EMBO reports

Mitochondrial transfer from MSCs to T cells induces Treg differentiation and restricts inflammatory response

Angela C. Court, Alice Le-Gatt, Patricia Luz-Crawford, Eliseo Parra-Crisóstomo, Victor Aliaga-Tobar, Luis Federico Bátiz, Rafael A. Contreras, María Ignacia Ortúzar, Mónica Kurte, Roberto Elizondo-Vega, Vinicius Maracaja-Coutinho, Karina Pino-Lagos, Fernando E. Figueroa and Maroun Khoury

Review timeline:

Submission date:	7 March 2019
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Editorial Decision:	14 October 2019
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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 26 April 2019

Thank you for submitting your manuscript entitled 'Organelle-based therapy for immune diseases: mitochondrial transfer elicits Tregs reprogramming' to EMBO Reports. We have now received two referee reports, which are included below. In light of these comments, we unfortunately had to conclude that we cannot offer publication in EMBO Reports.

I would like to apologize for this unusual delay in getting back to you. Given this busy time of the year, it took longer than anticipated to receive the referee reports. The referees appreciate the topic - the effects of mitochondrial transfer from MSCs to PBMCs on differentiation and function of Tregs. However, they also raise a significant number of concerns regarding the conclusiveness of the dataset and the methodologies used. I realize that ref #2 is more positive on the overall scope of the manuscript but we have to take into account that referee #1 has a stronger technical expertise on the methods used in the manuscript. Given the discrepancy in the referee reports, I consulted with two external expert advisors in the field to get further input on the technical concerns and both advisors raised similar concerns as referee #1 and did not recommend publication. Given these comments from the good experts in the field, I am afraid we cannot offer publication here.

Thank you in any case for the opportunity to consider this manuscript. I am sorry that I cannot communicate more positive news, but nevertheless hope that you will find our referees' comments helpful.

REFEREE REPORTS

Referee #1:

The authors propose to assess effect of organelles transfer to PBMC and lymphoid cells. They visualize in vitro dose dependent from mitochondria-labelled MSCs mainly to T helper CD4+ (57%) rather than T cytotoxic CD8+ lymphocytes (19%). Did the author check B cells, pDC, as well as monocytes?
The author visualize mitochondria in T cells, as has been described before. Are the mitochondria biologically active and are they functional ? did the author assess number, size, fusion of mitchondria in target cells ?

Artificial transfer of isolated MSC-derived mitochondria increased the expression of mRNA transcripts involved in T cell activation and T-regulatory cell differentiation. Did the author validate other markers of Tregs phenotype (Lag, GITR, CTLA4 ...) as well as methylation of foxp3 promotor in oder to distinguish activated T cell to Treg? Moreover, the experimental conditions after 5 days under PHA activation alter the read out of the mito transfer and makes it difficult to understand the results , showing that both MitoTneg and MitoTpos T cells inhibited the PBMCs proliferation.

In vivo : In a GVHD mouse model, transplantation of MSC have been shown to prevent GVHD. "Mitocepted" PBMC led to improvements in survival, but we need to know what is the relevant T cells population (or monocytes?) involved in the effect. Mice experiments are well conducted, but we are lacking N{degree sign} for ethical agreement for animal experiments. A second inflammatory model to demonstrate impact T cell function in vivo (for ex EAE) would be relevant.

Referee #2:

The manuscript by Court et al presents findings that transfer of mitochondria from MSCs to PBMCs leads to the differentiation of T reg cells. Transferring mitochondria to PBMCs changes nuclear gene expression, increasing FOXP3 expression which presumably drives the differentiation of T regs. The Tregs are function, capable of suppressing T cell proliferation and GVHD in a humanized mouse model.

Overall the manuscript is well written and figures are well presented. Some improvements in presentation of the data are necessary. Comments, mostly minor:

1. For flow cytometry, it is necessary to indicate whether single cells are gated on, particularly for data presented in Figure 1.
2. Figure 1K. The positive control for mouse-specific gene is missing.
3. Figure 3D is hard to read the labels in the bars.
4. Figure 3C. If possible, can clusters in the heatmap be further analyzed?
5. n is missing from most figure legends
6. Figure 4 and other bar graphs in manuscript. Data from multiple experiments should be presented when possible with data points shown as well as bars.
7. Figure 5. More complete data set should be presented. Quantitiatve data across different experiments should be shown for representative graphs show in A and B. This can be shown in supplemental if preferred.
8. Figure 5C has to be clarified what data is being shown; biological replicates, technical replicates, individual data points must be shown.
9. Figrue 5c is missing the positive control of T regs. How is this suppression relative to the suppression mediated by T regs?
10. Figure 6. The individual data points for the human studies are appreciated - does each point represent an individual mouse? These details need to be included in all figure legends.
11. Similar data presentation should be used for the mouse studies in Figure 6. Individual points and clarity on what data is presented.
12. Is the gain in T reg phenotype specific to MSC mitochondria, or do Tregs develop when there is a greater number of mitochondria in the cell?
13. In the Treg assays how is the difference in proliferation controlled for in the differentiation phenotype?
14. Data presented in Figure S2 is interesting. Individual data points must be shown and clarification of whether they are technical or biological replicates are necessary.

15. Can the authors explain why discuss why there is an increase in maximal OCR and glycolysis (though the increase in "basal" glycolysis is not very convincing unless shown for multiple experiment). It be helpful to show the ECAR tracings for S2A and the OCR tracings for S2C to better interpret the data.
16. Red/green contrast for Figures S2 and the heat map are not colorblind friendly.

Additional Correspondence 2 May 2019

We greatly appreciate your expert editorial guidance and the referees' constructive comments including that "the animal experiments are well conducted" #Ref1, and that the "manuscript is well written, and figures are well presented" #Ref-2. We also acknowledge the extra effort that you invested by considering additional external experts.
While we do not contest the methodology followed for making the present decision, we would like if possible, to underscore some of the data already in the report as well as relevant point-by-point arguments and evidence that robustly reflect s the quality of this work while increasing the clarity of the message we feel is important to transmit.
We hope that you are able to have a quick glance at the attached document. We believe that these elements, in addition to the very insightful and constructive reviewer comments might warrants a consideration for a revised resubmission in EMBO reports.

2nd Editorial Decision 6 May 2019

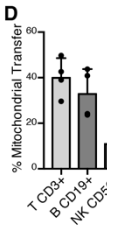
Thank you for submitting a point-by-point response. I have now taken a careful look at everything and discussed them with my colleagues. I appreciate that you can address the technical concerns of the referees. You are welcome to submit a revised manuscript, in which case we would send it to the referees. I would like to emphasize that we need strong support from the referees to consider publication here.

1st Revision - authors' response 27 August 2019

Referee #1

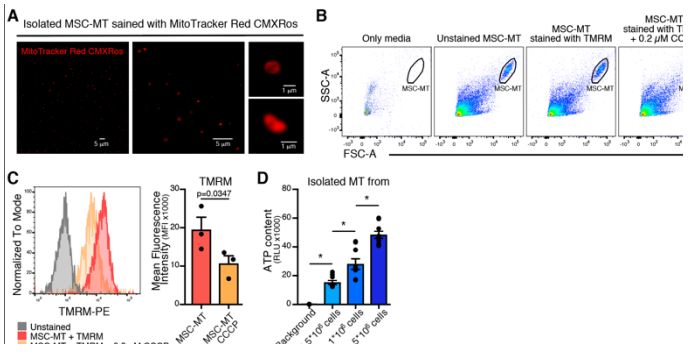
The authors propose to assess effect of organelles transfer to PBMC and lymphoid cells. They visualize in vitro dose dependent from mitochondria-labelled MSCs mainly to T helper CD4+ (57%) rather than T cytotoxic CD8+ lymphocytes (19%). **Did the author check B cells, pDC, as well as monocytes?**

- As we showed in Figure 1D, mitochondrial transfer does occur from UC-MSCs to B cells, positively marked for CD19. (CD19+ cells). We have changed the labels to make it clearer in **Figure 1D**. Using the same experimental design, we have proved MitoT to natural killer (CD45+ CD56+) cells. These data have now been included in **Figure 1D and Supplemental Figure 1B.**
- Regarding plasmacytoid dendritic cells (pDCs), we did evaluate and proved MitoT to dendritic (CD45+ CD11c+) cells by co-culture with UC-MSCs (n=3), with almost absolute MitoT. This interesting result let to the initiation of a side project with a new collaboration group that is not part of the current work. Since the scope of this work focus on transfer to lymphocytes, therefore we have chosen not to include them in the manuscript.



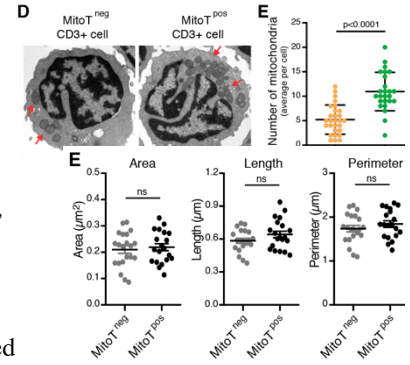
The author visualize mitochondria in T cells, as has been described before. Are the mitochondria biologically active and are they functional?

- We appreciate the reviewer this key question which prompted further analysis on the status of the mitochondria. Using MitoTracker Red CMXRos (a dye that stains MT maintaining their mitochondrial membrane potential (MMP)), TMRM (a dye that accumulates in



active MT with an intact MMP) and measurements of ATP production, as previously described by other teams, we were able to demonstrate that the MT we transfer to T lymphocytes are active and functional. These data have now been included in **Supplemental Figure 2 A-D.**

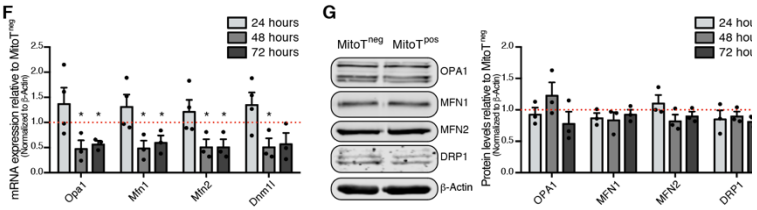
Did the author assess number, size, fusion of mitchondria in target cells?



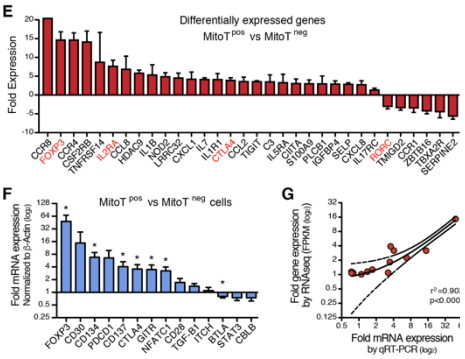
- As we showed in **Figure 2 D-E,** we did evaluate the number of mitochondria in target T CD3+ cells, proving a significant increase in MitoT^{pos} cells compared to MitoT^{neg} control cells.

- To determine the status of the mitochondrial network within the MitoT^{pos} CD3+ cells, we performed further transmission electron microscopy (TEM) analysis, measuring different mitochondrial parameters such as area, length, and perimeter. No significant differences were observed when comparing MitoT^{pos} to MitoT^{neg} CD3+ cells. These analyses have now been added to **Supplemental Figure 2E**

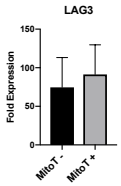
- Regarding fusion of mitochondria we appreciate the relevant argument raised by the reviewer. In order to evaluate fusion/fission of the exogenous MT within the MitoT^{pos} CD3+ cells, we assessed the changes on the mRNA expression and protein levels of mitochondrial mediators that regulates fusion (OPA1, MFN1 and MFN2) and fission (such DRP1 and p-DRP1 (Ser 616)), after 24, 48 and 72 hours post-mitoception. These analyses have now been added to **Supplemental Figure 2F-G**



Artificial transfer of isolated MSC-derived mitochondria increased the expression of mRNA transcripts involved in T cell activation and T-regulatory cell differentiation. Did the author validate other markers of Tregs phenotype (Lag, GITR, CTLA4 ...) as well as methylation of foxp3 promotor in oder to distinguish activated T cell to Treg?



- As we showed in **Figure 3 E-F**, mRNA levels of Treg phenotype markers, GITR and CTLA4, significantly increase in CD3+ MitoT^{pos} compared to MitoT^{neg} cells, not only by RNAseq analysis but validated by qRT-PCR. We also showed that RORC, that encodes for RAR-related orphan receptor gamma (RORγ) - a negative regulator of Treg differentiation -, was differentially decreased in MitoT^{pos} cells. These data are highlighted in red in **Figure 3E**.

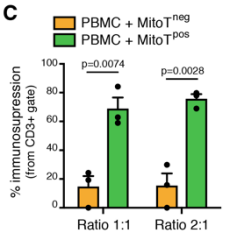


- Conversely, LAG3 mRNA expression levels, by RNAseq analysis, showed no significant differences between both groups analyzed, therefore, we did not include it on the manuscript.

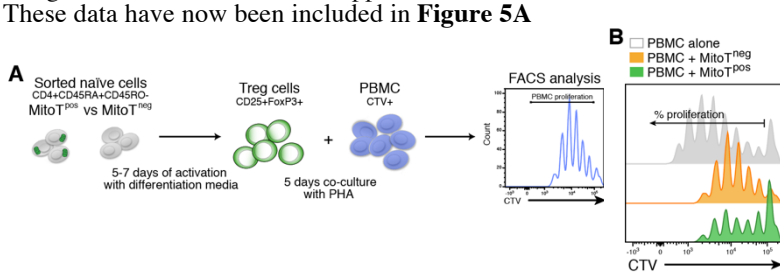
- It is reported that FOXP3 expression can be upregulated transitionally by human conventional T cells upon activation, but its expression is temporal and does not support suppressive function (Baron et al. J Eur Immunol, 2007). In addition, the methylation status is used to compare Treg cell populations and their cellular commitment, as natural Treg show highly demethylated Foxp3 promoter, an indication of high suppressive activity. However, our study shows that MitoT-induced Treg cells: express Foxp3 (30%), display expression of canonical Treg markers (ICOS, CTLA4, GITR, OX40, TGFb) associated with highly modulatory function, and most important, are able to inhibit T cell proliferation. Furthermore, our MitoT-induced Tregs show high levels of CD137, a molecule recently proposed as a marker to distinguish stable human Treg cells (Nowak, et al. Frontiers, 2018). Based on the above, we believe that MitoT-induced CD4+FoxP3+ T cells correspond to bona fide Treg cells.

Moreover, the experimental conditions after 5 days under PHA activation alter the read out of the mito transfer and makes it difficult to understand the results, showing that both MitoTneg and MitoTpos T cells inhibited the PBMCs proliferation.

- For the immunosuppression assay (Figure 5), we tested the suppressive function of our MitoT-induced Treg cells using PBMCs from a different donor activated with PHA. We showed that MitoT^{pos}-Tregs significantly increase the percentage of immunosuppression, as shown in **Figure 5C** (measured as reduction on proliferation of new PBMCs) compared to MitoT^{neg} Treg-induced cells. The result was replicated in three independent biological samples. This shows that PHA activation did not alter the read out since differential proliferation was observed.

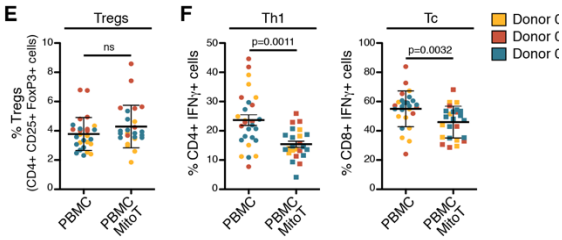


- In order to disolay the immunosuppression experimental setting and results in a clearer presentation, we have generated a schematic representation of the experimental design used for this assay and changed the proliferation histogram to make the immunosuppression effect of MitoT^{pos} Treg-induced cells more visible. These data have now been included in **Figure 5A and Figure 5B.**

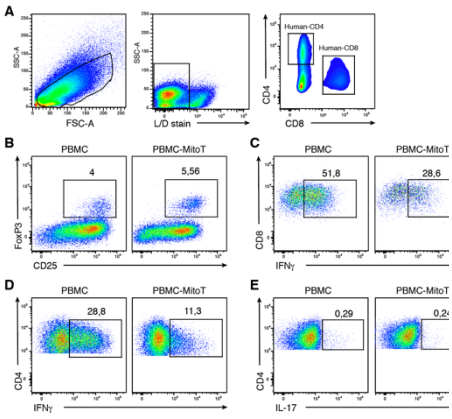


In vivo : In a GVHD mouse model, transplantation of MSC have been shown to prevent GVHD. "Mitocepted" PBMC led to improvements in survival, but we need to know what is the relevant T cells population (or monocytes?) involved in the effect.

- As we showed in **Figures 6 E-F and M** we analyzed subpopulations of human T cells following transplantation of PBMC-MitoT or control PBMC cells, including Tregs, Thelper and T cytotoxic cells. For further clarity, T cell subpopulation titles have now been added to each dot plot graph presented in **Figure 6.**

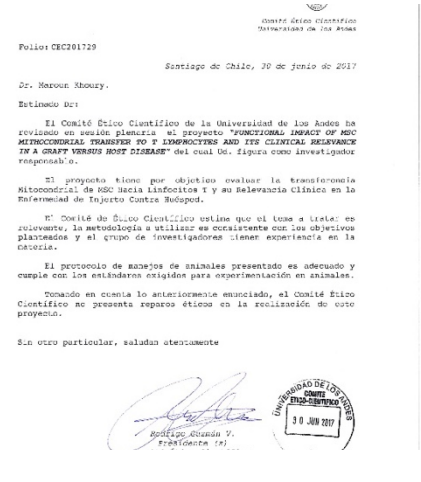


- We have also included representative FACS plots for each different T cell populations to indicate whether single cells are gated on, data now included in **Supplemental Figure 7.**



Mice experiments are well conducted, but we are lacking N {degree sign} for ethical agreement for animal experiments.

- The scientific-ethics committee from Universidad de los Andes approved the animal use for our studies in Graft vs Host disease mouse model on June 30th, 2017, number CEC201729. This information has now been included in **Materials and Methods** section:



Folio: CEC201729

Santiago de Chile, 30 de junio de 2017

Dr. Maroun Khoury.

Estimado Dr:

El Comité Ético Científico de la Universidad de los Andes ha revisado en sesión plenaria el proyecto *"FUNCTIONAL IMPACT OF MSC MITOCONDRIAL TRANSFER TO T LYMPHOCYTES AND ITS CLINICAL RELEVANCE IN A GRAFT VERSUS HOST DISEASE"* del cual Ud. figura como investigador responsable.

El proyecto tiene por objetivo evaluar la transferencia Mitocondrial de MSC hacia Linfocitos T y su Relevancia Clínica en la Enfermedad de Injerto Contra Huésped.

El Comité de Ético Científico estima que el tema a tratar es relevante, la metodología a utilizar es consistente con los objetivos planteados y el grupo de investigadores tienen experiencia en la materia.

El protocolo de manejos de animales presentado es adecuado y cumple con los estándares exigidos para experimentación en animales.

Tomando en cuenta lo anteriormente enunciado, el Comité Ético Científico no presenta reparos éticos en la realización de este proyecto.

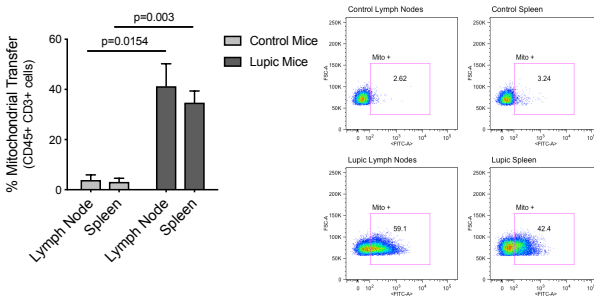
Sin otro particular, saludan atentamente

UNIVERSIDAD DE LOS ANDES
30 JUN 2017

"Animals were kept at specific pathogen-free animal facility of Universidad de los Andes with water and food ad libitum, according to international guidelines for animal care and protocols approved by the Institutional Animal Care and Use Committee (ARRIVE guidelines Animal Research), and approved by the local animal care committee (number CEC201729, June 30th 2017)".

A second inflammatory model to demonstrate impact T cell function in vivo (for ex EAE) would be relevant.

- We use a systemic lupus erythematosus (SLE) model to demonstrate that *ex-vivo* spleen and lymph node cells from SLE (lupic) mice (MRL/MpJ/Fas) had increased mitochondrial transfer from UC-MSCs compared to control healthy mice (MRL/MpJ) (n=3). SLE is considered an autoimmune disease with inflammatory disorder of the connective tissues. While the results are very promising, the present work focus on GVHD, to avoid *the* information dilution in two models that present different levels of advances, we decided not to include the SLE in the manuscript section and the strategy of a follow-up paper is privileged.



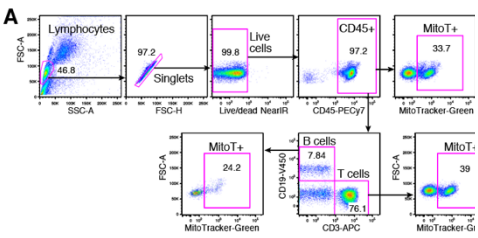
Referee #2:

The manuscript by Court et al presents findings that transfer of mitochondria from MSCs to PBMCs leads to the differentiation of T reg cells. Transferring mitochondria to PBMCs changes nuclear gene expression, increasing FOXP3 expression which presumably drives the differentiation of T regs. The Tregs are function, capable of suppressing T cell proliferation and GVHD in a humanized mouse model.

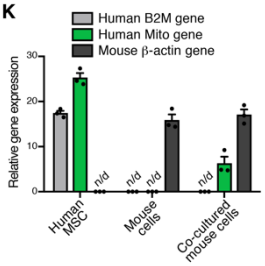
Overall the manuscript is well written, and figures are well presented. Some improvements in presentation of the data are necessary. Comments, mostly minor:

1. For flow cytometry, it is necessary to indicate whether single cells are gated on, particularly for data presented in Figure 1.

- We agree with the reviewer that showing the gating strategy used for data presented in Figure 1 will help to better understand the MitoT to different cell type populations. Representative FACS plots analysis have now been included in **Supplemental Figure 1A.**

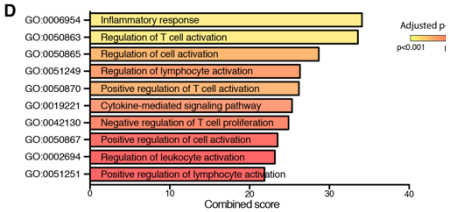


2. Figure 1K. The positive control for mouse-specific gene is missing.



- Using mouse-specific primers and the same cDNA samples used for figure 1K we assessed the mouse beta-actin levels by qPCR. These results have now been added to **Figure 1K.**

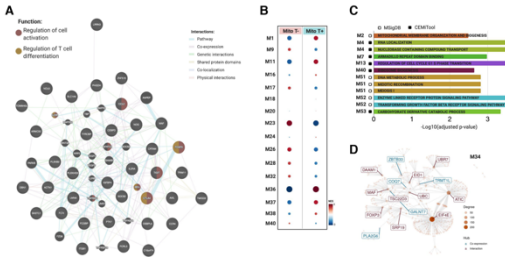
3. Figure 3D is hard to read the labels in the bars.



- We would like to thank this reviewer for this observation. Labels and bar color intensity have now been changed to replace **Figure 3D.**

4. Figure 3C. If possible, can clusters in the heatmap be further analyzed?

- In response to this question, we have included data showing further analysis on RNA-seq data from heatmap clusters, and Gene Set Enrichment Analysis to identify co-expression/interaction networks functional enriched pathways between MitoT^{pos} and MitoT^{neg} CD3+ T cells. These new data have now been included in **Supplemental Figure 4.**

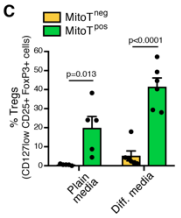


5. n is missing from most figure legends

- We have revised all figure legends to make sure the number of biological replicates were added for each figure.

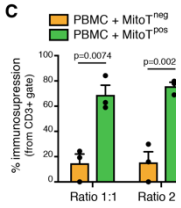
6. Figure 4 and other bar graphs in manuscript. Data from multiple experiments should be presented when possible with data points shown as well as bars.

- We appreciate the reviewer for this key point that will make our results look more defined and robust. We have now modified **Figure 4C and all other bar graph figures** in the manuscript to present data points for each independent experiment as well as the bar graph that represents the average of all data points.



7. Figure 5. More complete data set should be presented. Quantitiatve data across different experiments should be shown for representative graphs show in A and B. This can be shown in supplemental if preferred.

- We agree with the reviewer therefore we have included all data points to the bar graph presented in **Figure 5C**, corresponding to three independent experiments. Also, we have changed one of the representative FACS plots of figure 5C to **Supplemental Figure 4B** to make more distinct the effect of this immunosuppression assay.



8. Figure 5C has to be clarified what data is being shown; biological replicates, technical replicates, individual data points must be shown.

- We have addressed this point by showing all data points in **Figure 5C** and changing the figure legend to reflects the reviewer suggestion.

9. Figrue 5c is missing the positive control of T regs. How is this suppression relative to the suppression mediated by T regs?

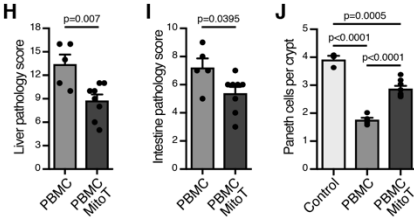
- We used as positive control for the immunosuppression assay the CD4+ T cells differentiated in Treg induction media (containing IL-2 + TGFb) without acquisition of Mitochondria (Mito^{neg} fraction). The induced Treg cells obtained using that gold standard protocol represent the adequate comparative condition. As the figure shows, this condition inhibits the % of PBMC under proliferation. We have now edited the **Figure legend** to better understand the data.

10. Figure 6. The individual data points for the human studies are appreciated - does each point represent an individual mouse? These details need to be included in all figure legends.

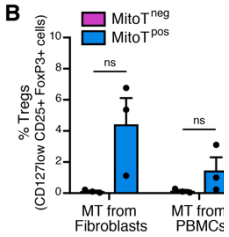
- As correctly pointed out by this reviewer, each point in Figure 6 represents an individual mouse. For **Figures 6 E-F and M**, we have changed the figure legends to point out a total of 25-27 mice per group from 3 independent *in vivo* experiments. Figure legends for the histopathological analysis, **Figures 6 G-L**, were changed as well to point out a total of 5-8 mice per group from 3 independent *in vivo* experiments.

11. Similar data presentation should be used for the mouse studies in Figure 6. Individual points and clarity on what data is presented.

- Figure 6 have now been modified to show all data points. Figure legends also have been changed to reflect the number of mice analyzed per experiment, as mentioned in reviewers' question n°10.



12. Is the gain in T reg phenotype specific to MSC mitochondria, or do Tregs develop when there is a greater number of mitochondria in the cell?



- The question raised here allowed us to acquaint the specific role of MSC-mitochondria in Treg differentiation. To address this question, we have performed two set of experiments following the same methodology used in Figure 4; in one set we mitocepted PBMCs using MT from human fibroblast and on the other we used MT from the same donor of PBMCs. Our results showed that neither other source of MT nor greater number of MT in the cell were sufficient to significantly differentiate naïve MitoT+ cells to Tregs (4.4 and 1.4 % of Tregs, respectively, compared to 19.8% of Tregs obtained with MSC-MT). This new data have now been included in **Supplemental Figure 4B.**

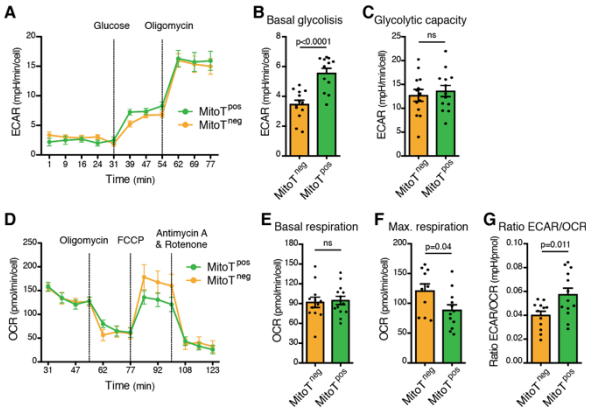
13. In the Treg assays how is the difference in proliferation controlled for in the differentiation phenotype?

- First, these assays were performed using *cell-sorted* human naïve CD4+ T cells, so no Tregs were present in the starting population (less than 0.5%). In addition, it is very well known that natural Tregs (if present) require high levels of co-stimulation, high levels of IL-2 and rapamycin (doi: 10.1007/978-1-61779-430-8_17). Thus, our final CD4+ T cell population containing MitoT-Treg cells does not have "contaminating" nTregs that could overgrow in culture.

14. Data presented in Figure S2 is interesting. Individual data points must be shown and clarification of whether they are technical or biological replicates are necessary.

- We agree and have changed the figure to show all data points and added more detailed figure legend descriptions to clarify number of biological replicates in each panel. New data is now presented in **Supplemental Figure 3**.

15. Can the authors explain why discuss why there is an increase in maximal OCR and glycolysis (though the increase in "basal" glycolysis is not very convincing unless shown for multiple experiment). It be helpful to show the ECAR tracings for S2A and the OCR tracings for S2C to better interpret the data.



- The basal energy metabolism of the cells was assessed by analyzing ECAR/OCR ratio. The increased ECAR/OCR ratio in MitoT^{pos} cells compared to MitoT^{neg} cells suggests a metabolic reprogramming of these cells from oxidative phosphorylation to aerobic glycolysis. This metabolic switch is described as one of the most striking changes to affect T cells when activating *(Pearce EL et al., 2010, Curr Opin Immunol* and *Patsoukis N. et al., 2016, Curr Trends Immunol)*. Maximal respiration is directly linked to the extra capacity of mitochondria to produce the necessary energy when a cell is under conditions of high levels of stress or work, and therefore is considered as important for cell survival. Regarding lymphocytes, it has been shown that effector T cells have a substantially lower maximal respiration than memory T cells *(Buck MD. et al., 2016, Cell* and *Van der Windt GJ et al., 2012, Immunity)*, therefore, we find relevant to show that in our experiments MitoT^{pos} cells had decreased maximum respiration compared to MitoT^{neg} cells. Altogether, our findings suggest that T cells may enter in an activated state when mitocepted. **Main text** have been modified to make our results more clear to understand and references have been added to the **discussion section**.
    - **Supplemental Figure 2** have now been changed to show ECAR tracings first, in S2A, and OCR tracings next, in S2D. Additionally, all data points have been included, as mentioned in reviewers' question n°14, to show multiple biological replicates (n=3 biological replicates, ran in quadruplicates).

16. Red/green contrast for Figures S2 and the heat map are not colorblind friendly.

- We would like to thank this reviewer for this important criterion. Red/green colors have now been changed and replaced for more colorblind safe color scheme, in **Figure S3 and the heat map.**

3rd Editorial Decision 14 October 2019

Thank you for submitting a revised version of your manuscript. Having read your response carefully, I sent it back to both of the original referees, whose comments are pasted below.

I would like to apologize for the delay in getting back to you, which was due to this busy time of the year.

As you can see, the referees find that the study is significantly improved and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

• Please address the remaining minor concerns of the referees by including the MRL lupus data in the manuscript and expanding the discussion.
• Please deposit your RNA sequencing data in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>) and make it publicly available.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

• We have noted that there are currently 7 keywords provided. We can accommodate maximally 5 keywords, therefore please eliminate 2 of them.
• We noted that there are 2 MKs in the author contributions section, which should be shortened to MKh and MKu.
• We noted that your ORCID iD is not linked. As of January 2016, new EMBO Press policy asks for corresponding authors to link to their ORCID iDs. You can read about the change under "Authorship Guidelines" in the Guide to Authors here: http://emboj.embopress.org/authorguide

In order to link your ORCID iD to your account in our manuscript tracking system, please do the following:

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• We noted the presence of the phrase "data not shown" on pages 6, 9 and 11, which we do not allow as per journal policy. Please either show the data or remove the statement.
• For technical reasons, our limit for expanded view (EV) figures is 5 (please see our author guidelines https://www.embopress.org/page/journal/14693178/authorguide#expandedview). You currently have 6 supplemental figures and a table provided as a single file. You can either combine the figures into 5 expanded view (EV) figures and provide the figures as single files. Then the remaining table would be called EV table 1. Alternatively, this could be turned into an appendix file, with the correct nomenclature "Appendix Figure S1" etc. and a table of contents added to the first page. Either way, please update the callouts in the text.
• Please add scale bars to Figure 1B top left panel and figure 4A.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The

synopsis includes a short standfirst summarizing the study in 1 or 2 sentences - as well as 2-5 one sentence bullet points that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your suggestions for a synopsis blurb and bullet points.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

REFEREE REPORTS

Referee #1:

The authors has improved the manuscript, and corrected the requested for quality of the figures as well as legend. One important issue concerned the specificity of the effect of mitochondrial transfer in vivo and a second model was requested. The author have data on MRL lupus model, and this should be included in the manuscript to strength the message

Referee #2:

The authors have nicely addressed the reviewers comments. The paper is suitable for publication.

My only suggestion would be, if the authors desire, to be a bit more speculative on the role of mitochondria in cellular programming, because I think this is a really exciting area (I find the discussion a little on the rehashing the results side). Maybe some discussion in context of Navdeep Chandell's recent paper (Weinberg et al) on mito programming in T regs.

2nd Revision - authors' response 11 November 2019

We are very delighted with the reviewer recommendation . We would like to thank you for considering our work and for the very fair reviewing process that EMBO reports has provided us.

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: Maroun Khoury and Fernando E. Figueroa
Journal Submitted to: EMBO Reports
Manuscript Number: 2019-48052

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?	Sample size was chosen according to minimum of three independent replicates use, and a larger number was included in some cases to allow more robust analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size for animal studies was chosen following previously reported studies that have used the GvHD mouse model; at least 8-10 independent mice per experimental set, repeating three times each experimental set (n=3 biological replicates)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes, animals were ramdomized previous injection of control or MitoT PBMCs.
For animal studies, include a statement about randomization even if no randomization was used.	Statement is included in materials and methods section.
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, when performing histopathological analysis, slides were examined systematically and in a blinded fashion. Statement in included in materials and methods section.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Statement is included in materials and methods section.
5. For every figure, are statistical tests justified as appropriate?	Yes, for every figure we included the number of independent biological replicates, graph representation and the type of statistical test used: Unpaired, two-tailed Student's t-test was used to compare two samples; One-way ANOVA was used to analyzed more than two samples.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes

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http://www.antibodypedia.com
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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
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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/

Is there an estimate of variation within each group of data?	Yes, we showed standard error of the mean (SEM) for each group of data analized, as described in each figure legend.
Is the variance similar between the groups that are being statistically compared?	Statitsical analysis was done using the software GraphPad Prism which calulates the F value for variances, and data was presented as data points for each independent experiments.

C- Reagents

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).	Detailed information of all the antibodies used in this study has been included in materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Information regarding animal models is included in materials and methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Statement is included in materials and methods section.
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.	Animal studies are in compliance with ARRIVE guidelines, as stated in materials and methods section.

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	The RNA sequencing data will be deposited in a public database upon final approval of the present manuscript.
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