showed in Fig.1a, one would expect to see no difference in Th2 or Th17 survival in the presence of miR-148a antagomir.

2. In Fig.5C, the authors demonstrated that adding IFNg did not lead to the upregulation of miR-148a in Th1 cells. However, it is puzzling as to why such a treatment would result in the downregulation of miR-148a in WT Th1 cells (comparing the first and the third column). Any explanation?

3. In Fig.5H, the authors have performed ChIP study to show the binding of Twist to the upstream of miR-148a with IFNg as a positive control. Even though both genes can be bound by Twist but the biological consequences of such interactions seem to very different. While it was previously shown that Twist could inhibit IFNg production depending on complex formation with Runx3 (Pham et al., 2012. JI), Twist plays a positive role in miR-148a induction in the current manuscript. Any mechanistic insights?

MINOR POINTs

1. The results of intracellular staining of Bim protein in Fig.2 should be shown with all the bar graphs in addition to the one with antagomir treatment since Bim is the major target focused in this study.

2. Intracellular staining of Bcl2 might not be sensitive enough and truthfully represent the differences between treatments. The authors should perform the WB study to clearly determine whether or not Bcl2 is also targeted by miR-148a as suggested by previous studies (Zhang el al. 2011 Cell Death Differ).

Reviewer: 2

European Journal of Immunology

Comments to the Author

Comments:

Haftmann et. al describe an abundant expression of miR-148a in repeatedly activated Th1 cells induced by Twist1 and T-bet. miR-148a regulates expression of the proapoptotic gene Bim thereby resulting in a decreased Bim/Bcl2 ratio. The authors conclude that miR- 148a controls survival of Th1 cells by regulating Bim expression. There are several concerns regarding the interpretation of the presented data.

1. The authors assess the expression level of miR-148a in both, once and repeatedly activated Th1, Th2 and Th17 cells. However, the authors did not provide information about the detailed culture conditions in the material and method part. Likewise, the frequencies of once and repeatedly activated Th2 and Th17 cells after culture were not depicted.

2. The authors assess the expression level of miR-148a in Th1 cells generated in vivo using the LCMV model. The expression level of IFNγ should also be assessed in those differentiated Th1 cells for control of the Th1 effector cell state.

3. The once and repeatedly activated Th1 cells generated under the culture conditions are not homogenous populations. Modern technology such as cytokine secretion assays could allow isolating pure Th1 cells. It would be much informative to assess the expression of miR-148a in pure once and repeatedly activated Th1 cells in vitro and in vivo.

4. The constant activation culture conditions the authors use to generate repeatedly activated Th1 cells might induce apoptosis and interfere with the expression of miR-148a. Therefore, in order to exclude the influence of the cell culture on cell viability, experiments using sorted viable cells should be performed to assess the expression of miR-148a in different Th subtypes. Furthermore, sorted viable cells should be used to assess the expression of Bim, Tbx21 and Twist1.

5. The authors had observed a decreased number of viable repeatedly activated Th1 cells under the treatment with a miR-148a antagomir and proposed that this is associated with a high apoptotic ratio in these cells. However, the effect of miR-148a on the cell proliferative capacity should be excluded before making the conclusion that the authors draw.

6. The authors observe that ectopic miR-148a down-regulates reporter gene expression by targeting the Pten 3'UTR. However the treatment with miR-148a antagomirs in repeatedly activated Th1 cells did not change the endogenous expression of Pten. Similar observations have been made for the expression of Bach2 in those cells. The authors claim that both genes in repeatedly activated Th1 cells are not the physiologic targets of miR-148a. The authors should discuss this further to explain the selective targeting of miRNAs.

7. The authors measure the expression of miR-148a in memory cells from mixed synovial fluids of patients with RA, PsA and JRA. However, the etiology and pathogenesis of these three diseases are quite different and, furthermore, only a small portion of the memory T cells are IFNγ- producing cells. The authors should indicate the frequency of Th1 and Th17 cells in their samples and perform the analysis of miR-148a expression using pure Th1 cells from synovial fluid of one type of disease. Otherwise, it is impossible to interpret the data.

European Journal of Immunology

Reviewer: 3

Comments to the Author

The manuscript “miR-148a is upregulated by Twist1 and T-bet and promotes the survival of T helper 1 cells by regulating the proapoptotic gene Bim” by Haftmann et al. describes a novel regulatory network of Th1 cell survival driven by Bim negative regulation by miR-148a, which is in turn regulated by Twist and T-bet transcription factors. The authors make use of suitable approaches to draw conclusions that could be relevant for understanding chronic inflammatory disease. However, some additional data and clarifications, most notably regarding statistical analysis, should be needed to support the claims raised by the authors, as detailed below,

1. Could the authors discuss why they became interested in miR-148a to start with? According to previously published data (Kuchen et al 2010), miR-148a expression is highest in pancreas and shows very modest expression levels (about 60 times lower) in Th1 cells. Therefore a priori it would not seem a very attractive candidate to regulate Th1 biology.

2. Along the same lines, could the authors specify in the text the features that made Bim an interesting potential target of miR-148a?

3. Statistical analysis should be reinforced and clarify throughout the manuscript. For instance:

- Please provide stats for Figure 1B and Figure 1C
- Figure 2H apparently shows quantification of FACS data shown in Figure 2G, and should show the mean or median value of all experiments performed. However Figure legend says “Depicted is one experiment representative of two independent experiments with n=3”. Why only a representative experiment is represented in the bar graph? Please clarify the n to which the graph and the statistical values refer. Same clarification is required for Figure 4C, Figure 5D.

4. Figures 5B and 5C both apparently show miR-148a expression in Tbx21+/+ versus Tbx-/- Th1 cells. However in 5B graph fold difference seems no less than 5-6 fold (roughly from 6 to 40) while in 5C is very subtle (less than 2 fold, from 1.5 to 25). Why is this?.

5. The results section would benefit from more detailed description and including conclusions.

6. The discussion section could be reduced (for instance, the Pten paragraph). However, the authors do not discuss on the physiological meaning of miR-148a being regulated both by Twist and T-bet.

European Journal of Immunology

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First revision – authors' response – 24 June 2014

Reviewer #1:

The manuscript by Haftmann et al. demonstrated a role of miR-148a in promoting the survival of Th1 cells through targeting Bim. Moreover, the authors have shown that both Twist and T-bet are required for the induction of miR-148a in repeatedly stimulated Th1 cells. Specifically, by using ChIP approach, it was shown that Twist can directly bind to the upstream of the miR-148a gene, implying that Twist can directly promote the expression of miR-148a in Th1 cells. Overall, the study was performed in a straightforward manner and the data present in the manuscript are mostly of good quality. However, there is an obvious disconnection between the known negative role of Twist in controlling Th1 responses and the observation of Twist-dependent miR-148a induction in promoting the persistence of repeatedly activated Th1 cells described in this current study. There was no explanation or even a discussion provided by the authors. Moreover, it has been recently shown that miR-148a could function as an oncomiR through targeting Bim in Glioblastoma (Kim et al., 2014 Cancer Res), so that aspect of the paper, while still important in the context of Th1 cells, is unlikely to add to the general appeal of the work. Therefore, the novelty of the paper relies very heavily on the observation of Twist- (or T-bet-) dependent miR-148a induction, for which there are also issues and concerns remained to be addressed in this current manuscript.

Response: We politely disagree with the reviewer with respect to the originality of the work. Here we show for the first time, that miR-148a is upregulated selectively in repeatedly restimulated Th1 cells, under the control of T-bet and Twist1, and that this results in downregulation of Bim, but not Bcl2, thus an improved Bim/Bcl2 ratio and in improved survival. We demonstrate for the first time that this survival is dependent on downregulated Bim, and not on other potential targets of miR-148a, like Pten. It should be noted that it turns out more and more, that target specificity of miRNAs is context dependent, and targets in one cell are not necessary targets in every cell expressing the respective miRNA.

We, and later others, had shown before, that Twist1 is selectively upregulated in repeatedly restimulated Th1 cells, under the control of Stat4, NFAT and NFkB [1, 2]. In a murine model of arthritis, Twist1 ameliorated immunopathology. In vitro, Twist1 downregulated expression of proinflammatory cytokines. This is the "control of Th1 immune responses" the referee refers to. We now show, that Twist1 indirectly supports survival of the Th1 cells. This identifies Twist1 as a master switch of chronicity, dampening effector functions of Th1 cells to minimize immunopathology, but also ensuring the persistence of Th1 cells, to respond to the antigenic challenge permanently. We have now included these remarks into the discussion (page 7).

SPECIFIC EXPERIMENTAL POINTS

1. In Fig.4, the authors have shown that treatment of miR-148a antagomir could result in reduced survival in repeatedly stimulated Th1 cells. In addition to using scramble antagomir as controls, I think it is important to treat Th2 and Th17 cells with the same miR-148a antagomirs in these experiments as

European Journal of Immunology

controls. Since miR-148a is not induced in repeatedly stimulated Th2 and Th17 cells as the authors showed in Fig.1a, one would expect to see no difference in Th2 or Th17 survival in the presence of miR-148a antagomir.

Response: We have done the experiments suggested. In the new Fig. S3 we demonstrate that inhibition of miR-148a with antagomirs does not affect the viability of repeatedly activated Th2 and Th17 cells.

2. In Fig.5C, the authors demonstrated that adding IFNg did not lead to the upregulation of miR-148a in Th1 cells. However, it is puzzling as to why such a treatment would result in the downregulation of miR-148a in WT Th1 cells (comparing the first and the third column). Any explanation?

Response: The suppressive effect of IFNγ on miR-148a expression in once activated WT Th1 cells is suggestive, but statistically not significant. It is in line with previous observations that IFNγ in Th1 cells induces the expression of pro-apoptotic proteins, including Bim [3]. The promotor of miR-148a contains several conserved Stat-binding sites, the role of which we have not analysed so far. It can be speculated that Stat1-activation by IFNγ modulates expression of miR-148a.

3. In Fig.5H, the authors have performed ChIP study to show the binding of Twist to the upstream of miR-148a with IFNg as a positive control. Even though both genes can be bound by Twist but the biological consequences of such interactions seem to very different. While it was previously shown that Twist could inhibit IFNg production depending on complex formation with Runx3 (Pham et al., 2012. JI), Twist plays a positive role in miR-148a induction in the current manuscript. Any mechanistic insights?

Response: Pham et al. have shown that Twist1 physically interacts and represses the T-bet and Runx3 mediated IFNγ expression in Th1 cells [2]. The IFNγ promotor contains binding sites for T-bet and Runx3, and for Twist1 (see also Fig. 5H). The miR-148a promotor only contains binding sites for Twist1, an E-Box motif 2.1 kb upstream of the miR 148a gene (Fig. 5H), but not for T-bet [4] and no Runx binding motifs (TG(C/T)GG(C/T)) according to Lotem et al. [5] in close vicinity of the Twist1 binding site. So far, we have no clue about the partners of Twist1 in the transcriptional complex promoting miR-148a expression. It can be noted that in as yet unpublished work from our group, comparing the transcriptomes of Twist1 deficient and sufficient Th1 cells, we find as well down- as upregulated genes differentially expressed.

MINOR POINTs

1. The results of intracellular staining of Bim protein in Fig.2 should be shown with all the bar graphs in addition to the one with antagomir treatment since Bim is the major target focused in this study.

Response: In Fig. 2G we exemplify the cytometric quantitation of Bim and Bcl2 expression in individual, repeatedly restimulated Th1 cells. The stainings show a Gaussion distribution and therefore differences

European Journal of Immunology

are evaluated as differences in “mean fluorescence intensities” (MFIs) of the populations. In Fig. 2H, we summarize the results of 2 independent experiments with three replicates in one and two repelicates in the other experiment (total n=5), by relating the MFI of cells treated with control antagomirs, set to 1, to MFI of cells treated with antagomir-148a. The error bars indicate variability within the control group and between the experimental group and the controls. We think that this presentation best reflects the differences between cells treated or not with antagomir-148a.

2. Intracellular staining of Bcl2 might not be sensitive enough and truthfully represent the differences between treatments. The authors should perform the WB study to clearly determine whether or not Bcl2 is also targeted by miR-148a as suggested by previous studies (Zhang el al. 2011 Cell Death Differ).

Response: We now have added a WB for Bcl2 protein in “Fig. A for referees”, by probing the filter shown in Fig. 3D with anti-Bcl2. There is no difference between antagomir-scr and antagomir-148a treated repeatedly activated Th1 cells.

Reviewer #2:

Haftmann et. al describe an abundant expression of miR-148a in repeatedly activated Th1 cells induced by Twist1 and T-bet. miR-148a regulates expression of the proapoptotic gene Bim thereby resulting in a decreased Bim/Bcl2 ratio. The authors conclude that miR- 148a controls survival of Th1 cells by regulating Bim expression. There are several concerns regarding the interpretation of the presented data.

1. The authors assess the expression level of miR-148a in both, once and repeatedly activated Th1, Th2 and Th17 cells. However, the authors did not provide information about the detailed culture conditions in the material and method part. Likewise, the frequencies of once and repeatedly activated Th2 and Th17 cells after culture were not depicted.

Response: We have now included a detailed description of the culture conditions in the material and methods section (page 10). In addition to Th1, we now provide the frequencies of once and repeatedly activated Th2 and Th17 cells expressing IL-4 and IL-17, respectively (new Fig. S1). The data show that these cells maintained their cytokine memory.

2. The authors assess the expression level of miR-148a in Th1 cells generated in vivo using the LCMV model. The expression level of IFNγ should also be assessed in those differentiated Th1 cells for control of the Th1 effector cell state.

European Journal of Immunology

Response: On day 6 after infection with LCMV-WE, 87% of the virus specific Th cells are IFNγ producers (Fig. B for referees). As depicted in the new Fig. 1C and 1D the virus specific Th cells are T-bet positive, indicating that they are Th1 cells.

3. The once and repeatedly activated Th1 cells generated under the culture conditions are not homogenous populations. Modern technology such as cytokine secretion assays could allow isolating pure Th1 cells. It would be much informative to assess the expression of miR-148a in pure once and repeatedly activated Th1 cells in vitro and in vivo.

Response: We politely disagree with the referee with respect to the homogeneity of the Th cells generated by us. As we show in “Fig. C for referees”, once activated Th1 cells already show a uniform upregulation of T-bet, and about 85% of them express IFNγ upon restimulation (Fig. S1A). After repeated activation, 99% of the cells express IFNγ. We considered it not necessary to use the IFNγ-secretion assay, which we had originally developed [6], since the decisive parameter for miR-148a expression is expression of T-bet and not INFγ expression. T-bet expression was uniform from the beginning.

4. The constant activation culture conditions the authors use to generate repeatedly activated Th1 cells might induce apoptosis and interfere with the expression of miR-148a. Therefore, in order to exclude the influence of the cell culture on cell viability, experiments using sorted viable cells should be performed to assess the expression of miR-148a in different Th subtypes. Furthermore, sorted viable cells should be used to assess the expression of Bim, Tbx21 and Twist1.

Response: We have of course determined the viability of T cells in all the cultures analyzed in this study, and provided the information in the manuscript, wherever necessary. Dead cells were excluded from analysis upfront, by density gradient centrifugation.

5. The authors had observed a decreased number of viable repeatedly activated Th1 cells under the treatment with a miR-148a antagomir and proposed that this is associated with a high apoptotic ratio in these cells. However, the effect of miR-148a on the cell proliferative capacity should be excluded before making the conclusion that the authors draw.

Response: As shown in “Fig. D for referees”, antagomir mediated inhibition of miR-148a had no measureable effect on the proliferative capacity of repeatedly activated Th1 cells, as determined by CFSE-dilution.

6. The authors observe that ectopic miR-148a down-regulates reporter gene expression by targeting the Pten 3’UTR. However the treatment with miR-148a antagomirs in repeatedly activated Th1 cells did not change the endogenous expression of Pten. Similar observations have been made for the expression of

Bach2 in those cells. The authors claim that both genes in repeatedly activated Th1 cells are not the physiologic targets of miR-148a. The authors should discuss this further to explain the selective targeting of miRNAs.

Response: In the field of miRNA research, there is a growing understanding that regulation of gene expression by miRNAs is context dependent. The present data confirm the value of analyzing gene regulation by miRNA in the physiological context, here of repeatedly restimulated Th1 cells. By downregulating the physiological concentrations of miR-148a in repeatedly restimulated Th1 cells with antagomirs, we show that these physiological concentrations do suffice to regulate Bim, but not to regulate Pten, Bach2 and Bcl2, all of which are described targets of miR-148a in other cells. That this is a concentration dependent phenomenon becomes clear from the ectopic overexpression of miR-148a in Th1 cells. In Th1 cells overexpressing miR-148a, not only Bim but also Pten is regulated. We now discuss context dependency of miRNA regulation in more detail in the manuscript (Page 8 and Page 9).

7. The authors measure the expression of miR-148a in memory cells from mixed synovial fluids of patients with RA, PsA and JRA. However, the etiology and pathogenesis of these three diseases are quite different and, furthermore, only a small portion of the memory T cells are IFNγ- producing cells. The authors should indicate the frequency of Th1 and Th17 cells in their samples and perform the analysis of miR-148a expression using pure Th1 cells from synovial fluid of one type of disease. Otherwise, it is impossible to interpret the data.

Response: Due to low cell numbers derived from the biopsies, we were not able to characterize the isolated T cells further, which we have analysed here. However, there are several lines of evidence that synovial fluids of RA, PsA and JIA joints are enriched in Th1 cells. We had shown before that T cells from such synovia express very high levels of Twist1 [1] (the present manuscript, Fig. 6D). And, as shown in the new “Fig. E for referees”, CD3+CD4+CD45RO+ T cells isolated from synovia of RA, PsA and JIA, show a significantly higher mRNA expression of CXCR3 and CCR5, and lower expression of CCR6, than their counterparts from blood. This is in line with data from other groups, e.g. showing that 50% of T cells from synovial fluid express INFγ+ after restimulation ex vivo, as compared to 10% in peripheral blood [7]. Cells expressing IL-17 after restimulation were below 2%, both in synovial and in peripheral blood CD4+ T cells. In vivo, however, not many Th cells of synovia do express cytokines [8].

Reviewer #3:

The manuscript “miR-148a is upregulated by Twist1 and T-bet and promotes the survival of T helper 1 cells by regulating the proapoptotic gene Bim” by Haftmann et al. describes a novel regulatory network of Th1 cell survival driven by Bim negative regulation by miR-148a, which is in turn regulated by Twist and T-

European Journal of Immunology

bet transcription factors. The authors make use of suitable approaches to draw conclusions that could be relevant for understanding chronic inflammatory disease. However, some additional data and clarifications, most notably regarding statistical analysis, should be needed to support the claims raised by the authors, as detailed below,

1. Could the authors discuss why they became interested in miR-148a to start with? According to previously published data (Kuchen et al 2010), miR-148a expression is highest in pancreas and shows very modest expression levels (about 60 times lower) in Th1 cells. Therefore a priori it would not seem a very attractive candidate to regulate Th1 biology.

Response: Although it is hard to understand how our motiv could contribute to acceptance of the manuscript for publication, our rationale is easy to explain, and is justified by the data presented here. In a hypothesis-generating approach, we have determined the miRNA transcriptomes of once and repeatedly restimulated Th1, Th2 and Th17 cells. Since we are interested to understand the molecular adaptation of Th1 cells to chronic inflammation, we focused on those miRNAs, which were differentially and selectively expressed in repeatedly restimulated Th1 cells. One out of about 30 such miRNAs was miR 148a. MiR-148a was of immediate interest, since among its predicted targets are the proapoptotic genes Pten and Bim.

2. Along the same lines, could the authors specify in the text the features that made Bim an interesting potential target of miR-148a?

Response: As indicated in the manuscript, a systematic target identification approach using target predicition algorithms in combination with global transcriptome data analysis of once and repeatedly activated Th1 cells (Table S1) identified Bim as a predicted target of miR 148a.

3. Statistical analysis should be reinforced and clarify throughout the manuscript. For instance:
- Please provide stats for Figure 1B and Figure 1C
- Figure 2H apparently shows quantification of FACS data shown in Figure 2G, and should show the mean or median value of all experiments performed. However Figure legend says “Depicted is one experiment representative of two independent experiments with n=3”. Why only a representative experiment is represented in the bar graph? Please clarify the n to which the graph and the statistical values refer. Same clarification is required for Figure 4C, Figure 5D.

Response: We have now reinforced and clarified the statistical analysis.
For the new Fig. 1B, we have measured 3 additional experiments for miR 148b and miR-152, and now provide statistical evaluation.

European Journal of Immunology

For the representative T-bet staining in the new Fig 1C on day 7, we provide the statistical evaluation shown as the MFI of T-bet in the new Fig. 1D.

For Fig. 2H, we now pooled the two experiments with one experiment consisting of n=3 and one experiment consisting of n=2, resulting in n=5.

For the new Fig. 4C, we pooled four experiments, with one experiment consisting of n=3 and three experiments consisting of n=1 resulting in n=6.

For the new Fig. 5D, we pooled three experiments and indicate the sum of n for every group in the figure legend in the manuscript.

4. Figures 5B and 5C both apparently show miR-148a expression in Tbx21+/+ versus Tbx-/- Th1 cells. However in 5B graph fold difference seems no less than 5-6 fold (roughly from 6 to 40) while in 5C is very subtle (less than 2 fold, from 1.5 to 25). Why is this?.

Response: The experiments shown in Fig. 5B and 5C indeed differ with respect to the relative differences in miR-148a expression between Tbx21 deficient and sufficient Th cells. Nevertheless there is always a significantly lower expression of miR-148a in Tbx21-deficient cells. The differences in the relative differences may be due to the fact that both sets of experiments were conducted at different time points, by different persons, with different batches of reagents.

5. The results section would benefit from more detailed description and including conclusions.

Response: We have now edited the results section, and described all experiments in detail, including the conclusions drawn.

6. The discussion section could be reduced (for instance, the Pten paragraph). However, the authors do not discuss on the physiological meaning of miR-148a being regulated both by Twist and T-bet.

Response: We have now edited the discussion section as well, and included remarks on the context-dependency, as well as the relative contributions of T-bet and Twist1 to the regulation of expression of miR-148a, as requested by this referee and referee 2 (see above).

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Second Editorial Decision – 11 August 2014

Dear Dr. Mashreghi,

Thank you for submitting your revised manuscript ID eji.201444633.R1 entitled "miR-148a is upregulated by Twist1 and T-bet and promotes the survival of T helper 1 cells by regulating the proapoptotic gene Bim" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referees are included at the bottom of this letter.

Unfortunately, two of the referees were not satisfied with the revisions made and further major revision is requested. The journal does not encourage multiple rounds of revision and you should fully address the concerns of the referee in this final round of revision.

You should also pay close attention to the editorial comments included below.

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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,
Karen Chu

On behalf of Prof. David Gray

Dr. Karen Chu
Editorial Office
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Reviewer: 1

Comments to the Author
The authors have addressed several points raised. Nonetheless, there are two issues remained to be clarified.

1. In response to my original comment #1, the authors now provided additional experimental data to demonstrate that inhibition of miR-148a with antagomirs does not affect the viability of repeatedly activated Th2 and Th17 cells. However, it is unclear to me as to why the controlled cells in those conditions survived much better than those in Th1 condition (~90% in Fig. S3B and S3E vs. <60% in Fig. 4C) especially considering the fact that the Bim/Bcl2 ratios in those cells were much higher (Th2: ratio >2 in Fig. S3C) or similar (Th17: ratio= ~1 in Fig. S3F) compared to the controlled cells in the Th1 condition (ratio=1 in Fig. 2H). These results raise a question as to whether the difference in Bim/Bcl2 ratio is sufficient to explain the survival phenotypes observed in this study. Any explanation?

European Journal of Immunology

2. In response to my original comment #2, the authors provided additional reference demonstrating a role of IFNg in inducing Bim expression implying IFNg-mediated downregulation of miR-148 could potentially account for the aforementioned phenotype. However, it is known that IFNg signaling could lead to the upregulation of T-bet and T-bet is necessary for miR-148 induction in repeatedly stimulated Th1 cells as shown by the authors. It thus remained puzzling as to why IFNg treatment would result in the seemly downregulation (albeit statistically insignificant) of miR-148 in WT Th1 cells. If the authors could not provide any satisfactory answer or meaningful discussion, it is probably better to remove this panel (Fig. 5C) entirely since it is confusing and essentially just a negative result.

Reviewer: 2

Comments to the Author
The authors have addressed a few of the previous concerns. Surprisingly, they have opted to either not address others or have addressed them in their rebuttal letter only without revising their manuscript. This is a bit puzzling and difficult to understand as my review was not meant as a personal request for explanation but rather as a critical concern of the logical design of the study and the stringency of the presentation of the data. The authors do not have to comply with my suggestions, but they have to accept that the concerns identified in the first review have not eased by largely refraining from dealing with them in the manuscript.

Specific comments:

1. In the manuscript title it is claimed that “miR-148a is upregulated by Twist1 and T-bet and promotes the survival of T helper 1 cells by regulating the proapoptotic gene Bim”. However, as the authors show in Fig. S1A, only around 85% of the cells are positive for IFNγ. Th1 cells are defined by IFNγ production. Thus, the cells they report on contain a reasonably large fraction of non-Th1 cells. This is a major problem, in particular as there are techniques that would help to circumvent this contamination. Even if one would allow to take T-bet expression as a surrogate for Th1 cells, it remains unclear to the reader how pure the cells are as the frequency of T-bet is not indicated in “Fig. C for referees”. The referees do not need a figure but the paper must be understandable for the potential readership. In essence, the authors need to assess the expression of miR-148a in pure population of Th1 cells sorted based on IFNγ production from once and repeatedly activated Th1 cells for the in vitro and in vivo experiments.

2. In the revised manuscript, the authors characterized the expression of CXCR3, CCR5, and CCR6 in CD4 memory T cells from synovia of patients with RA, PsA and JIA. However, as I have mentioned in my previous comment, the etiology and pathogenesis and in particular the role of T cells and the nature of T cells identified within the synovium of these diseases are completely different. The analysis of a pooled

European Journal of Immunology

group of patients with “arthritis” is meaningless. Furthermore, clinical data should be included to allow for further interpretation of the data.

Reviewer: 3

Comments to the Author
The manuscript “miR-148a is upregulated by Twist1 and T-bet and promotes the survival of T helper 1 cells by regulating the proapoptotic gene Bim” by Haftmann et al. have now been revised and many of the reviewers concerns have been addressed. The claims of the work are now better substantiated and the clarity has been improved.

Second revision – authors’ response – 12 September 2014

Reviewer 1
The authors have addressed several points raised. Nonetheless, there are two issues remained to be clarified.

1. In response to my original comment #1, the authors now provided additional experimental data to demonstrate that inhibition of miR-148a with antagomirs does not affect the viability of repeatedly activated Th2 and Th17 cells. However, it is unclear to me as to why the controlled cells in those conditions survived much better than those in Th1 condition (~90% in Fig. S3B and S3E vs. <60% in Fig. 4C) especially considering the fact that the Bim/Bcl2 ratios in those cells were much higher (Th2: ratio >2 in Fig. S3C) or similar (Th17: ratio= ~1 in Fig. S3F) compared to the controlled cells in the Th1 condition (ratio=1 in Fig. 2H). These results raise a question as to whether the difference in Bim/Bcl2 ratio is sufficient to explain the survival phenotypes observed in this study. Any explanation?

Response: For the induction of apoptosis we reactivated Th cells by aCD3/aCD28 stimulation. It is well known that Th1 cells are more susceptible to activation induced cell death when compared to Th21-3 and Th17 cells4-6. Therefore, the better survival of Th2 and Th17 (~ 90 %), as compared to Th1 (~60 %), after reactivation is expected. However, unlike in repeatedly activated Th1 cells, the survival of Th2 and Th17 cells was not compromised by Antagomir-148a treatment, suggesting that only the survival of Th1 cells depends on miR-148a mediated suppression of Bim. We agree with the reviewer that in repeatedly activated Th2 and Th17 cells other survival mechanisms than the Bim/Bcl-2 pathway, contribute to their viability. We have now discussed this issue in the discussion, page 8. However, for repeatedly activated Th1 cells, the survival rates observed do depend on the Bim/Bcl2 ratio, as we show in Fig. 4F, by complementing the inhibition of miR-148a with the inhibition of Bim by siRNA.

European Journal of Immunology

2. In response to my original comment #2, the authors provided additional reference demonstrating a role of IFNγ in inducing Bim expression implying IFNγ-mediated downregulation of miR-148 could potentially account for the aforementioned phenotype. However, it is known that IFNγ signaling could lead to the upregulation of T-bet and T-bet is necessary for miR-148 induction in repeatedly stimulated Th1 cells as shown by the authors. It thus remained puzzling as to why IFNγ treatment would result in the seemly downregulation (albeit statistically insignificant) of miR-148 in WT Th1 cells. If the authors could not provide any satisfactory answer or meaningful discussion, it is probably better to remove this panel (Fig. 5C) entirely since it is confusing and essentially just a negative result.

Response: We agree with the reviewer and therefore removed the figure 5C from the manuscript.

Reviewer: 2

Comments to the Author
The authors have addressed a few of the previous concerns. Surprisingly, they have opted to either not address others or have addressed them in their rebuttal letter only without revising their manuscript. This is a bit puzzling and difficult to understand as my review was not meant as a personal request for explanation but rather as a critical concern of the logical design of the study and the stringency of the presentation of the data. The authors do not have to comply with my suggestions, but they have to accept that the concerns identified in the first review have not eased by largely refraining from dealing with them in the manuscript.

Specific comments:

1. In the manuscript title it is claimed that “miR-148a is upregulated by Twist1 and T-bet and promotes the survival of T helper 1 cells by regulating the proapoptotic gene Bim”. However, as the authors show in Fig. S1A, only around 85% of the cells are positive for IFNγ. Th1 cells are defined by IFNγ production. Thus, the cells they report on contain a reasonably large fraction of non-Th1 cells. This is a major problem, in particular as there are techniques that would help to circumvent this contamination. Even if one would allow to take T-bet expression as a surrogate for Th1 cells, it remains unclear to the reader how pure the cells are as the frequency of T-bet is not indicated in “Fig. C for referees”. The referees do not need a figure but the paper must be understandable for the potential readership. In essence, the authors need to assess the expression of miR-148a in pure population of Th1 cells sorted based on IFNγ production from once and repeatedly activated Th1 cells for the in vitro and in vivo experiments.

European Journal of Immunology

Response: As we show in our manuscript, expression of miR-148a is regulated by T-bet, not by IFNγ. Although originally, Th1 cells had been defined as IFNγ expressing cells, we think that meanwhile T-bet is accepted as an alternative marker, since it is the “master” transcription factor of Th1 cells7, 8. We have now modified Fig. S2A to show more clearly that in our Th1 cultures, already after 1 restimulation, all cells have uniformly upregulated expression of T-bet. Expression of T-bet is further upregulated in repeatedly activated Th1 cells (Fig. 5A).

As the reviewer requested, we now also separated IFNγ secreting and non-secreting Th1 cells after 1 week of in vitro culture, representing ~ 80% versus ~ 20% of the differentiated cells. MiR-148a expression in 1 week IFNγ secreters and non-secreters was the same (new Fig. S2B), and less than that of repeatedly activated Th1 cells (Fig. 1A), which all were IFNγ secreters (Fig. S1A). This shows that miR-148a expression is not a property of IFNγ secreters, but of repeatedly restimulated Th1 cells.

2. In the revised manuscript, the authors characterized the expression of CXCR3, CCR5, and CCR6 in CD4 memory T cells from synovia of patients with RA, PsA and JIA. However, as I have mentioned in my previous comment, the etiology and pathogenesis and in particular the role of T cells and the nature of T cells identified within the synovium of these diseases are completely different. The analysis of a pooled group of patients with “arthritis” is meaningless. Furthermore, clinical data should be included to allow for further interpretation of the data.

Response: Whether or not the roles of T cells and the nature of T cells isolated from synovia of patients with different inflammatory rheumatic diseases are similar or different, is not clear at this time point, we think. We agree that it would be interesting to dissect the differences and commons in more detail and relate that to clinical phenotypes and types of diseases. This is, however, far beyond the scope of this manuscript. Here we use those T cells to demonstrate a simple correlation between expression of TWIST1 and expression of miR-148a. We had shown before, that expression of TWIST1 is significantly upregulated, up to 100-fold, in Th cells isolated from inflamed tissues of patients with a variety of chronic inflammatory diseases 9. T cells from other tissues, e.g. blood or healthy colon do not show this upregulation. We had also shown that upregulation of Twist1 expression is dependent on repeated restimulation of Th cells and on the Th1-inducing signal transducer STAT4, i.e. is Th1 specific9. We have now changed our discussion (page 7) to clarify our intentions.

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7. Szabo, S.J. et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100, 655-669 (2000).
8. Rebhahn, J.A. et al. An animated landscape representation of CD4(+) T-cell differentiation, variability, and plasticity: Insights into the behavior of populations versus cells. European journal of immunology 44, 2216-2229 (2014).
9. Niesner, U. et al. Autoregulation of Th1-mediated inflammation by twist1. The Journal of experimental medicine 205, 1889-1901 (2008).

Third Editorial Decision – 8 October 2014

Dear Dr. Mashreghi,

Thank you for submitting your revised manuscript ID eji.201444633.R2 entitled "miR-148a is upregulated by Twist1 and T-bet and promotes the survival of T helper 1 cells by regulating the proapoptotic gene Bim" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referees are included at the bottom of this letter.

Although the referees have recommended publication, some revisions to your manuscript have been requested. Therefore, I invite you to respond to the comments of referee 2 and revise your manuscript accordingly - you will see that referee 2 feels strongly about this single point.

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.*

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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. We look forward to receiving your revision.

Yours sincerely,
Karen Chu

on behalf of Prof. David Gray

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Reviewer: 1

Comments to the Author
The authors have addressed my previous comments.

Reviewer: 2

Comments to the Author
In response to my original comment #2, the authors provided additional information demonstrating the necessity to perform the correlation analysis of miR-148a and Twist1 expression in activated T cells from patients suffering from PsA, RA and JIA. However, as I have mentioned in my previous comment, the etiology and pathogenesis and in particular the role of T cells and the nature of T cells identified within the synovium of these diseases are completely different. It is incorrect - and not arguable - to pretend as if every clinically overt arthritis is based on the very same mechanisms. This argument lacks insight into human arthritides and respect for the diversity of human diseases - which is in sharp contrast to the rather uniform nature of a given animal model. Therefore, if the authors cannot provide any satisfactory data

referring to the “pure” patient groups, it is advised to remove this figure completely since it will provide incorrect information to the readers.

Third revision – authors’ response – 3 November 2014

Reviewer 2:

Comments to the Author

In response to my original comment #2, the authors provided additional information demonstrating the necessity to perform the correlation analysis of miR-148a and Twist1 expression in activated T cells from patients suffering from PsA, RA and JIA. However, as I have mentioned in my previous comment, the etiology and pathogenesis and in particular the role of T cells and the nature of T cells identified within the synovium of these diseases are completely different. It is incorrect - and not arguable - to pretend as if every clinically overt arthritis is based on the very same mechanisms. This argument lacks insight into human arthritides and respect for the diversity of human diseases - which is in sharp contrast to the rather uniform nature of a given animal model. Therefore, if the authors cannot provide any satisfactory data referring to the “pure” patient groups, it is advised to remove this figure completely since it will provide incorrect information to the readers.

Response:
We now provide “pure” groups, samples from healthy controls or samples from patients with rheumatoid arthritis (RA, Figure 6 A-C). We have removed two samples, one from a patient with psoriatic arthritis (PsA) and another with juvenile idiopathic arthritis (JA).

Further, to be more explicit on the correlation analysis of miR-148a and Twist1 expression, we have annotated each sample with the corresponding disease and moved former Figure 6D into the Supporting Information 6B. As we have already stated in the discussion (page 8) we do not want to draw a common conclusion across diseases. We hope that this is an appropriate way to present the data and to make the claim that memory T cells isolated from the inflamed tissue show elevated expression miR-148a and twist1 expression. We think that this is highly remarkable and worth reporting to the readership of EJI.

Peer review correspondence

European Journal of Immunology

Fourth Editorial Decision – 7 November 2014

Dear Dr. Mashreghi,

It is a pleasure to provisionally accept your manuscript entitled "miR-148a is upregulated by Twist1 and T-bet and promotes Th1 cell survival by regulating the proapoptotic gene Bim" 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,
Karen Chu

on behalf of Prof. Iain McInnes

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