EMBO reports

miR-132 suppresses transcription of ribosomal proteins to promote protective Th1 immunity

James P. Hewitson, Kunal M. Shah, Najmeeyah Brown, Paul Grevitt, Sofia Hain, Katherine Newling, Tyson V. Sharp, Paul M. Kaye, and Dimitris Lagos

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 28 June 2018

Thank you for the submission of your manuscript to EMBO reports. I have read and discussed your manuscript with my colleagues, and I am sorry to say that we cannot offer publication of your work in our journal. However I have taken the liberty to also discuss your study with my colleague Andrea Leibfried at our sister journal LSA, and she would be happy to offer peer review if you were to transfer your manuscript there.

We appreciate that in your manuscript you describe that miR-132 is up-regulated during CD4+ T cell activation in vitro and in vivo (in Leishmania-infected mice). Using miR-212/132-deficient mice you then show global up-regulation of ribosomal protein genes in CD4+ T cells from chronically infected spleens of these mice. Next, you identify the TFIID cofactor BTAF1 as a direct target of miR-132 in CD4+ T cells, and demonstrate that by regulating its levels (and of the already known target p300) miR-132 promotes the suppression of ribosomal protein expression. Next, you report that this affects IL-10 levels in activated CD4+ T cells, as depletion of the miR-212/132 cluster increases IL-10 production, and thereby promotes the TH1 anti-inflammatory status of T cells. Finally, you show that miR-132-/- mice show increased Leishmania burden, which correlates with increased IL-10 expression in CD4+ T cells.

However, we also note it has been reported before that miR-132 targets p300, that miR-132/212-/- mice show elevated levels of IL-10-producing CD4+ T cells (PMID 25862525), that miR-132-3p is up-regulated in activated CD4+ T cells (PMID 21788445, 28615644), and that increasing IL-10 levels reduce pathogen clearance (also in the context of Leishmania infection). These previous findings impact on the conceptual novelty of the present report, although we appreciate that you now link this to the transcriptional regulation of ribosomal proteins. However, also as it remains unclear why mainly IL-10 expression is affected by miR-132 loss-of-function in CD4+ T cells, we do not think that the report provides the conceptual advance and broader impact we are looking for in an EMBO reports paper. We have therefore decided not to proceed with in-depth peer review.

That being said, as mentioned above, I discussed your work with Andrea Leibfried, executive editor of our new open-access journal Life Science Alliance. Life Science Alliance is launched as a partnership between EMBO Press, Rockefeller Press, and Cold Spring Harbor Laboratory Press, and publishes work that is of high value to the respective communities across all areas in the life sciences (see: http://www.life-science-alliance.org). I am glad to say that Andrea is interested in the publication of the manuscript at Life Science Alliance and she would be pleased to send your manuscript in its current form out for formal peer-review. No reformatting is required.

I very much hope that you are interested in this option - please use the link below for transfer.

Resubmission 29 June 2018

Thank you for your letter. I appreciate your reasoning. I also appreciate that you are bombarded by authors arguing the novelty of their manuscripts. However, I genuinely think that I should draw your attention to points below:

- Linking miR-132 to ribosomal protein expression does not only represent a new function of miR-132 (I believe its main function), but it is also a new mechanism of RP expression regulation. I think this is the major conceptual contribution of our work, especially given the centrality of the ribosome and how little we know about the role of ribosomal protein regulation in CD4+ T cell biology in vivo. The link to p300 (that we previously published) and BTAF1 goes further in-depth with regards to transcriptional networks that mediate this effect in CD4+ T cells. Of note, to my knowledge this is the first report linking BTFIID (a fundamental transcriptional unit) to CD4+ T cells.

- Identifying increased RP mRNAs levels as a hallmark of IL-10+ Th1 cells provides the lacking insight into why miR-132 selectively promote generation of these cells, which you correctly point remains poorly understood. As mentioned in our manuscript we believe that this enhanced RP expression results in premature differentiation of CD4+ T cells in vivo characterised by acquisition of IL10 expression and compromised immunity. This is a fundamentally different concept to that described in PMID: 25862525, where miR-212 specifically (and not miR-132) is reported to suppress cMAF. Note that we do not find any evidence for this specificity, neither targeting of cMAF in our hands.

- Although references PMID 21788445, 28615644 agree with our results with regards to miR-132 induction upon TCR engagement, these studies are performed in vitro. I am sure you agree that not all miRNAs that are regulated in vitro in an immune cell have in vivo phenotypes (or are even regulated in vivo). To my knowledge our work is the first to demonstrate that miR-132 deficiency promotes protective immunity in vivo. This represents a conceptual departure from early studies on miR-132 (in vitro or in acute infection models) supporting the thesis that miR-132 is an anti-inflammatory miRNA. Taken together with these previous studies, our study reveals that miR-132 has context dependent functions in immune system. In fact, this manuscript combined with our earlier work (Lagos et al 2010, Nat Cell Biol) demonstrates that even the same miRNA/target pair (miR-132/p300) can have opposing effects in immune outcomes (pro vs anti-inflammatory) depending on the cell type and infectious model context.

Given the above, my view is that our work represents a clear conceptual advancement in our understanding of miRNA-driven networks in Th1 immunity. Based on that, I would like to ask you whether it would be possible to reconsider your decision. I appreciate that your time is precious and sincerely thank you in advance for your attention to our work.

Additional Correspondence 2 July 2018

I have now heard back from the advisor, and we have decided to have the manuscript reviewed. I will be back with you, once I have received the referee reports.

2nd Editorial Decision 31 July 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think the manuscript is of interest, but requires further revisions to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript, and/or a rebuttal letter. As the reports are below, I will not detail them here. Point 7 of referee #3 would be very nice to see addressed experimentally, but we understand if you would be not able to provide these data within the timeframe defined below.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact me if a 3-months time frame is not sufficient so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

For more details please refer to our guide to authors:
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Important: All materials and methods should be included in the main manuscript file.

See also our guide for figure preparation:
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Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See:
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Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://embor.embopress.org/authorguide#livingorganisms

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.
I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The manuscript describes in a brief definitive report format a new link between microRNA, Ribosomal protein gene expression and the immunoregulatory status of Th1 cells. This regulatory circuit promotes anti-parasite immunity. Loss of microRNA 132 ultimately leads to elevated IL-10 production and this immunosuppressive cytokine is known to limit anti-parasite responses.

The effect of the microRNA on RP gene expression are indirect and appear to be mediated by transcriptional co-regulators.

It could be that some of the in vivo effects seen in KO mice arise from roles of the microRNA in non-T cells. This could be discussed more clearly.

Overall this is a well conducted set of experiments and an easy to read manuscript, with good quality figures, which provides new mechanistic insight.

Referee #2:

In the present manuscript, Hewitson et al. are studying the involvement of miR-132 as a molecular regulatory mechanism of CD4 T-cell activation in the context of a parasitic infection. This manuscript is of general interest for the scientific community and of particular interest for molecular biologists and immunologists. While the findings depicted in the paper are novel and interesting, the presented data are mostly correlative. Therefore, the main conclusions are not fully supported by the experimental data. Finally, lack of a clear link between the first (figs 1-3) and second part (figs 4, 5) of this manuscript represents its weakest aspect.

Major points:

1. The authors did not rule out the role of miR-212 in the described mechanism. Indeed, miR-132-/- mice are deficient for both miR-132 and miR-212. Thus, it would be important to also assess the role of miR-212. Specifically in Fig. 1 B, miR-212 is not depicted while it is even more upregulated upon activation than miR-132 (Fig. 1A). miR-212 is also absent from Fig. 3. Completing this figure with a miR-212 mimics experiment assessing the role of miR-212 on ribosomal proteins, BTAF and p300, would be a good way to strengthen this part of the manuscript.

2. This is somehow puzzling that the conclusions that have led the authors to study a parasitic infection are deriving from experiments made with MEFs (figure 3). At least, they should have used lymphocyte cell lines rather than MEFs. miR-132 and miR-212 mimic experiments on ex vivo CD4 T cells from miR132-/- mice would strengthen strongly the conclusions of this manuscript.

3. While there is a correlation between the upregulation of IL-10 production by CD4 T cells from miR-132-/- mice and the better parasite clearance observed in IL-10-/- mice, such a correlation does not demonstrate that it is specifically the IL-10 produced by CD4 T cells that is responsible for the observed results. The ideal way to fully demonstrate that point would be to restrict IL-10 deficiency to CD4 T cells. At least, it is necessary to show that other potential IL-10 producers are not or at least less affected than CD4 T cells in miR-132-/- mice.

4. There is no experiment showing directly that IL-10 is responsible for the lack of clearance of the parasite in miR-132-/- infected mice. In vivo IL-10 neutralization experiments should be done to demonstrate that point.

5. There is no direct link between the upregulation of ribosomal proteins in miR-132-/- mice and the

lack of parasite clearance. Experiments assessing that point would strengthen strongly the main conclusions of the paper. The authors claim: "Of note, due to the impossibility of concurrent physiological knockdown or over-expression of RPs, the functional relevance of this family of proteins to Th1 responses in vivo has remained elusive." Btaf1-/- mice are available to assess that point. It is also possible to treat mice with phenylephrine and to infect them.

6. The significance of the decreased spleen and liver weight (Fig. 5E and F) should be discussed. Indeed, I would have expected a mouse with chronic parasitic infection to have enlarged spleen and liver.

Minor points:
1. There is sometimes a lack of consistency in the figure labels that can make the manuscript difficult to read.
2. The building of the figures is sometimes tricky. This is not always obvious which legend is related to which panel.
3. Line 228 and 229: "This was accompanied by a greater fold increase in production of IL-10 (38-54%) by miR-132-/- IFNγ+ CD4 T cells compared to wild-type cells (Fig. 4B and Fig. EV4A)". It is not clear what these 38-54% are referring to? If this is the % of IL-10+ cells within IFNg+ cells, this does not match the dot plots shown in Fig. EV4A.

Referee #3:

This manuscript explores the role of miR-132 in T cells using a Leishmania (Ld) infection model in mice. The authors demonstrate that miR-132 is induced during T cell activation, and represses a large number of genes, including many genes that encode ribosome proteins (RPs). The authors also identify BTAF1 and p300 as targets of miR-132 in this context and provide some evidence that these targets regulate RP genes in activated miR-132-/- T cells. Further, miR-132-/- T cells take on an IL-10 regulatory phenotype prematurely during the infection, and miR-132-/- mice have some defect in clearing Ld.

This manuscript is well written and provides some novel insight into the role of miR132 in the T cell compartment. The connection between miR-132 and ribosome protein gene expression and IL-10 production during T cell activation is interesting yet rather preliminary in nature. There are many aspects of how these events are connected that remain unclear. The following points should be addressed to improve this manuscript.

1. Why does increased RP expression increase IL10 vs IFNg levels in miR132ko T cells? How is selective expression of IL10 regulated by increased RP gene expression?
2. Why do p300 and BTAF1 preferentially target RP genes for transcriptional regulation? Again, like point 1, it is unclear where the specificity comes from here.
3. The authors should look at ribosome protein levels by Western blot in addition to mRNA levels. The changes at the RNA level appear modest and differences at the protein level should be demonstrated.
4. Can overall translational output be assayed? Once would expect for this to be generally increased in the miR132ko T cells that have increased RP expression.
5. Ld levels in miR-132ko mice appear to be marginally increased. Is this biologically significant? For example, IL-10 has a much larger impact that is more convincing.
6. The authors should assay miR132 3p v 5p levels before and after T cell activation to confirm which strand is being expressed.
7. If possible, the authors should perform T cell transfer experiments where direct targets of miR132 are reduced with siRNA to study their impacts in vivo. Transfer of Wt and miR-132-/- T cells should also be performed. This would further support a cell intrinsic role for miR132 during the in vivo phenotypes and demonstrate functional relevance for the direct targets p300 and BTAF1.

Referee #1:

The manuscript describes in a brief definitive report format a new link between microRNA, Ribosomal protein gene expression and the immunoregulatory status of Th1 cells. This regulatory circuit promotes anti-parasite immunity. Loss of microRNA 132 ultimately leads to elevated IL-10 production and this immunosuppressive cytokine is known to limit anti-parasite responses.

The effect of the microRNA on RP gene expression are indirect and appear to be mediated by transcriptional co-regulators.

It could be that some of the in vivo effects seen in KO mice arise from roles of the microRNA in non-T cells. This could be discussed more clearly.

Response: We would like to thank the Reviewer for the positive evaluation of our work.
We have now added the following statement in the concluding paragraph of the manuscript: "Although we cannot exclude that other cell types or mechanisms contribute to the observed increase in parasite loads in *miR-132*$^{-/-}$ mice, we propose that the effects of miR-132 deficiency on IL-10 expression in IFNg^{+} CD4^{+} T cells significantly contribute to reduced protective inflammation and enhanced susceptibility of *miR-132*$^{-/-}$ mice to infection."

Overall this is a well conducted set of experiments and an easy to read manuscript, with good quality figures, which provides new mechanistic insight.

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In the present manuscript, Hewitson et al. are studying the involvement of miR-132 as a molecular regulatory mechanism of CD4 T-cell activation in the context of a parasitic infection. This manuscript is of general interest for the scientific community and of particular interest for molecular biologists and immunologists. While the findings depicted in the paper are novel and interesting, the presented data are mostly correlative. Therefore, the main conclusions are not fully supported by the experimental data. Finally, lack of a clear link between the first (figs 1-3) and second part (figs 4, 5) of this manuscript represents its weakest aspect.

Response: We would like to thank the Reviewer for the constructive comments, which strengthened our manuscript.

Major points:

1. The authors did not rule out the role of miR-212 in the described mechanism. Indeed, miR-132-/- mice are deficient for both miR-132 and miR-212. Thus, it would be important to also assess the role of miR-212. Specifically in Fig. 1 B, miR-212 is not depicted while it is even more upregulated upon activation than miR-132 (Fig. 1A). miR-212 is also absent from Fig. 3. Completing this figure with a miR-212 mimics experiment assessing the role of miR-212 on ribosomal proteins, BTAF and p300, would be a good way to strengthen this part of the manuscript.
Response: We performed the requested experiments and show that mimics of miR-212-3p also suppress p300, BTAF, and RP expression (NEW **Figs. EV3A-C**). This is consistent with the fact that miR-132-3p and miR-212-3p have the same seed sequence. We have acknowledged the contribution of miR-212-3p to the observed effects in the text and abstract. The potential contribution of miR-212-5p to the observed effects was addressed in Fig. EV2G of the original manuscript.

2. This is somehow puzzling that the conclusions that have led the authors to study a parasitic infection are deriving from experiments made with MEFs (figure 3). At least, they should have used

lymphocyte cell lines rather than MEFs. miR-132 and miR-212 mimic experiments on ex vivo CD4 T cells from miR132-/- mice would strengthen strongly the conclusions of this manuscript.
Response: We follow the Reviewer's recommendation and show that over-expression of miR-132 mimics in the EL4 mouse T cell line results in suppression of p300 and BTAF1 (NEW **Fig. EV3C**). We also clarify that our experiments with *L. donovani* infected mice and $CD4^+$ T cells (Figs 4 and 5) follow directly from Figures 1 and 2, in which we analysed expression and effects of miR-132-3p in $CD4^+$ T cells *in vitro* and from mice infected with *L. donovani*. In Fig 3, we wanted to demonstrate that the effects on BTAF1, p300, and RP expression are not only restricted to $CD4^+$ T cells. This also provided a cell type that is more easily transfectable than naïve $CD4^+$ T cells and allowed us to probe further RP regulation by miR-132.

3. While there is a correlation between the upregulation of IL-10 production by CD4 T cells from miR-132-/- mice and the better parasite clearance observed in IL-10-/- mice, such a correlation does not demonstrate that it is specifically the IL-10 produced by CD4 T cells that is responsible for the observed results. The ideal way to fully demonstrate that point would be to restrict IL-10 deficiency to CD4 T cells. At least, it is necessary to show that other potential IL-10 producers are not or at least less affected than CD4 T cells in miR-132-/- mice.
Response: We sorted myeloid cell subpopulations from infected mice and show that miR-132 deficiency does not affect IL-10 expression in myeloid cells from infected mice (NEW **Fig. EV5F**). We note in the text that we cannot exclude that miR-132 affects IL-10 in other IL-10-producing cell types (e.g. ILCs, B cells). We also note previous publications that demonstrate the functional significance of Th1-derived IL-10 in the context of *L. donovani* infection when compared to IL-10 produced by Tregs (Jankovic D. et al., JExpMed, 2007) or myeloid cells (Ranatunga D. et al., PNAS, 2009).

4. There is no experiment showing directly that IL-10 is responsible for the lack of clearance of the parasite in miR-132-/- infected mice. In vivo IL-10 neutralization experiments should be done to demonstrate that point.
Response: Ideally, to address this point we would need to reduce IL-10 expression in *miR-132*$^{-/-}$ $CD4^+$ T cells to levels found in WT cells. We performed pilot experiments as suggested by the Reviewer to explore IL-10R blockade as a means of reversing the effects of miR-132. As shown below and in agreement with previous reports (Murray HW et al., JID, 2003), treatment with anti-IL-10R-blocking antibodies results in a dramatic reduction of parasite loads. The effect of IL-10R blockade on parasite load is significantly more profound than that of miR-132 deficiency, which would significantly limit interpretation of such experiments. Additional experiments aiming to only partially block IL-10R could take an extensive amount of time without guarantee of achieving the intended outcome (partial IL-10R blockade).

We agree with the Reviewer that without a means of reducing IL-10 expression in *miR-132*$^{-/-}$ $CD4^+$ T cells to levels found in WT cells, we cannot exclude that other mechanisms contribute to the observed effects. To reflect this limitation and address the Reviewer's concern, we have now modified our conclusion as below:
"Although we cannot exclude that other cell types or mechanisms contribute to the observed increase in parasite loads in *miR-132*$^{-/-}$ mice, we propose that the effects of miR-132 deficiency on IL-10 expression in $IFNg^+$ $CD4^+$ T cells significantly contribute to reduced protective inflammation and enhanced susceptibility of *miR-132*$^{-/-}$ mice to infection."

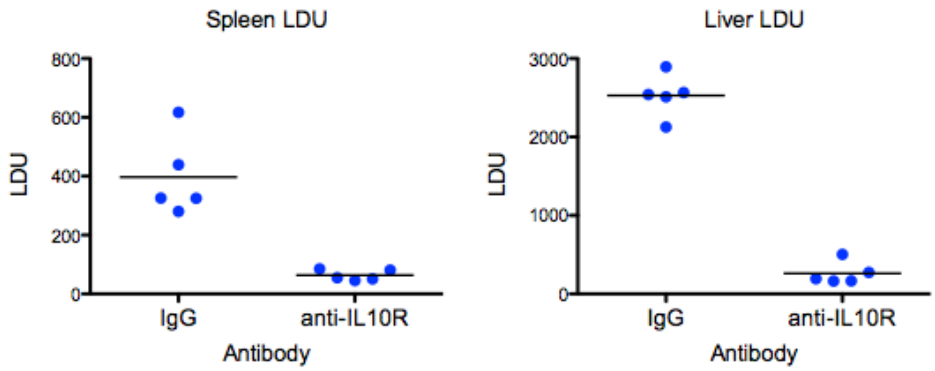

Figure R1: Mice were infected with *L. donovani* and received anti-IL10R (Clone: 1B1.3A from Bio X Cell) or IgG isotype control injections at day 0, 14, and 21 p.i. at 0.5mg mAb/injection. Spleen (left panel) and liver (right panel) LDUs were analysed at day 28 p.i.

5. There is no direct link between the upregulation of ribosomal proteins in miR-132-/- mice and the lack of parasite clearance. Experiments assessing that point would strengthen strongly the main conclusions of the paper. The authors claim: "Of note, due to the impossibility of concurrent physiological knockdown or over-expression of RPs, the functional relevance of this family of proteins to Th1 responses in vivo has remained elusive." Btaf1-/- mice are available to assess that point. It is also possible to treat mice with phenylephrine and to infect them.
Response: We appreciate that the link between RP expression and parasite clearance is indirect. We now discuss this clearly in the text (lines 269-273). Treatment with phenylephrine could have pleiotropic, predominantly cardiovascular, non-immune effects (Cavalli A *et al.*, PNAS, 1997; Tejero-Taldo MI *et al.*, J Mol Cell Cardiol., 2002; Vecchione C *et al.*, Ciruclation 2002; Peng C *et al.*, Mol Biosyst, 2017; Cheng X., et al., Sci Rep, 2018) that would prevent interpretation of chronic infection experiments such as the ones presented in our manuscript. Only the ES cells of the *Btaf1*$^{-/-}$ are available (http://www.informatics.jax.org/allele/MGI:5002959). Even if we generated *Btaf1*$^{-/-}$ mice using the ES cells, they would have to be crossed to conditional *p300*$^{-/-}$ mice to recapitulate the dependence on both proteins for the observed effect of miR-132 on RP expression (as seen in new **Fig. 3E**). We believe that these time-consuming experiments could certainly be the topic of future work but they are beyond the scope of this manuscript.

6. The significance of the decreased spleen and liver weight (Fig. 5E and F) should be discussed. Indeed, I would have expected a mouse with chronic parasitic infection to have enlarged spleen and liver.
Response: The extent of hepatosplenomegaly during *L. donovani* infections is often proportional to parasite load. This is due to both the protective and pathological aspects of the immune response to the parasite, and not always the case in immunodeficient models. For example, RagKO mice display excessive parasite loads with minimal hepatosplenomegaly. In our manuscript, we interpret the co-occurrence of reduced organ size and increased parasite burden in miR-132$^{-/-}$ mice as a demonstration of miR-132 being a determinant of protective immunity rather than pathologic inflammation.

Minor points:

1. There is sometimes a lack of consistency in the figure labels that can make the manuscript difficult to read.
2.The building of the figures is sometimes tricky. This is not always obvious which legend is related to which panel.
Response: We have extensively reviewed and revised all our Figure legends and labels and clarified areas of potential confusion.

3. Line 228 and 229: "This was accompanied by a greater fold increase in production of IL-10 (38-54%) by miR-132-/- IFNγ+ CD4 T cells compared to wild-type cells (Fig. 4B and Fig. EV4A)". It is not clear what these 38-54% are referring to? If this is the % of IL-10+ cells within IFNg+ cells, this

does not match the dot plots shown in Fig. EV4A.
Response: We have removed the percentages shown in brackets as we see this can indeed cause confusion. The percentages mentioned in the original text were not the % of IL-10-expressing $IFNg^+$ cells. They referred to the difference in % of IL-10-expressing $IFNg^+$ cells between WT and *miR-132*$^{-/-}$ mice as a proportion of the % of IL-10-expressing $IFNg^+$ cells in WT cells.

Referee #3:

This manuscript explores the role of miR-132 in T cells using a Leishmania (Ld) infection model in mice. The authors demonstrate that miR-132 is induced during T cell activation, and represses a large number of genes, including many genes that encode ribosome proteins (RPs). The authors also identify BTAF1 and p300 as targets of miR-132 in this context and provide some evidence that these targets regulate RP genes in activated miR-132-/- T cells. Further, miR-132-/- T cells take on an IL-10 regulatory phenotype prematurely during the infection, and miR-132-/- mice have some defect in clearing Ld.

This manuscript is well written and provides some novel insight into the role of miR132 in the T cell compartment. The connection between miR-132 and ribosome protein gene expression and IL-10 production during T cell activation is interesting yet rather preliminary in nature. There are many aspects of how these events are connected that remain unclear. The following points should be addressed to improve this manuscript.

Response: We would like to thank the Reviewer for the constructive comments, which strengthened our manuscript.

1. Why does increased RP expression increase IL10 vs IFNg levels in miR132ko T cells? How is selective expression of IL10 regulated by increased RP gene expression?
Response: In our data, the extent of the effect of miR-132 depletion varies for different RPs (Fig. 1). Similarly, the effect of $CD4^+$ T cell activation seems to affect individual RPs to different extent (Fig. 1H). This means that the ribosomal protein composition of the ribosome changes during $CD4^+$ T cell activation and that this RP landscape is further altered in *miR-132*$^{-/-}$ cells. This is consistent with work demonstrating that ribosomes are not static units and specialised ribosomes can target specific mRNAs for translation (Xue S and Barna M, Nat Rev Mol Cell Biol. 2012; Segev N and Gerst JE, JCB, 2018). In our data, IL-10 mRNA levels are similar between WT and *miR-132*$^{-/-}$ $CD4^+$ T cells (NEW **Fig. 4C**), which is consistent with regulation occurring at the level of translation. A possible explanation of why the observed changes in RP expression affect IL-10 translation but not IFNg could be that more lowly expressed proteins (such as IL-10 in Th1 cells) are more sensitive to changes in the composition of the ribosome rather than highly expressed proteins (such IFNg), or that other indirect mechanisms contribute to regulation of IFNg in *miR-132*$^{-/-}$ T cells. We now discuss this in our manuscript. Overall, elucidating the mechanisms underpinning ribosomal dynamics in activated $CD4^+$ T cells and how these are altered by miR-132 is a fascinating challenge, which we believe is beyond the remit of our current manuscript and should be the topic of future studies.

2. Why do p300 and BTAF1 preferentially target RP genes for transcriptional regulation? Again, like point 1, it is unclear where the specificity comes from here.
Response: We speculate that this is due to two reasons: 1) occupancy of the two transcriptional co-activators across the genome, especially in highly transcribed genes such as the RP genes, and 2) the specific combination of concurrent down-regulation of both of these proteins by miR-132. Although we believe that addressing the first point through extensive mapping of p300 and BTAF1 targets is beyond the scope of this manuscript, we performed experiments that show that suppression of RP expression by miR-132 requires its effect on both p300 and BTAF1 (NEW **Fig. 3E**). Interestingly, through these experiments we identified miR-132-mediated effects on RP expression that were abolished upon knockdown of either p300 or BTAF1, but also effects that were specifically dependent on p300 (e.g. miR-132-mediated suppression of Rps9) or BTAF1 (e.g. miR-132-mediated suppression of Rpl18).

3. The authors should look at ribosome protein levels by Western blot in addition to mRNA levels.

The changes at the RNA level appear modest and differences at the protein level should be demonstrated.
Response: We confirmed that the effects were also observed at the protein level using Rps10 and Rpl27 as representative ribosomal proteins affected by miR-132 (NEW **Fig. EV3B**).

4. Can overall translational output be assayed? Once would expect for this to be generally increased in the miR132ko T cells that have increased RP expression.
Response: We used puromycin incorporation as a way of measuring translational output (Schmidt EK, *et al.*, Nat Methods, 2009). We show that miR-132-3p and miR-212-3p transient over-expression cause a reduction in translation rates in MEFs (NEW **Fig. 3F**). This functionally validates the observed RP suppression by these miRNAs. This demonstrates that the miR-132/212 cluster can alter translational outputs. We are cautious regarding the interpretation of these findings in the context of long-term experiments *in vivo*. As discussed above, an increase in expression of some RPs could lead to alteration of ribosome composition that might affect global translation rates or alternatively target specific mRNAs. Furthermore, given previously reported negative and positive autoregulatory loops between RP expression and translational efficiency (Zhao *et al.*, MCB, 2003; Warner JR and McIntosh KB, Mol Cell, 2009; Betney R *et al.*, RNA, 2010), our findings reported in this manuscript set the basis for future experiments, in which ribosome dynamics and translational outputs are assayed in detail over time in *WT* and *miR-132*$^{-/-}$ CD4^{+} T cells isolated from infected mice.

5. Ld levels in miR-132ko mice appear to be marginally increased. Is this biologically significant? For example, IL-10 has a much larger impact that is more convincing.
Response: We agree that IL-10 deletion across all cell lineages has a more profound effect than miR-132 deletion. This is likely due to the fact that miR-132 does not completely suppress IL-10 expression. We state that the effect of miR-132 on LDUs is modest (2-fold on average). However, as this is accompanied with a change in pathology (reduced hepatosplenomegaly), we consider it as biologically significant, meaning that it reflects a miR-132-driven immune mechanism that determines infection outcomes.

6. The authors should assay miR132 3p v 5p levels before and after T cell activation to confirm which strand is being expressed.
We determined the levels of miR-132-3p and miR-132-5p (formerly miR-132*) in CD4^{+} T cells sorted from spleens of mice infected with *L donovani* (day 28). Below, we show relative levels to a housekeeping RNA (U6) and Ct values for miR-132-3p and miR-132-5p. These demonstrate very low to negligible expression of miR-132-5p in CD4^{+} T cells. This is in agreement with the overall abundance of the two strands as depicted in miRbase (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0000158).

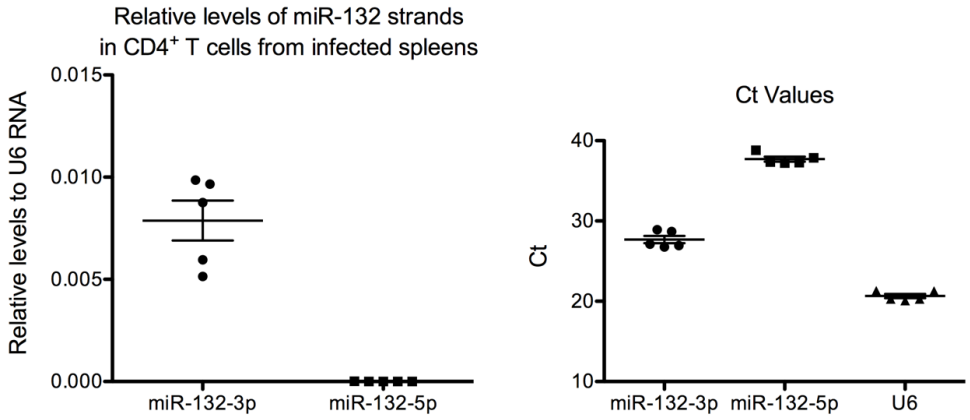

Figure R2: CD4^{+} T cells were purified from spleens of C57BL6 mice infected with *L. donovani* (28 days). Levels of miR-132-3p and miR-132-5p were measured by qRTPCR and normalised to U6 RNA (left panel). Threshold cycle (Ct) values used to calculate miR-132 strand expression are shown in the left panel.

7. If possible, the authors should perform T cell transfer experiments where direct targets of miR132

are reduced with siRNA to study their impacts in vivo. Transfer of Wt and miR-132-/- T cells should also be performed. This would further support a cell intrinsic role for miR132 during the in vivo phenotypes and demonstrate functional relevance for the direct targets p300 and BTAF1.
Response: We appreciate the significance of the proposed experiments. The first experiment would require generation of primary $CD4^+$ T cells with concurrent stable knockdowns for both p300 and BTAF1 that would persist in mice for at least 21 days (earliest timepoint when we observe differences). We believe that it is beyond the scope and timeframe of this manuscript.
Regarding adoptive transfers of WT or *miR-132*$^{-/-}$ $CD4^+$ T cells, we explored using of *Rag2*$^{-/-}$ mice as hosts for $CD4^+$ T cell transfers. However, we found that the use of *Rag2*$^{-/-}$ hosts is of limited value when exploring effects on *L. donovani* parasite load or IL-10 expression in Th1 cells (requires prolonged exposure to pathogen), as infection is cleared upon adoptive $CD4^+$ T cell transfer (we could not detect any parasites at day 21 nor 28 in competitive or single $CD4^+$ T cells transfers – not shown). Work using alternative transfer models (e.g. TCR KO mice) could potentially address this issue but we believe it would be beyond the scope of this manuscript. Our *in vitro* experiments with purified naïve $CD4^+$ T cells and Phenylephrine (Fig. 4C) demonstrated that the effects on IL-10 can be recapitulated in a $CD4^+$ T cell-intrinsic context. In addition, the effects of miR-132 effects on p300, BTAF1, and RP expression are T cell intrinsic.

To compensate for the absence of the experiments suggested by the Reviewer and to accurately represent our findings we have modified the conclusion of our manuscript to include this sentence: "Although we cannot exclude that other cell types or mechanisms contribute to the observed increase in parasite loads in *miR-132*$^{-/-}$ mice, we propose that the effects of miR-132 deficiency on IL-10 expression in $IFNg^+$ $CD4^+$ T cells significantly contribute to reduced protective inflammation and enhanced susceptibility of *miR-132*$^{-/-}$ mice to infection." We also note previous publications that demonstrate the functional significance of Th1-derived IL-10 in the context of *L. donovani* infection when compared to IL-10 produced by Tregs (Jankovic D. et al., JExpMed, 2007) or myeloid cells (Ranatunga D. et al., PNAS, 2009).

3rd Editorial Decision 30 January 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study (which unfortunately took much longer than expected), you will find below. As you will see, the referees now support the publication of your manuscript in EMBO reports. However, referee #2 has one further suggestion we ask you to take up and to address in a final revised version of your manuscript.

Further, I have these editorial requests:

- Please provide a more comprehensive title with not more than 100 characters (including spaces).

- As they are significantly cropped, please provide the source data for the entire Western blots shown in the manuscript (including the EV figures). The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

- Please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #2:

In their revised manuscript, Hewitson et al. have addressed most of my concerns. I would suggest a last minor modification regarding major point 4 that will make the manuscript suitable for publication according to me: I find the IL-10 receptor neutralization experiment quite convincing. As I think that it is a really important point, the related figure should be part of the main manuscript. I would suggest adding this experiment to Fig. 5.

The major point 5 regarding a direct link between RP expression and parasitic clearance was among the critical points of my previous comments. The suggested experiments are not provided. However I understand the lack of availability of Btaf1-/- mice. Moreover, the authors' arguments regarding the difficulties of a potential treatment with phenylephrine are convincing. Therefore, I find the discussion of that point satisfying.

Referee #3:

My concerns have been adequately addressed.

2nd Revision - authors' response 1 February 2019

We now include the IL-10R neutralisation data in Fig. 5A as requested by Reviewer 2.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dimitris Lagos
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-46620V4

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For all animal experiments size calculations guided experimental design based on pilot experiments, which served as indicators of expected effects and varinace within groups.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For all animal experiments size calculations guided experimental design based on pilot experiments. Initial size calculations were done for Power=0.80 and Type I error rate 5% using: http://powerandsamplesize.com/Calculators/Compare-2-Means/2-Sample-Equality
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For infection experiments, animals were randomly selected to be infected or remain naïve
For animal studies, include a statement about randomization even if no randomization was used.	For infection experiments, animals were randomly selected to be infected or remain naïve
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes, for example LDU measurments were done blindly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Investigators measuting LDUs were blind to the background of the animal (WT or KO)
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Where possible, normal distribution was confirmed with appropriate statistical tests (Graphpad Prism).
Is there an estimate of variation within each group of data?	Yes, as seen by the presented graphs.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	total CREB (clone 48H2), p-CREB S133 (87G3), BTAF1 (rabbit pAb #2637; all Cell Signaling Technology), p300 (clone NM11), Rpl27 (14980-1-AP, Proteintech), Rps9 (14894-1- AP, Proteintech), β-actin (AC-15), GAPDH (9484; all Abcam). CD45.1 APC (clone A20); CD45.2 BV786 (104); CD3 FITC (145-2C11); B220 FITC (RA3-6B2); TCRβ PE-Cy7 (H57-597); MHCII alexa700 (M5/114.15.2); Ly6G PE-Cy7 (1A8); CD11b PB and APC (M1/70); CD11c PerCP/Cy5.5 (N418); F4/80 FITC and alexa647 (BM8); CD44 FITC (IM7); CD62L PE (MEL-14); CD8α APC (53-6.7); CD4 PE and PerCP/Cy5.5 (RM4-5); IFNγ FITC (XMG1.2); IL-10 PE (JES5-16E3).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines are routinely tested for mycoplasma. MEFs were isolated from C57BL6 mice and cultured.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Female C57BL/6 CD45.1, CD45.2, and RAG2-/- mice were obtained from Charles River (UK). Mir212/132-/- mice (complete knockouts) were provided by Dr Richard Goodman (Vollum Institute, Oregon Health & Science University, USA). IL-10-/- mice were provided by Dr Anne O'Garra (Francis Crick Institute, UK) and were crossed with WT CD45.2 C57BL/6 mice to generate IL-10+/- heterozygotes. All mice were bred in house, maintained under specific pathogen-free conditions and used at 6 – 12 weeks of age.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal care and experimental procedures were regulated under the Animals (Scientific Procedures) Act 1986 (revised under European Directive 2010/63/EU) and were performed under UK Home Office License (project licence number PPL 60/4377 with approval from the University of York Animal Welfare and Ethical Review Body).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	GEO number GSE125268
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.