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Peer Review Information

Journal: Nature Immunology
Manuscript Title: Impaired mitochondrial oxidative phosphorylation limits the self-renewal of T-cells exposed to persistent antigen
Corresponding author name(s): Craig Thompson

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A29271
Message: 13th Mar 2020

Dear Craig & Santosh,

Your manuscript entitled, "Impaired mitochondrial oxidative phosphorylation limits the self-renewal of T-cells exposed to persistent antigen" has now been seen by 2 referees. Both referees were actually quite supportive of the study, but they expressed that a few additional experiments should be performed to strengthen the study findings. I am pasting below their specific comments. Q1 suggests that experiments using a mitochondrial targeted antioxidant like Mito-tempo in vitro and in vivo is needed. Q2 notes that cell viability needs to be examined and that Glyco-Stress tests need to be performed as well. Any mechanistic insights would also be welcome.

We therefore invite you and your colleagues to revise the manuscript to address these concerns posed by the referees. Please take into account all reviewer and editor comments, please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

* Include a “Response to referees” document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it

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conforms to our Article format instructions at
http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

The Reporting Summary can be found here:
https://www.nature.com/documents/nr-reporting-summary.pdf

You may use the link below to submit your revised manuscript and related files:

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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit <a
href="http://www.springernature.com/orcid">www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Stay well!

Laurie

Laurie A. Dempsey, Ph.D.
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Referee expertise:

Referee #1:

Referee #2:

Referee #3:

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:

This is an interesting paper linking mitochondrial dysfunction to exhausted T cell phenotype. I like the connection of mitochondrial ROS ot the high levels of expression of the exhaustion-associated transcription factor TOX. Overall the paper is well done and will be of interest for metabolism, immunotherapy and T cell biology field.

(1) The authors should try mito-tempo in addition to NAC in vitro and in vivo. This would link mitochondrial ROS to the exhaustion pathway. This is a key experiment to support their hypothesis.
(2) Could the authors examine whether mitochondrial complex III superoxide suppressors diminish TOX levels in vitro? There is a commercially available reagent that decreases complex III superoxide levels (PMCID: PMC4618194). Complex III is a major site of ROS generation.
(3) Is increasing intracellular hydrogen peroxide sufficient to induce exhaustion phenotype (high TOX)? They should try Paraquat and examine TOX levels.
(4) Can the authors provide some pathways that link mitoROS during exhaustion to pathways that control TOX?
(5) Supplemental Figure 3c should be moved to main figure. It is an important genetic experiment.

Reviewer #2:
Remarks to the Author:
Vardhana et al investigated the limitations of immune checkpoint blockade therapy (anti-PD-1), using an in vitro model of chronic antigen stimulation to simulate “exhausted” CD8+ T cells. Given the evidence that suggests the efficacy of anti-PD-1 therapy could be related to metabolic rewiring in T cells, defining the molecular mechanisms that underlie metabolic dysfunction in exhausted T cells could inform novel treatment strategies for boosting anti-tumor immunity in non-responders. Vardhana et al show that exhausted T cells are highly glycolytic despite a poor proliferative capacity, consistent with a loss of progenitor-like cellular transcription factors. Specifically, chronically stimulated T cells exhibited reduced mitochondrial beta-oxidation capacity, which lowered ATP and other tri-phosphate nucleotides availability and compromised the cellular pools of nucleotide triphosphates. Reactive oxygen species (ROS) were elevated in chronic antigen stimulation, which could contribute to mitochondrial dysfunction and impaired proliferation. Notably, N-acetylcysteine restored defects in chronically stimulated T cells

and synergized with anti-PD-L1 therapy both in vitro and in vivo, suggesting a novel strategy for improving PD-L1 efficacy in both responders and non-responders. The topic is interesting and important, and the studies are on the whole well performed.

Major points:

The paper hinges on the in vitro restimulation conditions as a model for chronic in vivo stimulation. While these data on the whole look convincing, restimulations can lead to significant cell death in vitro and the overall measure of "population doublings" is not really sufficient to describe the conditions. The authors should show that degree of cell death as well as proliferation and viable cell numbers over time. If the assays are performed on a highly selected end population rather than a qualitatively different population of cells that have undergone a specific adaptation, some conclusions may be limited.

One interesting possibility is if these populations have different p53 signaling/DNA damage (nuclear or mitochondrial)/etc., which could tie in with the ROS differences already observed by the authors and provide more mechanistic detail.

The attention to detail e.g. measuring glucose up take as well as lactate secretion over time is a strength of this paper. In Figure 1, the authors discuss the "enhanced glycolysis" observed in the chronic cells. This claim would be supported much better by a Glyco Stress test on the Seahorse Extracellular Flux Analyzer; for example, do these cells have increased glycolytic capacity, superior acute response to glucose, or both? It would also be interesting to know if these cells have higher levels of glucose transporters and glycolytic enzymes.

The signals that lead to the exhausted state are not well explored. These could lead to mechanistic insight of exhaustion and could be genetically tested at more depth to complement an otherwise heavily inhibitor-dependent study.

Minor points:

The in vitro model of exhaustion could be much stronger if shown that chronically stimulated cells have decreased CTL killing efficiency, not only decreased cytokine production and proliferation.

Memory-like subsets are not the best comparison for the datasets discussed in Figure 1i. Memory T cell subsets are inherently low in glycolytic metabolism and does not seem like a fair comparison to the chronically stimulated cells. How would this data have compared to activated effector T cells which are also highly glycolytic? The authors could compare acute vs chronic not just at the end of the response, but at multiple time points. Since memory cells are resting and non-cycling, what is the importance of a higher "glycolysis score"?

References should be added for the brief discussion of TOX vs. TCF-1.

The claim of "reliance on glycolysis for ATP production" from Figure 2c would be better supported by a Seahorse Mito Fuel Flex test, to directly test these cells' dependence on glucose for ATP synthesis. This could also create an opportunity to address dependency on glutamine flux, which was otherwise overlooked in this manuscript.

Supplemental Figure 3a-c was an interesting approach. However, confirmation of mitochondrial localization of mitoLbNOX should be provided. Supplemental Figure 3b is not entirely convincing. How many times was the experiment repeated? Is there any statistical difference in ROS staining intensity between the vector and the recombinant enzymes? Additionally, it would be helpful to include "chronic" samples as a comparison for the panels in Supplemental Figures 3e+f.

Page 13: Data in Figure 3H are used to support the statement "Thus, the loss of mitochondrial oxidative phosphorylation is sufficient to impair T-cell proliferative capacity and activate exhaustion-associated gene expression". The results are not so clear cut and are limited to TCF1 vs TOX expression. These data are overinterpreted and the conclusion should be toned down.

Author Rebuttal to Initial comments

Response to reviewers

We thank the reviewers for the constructive comments on the manuscript. The experiments they suggested have significantly strengthened our finding that chronic antigen-driven mitochondrial dysfunction is required for development of both the functional and transcriptional alterations that define terminally exhausted T-cells and can be therapeutically targeted to enhance anti-tumor immune responses.

The additional experiments address three major themes that emerged from the reviewers. First, we provide additional experiments to confirm that mitochondrial oxidative stress contributes to T-cell dysfunction during chronic stimulation. The new data confirm that depleting endogenous anti-oxidant pools using diamide is sufficient to decrease TCF-1 expression and activate TOX expression, while mitochondrial anti-oxidants such as MitoTEMPO are sufficient to maintain T-cell function during chronic stimulation.

Second, we provide a molecular mechanism by which mitochondrial dysfunction can activate the exhaustion-associated gene expression program. Sustained nuclear NFAT has been shown to bind to the TOX promoter and activate TOX expression; accordingly, constitutively nuclear NFAT recapitulates many transcriptional features of T-cell exhaustion. By showing that chronic stimulation-dependent activation of NFAT gene targets is reversed by N-acetylcysteine, we provide a molecular mechanism by which mitochondrial ROS can activate TOX expression. Consistent with these findings, analysis of single-cell RNA-sequencing data demonstrated a strong correlation between NFAT target genes and oxidative stress-associated genes in

intratumoral T-cells with patients; as a result, NFAT target genes were enriched in terminally exhausted T-cells.

Finally, we provide additional data to support the metabolic phenotype associated with chronic TCR stimulation. The new data show that the enhanced rate of glycolysis in chronically stimulated T-cells is present from two days following stimulation, is associated with increased expression of the Glut1 transporter, and is operating at a maximal level such that it cannot be further increased by inhibition of mitochondrial oxidative phosphorylation. Consistent with being completely dependent on glycolytic ATP, we also observed a near-complete elimination of glucose-dependent spare respiratory capacity in chronically stimulated T-cells.

Response to Reviewer #1.

(1) The authors should try mito-tempo in addition to NAC in vitro and in vivo. This would link mitochondrial ROS to the exhaustion pathway. This is a key experiment to support their hypothesis.

We thank the reviewers for this excellent suggestion. Our new data demonstrates that the benefit of antioxidants during chronic stimulation is not restricted to N-Ac, as both alternative free radical scavengers such as Trolox and mitochondrially targeted antioxidants such as MitoTEMPO can restore T-cell function during chronic stimulation (New Supplementary Fig. 7d). Unfortunately, due to current constraints resulting from the COVID-19 epidemic we cannot initiate any experiments to confirm the efficacy of MitoTEMPO or Trolox-treated cells *in vivo*.

(2) Could the authors examine whether mitochondrial complex III superoxide suppressors diminish TOX levels in vitro? There is a commercially available reagent that decreases complex III superoxide levels (PMCID: PMC4618194). Complex III is a major site of ROS generation.

We appreciate this excellent suggestion by the reviewer, as complex III is a key site of ROS generation activated T-cells. While we did not specifically use inhibitors of complex III superoxide production, the ability of MitoTEMPO, which neutralizes mitochondrial superoxides, and Trolox, which neutralize hydroxyl groups likely generated downstream of hydrogen peroxide, support the hypothesis that excessive generation of superoxides at complex III contribute to the exhausted T-cell phenotype (New Supplementary Fig. 7d).

(3) Is increasing intracellular hydrogen peroxide sufficient to induce exhaustion phenotype (high TOX)? They should try Paraquat and examine TOX levels.

We appreciate this suggestion by the reviewer. In addition to blocking endogenous glutathione production with BSO, we now show that either reducing the exogenous scavenging of free radicals by decreasing BME or increasing the consumption of glutathione stores using diamide to generate intracellular disulfide bonds was sufficient to reduce T-cell proliferation, decrease TCF-1 expression and increase TOX expression (New Figure 4e, Supplementary Fig. 4g).

(4) Can the authors provide some pathways that link mitoROS during exhaustion to pathways that control TOX?

We thank the reviewer for this suggestion, which enabled us to provide a potential mechanism by which mitochondrial dysfunction during chronic T-cell stimulation activates TOX. Upstream regulators of TOX expression have not been identified with the exception of NFAT, which binds to the TOX promoter and can independently activate TOX expression[1]. Nuclear NFAT translocation has been shown to require mitochondrial ROS[2]. Accordingly, we found that NFAT target genes were highly enriched during chronic T-cell stimulation in a manner that was reversed in the presence of N-acetylcysteine (New Supplementary Fig. 4c, 6c-d). Nuclear NFAT translocation is promoted by the calcium-dependent phosphatase calcineurin; intracellular calcium elevation also promotes mitochondrial oxidative phosphorylation, effectively coupling TCR-dependent NFAT mobilization to mitochondrial function. Our new data demonstrates that chronically stimulated T-cells maintain elevated intracellular calcium pools (New Supplementary Fig. 4b); this provides a mechanism by which chronic TCR signaling, through sustained elevated intracellular calcium, leads to persistent nuclear NFAT and activation of TOX. Consistent with data obtained using our model system, analysis of single-cell RNA-sequencing of intratumoral T-cells isolated from melanoma patients demonstrated a strong correlation between enrichment of NFAT target genes and genes related to oxidative stress; as a result, NFAT target genes were most significantly enriched in terminally exhausted T-cells (New Supplementary Fig. 4d,e). This data supports a role for NFAT in activating the ROS-dependent, TOX-driven exhaustion program.

(5) Supplemental Figure 3c should be moved to main figure. It is an important genetic experiment.

We appreciate the reviewer's attention to this experiment. While restoring the NAD+/NADH ratio using LbNOX did provide some benefit to T-cells during chronic stimulation, the recovery

was incomplete. We suspect that this was because the NADH oxidase activity that LbNOX provides is uncoupled from ATP synthesis, leaving these cells with a persistent bioenergetic defect. Conversely, N-Ac supplementation increased ADP-coupled oxidative phosphorylation and thus more effectively reversed T-cell dysfunction. For this reason and due to space constraints, we have emphasized the benefit of N-acetylcysteine in the main figures and kept the LbNOX experiment in the supplement.

Response to Reviewer #2:

The paper hinges on the in vitro restimulation conditions as a model for chronic in vivo stimulation. While these data on the whole look convincing, restimulations can lead to significant cell death in vitro and the overall measure of "population doublings" is not really sufficient to describe the conditions. The authors should show that degree of cell death as well as proliferation and viable cell numbers over time. If the assays are performed on a highly selected end population rather than a qualitatively different population of cells that have undergone a specific adaptation, some conclusions may be limited.

We appreciate this key insight by the reviewer. They are absolutely correct in noting that persistent antigenic stimulation can cause cell death *in vitro.* This may also contribute to the decreased accumulation of antigen-specific cells during chronic viral infections and within tumors along with diminished proliferative capacity. We observe a small, but reproducible decrease in cell viability during chronic stimulation (New Supplementary Fig. 1m). However, the primary defect leading to the reduced accumulation of chronically stimulated cells is a loss of proliferative capacity, as shown in the newly generated Supplementary Fig. 1n. Cell Trace Violet staining reveals that the chronically stimulated T-cells lose proliferative capacity about 1-2 generations earlier than acutely stimulated T-cells and uniformly activate TOX expression. We thank the reviewer for the opportunity to clarify this key area.

One interesting possibility is if these populations have different p53 signaling/DNA damage (nuclear or mitochondrial)/etc., which could tie in with the ROS differences already observed by the authors and provide more mechanistic detail.

We thank the reviewer for this interesting suggestion. While we do observe an increase in ROS during chronic stimulation, this only mildly reduces cell viability during the first eight days of chronic stimulation (New Supplementary Fig. 1m). Consistent with these findings, analysis of RNA-seq our *in vitro*

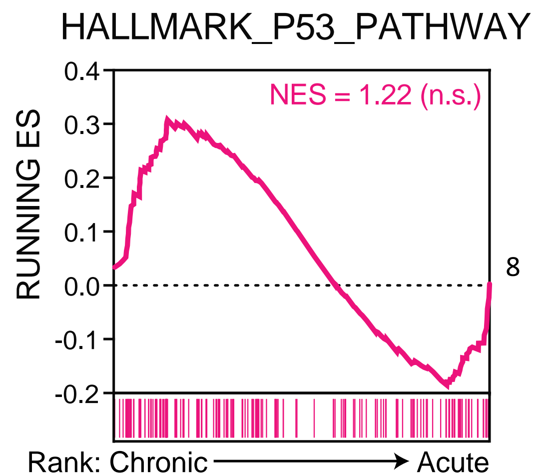

Figure 1.

co-culture system did not show significant enrichment of p53 target genes after eight days of stimulation (**Figure 1**). Similarly, analysis of tumor-infiltrating T-cells from Scott et al showed enrichment of NFAT, but no enrichment of p53 binding motifs at loci with increased chromatin accessibility in intratumoral T-cells as compared to effector T-cells at day 7 (Supplementary Fig. 4a, **Table 1** below). While we cannot exclude a role for p53 stabilization in mediating late transcriptional or epigenetic changes during chronic T-cell stimulation, our analysis does not suggest that p53 is involved in the transcriptional or functional effects of chronic stimulation that we observe at earlier timepoints, and as such, we have not included this data in the revised submission.

Motif Name	**Consensus**	**P-value**	**% of Target**	**% of Background**
NFAT(RHD)/Jurkat-NFATC1-ChIP-Seq/Homer	ATTTTCCATT	1e-642	48.89%	19.99%
NFAT:AP1(RHD,bZIP)/Jurkat-NFATC1-ChIP-Seq/Homer	SARTGGAAAAWRTGAGTCAB	1e-624	20.76%	3.73%
p53(p53)/mES-cMyc-ChIP-Seq/Homer	ACATGCCCGGGCAT	1	0.24%	0.33%
p53(p53)/Saos-p53-ChIP-Seq/Homer	RRCATGYCYRGRCATGYYYN	1	2.30%	2.63%

Table 1.

The attention to detail e.g. measuring glucose up take as well as lactate secretion over time is a strength of this paper. In Figure 1, the authors discuss the "enhanced glycolysis" observed in the chronic cells. This claim would be supported much better by a Glyco Stress test on the Seahorse Extracellular Flux Analyzer; for example, do these cells have increased glycolytic capacity, superior acute response to glucose, or both? It would also be interesting to know if these cells have higher levels of glucose transporters and glycolytic enzymes.

We appreciate this opportunity to clarify the glycolytic phenotype observed during chronic T-cell stimulation. We know based on analysis of the extracellular fluid that chronically stimulated T-

cells consume glucose and excrete lactate at increased rates compared to acutely stimulated cells and have confirmed by isotope tracing that the lactate produced by T-cells is almost entirely glucose-derived (New Supplementary Fig. 1h). In combination with the data showing that the fraction of glucose excreted as lactate is increased during chronic stimulation this is consistent with increased rates of aerobic glycolysis. While we did not perform a Glycolytic Stress Test using the proprietary Seahorse Analyzer, we now provide additional extracellular flux data demonstrating that 1) The increased extracellular acidification rate is dependent on available glucose and eliminated when glucose uptake is blocked by 2-DG, and 2) The capacity of chronically stimulated T-cells to further increase their glycolytic rate when mitochondrial oxidative phosphorylation is blocked with oligomycin is completely lost, consistent with chronically stimulated cells operating at maximal glycolytic capacity (New Supplementary Fig. 1j,k). We also provide new data showing marked upregulation of Glut1 transporter expression that allows for the increased rates of glycolytic flux that we observe (New Supplementary Fig. 1g). We thank the reviewers for the opportunity to offer this clarification.

The signals that lead to the exhausted state are not well explored. These could lead to mechanistic insight of exhaustion and could be genetically tested at more depth to complement an otherwise heavily inhibitor-dependent study.

We thank the reviewer for this suggestion, which enabled us to provide a potential mechanism by which mitochondrial dysfunction during chronic T-cell stimulation promotes exhaustion. Given the recent finding NFAT binds to the TOX promoter and can independently activate TOX expression, as well as established data demonstrating that nuclear NFAT translocation requires mitochondrial ROS, we asked whether persistent NFAT activity was observed during chronic T-cell stimulation. Indeed, we found that NFAT target genes were highly enriched during chronic T-cell stimulation in a manner that was reversed in the presence of N-acetylcysteine (New Supplementary Fig. 4c, 6d).

Nuclear NFAT translocation is promoted by the calcium-dependent phosphatase calcineurin; intracellular calcium elevation also promotes mitochondrial oxidative phosphorylation, effectively coupling TCR-dependent NFAT mobilization to mitochondrial function. Our new data demonstrates that chronically stimulated T-cells maintain elevated intracellular calcium pools (New Supplementary Fig. 4b); this provides a mechanism by which chronic TCR signaling, through sustained elevation of intracellular calcium, leads to persistent nuclear NFAT and activation of TOX. Consistent with data obtained using our model system, analysis of single-cell RNA-sequencing of intratumoral T-cells isolated from melanoma patients demonstrated a strong correlation between enrichment of NFAT target genes and genes related to oxidative stress; as a result, NFAT target genes were most significantly enriched in terminally exhausted T-cells

(New Supplementary Fig. 4d,e). This data supports a role for NFAT in activating the ROS-dependent, TOX-driven exhaustion program.

Minor points:
The in vitro model of exhaustion could be much stronger if shown that chronically stimulated cells have decreased CTL killing efficiency, not only decreased cytokine production and proliferation.

We appreciate the opportunity to provide additional evidence demonstrating decreased killing efficiency of chronically stimulated T-cells. In addition to a decreased ability to kill clonogenic B16 melanoma cells during 24 hours of 2-d culture (revised Fig. 7b), we now also provide data using a luciferase reporter system to demonstrate impaired tumor cell killing across antigen doses (New Supplementary Fig. 1f). We thank the reviewer for the opportunity to demonstrate this functional effect of chronic stimulation more thoroughly.

Memory-like subsets are not the best comparison for the datasets discussed in Figure 1i. Memory T cell subsets are inherently low in glycolytic metabolism and does not seem like a fair comparison to the chronically stimulated cells. How would this data have compared to activated effector T cells which are also highly glycolytic? The authors could compare acute vs chronic not just at the end of the response, but at multiple time points. Since memory cells are resting and non-cycling, what is the importance of a higher "glycolysis score"?

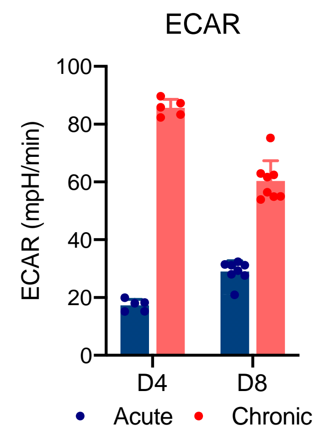

Figure 2.

We appreciate the opportunity to clarify the nomenclature used to describe the single-cell RNA-seq datasets. The CD8 T-cell sub-clusters defined in the dataset in Figure 1i are all intratumoral exhausted T-cells, differentiated by transcriptional signatures more consistent with a "terminally" exhausted as compared to a more "progenitor-like" exhausted state. The data shown in revised Figure 1i therefore indicates that within the exhausted T-cell compartment, terminally exhausted, non-self-renewing T-cells are enriched in glycolytic genes compared to self-renewing, progenitor-like exhausted T-cells. We have clarified both the text and figure to reflect this. Additionally, Yost et al utilized an alternative clustering approach in which conventional effector cells, which are traditionally glycolytic, are a separate cluster from exhausted T-cells. As shown in revised Supplementary Fig. 1o, exhausted T-cells from this dataset were enriched in glycolytic genes compared to traditional effector cells. We do

show in revised Figure 1e-f that chronically stimulated T-cells maintain higher rates of glucose consumption and lactate excretion relative to acutely stimulated T-cells throughout the response; we attach additional extracellular acidification rate data in **Figure 2** but have not included this in the revised manuscript due to space constraints.

References should be added for the brief discussion of TOX vs. TCF-1.

We thank the reviewer very much for pointing out this error and have provided additional references to highlight the key roles of TOX and TCF-1 during T-cell exhaustion.

The claim of "reliance on glycolysis for ATP production" from Figure 2c would be better supported by a Seahorse Mito Fuel Flex test, to directly test these cells' dependence on glucose for ATP synthesis. This could also create an opportunity to address dependency on glutamine flux, which was otherwise overlooked in this manuscript.

We thank the reviewers for the opportunity to offer this clarification. While we did not perform the Mito Fuel Flex test specifically, we now provide additional data demonstrating that while a significant proportion of the spare respiratory capacity of acutely stimulated T-cells is derived from glucose, essentially none of the spare respiratory capacity of chronically stimulated T-cells is glucose-dependent (New Supplementary Fig. 2b). This further establishes that the overwhelming majority of ATP production in chronically stimulated cells is glycolytic in origin.

Supplemental Figure 3a-c was an interesting approach. However, confirmation of mitochondrial localization of mitoLbNOX should be provided. Supplemental Figure 3b is not entirely convincing. How many times was the experiment repeated? Is there any statistical difference in ROS staining intensity between the vector and the recombinant enzymes? Additionally, it would be helpful to include "chronic" samples as a comparison for the panels in Supplemental Figures 3e+f.

This experiment was repeated twice with biological duplicates. We did not generate mitochondrial and cytoplasmic isolates from these samples and so cannot confirm mitochondrial localization, but this has been confirmed in other cell types within the lab (data not shown). We have replaced Supplementary Fig. 3e with an alternate experiment in which we include a chronically stimulated sample as a control and included qPCR data for chronically stimulated cells in Supplementary Fig. 3f.

Page 13: Data in Figure 3H are used to support the statement "Thus, the loss of mitochondrial oxidative phosphorylation is sufficient to impair T-cell proliferative capacity and activate exhaustion-associated gene expression". The results are not so clear cut and are limited to TCF1 vs TOX expression. These data are overinterpreted and the conclusion should be toned down.

We thank the reviewer for pointing this out and have removed this statement so as not to overinterpret the data.

References for reviewer responses:

1. Khan, O. *et al.* TOX transcriptionally and epigenetically programs CD8(+) T cell exhaustion. *Nature* **571**, 211-218 (2019).

2. Sena, L.A. *et al.* Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. *Immunity* **38**, 225-236 (2013).

Decision Letter, first revision:

Subject: Nature Immunology - NI-A29271A pre-edit

Message: Our ref: NI-A29271A

24th May 2020

Dear Craig & Santosh,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Impaired mitochondrial oxidative phosphorylation limits the self-renewal of T-cells exposed to persistent antigen" (NI-A29271A). I am attaching the edited manuscript. The manuscript is generally well-written, but my biggest concern is that it's a bit longer than is usually allowed (see below) - over 5400 words for the combined Introduction-Results-Discussion, so some trimming will be necessary. Try to get it below 5000.

I have made changes marked in tracked-changes, queries in red and comments are embedded throughout the manuscript, so please have the view comments option enabled.

Please follow the instructions provided here and in the attached files, as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

When you upload your final materials, please include a point-by-point response to the

points below. We won't be able to proceed further without this detailed response.

General formatting:

Our standard word limit is 4000 words for the Introduction, Results and Discussion. Your current manuscript exceeds this limit by 1282 words, and you will need to cut substantially.

Please include a separate "Data availability" subsection at the end of your Online Methods. This section should inform our readers about the availability of the data used to support the conclusions of your study and should include references to source data, accession codes to public repositories, URLs to data repository entries, dataset DOIs, and any other statement about data availability. We strongly encourage submission of source data (see below) for all your figures. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, these should be included in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf.

Your abstract must be fewer than 150 words and should not include citations.

As a guideline, Articles allow up to 50 references in the main text. An additional 20 references can be included in the Online Methods. Only papers that have been published or accepted by a named publication or recognized preprint server should be in the numbered list. Published conference abstracts, numbered patents and research data sets that have been assigned a digital object identifier may be included in the reference list.

Place Methods-only references after the Methods section and continue the numbering of the main reference list (i.e., do not start at 1).

Genes must be clearly distinguished from gene products (e.g., "gene Abc encodes a kinase," not "gene Abc is a kinase"). For genes, provide database-approved official symbols (e.g., NCBI Gene, http://www.ncbi.nlm.nih.gov/gene) for the relevant species the first time each is mentioned; gene aliases may be used thereafter. Italicize gene symbols and functionally defined locus symbols; do not use italics for proteins, noncoding gene products and spelled-out gene names.

Figures and Tables:

All figures and tables, including Extended Data, must be cited in the text in numerical order.

Might consider splitting Supplementary Fig. 1 into two separate figures.
Figure legends should be concise. Begin with a brief title and then describe what is presented in the figure and detail all relevant statistical information, avoiding inappropriate methodological detail.

All relevant figures must have defined error bars.

Graph axes should start at zero and not be altered in scale to exaggerate effects. A 'broken' graph can be used if absolutely necessary due to sizing constraints, but the break must be visually evident and should not impinge on any data points.

Cropping of gel and/or blot images must be mentioned in the figure legend. Gel pieces should be separated with white space (do not add borders). Please ensure that all blots and gels are accompanied by the locations of molecular weight/size markers; at least one marker position must be present in all cropped images. Please also supply full scans of all the blots and gels as Source Data, as instructed below.

All bar graphs should be converted to a dot-plot format or to a box-and-whisker format to show data distribution. All box-plot elements (center line, limits, whiskers, points) should be defined.

Statistics and Reproducibility:

The Methods must include a statistics section where you describe the statistical tests used. For all statistics (including error bars), provide the EXACT n values used to calculate the statistics (reporting individual values rather than a range if n varied among experiments) AND define type of replicates (e.g., cell cultures, technical replicates). Please avoid use of the ambiguous term "biological replicates"; instead state what constituted the replicates (e.g., cell cultures, independent experiments, etc.). For all representative results, indicate number of times experiments were repeated, number of images collected, etc. Indicate statistical tests used, whether the test was one- or two-tailed, exact values for both significant and non-significant P values where relevant, F values and degrees of freedom for all ANOVAs and t-values and degrees of freedom for t-tests.

The reproducibility checklist must be complete, accurate and up to date.

Supplementary Information:
All Supplementary Information must be submitted in accordance with the instructions in the attached Inventory of Supporting Information, and should fit into one of three categories:

25 EXTENDED DATA: Extended Data are an integral part of the paper and only data that directly contribute to the main message should be presented. These figures will be integrated into the full-text HTML version of your paper and will be appended to the online PDF. There is a limit of 10 Extended Data figures, and each must be referred to in the main text. Each Extended Data figure should be of the same quality as the main figures, and should be supplied at a size that will allow both the figure and legend to be presented on a single legal-sized page. Each figure should be submitted as an individual .jpg, .tif or .eps file with a maximum size of 10 MB each. All Extended Data figure legends must be provided in the attached Inventory of Accessory Information, not in the figure files themselves.

26 SUPPLEMENTARY INFORMATION: Supplementary Information is material that is essential background to the study but which is not practical to include in the printed version of the paper (for example, video files, large data sets and calculations). Each item must be referred to in the main manuscript and detailed in the attached Inventory of

Accessory Information. Tables containing large data sets should be in Excel format, with the table number and title included within the body of the table. All textual information and any additional Supplementary Figures (which should be presented with the legends directly below each figure) should be provided as a single, combined PDF. Please note that we cannot accept resupplies of Supplementary Information after the paper has been formally accepted unless there has been a critical scientific error.

All Extended Data must be called you in your manuscript and cited as Extended Data 1, Extended Data 2, etc. Additional Supplementary Figures (if permitted) and other items are not required to be called out in your manuscript text, but should be numerically numbered, starting at one, as Supplementary Figure 1, not SI1, etc.

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Laurie

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Reviewer #2:
Remarks to the Author:
The authors have addressed my concerns.

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Dear Craig & Santosh,

I am delighted to accept your manuscript entitled "Impaired mitochondrial oxidative phosphorylation limits the self-renewal of T-cells exposed to persistent antigen" for publication in an upcoming issue of Nature Immunology.

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Kind regards & stay well,

Laurie

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