European Journal of Immunology

Peer review correspondence

Rewiring cellular metabolism via the AKT/mTOR pathway contributes to host defence against Mycobacterium tuberculosis in human and murine cells

Ekta Lachmandas, Macarena Beigier-Bompadre, Shih-Chin Cheng, Vinod Kumar, Arjan van Laarhoven, Xinhui Wang , Anne Ammerdorffer, Lily Boutens, Dirk de Jong, Thirumala-Devi Kanneganti, Mark S. Gresnigt, Tom H.M. Ottenhoff, Leo A.B. Joosten, Rinke Stienstra, Cisca Wijmenga, Stefan H.E. Kauffman, Reinout van Crevel and Mihai G. Netea

Corresponding author: Ekta Lachmandas, Department of Medicine, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

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Handling Executive Committee member: Prof. Maria Yazdanbakhsh

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 1 February 2016

Dear Prof. Netea, Ms. Lachmandas,

Please accept my apologies for the delay in processing the peer review of your manuscript ID eji.201546259 entitled "Rewiring cellular metabolism via the AKT/mTOR pathway is central for host defence against Mycobacterium tuberculosis" which you submitted to the European Journal of Immunology. The timing of the submission made it challenging to secure 2 referee reports in a timely manner; thereafter there was a divergence of opinion among the referees as to the revisions necessary, which we discussed internally before coming to a decision.

The comments of the referees are included at the bottom of this letter. You will see that both referees feel your submission is timely and we feel that your work nicely complements the article cited by one of the referees:

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http://www.nature.com/articles/srep18176

However, the universal concern between the 2 referees is that although the data as a whole are convincing, they are not entirely surprising. Moreover, the most interesting part of the story, the link to glycolysis and metabolism, lacks the strongest data. You will see that the 2 referees had differing opinions on how to enhance the credibility and impact of this part of the story.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

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

Yours sincerely,
Laura Soto Vazquez and Karen Chu

On behalf of Prof. Maria Yazdanbakhsh

Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

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Reviewer: 1

Comments to the Author

Summary:
The authors use human and mouse models to examine the metabolic alterations induced by Mycobacterium tuberculosis. Specifically, they focus on the metabolic switch from oxidative metabolism to aerobic glycolysis (Warburg effect). They find through analysis of published datasets that human PBMC from active Tb patients display an increased glycolytic signature and decreased TCA cycle, suggesting possible increased aerobic glycolysis. The authors find that exposure of PBMC in vitro to Mtb induced aerobic glycolysis, mirroring exposure to purified TLR agonists. This affect is in part mediated through activation of AKT-mTOR (mTORC1) pathways, as in vitro inhibition can blunt this metabolic switch. The authors find that TLR2, but not TLR4 or NOD pathways, mediate this metabolic switch in PBMC. In vivo, the authors treat mice exposed to Mtb with rapamycin, an mTOR/autophagy inhibitor, and find modest reductions in a range of inflammatory cytokines from splenocytes stimulated with MtB extract.

Together, these findings provide good evidence that Mtb can exert a metabolic switch from Ox/Phos metabolism to aerobic glycolysis. This finding has been well-described in models of cancer, T-cell activation, and exposure to TLR/PAMPS, but has not been specifically delineated for Mtb. Indeed, aerobic glycolysis appears to be a fairly conserved pathway that activated/replicating cells require for anabolic support. As such, these findings are not entirely surprising. The more interesting findings involve the pathways required for this metabolic switch, which appear to be mediated by TLR2 signaling and AKT/mTOR. These data are fairly well-explored and supported, with some modest points noted below. However, the primary issue is the relevance of these findings to actual infection. The only in vivo data (Figure 7) found no affect of rapamycin on Mtb burden, with mild decreases in many cytokines from splenocytes. This paper could be significantly strengthened if further in vivo evidence was provided to support the authors in vitro data. In the abstract, the authors state “We examined whether metabolic reprogramming towards aerobic glycolysis is important for host response to Mtb” This question is a relevant one, and further focus on the in vivo changes and requirements for metabolism would strengthen this work. Additionally, the authors often use PBMC and find alterations in both T-cell and monocyte-associated cytokines (Figure 4), but do not pursue which (or both) of these cells are being altered by Mtb exposure, and which (or both) are relevant to Mtb disease control. Some further clarification would strengthen the manuscript and increase the relevance.

General Comments:
For many (but not all) of the experiments, the authors compare Mtb exposure to unexposed. It would be helpful to have comparisons throughout, such as LPS (which, as the authors note, has been previously described to stimulate similar results). For example, the Seahorse analysis in Figure 2, how do these

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parameters compare with classic TLR stimulation? Are there unique aspects of Mtb driven metabolic changes compared with more traditional activators? For Figure 3, the Mtb induction of aerobic glycolysis (as read out by lactate) is dependent on p-AKT/mTOR…what about other stimuli? Overall, the broader point is, what is similar and different between Mtb versus other well-described controls and/or model infectious organisms.

For the NOD experiments, the data from human NOD2-/- PBMC is clean and strong. However, with the shift to mouse NOD deficient cells, the induction of lactate appears modest in this experiment, even for the control WT. Are the increased in lactate significantly different (control vs Mtb treated?) This should be repeated, as it is certainly possible that human and mouse differ. Along these lines, similarly for Figure 6 the authors use an LPS antagonist for human PBMC to determine that TLR4 is not required, then switch to mouse TLR2 KO to determine the requirement for TLR2. While this is a strength of the work, utilizing both human and mouse, the authors should directly compare human to human and mouse to mouse. What do the TLR4 KO mice look like for this assay? Similar to discussion above, how does this specific requirement for TLR2 compare to other model infectious stimuli?

The primary issue lays in the relevance of these findings for Mtb infection. In Figure 7, the authors treat for 28 days with rapamycin and examine splenic cytokine production, which appears mildly decreased for many cytokines. Of note, many of the graphs in panel B do not show stars to indicate significance or not, but the text indicates these changes are significant. If aerobic glycolysis is important for the response to Mtb (either through mono/macrophages and/or T-cells), data should be shown regarding these cells after 28 days…are they shifted away from aerobic glycolysis? What are the effects on the immune response to Mtb at infected sites? Other lines of evidence should be used, in addition to rapamycin, to corroborate these findings.

Minor Points:
For Figure 1, the authors rely on published datasets. A more complete description of the human dataset would be helpful (what specimens/cells were analyzed.) Additionally, it should be clearly stated for all portions of Figure 1 that the data is derived from published datasets.
Figure 1 legend, “is plotted along PC1 for glycolysis and PC1 (should read PC2) for TCA”

Throughout the manuscript, many of graphs have a background of grey, which (in this reviewer’s opinion) is visually distracting.

Supplemental Figures: No legends are provided.

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Reviewer: 2

Comments to the Author

The paper of Lachmandas et al describes that infections with Mycobacterium tuberculosis induces glycolysis in human PBMC and monocytes. They further show that stimulation with Mtb is able to induce glycolysis and that this effect is dependent on TLR2 but not NOD2 signalling. Induction of glycolysis is reduced by ablation of the mTOR pathway as is cytokine expression in the context of experimental MTB infection. The paper is well written and these data are in accordance with a more general pattern according to which immune activation induces glycolysis. The study is well presented and adds evidence to the fundamental connection between immune activation and glycolysis and is suitable to the readership of EJI. All experiments performed show appropriate statistical analysis and the conclusions drawn are supported by the presented data. In addition, the results nicely complement a recent study by Shi et al reporting similar results in the lungs of Mycobacterium tuberculosis infected mice without providing the link to TLR2, a definite strength of the paper by Lachmandas et al. However, the study of Shi et al should be included in the current reference list.

My main concern with this study is that the link provided between mTOR activity as the main driver of glycolysis is rather weak. While Mtb exposure appears to activate the mTOR1 pathway, blockade by rapamycin, torin and wortmannin does not completely ablate lactate production and has no effect on monocyte derived cytokines. In addition treating Mtb infected mice with rapamycin appears to only significantly reduce TNFa production. Hence it is possible that mTOR activity may play a partial role in the induction of glycolysis and cytokine expression in response to Mtb. It may benefit the paper that the authors consider performing the infection experiments with simultaneous blockade of glycolysis with 2-DG. The title of the paper could then be adapted accordingly.

Minor points:
The statistical analysis is missing in Figure 2C
The supplementary figures show low resolution and the figure legends appear to be missing

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First revision – authors' response – 12 July 2016

Reviewer: 1

1. The primary issue is the relevance of these findings to actual infection. The only in vivo data (Figure 7) found no effect of rapamycin on Mtb burden (because rapa is bactericidal), with mild decreases in many cytokines from splenocytes. This paper could be significantly strengthened if further in vivo evidence was provided to support the authors in vitro data. In the abstract, the authors state "We examined whether metabolic reprogramming towards aerobic glycolysis is important for host response to Mtb" This question is a relevant one, and further focus on the in vivo changes and requirements for metabolism would strengthen this work.

We thank the reviewer for pointing out to this important aspect that we have now detailed in the revised manuscript. Since the initial submission of our manuscript, two independent studies have reported the in-vivo importance of glycolysis in murine models [1, 2]. These findings strongly support our data and we have therefore discussed these papers in our manuscript (Lines 68-71). Our manuscript remains unique as it illustrates these changes in-vivo in humans whilst also investigating which TLR and intracellular signalling pathways lead to the induction of glycolysis and the subsequent functional consequences such as cytokine production.

2. Additionally, the authors often use PBMC and find alterations in both T-cell and monocyte-associated cytokines (Figure 4), but do not pursue which (or both) of these cells are being altered by Mtb exposure, and which (or both) are relevant to Mtb disease control. Some further clarification would strengthen the manuscript and increase the relevance.

We agree that this is a relevant point. To address this, we first stimulated PBMCs with Mtb lysates for two hours and then isolated purified fractions of CD14+ monocytes and CD3+ T cells from the stimulated PBMCs. Western blot analysis from the resulting lysates revealed that the mTOR targets of p70 S6K and p 4EBP1 were up-regulated especially in monocytes, but not T cells (Figure 3D). This suggests that the initial metabolic reprogramming occurs in monocytes. (Lines 322-325). As observed in PBMCs (Figure 3C), rapamycin also inhibits p70 S6K and p 4EBP1 activation in monocytes (Supplementary Figure 3B).

3. For many (but not all) of the experiments, the authors compare Mtb exposure to unexposed. It would be helpful to have comparisons throughout, such as LPS (which, as the authors note, has been previously described to stimulate similar results). For example, the Seahorse analysis in Figure 2, how do these parameters compare with classic TLR stimulation? Are there unique aspects of Mtb driven metabolic changes compared with more traditional activators? For Figure 3, the Mtb induction of aerobic glycolysis (as read out by lactate) is dependent on p-AKT/mTOR…what about other stimuli? Overall, the broader

point is, what is similar and different between Mtb versus other well-described controls and/or model infectious organisms.

It is an interesting aspect to compare how Mtb and other stimuli such as LPS alter host metabolism. We have now included data in Supplementary Figure 2A where we demonstrate that the OCR / ECAR ratio for Mtb is indeed similar to what is described in literature for LPS. Line 303, Supplementary Figure S2A

Simultaneously we are also assessing in detail how different stimuli specifically alter host metabolism, however we find this data preliminary and beyond the scope of this manuscript.

4. For the NOD experiments, the data from human NOD2-/- PBMC is clean and strong. However, with the shift to mouse NOD deficient cells, the induction of lactate appears modest in this experiment, even for the control WT. Are the increased in lactate significantly different (control vs Mtb treated?) This should be repeated, as it is certainly possible that human and mouse differ.

In Figure 5b, the increase in lactate by Mtb stimulation of human PBMCs (500,000 cells / well) is approximately two-fold. Similarly, the increase of lactate release is 1.5 to 2-fold higher in Mtb stimulated WT mice (100,000 cells / well), and no effects of NOD2 deficiency was observed. Due to the lack of differences in lactate production in Mtb stimulated WT and NOD deficient cells in both humans and mice we decided to focus on other pattern recognition pathways.

5. Along these lines, similarly for Figure 6 the authors use an LPS antagonist for human PBMC to determine that TLR4 is not required, then switch to mouse TLR2 KO to determine the requirement for TLR2. While this is a strength of the work, utilizing both human and mouse, the authors should directly compare human to human and mouse to mouse. What do the TLR4 KO mice look like for this assay? Similar to discussion above, how does this specific requirement for TLR2 compare to other model infectious stimuli?

Bartonella LPS is a very efficient TLR4 antagonist which completely blocks TLR4 signaling, as demonstrated by Popa et al [3]. We agree with the reviewer that an additional genetic control would make the picture complete, but because the experiments using Bartonella LPS gave such clear data, we considered it very unlikely that experiments in TLR4 knockout mice would have any impact on the conclusions.

Furthermore it is indeed an interesting question to pose, is TLR2 also a requirement for the induction of glycolysis by other stimuli? This could well be the case for Gram positive bacteria such as Staphylococcus aureus and Streptococcus pneumoniae, however whilst we speculate this in the discussion (Lines 498-

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500) we find it beyond the scope of this manuscript, which focuses primarily on Mtb, to perform the detailed experiments needed to be able to answer this question appropriately.

6. The primary issue lays in the relevance of these findings for Mtb infection.
In Figure 7, the authors treat for 28 days with rapamycin and examine splenic cytokine production, which appears mildly decreased for many cytokines. Of note, many of the graphs in panel B do not show stars to indicate significance or not, but the text indicates these changes are significant. If aerobic glycolysis is important for the response to Mtb (either through mono/macrophages and/or T-cells), data should be shown regarding these cells after 28 days…are they shifted away from aerobic glycolysis? What are the effects on the immune response to Mtb at infected sites? Other lines of evidence should be used, in addition to rapamycin, to corroborate these findings.

In addition to rapamycin, a more specific mTORC1 inhibitor, torin, has been used to determine whether the effects of rapamycin on inhibition of lactate (Figure 3G) and cytokine production (Figure 4B). In most cases torin yields very similar results to rapamycin, supporting the notion that mTOR is responsible, at least in part, for the shift towards glycolysis.

It would be interesting as suggested to look into detail at the effects of the rapamycin or torin on the immune response at Mtb infected sites. These detailed in-vivo studies are warranted and will need to be assessed in future studies.

Minor Points:
1. For Figure 1, the authors rely on published datasets. A more complete description of the human dataset would be helpful (what specimens/cells were analyzed.)
We have elaborated on this aspect in the revised manuscript, please see lines 236 – 238.

2. Additionally, it should be clearly stated for all portions of Figure 1 that the data is derived from published datasets. Figure 1 legend, “is plotted along PC1 for glycolysis and PC1 (should read PC2) for TCA”.

For figure 1 A and B the first principal component (PC1) of the glycolysis pathway is plotted against the first principal component (PC1) of the TCA cycle pathway, PC2 for either pathway is not used. To improve clarity, we have elaborated on this figure in lines 245-250.

Both figure legends and results section state that various parts of Figure 1 are derived from published data sets.

3. Throughout the manuscript, many of graphs have a background of grey, which (in this reviewer’s opinion) is visually distracting.

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These graphs have been remade and the grey background has been removed from all graphs.

4. Supplemental Figures: No legends are provided.
Figure legends for all supplementary figures have now been included at the end of the manuscript.

Reviewer: 2

1. The study of Shi et al should be included in the current reference list.
Thank you for pointing that out, we have now included the study of Shi et al. as part of our reference list. (lines 68-71)

2. My main concern with this study is that the link provided between mTOR activity as the main driver of glycolysis is rather weak. While Mtb exposure appears to activate the mTOR1 pathway, blockade by rapamycin, torin and wortmannin does not completely ablate lactate production and has no effect on monocyte derived cytokines.

The point raised by the reviewer is valid and several aspects could account for it: firstly, a complex biological pathway such as glycolysis is likely to be regulated by additional pathways; secondly, the efficacy of any inhibitors is of course not complete either. This is reflected in a change of title and throughout various parts of the manuscript. We have included this in our discussion (Lines 481-485).

3. In addition, treating Mtb infected mice with rapamycin appears to only significantly reduce TNFa production. Hence it is possible that mTOR activity may play a partial role in the induction of glycolysis and cytokine expression in response to Mtb. It may benefit the paper that the authors consider performing the infection experiments with simultaneous blockade of glycolysis with 2-DG. The title of the paper could then be adapted accordingly.

We agree with the reviewer, and we have now discussed in the revised manuscript the experiment suggested by the reviewer, which has been recently published by Gleeson et al [1]. This study demonstrates that blocking glycolysis with 2-DG or galactose does indeed lead to reduced mycobacterial killing and this supports our conclusions (Lines 68-71)

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Minor points:

4. The statistical analysis is missing in Figure 2C

Thank you for pointing that out, the statistical analysis has now been provided.

5. The supplementary figures show low resolution and the figure legends appear to be missing.

We have added higher resolution supplementary figures and included the figure legends.

References

1 Gleeson, L. E., Sheedy, F. J., Palsson-McDermott, E. M., Triglia, D., O'Leary, S. M., O'Sullivan, M. P., O'Neill, L. A. and Keane, J., Cutting Edge: Mycobacterium tuberculosis Induces Aerobic Glycolysis in Human Alveolar Macrophages That Is Required for Control of Intracellular Bacillary Replication. J Immunol 2016. 196: 2444-2449.

2 Shi, L., Salamon, H., Eugenin, E. A., Pine, R., Cooper, A. and Gennaro, M. L., Infection with Mycobacterium tuberculosis induces the Warburg effect in mouse lungs. Sci Rep 2015. 5: 18176.

3 Popa, C., Abdollahi-Roodsaz, S., Joosten, L. A., Takahashi, N., Sprong, T., Matera, G., Liberto, M. C., Foca, A., van Deuren, M., Kullberg, B. J., van den Berg, W. B., van der Meer, J. W. and Netea, M. G., Bartonella quintana lipopolysaccharide is a natural antagonist of Toll-like receptor 4. Infect Immun 2007. 75: 4831-4837.

Second Editorial Decision – 1 August 2016

Dear Prof. Netea, Ms. Lachmandas,

It is a pleasure to provisionally accept your manuscript entitled "Rewiring cellular metabolism via the AKT/mTOR pathway contributes to host defence against Mycobacterium tuberculosis" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

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We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

on behalf of Prof. Maria Yazdanbakhsh

Dr. Karen Chu
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu