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Redox homeostasis in *Mycobacterium tuberculosis* is modulated by a novel actinomycetes-specific transcription factor

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1st Editorial Decision 7th Aug 2020

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received a full set of reviewer reports, which are included below for your information. Based on these comments, we unfortunately had to conclude that the study is not a sufficiently strong candidate for publication in The EMBO Journal.

As you can see, while all reviewers find the proposed model of AosR function interesting, they also raise a number of substantive concerns regarding the experimental setup, data analysis and interpretation, and they find that the main conclusions of the study are not conclusively supported by the provided data. Given these opinions from good experts in the field, and since these concerns affect the core aspects of the study and would require extensive revision work with an uncertain outcome, I am afraid that we cannot offer further proceedings towards publication in The EMBO Journal.

Thank you in any case for the opportunity to consider this manuscript. I regret that I cannot communicate more positive news, but I nevertheless hope that you will find our reviewers' comments helpful for further improvement of the manuscript.

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Referee #1:

The authors identify AosR/Rv1332 as an actinomycete-specific TF involved in M. tuberculosis response to oxidative stress in vitro and in vivo. They report that AosR interacts with SigH to regulate a cysteine biosynthesis operon involving CysM and other proteins.
This is a potentially interesting study, however several conclusions are poorly supported by the data and the exact mechanism by AosR interacts with SigH, and how AosR/SigH-mediates regulation of the CysM pathway is not elucidated.

Major comments
- The authors claim that their « data indicates that oxidative stress alters the conformation of AosR through the formation of a single intrasubunit disulphide bond, which in turn facilitates its interaction with an extracytoplasmic sigma factor, SigH. » This claim is not supported by the data at

all. There is no demonstration of conformational change in AosR that mediates interaction with SigH. More generally, most of the scenario depicted in Fig 6d is not supported by the data. A thorough structural study of AosR and SigH in normal and oxidized conditions should be performed.
- Fig 1f/Line 94, the C-ter end is said to be unstructured, yet the authors show an alpha-helix for residues 1-22. This is contradictory.
- It is quite an achievement to have generated antibodies against so many Mtb proteins (AosR, ClpS, Rv1333, CysO, CysM, CysK1, CysK2, GroEL1, SigA), and this could be very useful to the community. Yet, the specificity of these antibodies must be reported in details; whole WB must be provided, with appropriate controls. Please also provide more technical details on how these Abs were generated.
- Most of the second part of the study is based on CysM while mec is left aside; this is surprising since overexpression with CysM is not functional (3e). Furthermore, cysM is not found as induced in the RNAseq experiment (Table S6) while mec and cysO are; how do the authors explain this apparent discrepancy and justify they focus on CysM?
- In Fig 4l, the authors show that an aosR-KO mutant is less attenuated in mice treated with a NO-inhibitor than in control animals; however from data in S4d, they conclude that aosR is NOT involved in response to NO stress. Can the authors reconcile these apparently contradictory data sets?
- Statistics are sometimes weird; in 4k for example, in phox-KO cells, how can it be that there are *** between Rv and cysM-KO and only one * between cysM-KO and the complemented strain, while all means look the same? Same applies for phox-KO data in 4j, 4l, and for the NO-inhibitor in 4l. Displaying full data points appears necessary here. More generally speaking, it would be wise to display histograms containing all data points for all experiments reporting CFUs.
- In Fig 5g-h, loading controls are necessary.
- The mechanism of AosR interaction with SigH is not investigated and the authors cannot conclude that the two Cys residues in AosR are involved. At the very least, pull down experiments with the AosR (CxxxC -> AxxxA) mutant should be performed.
- Fig 5i, data should be complemented with a control consisting in CHP+DTT (without Cys), to assess whether the redox state of AosR alone modulates gene expression.
- It is not clear why the authors report on the effect of INH and Rif treatment in infected mice as the attenuated profile of the mutant looks identical in control and treated animals (which is surprising given the sensitivity profile of the mutant to INH in vitro showed in Fig 6a).
- Regarding Fig 6a, how can it be that 100% bacteria are recovered after treatment with INH at a 50xMIC dose?

Minor comments
- In extenso definition of AosR should appear as soon as possible.
- AosR is not strictly specific to actinomycetes. In particular it is present in Serratia marcescens and other non-actinomycetes. More generally speaking, the rationale for the selection of AosR as a candidate TF to study is odd: why concentrate on actinomycete-specific TFs? Other TFs that are essential in vivo but not in vitro might be of interest as well.
- In Figure 1 (b), it is surprising that Mtb does not grow in macrophages at all. What is the activation status of these cells? The Methods section is very incomplete.
- From the authors' data, it cannot be concluded that AosR is « essential » for in vivo survival, it is « required » or « involved in », at best.
- The selection of AosR is based on two publications from two labs. What is the fate of AosR in other screening studies by other labs, in particular in macrophages.
- Panels 1a and 1d could be removed.
- Fig 1i could be better explained. What is supposed to be concluded from this DC experiment?
- In Fig 2g and h, it looks like DCt data, not DDCt are displayed. Could the authors clarify? If data are

normalized to 16S, how can it be that all genes are at the same level in the -CHP condition? How were the genes chosen? Why Rv3529 is showed in g and not in h?
- In Table S6, please make reference to Fig 2g/h, not 2i that does not exist.
- Lines 201-3, replace Fig 5 (l-m, n-o) by Fig 4.
- Line 235-250 should be moved to the Discussion section as this is just a discussion of already reported data.

Referee #2:

In this manuscript, Khan and colleagues report the identification and characterization of a transcriptional activator Rv1332 (AosR). They demonstrate that AosR responds to peroxide stress with the formation of an intramolecular disulfide bond, which enables interaction with SigH and activation of an operon leading non-canonical cysteine biosynthesis. The AosR mutant is attenuated in peritoneal macrophages and mice. The survival defect in macrophages is rescued in phagocyte oxidase deficient macrophages that are also treated with an inhibitor of nitric oxide synthase, linking the attenuation in macrophages to the generation of reactive oxygen and nitrogen intermediates. In vitro, an aosR mutant fails to upregulate cysteine biosynthesis during oxidative stress and addition of cysteine overcomes the impact of deletion of aosR.
Overall this is a well-executed, comprehensive and interesting manuscript that provides new insight into a redox responsive transcription factor. The authors conclusions are for the most part supported by the presented data. I have one major and a few minor concerns that need to be addressed.
The conclusion that "cysteine flux regulated by AosR potentiates drug tolerance" is not supported by the data. In vitro the susceptibility of the mutant to INH is only ~ 2-fold higher than that of wild type H37Rv and only shown for a single concentration and single time point. This is not convincing. In vivo, the mutant does not seem to be significantly more susceptible to INH and Rif treatment than wild type as the impact of INH/Rif results in a similar reduction in CFU for wt and mutant compared to the CFU of each strain before treatment. Thus, that AosR affects drug tolerance is not convincingly demonstrated and the data and conclusion should be removed.
The authors interpret the in vivo data as "Rv1332 is specifically essential for mycobacterial survival within its host". This is incorrect. The mutant has a growth defect in mice compared to wild type Mtb but does not fail to survive, its titers never decline. The conclusion needs to be reworded.
AosR lacking the N-terminal domain and AosR from M. smegmatis did not complement the Mtb AosR Mutant. The authors need to show that these proteins were expressed at equal amounts as AosR.
In the discussion the authors wrote "Our data shows that the attenuated survival of Mtb due to the deletion of cysM or its regulator aosR is mitigated in phox -/- mice, confirming the specific requirement of this pathway in response to host-induced oxidative stress". However, if the growth defect observed in BalbC mice is directly linked to oxidative stress has not been interrogated. The authors have not infected phox-/- mice. Please rephrase.
Also in the discussion: "In murine Mtb infection models, the oxidative burst occurs 14 days post infection" - that is - to my knowledge - incorrect. The respiratory burst occurs in response to phagocytosis and does not happen only after 14 days. Either provide evidence for this statement or remove.

Referee #3:

This manuscript describes the characterization of a putative transcription factor encoded by Rv1332, here termed AosR, deletion of which makes Mtb more susceptible to oxidative stress in vitro, and leads to a growth defect in macrophages and mice. A large amount of data is presented that connects this TF to the expression of a gene cluster that harbors genes for an alternate cysteine biosynthesis pathway. Furthermore, evidence is presented that the N-terminal portion of the protein is necessary to prevent susceptibility to oxidative stress, and that a cysteine motive is involved. Furthermore it is shown that SigH also plays a role in the regulation of this operon. The genetic and protein work is sound and rigorous. On the whole the manuscript is interesting and easy to follow.

However, while the authors are commended for their large amount of rigorous work to characterize this putative transcription factor, the results are mostly overinterpreted and important controls to support the proposed mechanisms are missing.

1. The authors claim that "Cellular redox poise in M. tuberculosis is modulated by a novel actinomycetes-specific transcription factor", yet nowhere in the paper is this formally shown. In fact, the redox poise of the cells are not measured throughout this study. Instead, susceptibility to oxidative stress and rescue through cysteine addition is extrapolated that this putative TF is actually modulating redox poise. To support this claim, ratios of redox cofactors actually need to be determined.
2. It is proposed that Rv1335 is a transcription factor, yet no attempts are made to formally show this functionality, e.g. with gel shift assays. In addition, a consensus sequence for TF binding is also not determined, even though this could be done with the data at hand.
3. Another issue that needs further attention is the proposed decrease in cysteine biosynthesis. Cysteine is not measured and neither are redox factors like mycothiol or ergothionine. Also, it is unclear why the serine derived cysteine biosynthetic pathway is not compensating a potential decrease in cysteine...
4. The proposed drug phenotype needs more attention. First, in order to illustrate decreased persister survival in the presence of INH, an actual kill curve has to be shown over at least 15 days. Secondly, the in vivo drug data does not support their conclusion of increased susceptibility, because the mutant is by itself already more susceptible in vivo. The kill kinetics in mice look the same for WT and mutant. By week 4 there is already a ~1 log diff in KO and WT, this trend continues following drug treatment but is not exacerbated. Therefore lower CFU following treatment can't necessarily be concluded to imply decreased tolerance of KO as the kinetics appear to largely unchanged.
5. The language should be toned down. A lot of results are oversold. E.g. a 1 log difference in mouse burden is sold as: AosR is essential for survival in mice... Essentiality would be demonstrated by rapid clearance of the strain, yet it can grow in a mouse, albeit at a reduced rate. Also the word myriad is used in places, and it is clearly overstated.
6. Cysteine flux? What do you mean by cysteine flux? First of all cysteine is never measured, secondly cysteine flux doesn't make much sense. Do you means flux through cysteine biosynthesis? In the absence of metabolite data nothing can be claimed about flux.
7. Intra-disulphide bond and sigH interaction
The authors infer that the formation intra-disulphide bond is important for interaction with SigH (line17-18) but provide limited data to support this. Using in vitro and ex vivo assays, the authors demonstrate the CxxxC motif is important for protection against oxidative stress and for survival in macrophages, and biochemical data suggests these cysteine residues are capable of forming an intra-disulphide bond enabling AosR to act as redox rheostat. The interaction between AosR and SigH was identified by immunoprecipitation in both directions and shown to be redox dependent. This assay should also be performed using the AxxxA mutant to conclusively show that formation

of the intra-disulphide bond indeed facilitates this interaction, as other amino acids that could also be oxidized may influence this interaction.

More specific comments:
• Inconsistency in naming of mutant in the manuscript and supplement. In some instances written as RvΔ1332 (eg. Line 70 and Figure 1e, S4), elsewhere RvΔaosR (e.g. line 95, figure 1g, S5)
• Line 96: Msm - replace with M. smegmatis. Aside from the missing N terminus, what is the homology of AosRMsm with AosRMtb?
• Line 142-143/figure 2 - Rv3529 missing from fig 2h
• Line355 - no figure 5k. Should this be 6d?
• Line 360 - should this not a be a redox rheostat rather than a cysteine rheostat?

Figure 4
4f. Only shows RvΔcysM, and not RvΔaosR, whereas 4e. shows both, would be good to see how RvΔcysM and RvΔaosR compare in macrophage.
4j,k. Redundant as same conditions shown in 4i and 4m.

Author Correspondence 10th Aug 2020

Thank you for considering our manuscript for publication in EMBO J.
We would go through the comments carefully. However with the first reading it appears as though we can address most, if not all the comments by performing additional experiments. I was wondering if you would be open to resubmission of the manuscript if we successfully address the majority of the reviewers concerns?
Looking forward to hearing from you.

Editor Correspondence 11th Aug 2020

I would be open to a resubmission of the manuscript, if you find that you are able to address most of the referee comments in a satisfactory manner. I would suggest to prepare a preliminary point-by-point response to give me an insight into the planned revision scope. I would also like to discuss the revision plan with the reviewers to make sure that the proposed revision will satisfy their main concerns and to avoid an extended revision process with a negative outcome. If you find this approach reasonable, you can send me your preliminary point-by-point response to reviewer comments via email.

Author Correspondence 14th Aug 2020

Thank you for providing us with the opportunity to address the reviewer's concerns. We would like to thank all three reviewers for insightful and critical comments, which once we address would certainly strengthen the manuscript. In the past week, we have performed a few experiments that would address reviewers concerns regarding controls (data provided in the attached response). Based on the comments from all three reviewers, we believe we have to perform four important experiments.

1. While we have established interaction between AosR and SigH in response to oxidative stress, both Reviewer 1 and 3 have asked us to demonstrate loss of interaction between AosRAxxxA mutant and SigH, which we aim to perform through co-immunoprecipitation and/or SPR.

2. Reviewer 3 has suggested that we demonstrate the DNA binding ability of AosR. While we would attempt this experiment, we are not very sure we would be able to establish either SigH dependent or independent interaction of AosR with DNA under oxidative conditions through EMSA. However, we would make earnest efforts in this direction.

3. Reviewer 3 has asked for the quantification of cysteine. We have standardized protocols for the detection of six metabolic intermediates of the cysteine biosynthesis pathway (in collaboration with another group). They are; L-Serine, Ergothioneine, L-Cysteine, O-Acetyl-L-Serine, O-Phospho-L-Serine and S-Sulpho cysteine. We plan to estimate their levels in *wild type, KO, and complemented strains* under normal and oxidized conditions.

4. We also plan to measure the impact of AosR on the redox status of the cell by measuring mycothiol redox potential using a redox-sensitive GFP (1) and measuring NADH:NAD+ using peredox biosensor (2).

5. We have examined the survival of *Rv**D**aosR,* *Rv**D**aosR::aosR* and *Rv**D**cysM, Rv**D**cysM::cysM *in peritoneal macrophages independently. However as suggested by the Reviewer 3, we would perform peritoneal macrophage infection experiment with *Rv, Rv**D**aosR,* *Rv**D**aosR::aosR, Rv**D**cysM, and Rv**D**cysM::cysM *at the same time to evaluate relative survival differences between the two mutants.

Please find attached point by point response. We would very much appreciate, based on our response and explanations, if we are given an opportunity to submit a revised manuscript to EMBO J. Thank you and looking forward to your response.

Thank you again for providing a preliminary revision plan in response to the reviewer comments. I have now shared your revision proposal with reviewers #1 and #3 and received their input after a regrettable delay. I have included the assessments of both reviewers below and in the attached file. I am afraid that the comments from both reviewers indicate that the revision has a limited chance to fully satisfy reviewers' concerns in an overseeable timeframe. Therefore, I am unfortunately not in a position to invite a revised manuscript. I appreciate that you contacted me for a further discussion, and I am sorry that I could not deliver more positive news, but I do wish you all the best with the publication of this work at another venue.

Reviewer #3:

Their plan seems reasonable. Yet of course the outcome of their studies could change the story completely.

Referee #1:

- The authors claim that their « data indicates that oxidative stress alters the conformation of AosR through the formation of a single intrasubunit disulphide bond, which in turn facilitates its interaction with an extracytoplasmic sigma factor, SigH. » This claim is not supported by the data at all. There is no demonstration of conformational change in AosR that mediates interaction with SigH. More generally, most of the scenario depicted in Fig 6d is not supported by the data. A thorough structural study of AosR and SigH in normal and oxidized conditions should be performed.

Response:

Thank you for the comment. The conclusions were based on three independent sets of data-
(i) mBBr assay showed the presence of a single intrasubunit disulfide bond in AosR, which is dependent on the oxidative environment (Fig 1i),
(ii) Mutation of residues from CxxxC to AxxxA in AosR abrogates survival advantage in our in vitro oxidative stress (Fig 1h) and in our ex vivo experiment (Fig S5d).
(iii) Interaction of SigH with AosR is detected only under oxidative stress conditions(Fig 5d).
However, we agree that oxidative stress can also oxidize other amino acids. Thus we plan to perform a coimmunoprecipitation experiment to show loss of interaction between $AosR_{AxxxA}$ and SigH during normal and oxidized conditions. We also plan to attempt SPR binding experiments between AosR-SigH and $AosR_{AxxxA}$SigH in normal and oxidized conditions.

These proposed experiments are interesting to demonstrate the oxidation of the Cys residues in AosR is required for molecular interaction. Yet, it will not prove that a conformational change occurs.

Schematic presented in Fig 6d is based on the following experiments.
a. Disulfide bond formation in AosR in response to oxidative stress (Fig 1i).
b. Oxidative stress-dependent interaction between AosR and SigH (Fig 5d).
c. Increased transcription of mec, cysO and cysM upon oxidative stress (Fig 3h).
d. Using a reporter assay we show that the transcription is driven through a stressresponsive auxiliary promoter (Fig 4b).
e. Increased transcription of mec, cysO and cysM is dependent on AosR and SigH (Fig 3m and 5f)
f. While we have not proved directly that L-cysteine levels increase upon oxidative stress, results show that supplementation with cysteine rescues the phenotype (Fig 3c and 3d).
g. AosR mediated cysteine biosynthesis regulation in response to oxidative stress results in genome-wide transcription changes (Fig 2e). However, transcriptional changes of seven genes other than mec could be mitigated upon supplementation with cysteine (Fig 5i).

Most experiments include only some genes (usually 2) out of the 3-gene gene cluster, and the displayed genes vary from one experiment to the other. This is very annoying that the authors conclude on the role of the three genes as a whole based on these only partial experiments.

- It is quite an achievement to have generated antibodies against so many Mtb proteins (AosR, ClpS, Rv1333, CysO, CysM, CysK1, CysK2, GroEL1, SigA), and this could be very useful to the community. Yet, the specificity of these antibodies must be reported in detail; whole WB must be provided, with appropriate controls. Please also provide more technical details on how these Abs were generated.

Response:

Thank you for the comment. Please find the whole WB for all the antibodies that were used in this study. GroEL1 was previously generated in the lab. We plan to expand the supplementary methods section to provide more details regarding the
antibody generation. 400µg of purified His tagged protein in PBS was mixed with Freund's incomplete adjuvant (Sigma) in 1:1 ratio to a final volume of 1.2 ml. 200 µl of emulsion was injected subcutaneously at 2 sites into 6 mice. In the case of ClpS, Rv1333, CysK2, Coomassie-stained bands were excised from the gel, and the emulsion was prepared. Three booster doses were given every two weeks. Final bleed was collected 7 days following final injection and western blotting was performed to assess antibody specificity and sensitivity of the antibody.

I am sorry, but the blots shown are NOT to the expected standard at all. All blots should be shown over a complete scale in protein size. The very poor quality of the SigA blot casts strong doubt about the possible quality of the other blots.

- Most of the second part of the study is based on CysM while mec is left aside; this is surprising since overexpression with CysM is not functional (3e). Furthermore, cysM is not found as induced, in the RNAseq experiment (Table S6) while mec and cysO are; how do the authors explain this apparent discrepancy and justify they focus on CysM?
Response:
Increased production of L-cysteine would require the upregulation of all the genes involved in that particular pathway. CysM utilizes O-phospho-L-serine and CysO-SH as substrates to generate CysO-cysteine. Mec cleaves CysO-Cysteine adduct to liberate L-Cysteine and CysO (Fig 3a). Thus overexpression of CysM alone would not be sufficient to increase L-Cysteine levels in the cell. We would require overexpression of all three genes of the pathway for the functional complementation. We would modify the discussion to clarify this part.
In our RNA seq data, we found CysM levels also to be higher, however, it was not included in the list due to higher standard deviation in the transcript levels between the samples.
We focused on CysM since it is the cysteine synthase that converts O-phospho-L-serine and CysO-SH as substrates to generate CysO-cysteine. Furthermore, prior to the generation of cysM mutant, we have established upregulation of all three genes - mec, cysO and cysM upon oxidative stress (Fig 3h and 3i). We also established that the increased protein levels of CysO and CysM is dependent on oxidative stress (Fig 3j-l) and the presence of AosR (Fig 3m).

The rationale provided is NOT correct. Induction of only one gene in a pathway may be enough to control this pathway if that gene encodes an enzyme involved in a limiting step of the pathway. Therefore, we reiterate our request to include the three genes in all the study.

- In Fig 4l, the authors show that an aosR-KO mutant is less attenuated in mice treated with a NO-inhibitor than in control animals; however, from data in S4d, they conclude that aosR is NOT involved in response to NO stress. Can the authors reconcile these apparently contradictory data sets?
Response:
In vivo, in addition to Phox, iNOS also known to contribute to the intracellular ROS through the pathways depicted in Fig S11. This pathway is specific to the host. Our in vitro DETA NO experiment was performed only with the pathogen and hence it is DETA NO is not expected to contribute to oxidative stress.

I do not understand this paragraph. Using DETA NO usually intends to induce an oxidative stress.

- Statistics are sometimes weird; in 4k for example, in phox-KO cells, how can it be that there are *** between Rv and cysM-KO and only one * between cysM-KO and the complemented strain, while all means look the same? Same applies for phox-KO data in 4j, 4l, and for the NO-inhibitor in 4l. Displaying full data points appears necessary here. More generally speaking, it would be wise to display histograms containing all data points for all experiments reporting CFUs.
Response:
The difference in the significance despite similar-looking mean is due to the higher standard deviation in complemented strain, as can be seen with represented error bar in Fig 4 graphs. In Fig 4k- the mean±SD value are Rv:96333.33±3214.55, RvDaosR:59333.33±5033.33 and RvDaosR::aosR- 90666.67±11015.14. For clearer representation, we would present full data points bar graphs in the revised manuscript. We would be happy to provide all the source data as a supplementary file.

I leave it to the appreciation of the Editor; should the paper be ultimately accepted for publication, I strongly advise the data to be scrutinized by a professional statistician.

- In Fig 5g-h, loading controls are necessary.
Please find below the loading control for Fig 5g-h. We would modify the figures in a revised version of the manuscript to include this data.

OK. So, the blots now suggest that sigB deletion inhibits cysM expression in oxidative conditions, contrary to what is concluded in the manuscript.

- Fig 5i, data should be complemented with a control consisting in CHP+DTT (without Cys), to assess whether the redox state of AosR alone modulates gene expression.
Response:
The second sample in Fig 5i is RvDaosR+CHP without cysteine. We agree that the addition of CHP+DTT may mitigate each other's effects and can act as a control. Moreover, DTT addition may have unintended consequences as it is a strong reducing agent. An alternate control is to utilize the Rv sample without CHP or DTT. We have shown in Fig 2g that the transcriptional changes in all nine genes are dependent on the addition of CHP. Since this aspect has already been established in Fig 2g, we have performed the experiment described in Fig 5i only oxidative stress conditions. We can modify/improve our results section to bring out this aspect.

I do not understand this response. How to exclude that the reductive properties of Cys are not the origin of the observed effect? In the case of mec, couldn’t it be the effect of a retrocontrol by Cys.

I would like to sincerely thank you for your time and kind consideration.
We were quite disappointed to hear about the decision. We welcome reviewer 3's comments, who found our way forward reasonable and stated the fact that the results may change the conclusions. We agree with this comment in principle. However, in line with the Reviewer 3 suggestion, we have been working on gel-shift assays, SPR and immunoprecipitation experiments, and all the results provide strong support to our previously stated findings.

On the contrary, based on several points raised by reviewer 1, we feel that the reviewer may not have completely appreciated the novelty of our findings and the rationale of the study (comment #2 and 8). We also feel that the response to our explanations and working plan is quite harsh (comment #2 and 3) and at times unreasonable (comment #4). Moreover, there is an implicit insinuation about the sanctity of data, which is reflected in comment#3 and #6.

This work was selected for the best poster award at the EMBO-TB International conference 2020 by leading tuberculosis researchers, and received immense appreciation. We are working on finishing the experiments and in fact already obtained data that shows direct binding of AosR and SigH to the promoter region in a redox responsive manner (EMSA and SPR experiment). We also obtained data, which showed that interaction between SigH and AosR decreases significantly upon mutating CxxxC residues. We believe we would be able to address all the comments in the next two months.

Please find attached our point by point response to reviewer 1's comments. We are very keen to resubmit a revised version of the manuscript to EMBO J. We would appreciate your thoughts on this. Thank you and looking forward to your response.

I apologise for the delay in responding to your email. I have now had a chance to look into your points. I woudl like to point out that the decision was also based on the concerns by reviewer #3 regarding the unpredictable outcome of such a major revision. If you find that you are able to answer the concerns from all reviewers in a conclusive manner, I would not be opposed to reconsidering the revised version as a new submission, while sending it back to the same reviewers. I agree that the language reviewer #1 used was perhaps incensive, but based on this reviewer's expertise in Mtb physiology I would still want to hear their thoughts on such a revised version, while the final decision would not be based solely on their input.

Referee #1:
The authors identify AosR/Rv1332 as an actinomycete-specific TF involved in M. tuberculosis response to oxidative stress in vitro and in vivo. They report that AosR interacts with SigH to regulate a cysteine biosynthesis operon involving CysM and other proteins. This is a potentially interesting study, however, several conclusions are poorly supported by the data and the exact mechanism by which AosR interacts with SigH, and how AosR/SigH-mediates regulation of the CysM pathway is not elucidated.

Major comments
- The authors claim that their « data indicates that oxidative stress alters the conformation of AosR through the formation of a single intrasubunit disulphide bond, which in turn facilitates its interaction with an extracytoplasmic sigma factor, SigH. » This claim is not supported by the data at all. There is no demonstration of conformational change in AosR that mediates interaction with SigH.

Response:
Thank you for the comment. The inference of conformation change was drawn based on mBBr assay, which showed the formation of a single intrasubunit disulfide bond in AosR in response to the oxidative environment (Previously Fig 1i/Revised Fig 6d). However, we agree that this is not direct proof of conformation change. Thus we rephrased the sentence to "Oxidative stress ensues the formation of a single intrasubunit disulphide bond in AosR, which in turn facilitates its interaction with an extracytoplasmic sigma factor, SigH."

To validate the importance of disulfide bond formation for AosR-SigH interaction we performed Biolayer Interferometry (BLI) and co-immunoprecipitation experiments. BLI analysis was performed with purified SigH, AosR, and $AosR_{AxxxA}$ proteins and found that mutation of cysteine residues in the CxxxC motif abrogates the interaction of AosR with SigH (Fig 6g). Loss of interaction was further confirmed through the co-immunoprecipitation experiment (Fig 6h).

Comment:
More generally, most of the scenario depicted in Fig 6d is not supported by the data. A thorough structural study of AosR and SigH in normal and oxidized conditions should be performed.

Response:

We thank the reviewer for incisive comments that helped in making the manuscript focused. We agree that a few aspects of the model were not experimentally demonstrated in the previous version, such as:

a. Direct interaction between AosR-SigH.
b. Loss of interaction upon mutating the CxxxC motif in AosR.
c. Establishing a connection between AosR and oxidative stress on cysteine derived metabolites.
d. Direct binding of AosR to an auxiliary promoter.

To address these points:

1. We performed BLI analysis with purified AosR and SigH proteins. Results show that AosR interacts with immobilized SigH with ~10 fold higher affinity under oxidative conditions compared with the corresponding reductive conditions (Fig 5f & S10).
2. We performed BLI analysis with purified SigH with purified AosR and $AosR_{AxxxA}$ proteins and found that mutation of cysteine residues in the CxxxC motif abrogates the interaction

of AosR with SigH (Fig 6g). Loss of interaction was further confirmed through the co-immunoprecipitation experiment (Fig 6h).

3. We quantitated the total thiol content and major cysteine derived antioxidants- mycothiol and ergothioneine in *Rv*, *RvΔaosR*, *RvΔaosR::aosR* under normal and oxidized conditions with the help of LC-MRM MS/MS analysis (Fig 7a-c).
4. We performed an EMSA experiment with a 100 bp auxiliary promoter sequence to establish direct binding (Fig 5k).

Schematic presented in the revised Fig 8 (previous Fig 6d) is based on the following experiments.

a. Disulfide bond formation in AosR in response to oxidative stress (Previous Fig 1i/Revised Fig 6d).
b. Oxidative stress-dependent interaction between AosR and SigH (Previous Fig 5d/ Revised Fig 5f-g).
c. AosR and SigH directly bind to the *mec* promoter, more strongly under oxidative conditions (Fig 5k).
d. Increased transcription of *mec, cysO* and *cysM* upon oxidative stress (Previous Fig 3h/ Revised Fig 4g).
e. Using a reporter assay we show that the transcription is driven through a stress-responsive auxiliary promoter (Previous Fig 4b/ Revised Fig 5b&j).
f. Increased transcription of *mec, cysO* and *cysM* is dependent on AosR and SigH (Previous Fig 3m and 5f/ Revised Fig 4h & 5h)
g. Since L-cysteine levels are very low in the cell, we showed a relative increase in downstream products i.e. mycothiol and ergothioneine in *Rv* and *RvΔaosR::aosR* compared with *RvΔaosR* under oxidative conditions (Revised Fig 7b-c).
h. Supplementation with cysteine rescues the *RvΔaosR* growth defects (Previous Fig 3c and 3d/ Revised Fig 4c-d).
i. Increased production of cysteine through non-canonical cysteine biosynthesis protects mycobacteria cells from phagocyte-derived oxidative and nitrosative stress (Revised Fig 2).
j. AosR mediated cysteine biosynthesis regulation in response to oxidative stress results in genome-wide transcription changes (Previous Fig 2e/ Revised Fig 3e). However, transcriptional changes of seven genes other than *mec* could be mitigated upon supplementation with cysteine (Previous Fig 5i/ Revised Fig 7h).

To address the reviewer's concern, we have modified the figure legend to include few of the above points.

- Fig 1f/Line 94, the C-ter end is said to be unstructured, yet the authors show an alpha-helix for residues 1-22. This is contradictory.

Response:

We apologize for the mistake. The N-terminal region is largely unstructured up to 33 amino acids except for a helix-turn-helix motif (Previous Fig 1f & Fig S1a/ Revised 6b & Fig S1a). We have made the necessary corrections.

- It is quite an achievement to have generated antibodies against so many Mtb proteins (AosR, ClpS, Rv1333, CysO, CysM, CysK1, CysK2, GroEL1, SigA), and this could be very useful to the community. Yet, the specificity of these antibodies must be reported in detail; whole WB must be provided, with appropriate controls. Please also provide more technical details on how these Abs were generated.

Response:

Thank you for the comment. Whole WB for all the antibodies that were used in this study is provided as Fig S13. GroEL1 was previously generated in the lab. Further details on antibody generation have been included in the supplementary methods.

- Most of the second part of the study is based on CysM while mec is left aside; this is surprising since overexpression with CysM is not functional (3e). Furthermore, cysM is not found as induced in the RNAseq experiment (Table S6) while mec and cysO are; how do the authors explain this apparent discrepancy and justify they focus on CysM?

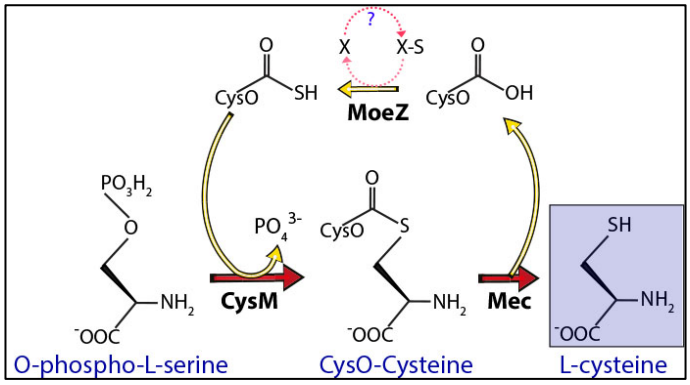
Response:

In our RNA seq data, we found CysM levels also to be higher, however, it was not included in the DEGs list due to higher standard deviation in the transcript levels between the samples.

We have re-worked the manuscript to focus majorly on AosR. Its target, CysM, is included in the manuscript only as a proof-of-concept (Fig 4i-k). We, therefore, have removed data sets that were peripheral to AosR; as our study is not on characterizing a pathway, rather regulation of a pathway (i.e. Cys biosynthesis from O-Phospho-Serine) by a specific TF.

Amongst the three genes, we focused on CysM since it is the **cysteine synthase** that converts O-phospho-L-serine and CysO-SH as substrates to generate CysO-cysteine. Furthermore, prior to the generation of *cysM* mutant, we established upregulation of all three genes - *mec, cysO*, and *cysM* upon oxidative stress (Previously Fig 3h-i/ Revised Fig 4g). We also established that the increased protein levels of CysO and CysM are dependent on oxidative stress (Fig 4h/ Revised Fig S8c) and the presence of AosR (Previously Fig 3m/ Revised Fig 4h).

Increased production of L-cysteine would require the upregulation of all the genes involved in the pathway. As depicted in the schematic, all three proteins involved in the pathway are intricately connected. The absence of any one of these three proteins (CysM, CysO, and Mec) would completely abrogate the synthesis of cysteine through this pathway.



The reaction carried out by CysM is not proven to be rate-limiting. In the absence of higher levels of CysO (a substrate) producing higher levels of CysM would not take the pathway forward. Thus, overexpression of CysM alone would not be sufficient to increase L-Cysteine levels in the cell. We would require overexpression of all three genes of the pathway for the functional complementation. To keep the manuscript more focused, we have removed CysM overexpression data from the revised manuscript.

- In Fig 4l, the authors show that an aosR-KO mutant is less attenuated in mice treated with a NO-inhibitor than in control animals; however, from data in S4d, they conclude that aosR is NOT involved in response to NO stress. Can the authors reconcile these apparently contradictory data sets?

Response:

We thank the reviewer for the critical comment. We re-visited our *in vitro* nitrosative susceptibility data and found that when plotted as percent survival with respect to day 0, the survival differences between *RvΔaosR* and *Rv* or *RvΔaosR::aosR* were significant (Revised Fig S3d). We have modified the results and discussion accordingly.

- Statistics are sometimes weird; in 4k for example, in phox-KO cells, how can it be that there are *** between Rv and cysM-KO and only one * between cysM-KO and the complemented strain, while all means look the same? Same applies for phox-KO data in 4j, 4l, and for the NO-inhibitor in 4l.

Response:

In line with the Reviewer's suggestion, we re-analyzed the statistics of all our data. We have now calculated the significance of all survival assays using one-way Anova (Graphpad Prism, post-hoc Tukey test). Additionally, we have provided the source data excel sheet for your kind perusal.

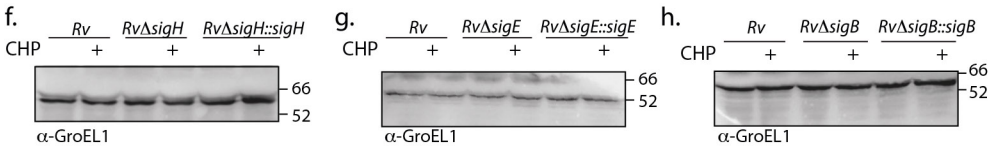
Displaying full data points appears necessary here. More generally speaking, it would be wise to display histograms containing all data points for all experiments reporting CFUs.

The figures have been modified in line with the suggestion.

- In Fig 5g-h, loading controls are necessary.

Please find below the loading control for Previous Fig 5g-h. In order to keep the focus, we have removed SigB and SigE data from the current manuscript (Previously Fig 5g and h). SigH data is provided along with the control (Revised Fig 5h).

Figure 5



- The mechanism of AosR interaction with SigH is not investigated and the authors cannot conclude that the two Cys residues in AosR are involved. At the very least, pull-down experiments with the AosR (CxxxC ->AxxxA) mutant should be performed.

Response.

To address this question, we electroporated *M. smegmatis* with pNit-SigH vector (containing 3X-FLAG tag at the N-terminus). This strain was further electroporated with an integrative vector or vector containing *aosR* or $aosR_{AxxxA}$ with C-terminus SBP-HA-S tag. The three strains were grown in the presence or absence of CHP stress, followed by immunoprecipitation of SigH with the help of anti-FLAG beads. Interaction with AosR or its mutant $AosR_{AxxxA}$ was evaluated by probing samples with an anti-HA antibody.

The interaction of AosR with SigH could only be detected upon peroxide stress and mutation of CxxxC significantly reduced the affinity (Revised Fig 6h). These results validate the importance of the redox environment as well as CxxxC motif of AosR in mediating AosR-SigH interaction, which in turn is important for combating host-induced peroxide stress.

- Fig 5i, data should be complemented with a control consisting in CHP+DTT (without Cys), to assess whether the redox state of AosR alone modulates gene expression.

Response:

The second sample in Previous Fig 5i/ Revised 7h is *RvΔaosR*+CHP without cysteine. We agree that the addition of CHP+DTT may mitigate each other's effects and can act as a control. However, DTT addition may have unintended consequences as it is a strong reducing agent. An alternate control is to utilize the *Rv* sample without CHP or DTT. We have shown in Previous

Fig 2g/Revised Fig 3g that the transcriptional changes in all nine genes are dependent on the addition of CHP. Since this aspect has already been established in Previous Fig 2g/Revised 3g, we have performed the experiment described in Previous Fig 5i/ Revised 7h only under oxidative stress conditions.

Comment #8: I do not understand this response. How to exclude that the reductive properties of Cys are not the origin of the observed effect? In the case of mec, couldn't it be the effect of a retrocontrol by Cys.

Response:

Reductive properties of cysteine indeed are responsible for rescue observed in other 8 genes, as suggested multiple times in the manuscript. We presume by "retrocontrol", the reviewer means feedback control. Till date there is no evidence that suggests feedback inhibition of *mec-cysO-cysM* by cysteine.

- It is not clear why the authors report on the effect of INH and Rif treatment in infected mice as the attenuated profile of the mutant looks identical in control and treated animals (which is surprising given the sensitivity profile of the mutant to INH in vitro showed in Fig 6a).

Response:

Thank you, point well taken. This particular concern has been raised by all three reviewers. As suggested, the data presented in the previous 6a and 6b is removed in the revised manuscript.

- Regarding Fig 6a, how can it be that 100% bacteria are recovered after treatment with INH at a 50xMIC dose?

Response:

The calculations were with respect to the untreated sample, which was considered 100%. The survival observed in the presence of antibiotics was only 1% (Previous Fig 6a). As suggested, the data presented previously 6a and 6b is removed in the revised manuscript.

Minor comments

- In extenso definition of AosR should appear as soon as possible.

Response:

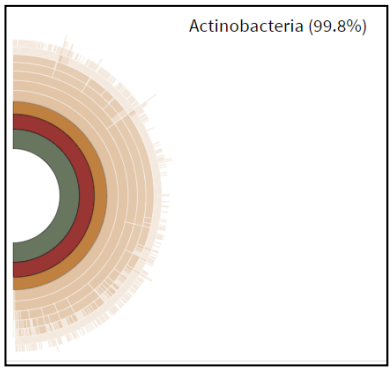
We thank the reviewer for the suggestion. We have incorporated the changes.

- AosR is not strictly specific to actinomycetes. In particular it is present in Serratia marcescens and other non-actinomycetes. More generally speaking, the rationale for the selection of AosR as a candidate TF to study is odd: why concentrate on actinomycete-specific TFs? Other TFs that are essential in vivo but not in vitro might be of interest as well.

Response:

We had previously analyzed our taxonomic group through the eggnog database 4.5 (http://eggnog45.embl.de/#/app/home) that suggested 100% orthologs belong to actinomycetes. However, the updated eggnog database 5 that was published last year (http://eggnog5.embl.de/#/app/results#ENOG502E4AX_datamenu) suggests 99.8% of orthologs belong to actinobacteria (Figure attached below). We did not find *Serratia marcescens* in the list generated through this database. Nevertheless, based on the eggnog analysis, it is apparent that rv1332 orthologs are highly enriched in actinomycetes. We have re-worded our results accordingly.

While we agree that all TFs that are *in vitro* non-essential but *in vivo* essential are of interest. We studied AosR because it was found to be majorly restricted to actinomycetes (99.8%), which we found intriguing. We felt that targeting such protein with inhibitors would have the least side effect on our general microbiome. Hence, such proteins are comparatively better drug targets for adjunct therapy. This is our rationale behind choosing AosR. We have modified the introduction to reflect the above rationale.



- In Figure 1 (b), it is surprising that *Mtb* does not grow in macrophages at all. What is the activation status of these cells? The Methods section is very incomplete.

Response:

Mtb grows very well in cell lines such as THP-1 and RAW cells. Since peritoneal macrophages are primary cells, we felt it is a better system to investigate *ex vivo* survival. As mentioned in Methods, peritoneal macrophages are extracted post-thioglycolate injection. We believe that the *Mtb* growth in peritoneal macrophages is inhibited because of its activation status. This phenomenon is reproducible in multiple experiments performed by different members of the lab (1). Our recently performed experiments followed a similar trend (Fig 4j). We have elaborated methods in the supplementary section.

- From the authors' data, it cannot be concluded that AosR is « essential » for in vivo survival, it is « required » or « involved in », at best.

Response:

We thank the reviewer for the suggestion. Point is well taken, we have re-worded essentiality throughout the manuscript.

- The selection of AosR is based on two publications from two labs. What is the fate of AosR in other screening studies by other labs, in particular in macrophages.

Response:

While many Tn-Seq studies have identified mycobacterium genes that are essential *in vitro*, there is only one study that has performed a similar experiment *in vivo* (2). Transposon mutant of *aosR* was found to be essential in this study as well inside macrophages (3). We have referred to this paper "In accordance with this, transposon mutants with insertions in *mec, cysO* and *cysM* exhibit attenuated survival inside the host (2-4)".

After the submission of this manuscript, another paper was published in which authors have identified genes that influence drug effects within the host using Tn-seq library (5). In their control group (no antibiotic treatment), AosR was represented to be essential for *in vivo* survival of *Mtb*. We have included the reference for this manuscript in our revised study.

- Panels 1a and 1d could be removed.

Response:

We believe panel 1a is important to provide the basis for the manuscript. Fig 1d is moved to Supplementary Fig S3f.

- Fig 1i could be better explained. What is supposed to be concluded from this DC experiment?

Response:

Thank you for you comment. We have provided more detailed explanation in the revised manuscript.

We were interested in investigating if the two cysteine residues (part of CxxxC motif) form an intrasubunit disulfide bond. To address this, recombinant AosR protein was incubated in monobromobimane (mBBr). mBBr reacts with the free thiol group (here cysteine) and results in the formation of an adduct that displays fluorescence, which would be highest under completely reduced conditions and lowest in completely oxidized conditions. Hence, there is a direct correlation between the intensity and the redox state of thiol groups.

As observed in Previous Fig 1i/Revised Fig. 6d, an increasing ratio of reduced to oxidized DTT was coupled to an increase in fluorescence, suggesting that these cysteines participate in the formation of an intrasubunit disulfide bond that is attenuable in response to the external redox environment (Previous Fig 1i/Revised Fig. 6d).

- In Fig 2g and h, it looks like DCt data, not DDCt are displayed. Could the authors clarify? If data are normalized to 16S, how can it be that all genes are at the same level in the -CHP condition? How were the genes chosen? Why Rv3529 is showed in g and not in h?

Response:

The values were calculated as follows:

- The Ct values obtained for gene of interest were subtracted from the respective 16S value (ΔCt).
- ΔCt value of the experimental group was subtracted from the control group to get ΔΔCt. As the fold change was calculated with respect to the control group, the value of all control groups are 1.
- Fold change in these graphs represents $2^{-\Delta\Delta Ct}$.

Since it is not experimentally possible to confirm all genes that were found to be differentially regulated, we choose 9 genes among the 26 operonic gene sets (Table S7). The basis of the choice of genes is explained in Fig S5.

In line with the suggestion, we have evaluated Rv3529 transcript changes for panel Previous Fig 2h/Revised Fig 3h.

- In Table S6, please make reference to Fig 2g/h, not 2i that does not exist.

Response:

We apologize for the mistake. Necessary changes have been made.

- Lines 201-3, replace Fig 5 (l-m, n-o) by Fig 4.

Response:

We apologize for the mistake. Necessary changes have been made.

- Line 235-250 should be moved to the Discussion section as this is just a discussion of already reported data.

Response:
We believe a certain level explanation would be necessary to provide the basis for Previous Figure 5i/Revised Figure 7h. However, we made it more focused.

Referee #2:

In this manuscript, Khan and colleagues report the identification and characterization of a transcriptional activator Rv1332 (AosR). They demonstrate that AosR responds to peroxide stress with the formation of an intramolecular disulfide bond, which enables interaction with SigH and activation of an operon leading non-canonical cysteine biosynthesis. The AosR mutant is attenuated in peritoneal macrophages and mice. The survival defect in macrophages is rescued in phagocyte oxidase deficient macrophages that are also treated with an inhibitor of nitric oxide synthase, linking the attenuation in macrophages to the generation of reactive oxygen and nitrogen intermediates. In vitro, an aosR mutant fails to upregulate cysteine biosynthesis during oxidative stress and addition of cysteine overcomes the impact of deletion of aosR.

Overall this is a well-executed, comprehensive and interesting manuscript that provides new insight into a redox responsive transcription factor. The authors conclusions are for the most part supported by the presented data. I have one major and a few minor concerns that need to be addressed.

The conclusion that "cysteine flux regulated by AosR potentiates drug tolerance" is not supported by the data. In vitro the susceptibility of the mutant to INH is only ~ 2-fold higher than that of wild type H37Rv and only shown for a single concentration and single time point. This is not convincing. In vivo, the mutant does not seem to be significantly more susceptible to INH and Rif treatment than wild type as the impact of INH/Rif results in a similar reduction in CFU for wt and mutant compared to the CFU of each strain before treatment. Thus, that AosR affects drug tolerance is not convincingly demonstrated and the data and conclusion should be removed.

Response:

We thank the reviewer for their highly encouraging comments. We agree with the reviewer's concern and have removed this data from the current version of the manuscript.

The authors interpret the in vivo data as "Rv1332 is specifically essential for mycobacterial survival within its host". This is incorrect. The mutant has a growth defect in mice compared to wild type Mtb but does not fail to survive, its titers never decline. The conclusion needs to be reworded.

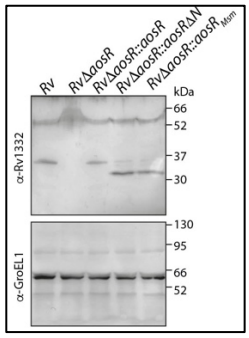
Response:

We have modified the lines to "Rv1332 is specifically required for mycobacterial growth within its host".

AosR lacking the N-terminal domain and AosR from M. smegmatis did not complement the Mtb AosR Mutant. The authors need to show that these proteins were expressed at equal amounts as AosR.

Response:

We agree this control is required. We have performed western blot recently to demonstrate equal expression of mutant proteins. Please find the attached western blot.



In the discussion the authors wrote "Our data shows that the attenuated survival of Mtb due to the deletion of cysM or its regulator aosR is mitigated in phox -/- mice, confirming the specific requirement of this pathway in response to host-induced oxidative stress". However, if the growth defect observed in BalbC mice is directly linked to oxidative stress has not been interrogated. The authors have not infected phox-/- mice. Please rephrase.

Response:

We agree with the reviewers comments. In the revised manuscript, we have included results of an animal experiment, wherein wild type B6, phox$^{-/-}$ and IFNγ$^{-/-}$ mice were infected with *Rv*, *RvΔaosR*, and *RvΔaosR::aosR* and monitored their survival at 4 weeks p.i. (Fig 2e & Fig S4c). Bacillary survival in the lungs of infected animals at day 1 indicated equal deposition. *RvΔaosR* displayed attenuated survival compared with *Rv* and *RvΔaosR::aosR* in WT mice (Fig 2e & Fig S4c). Importantly, the survival differences between the strains were abolished in phox$^{-/-}$ and IFNγ$^{-/-}$ mice (Fig 2e & Fig S4c). These results indicate that AosR detoxifies host-derived redox stress facilitating mycobacterial survival in the host.

Also in the discussion: "In murine Mtb infection models, the oxidative burst occurs 14 days post-infection" - that is - to my knowledge - incorrect. The respiratory burst occurs in response to phagocytosis and does not happen only after 14 days. Either provide evidence for this statement or remove.

Response:

We looked for this reference but could not find it. We have removed the sentence.

Referee #3:

This manuscript describes the characterization of a putative transcription factor encoded by Rv1332, here termed AosR, deletion of which makes Mtb more susceptible to oxidative stress in vitro, and leads to a growth defect in macrophages and mice. A large amount of data is presented that connects this TF to the expression of a gene cluster that harbors genes for an alternate cysteine biosynthesis pathway. Furthermore, evidence is presented that the N-terminal portion of the protein is necessary to prevent susceptibility to oxidative stress, and that a cysteine motive is involved. Furthermore, it is shown that SigH also plays a role in the regulation of this operon. The genetic and protein work is sound and rigorous. On the whole the manuscript is interesting and easy to follow.

However, while the authors are commended for their large amount of rigorous work to characterize this putative transcription factor, the results are mostly overinterpreted and important controls to support the proposed mechanisms are missing.

Response:

We thank the reviewer for their encouraging, critical, and insightful comments.

1. The authors claim that "Cellular redox poise in M. tuberculosis is modulated by a novel actinomycetes-specific transcription factor", yet nowhere in the paper is this formally shown. In fact, the redox poise of the cells are not measured throughout this study. Instead, susceptibility to oxidative stress and rescue through cysteine addition is extrapolated that this putative TF is actually modulating redox poise. To support this claim, ratios of redox cofactors actually need to be determined.

Response:

We agree with the concerns expressed by the reviewer. As suggested, we measured NADH and NAD^+ levels using LC-MRM MS/MS analysis (Revised Fig 7d-e). We found that the levels of both the redox cofactors NAD^+ and NADH were lower in *Rv*Δ*aosR,* probably a metabolic consequence of death. We further monitored the antioxidant response of *Rv* and *Rv*Δ*aosR* by measuring changes in mycothiol redox potential (E_{MSH}) using a non-invasive, highly sensitive redox biosensor specific to mycobacteria (6). The ratiometric fold change in *Rv* was modest (2 fold) as compared to *Rv*Δ*aosR* (5 fold). Moreover, while *Rv* was able to return to its ambient redox state within 80 min, *Rv*Δ*aosR* was compromised in its ability to do so (Revised Fig 7f). Together, the data suggest that AosR is crucial in maintaining intra-mycobacterial redox homeostasis.

It is proposed that Rv1335 is a transcription factor, yet no attempts are made to formally show this functionality, e.g. with gel shift assays. In addition, a consensus sequence for TF binding is also not determined, even though this could be done with the data at hand.

Response:

In line with the suggestion, we performed EMSA with AosR, SigH, and in combination using 100 bp *mec* promoter as the DNA probe under reduced and oxidized conditions.

We first demonstrated that the auxiliary promoter element necessary for stress-inducible response lies within the 100 bp region using luciferase assay (Revised Fig 5j). Next, we performed electrophoretic mobility shift assays (EMSA) with radiolabelled 100 bp fragment (double stranded) to examine the direct binding of AosR and SigH. EMSA indicated that both AosR and SigH bind 100 bp fragment both under reducing and oxidizing conditions. Importantly, the addition of both proteins together resulted in stronger binding under oxidizing conditions, compared with the reduced conditions indicating that the DNA binding is influenced by the redox environment (Fig 5k). We established the specificity of the observed shift in the EMSA by performing competition with fold excess of unlabelled 100 bp fragment (Fig 5k). Collectively, these results suggest that

AosR and SigH are independently essential for the regulation of non-canonical cysteine biosynthesis pathway and that the two factors come together in the cell's response to oxidative stress, acting concertedly to activate transcription of the *mom* cluster.

However, we could not find any consensus sequence with high confidence for Rv1332 binding in the promoter of genes enlisted in Table S7. This is in congruence with the major finding of our study that suggests transcriptional changes in *Rv* vs *RvΔaosR* in response to oxidative stress is due to the inability of mutant to upregulate *mec-cysO-cysM* dependent cysteine biosynthesis (Previous Fig 5i/ Revised fig 7h).

Based on the data we propose that AosR specifically augments the expression of *mec-cysO-cysM* through activation of an auxiliary promoter and other transcriptional changes are indirect-as a result of changes in the cysteine or cysteine metabolite levels.

3. Another issue that needs further attention is the proposed decrease in cysteine biosynthesis. Cysteine is not measured and neither are redox factors like mycothiol or ergothioneine.

Response:

Our efforts to measure cysteine through commercially available kits and mass spectrometry were unsuccessful, probably because the intracellular concentration of L-cysteine in *Mtb* is limiting in comparison with other amino acids (7).

As suggested, we quantified cysteine-derived highly abundant low molecular thiols, mycothiol, and ergothioneine using LC-MRM MS/MS analysis. The MSH levels were found to be similar in *Rv, RvΔaosR,* and *RvΔaosR::aosR* under normal conditions. While the addition of CHP resulted in ~30 and 15% and an increase in the MSH levels in *Rv* and *RvΔaosR::aosR*, we observed a ~50% decrease in *RvΔaosR* , indicating *Mtb* is unable to replenish MSH in the absence of AosR (Fig 7b). Similarly, levels of ergothioneine were found to be drastically lower in *RvΔaosR* (18%) as compared to *Rv* (~80%) and *RvΔaosR::aosR* (~70%) upon oxidative stress (Fig 7c).

In addition, we estimated the free thiol protein content in *Rv, RvΔaosR,* and *RvΔaosR::aosR* under normal and oxidized conditions using a commercially available kit (Invitrogen). As shown in Fig 7a, we observed a ~3 fold increase in the thiol content of *Rv* and *RvΔaosR::aosR* in oxidatively stressed cells compared with respective control groups. Importantly, the increase in thiol was found to be dependent on AosR (Fig 7a).

Also, it is unclear why the serine derived cysteine biosynthetic pathway is not compensating a potential decrease in cysteine…

We believe that *Mtb* elects to upregulate L-cysteine biosynthesis specifically through the CysM pathway. This is probably due to the higher stability of its acceptor substrate O-phospho-L-serine compared with the CysK1 substrate O-acetyl-L-serine[46], and higher resistance of donor thiocarboxylates to oxidation compared with thiols, the sulfur donor of CysK1[15]. In accordance with this, transposon mutants with insertions in *mec, cysO* and *cysM* exhibit attenuated survival inside the host[8,16,46].

4. The proposed drug phenotype needs more attention. First, in order to illustrate decreased persister survival in the presence of INH, an actual kill curve has to be shown over at least 15 days. Secondly, the in vivo drug data does not support their conclusion of increased susceptibility, because the mutant is by itself already more susceptible in vivo. The kill kinetics in mice look the same for WT and mutant. By week 4 there is already a ~1 log diff in KO and WT, this trend continues following drug treatment but is not exacerbated. Therefore lower CFU following treatment can't necessarily be concluded to imply decreased tolerance of KO as the kinetics appear to largely unchanged.

Response:

We agree with the reviewer's concern and have removed this data.

5. The language should be toned down. A lot of results are oversold. E.g. a 1 log difference in mouse burden is sold as: AosR is essential for survival in mice... Essentiality would be demonstrated by rapid clearance of the strain, yet it can grow in a mouse, albeit at a reduced rate. Also the word myriad is used in places, and it is clearly overstated.

Response:

Thank you for the comment. We have reworked the manuscript. The results of an animal experiment are inferred as "These data suggest that Rv1332 is specifically required for mycobacterial survival within its host." We have replaced the word myriad with "multiple".

6. Cysteine flux? What do you mean by cysteine flux? First of all cysteine is never measured, secondly cysteine flux doesn't make much sense. Do you means flux through cysteine biosynthesis? In the absence of metabolite data nothing can be claimed about flux.

Response:

We apologize for the use of the incorrect word/phrase. We removed the word "flux".

Our attempts to measure cysteine using a commercially available kit were unsuccessful. This is probably due to low cysteine levels that are important to protect cells from its cytotoxic effects. Nevertheless, we quantified total thiol content in *Rv, RvΔaosR*, and *RvΔaosR::aosR* that were either untreated or treated with CHP.

As shown in Revised Fig 7a, we observed a ~3 fold increase in the thiol content of *Rv* and *RvΔaosR::aosR* in oxidatively stressed cells compared with respective control groups. Importantly, the increase in thiol was found to be dependent on AosR.

7. Intra-disulphide bond and sigH interaction The authors infer that the formation intra-disulphide bond is important for interaction with SigH (line17-18) but provide limited data to support this. Using in vitro and ex vivo assays, the authors demonstrate the CxxxC motif is important for protection against oxidative stress and for survival in macrophages, and biochemical data suggests these cysteine residues are capable of forming an intra-disulphide bond enabling AosR to act as redox rheostat. The interaction between AosR and SigH was identified by immunoprecipitation in both directions and shown to be redox dependent. This assay should also be performed using the AxxxA mutant to conclusively show that formation of the intra-disulphide bond indeed facilitates this interaction, as other amino acids that could also be oxidized may influence this interaction.

Response:

To address this question, we performed an additional experiment, wherein we electroporated *M. smegmatis* with pNit-SigH vector (containing 3X-FLAG tag at the N-terminus). This strain was subsequently electroporated with an integrative vector or vector containing *aosR* or $aosR_{AxxxA}$ with C-terminus SBP-HA-S tag. The three strains were grown in the presence or absence of CHP stress, followed by immunoprecipitation of SigH with the help of anti-FLAG beads. Interaction with AosR or its mutant $AosR_{AxxxA}$ was evaluated by probing samples with anti-HA antibody.

The interaction of AosR with SigH could only be detected upon peroxide stress and mutation of CxxxC significantly reduced the affinity (Revised Fig 6h). These results validate the

importance of redox environment as well as the CxxxC motif of AosR in mediating AosR-SigH interaction, which in turn is important for combating host-induced peroxide stress.

More specific comments:
• Inconsistency in naming of mutant in the manuscript and supplement. In some instances written as RvΔ1332 (eg. Line 70 and Figure 1e, S4), elsewhere RvΔaosR (e.g. line 95, figure 1g, S5)

Response:
This was an uncharacterized protein, thus it had no annotated protein name, but only a Rv1332 number. We named it AosR only after establishing its role in combating oxidative stress. However, as suggested, to avoid confusion we have named it as AosR throughout in the revised manuscript.

• Line 96: Msm - replace with M. smegmatis. Aside from the missing N terminus, what is the homology of AosRMsm with AosRMtb?

Response:
Thank you, we have modified the manuscript accordingly.
Aside from the missing N-terminal region,
Identity between $AosR_{Msm}$ and $AosR_{Mtb.}$ - 67%
Homology (positives)- 81%

• Line 142-143/figure 2 - Rv3529 missing from fig 2h

Response:
We have included Rv3529 transcript changes in previous Fig 2h/ Revised 3h.

• Line355 - no figure 5k. Should this be 6d?

Response:
We apologize for the mistake. Necessary corrections have been made.

• Line 360 - should this not a be a redox rheostat rather than a cysteine rheostat?

Response:
In the revised manuscript, we quantitated the levels of major cysteine derived metabolites. Results show that levels of mycothiol and ergothioneine are regulated by AosR upon oxidative stress. Based on this data, we propose that AosR directly regulates cysteine levels in the cell, making it a "*cysteine rheostat*" (a direct role). Downstream effect of modulated cysteine levels in the cell are changes in the levels of redox cofactors including NADH, NAD+ and imbalance in the redox potential, which would also make AosR a "*redox rheostat*" (downstream consequence). Albeit, we would like to retain the term "*cysteine rheostat*" because data suggest AosR transcriptionally regulates the expression of genes involved in non-canonical cysteine biosynthesis.

Figure 4

4f. Only shows RvΔcysM, and not RvΔaosR, whereas 4e. shows both, would be good to see how RvΔcysM and RvΔaosR compare in macrophage.

Response:

We performed a peritoneal macrophage infection experiment with *Rv, RvΔaosR, RvΔaosR::aosR, RvΔcysM, and RvΔcysM::cysM* at the same time to evaluate relative survival differences between the two mutants (Fig 4j). Even though the survival of *RvΔcysM* was lower than *RvΔaosR* across multiple experiments, the difference was not statistically significant (One-way ANOVA, post-hoc Turkey).

4j,k. Redundant as same conditions shown in 4i and 4m

Response:

We have removed these panels.

References

1. Bhaskar, A., Kumar, S., Khan, M. Z., Singh, A., Dwivedi, V. P., and Nandicoori, V. K. (2020) Host sirtuin 2 as an immunotherapeutic target against tuberculosis. *Elife* **9**
2. Sassetti, C. M., and Rubin, E. J. (2003) Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A* **100**, 12989-12994
3. Rengarajan, J., Bloom, B. R., and Rubin, E. J. (2005) Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. *Proc Natl Acad Sci U S A* **102**, 8327-8332
4. Agren, D., Schnell, R., Oehlmann, W., Singh, M., and Schneider, G. (2008) Cysteine synthase (CysM) of Mycobacterium tuberculosis is an O-phosphoserine sulfhydrylase: evidence for an alternative cysteine biosynthesis pathway in mycobacteria. *J Biol Chem* **283**, 31567-31574
5. Bellerose, M. M., Proulx, M. K., Smith, C. M., Baker, R. E., Ioerger, T. R., and Sassetti, C. M. (2020) Distinct Bacterial Pathways Influence the Efficacy of Antibiotics against Mycobacterium tuberculosis. *mSystems* **5**
6. Bhaskar, A., Chawla, M., Mehta, M., Parikh, P., Chandra, P., Bhave, D., Kumar, D., Carroll, K. S., and Singh, A. (2014) Reengineering redox sensitive GFP to measure mycothiol redox potential of Mycobacterium tuberculosis during infection. *PLoS Pathog* **10**, e1003902
7. Laxman, S., Sutter, B. M., Wu, X., Kumar, S., Guo, X., Trudgian, D. C., Mirzaei, H., and Tu, B. P. (2013) Sulfur amino acids regulate translational capacity and metabolic homeostasis through modulation of tRNA thiolation. *Cell* **154**, 416-429

1st Revision - Editorial Decision 26th Feb 2021

Thank you for submitting a revised version of your manuscript. I apologise for the delay in the processing of your manuscript due to delayed submission of reviewer reports and protracted discussions within the team. Your revised study has now been seen by two of the original referees; reviewer #3 unfortunately was not able to submit their report. While reviewer #2 is generally satisfied with the revision, reviewer #1 indicates remaining concerns with the data analysis and presentation. I would like to invite you to address these points as outlined in the pre-decision discussion, as well as include the information requested by reviewer #2. I agree with reviewer #1 that addition of the complemented strain would be helpful in fig 7f. Please also present the data as the 390/488 ratio as requested by the reviewer to increase accessibility of the data. Addressing the last point regarding the use of alternative reducing agents will not not be required.

Furthermore, please address the remaining editorial issues before I can extend the official acceptance of the manuscript.

Please let me know if you have any further questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

Referee #1:

The authors have made efforts to address our previous comments and improve their manuscript. Yet several issues must be addressed for the conclusions to be fully supported by the data.

Major points

• Fig 1 a is useless; instead, a table with the selected TFs and the corresponding relevant references (ref. 9 for instance is inaccurate) would be helpful.
• Fig 1d,e - how do you explain the strain is less attenuated with time, and not significant at later time-points. The statistics have changed compared to previous version (e.g. day 112 in the lungs), this is quite puzzling.
• The absence of effect of DETA-NO is very surprising, do the authors have an explanation?
• Fig 7, the graphs shown do not match AT ALL with the raw data provided in the Source data file.

This is again very puzzling

• Fig 7f, the way data is shown is very misleading; instead of "fold change", the raw 390/488 ratio data should be shown, which would inform about the redox status of the cytosol in both strains. The complemented strain should be included here.
• Fig 7h, a reductive agent other than Cys should be included to formally prove that the observed effect is due to Cys and not to an unspecific redox change.

Other points

- Fig 1b, in text lines 80-81, indicate the survival is compromised at day 4 post-infection.
- Fig 1c is pointless.
- Legend to Fig 2e should be changed to CFUs/lung (?)
- Line 176 - cysO is not shown in the Fig S8b, please fix.
- Fig 5k, why is diamide used here while CHP was used in all other experiments?
- In the discussion, "redox stress" should rather be discussed as "oxidative stress"
- Line 372, ref. 16 & 46 are irrelevant to the claim.

Referee #2:

The authors strengthened their manuscript with additional data. However, they still claim, in the summary and throughout the manuscript that Rv1332 (AosR) "facilitates mycobacterial survival in the murine lungs and spleens". This is not supported by the data. The mutant grows more slowly in mouse lungs and spleens, but there is no evidence for a survival defect. This needs to be corrected throughout the manuscript.
Given the growth defect observed in mice and survival defect (decline in CFU) in peritoneal macrophages, it seems that peritoneal macrophages are not a good model for the in vivo infection. I don't expect the authors to repeat the experiment with other primary cells such as bone marrow derived macrophages, but it would be good to discuss the discrepancy between ex vivo and in vivo phenotype.
The authors must include the source, gender and age of the IFNg-/- and phox-/- mice in the methods section.

Referee #1:

The authors have made efforts to address our previous comments and improve their manuscript. Yet several issues must be addressed for the conclusions to be fully supported by the data.

Major points

• Fig 1 a is useless; instead, a table with the selected TFs and the corresponding relevant references (ref. 9 for instance is inaccurate) would be helpful.

Response:

We believe panel 1a is important to provide a pictorial representation for the basis for the manuscript. List of TFs illustrating their *in vitro* and *in vivo* essentiality on the basis of Tn-Seq data is present in Table S1.

Reference 9 (Sassetti, C. M. & Rubin, E. J. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A100, 12989-12994, doi:10.1073/pnas.2134250100 [pii] (2003).) was used to determine *in vivo* essentiality as presented in Table S1.

Comment.

• Fig 1d,e - how do you explain the strain is less attenuated with time, and not significant at later time-points.

Response:

During first two weeks of acute phase infection in mice, *Mtb* replicates actively and thereafter enters chronic phase of infection, wherein the growth is relatively limited. *aosR* deletion results in the attenuation of growth *in vivo* (the strain still grows) and hence as the time progresses, the differences in growth decreases.

Comment.

The statistics have changed compared to previous version (e.g. day 112 in the lungs), this is quite puzzling.

Response:

In our first version of the manuscript, we analyzed all the data using the student's t-test, as mentioned in that version of the manuscript. Thereafter, we received the reviews and one of the major comments was on statistical significance. Hence, we looked into the literature and realized that the majority of the manuscripts published in major journals analyze the data using "**One-way ANOVA post-hoc tukey test"**.

Hence, we reanalyzed all our bacillary survival data containing multiple strains using **One-way ANOVA post-hoc tukey test**. The change in the methodology resulted in apparent changes in the significance including Figs 1d,e. The method used for the analysis was clearly mentioned in the methods and legends of the revised manuscript. This was also very clearly stated in our previous response (as stated below).

"In line with the Reviewer's suggestion, we re-analyzed the statistics of all our data. We have now calculated the significance of all survival assays using one-way Anova (Graphpad Prism, posthoc Tukey test). Additionally, we have provided the source data excel sheet for your kind perusal".

Even though there are minor differences in the statistical significance due to change in the methodology used for analysis, it has no impact on the conclusions of the manuscript.

Comment.

• The absence of effect of DETA-NO is very surprising, do the authors have an explanation?

Response:

The survival of *RvΔaosR* is compromised compared with *Rv* and *RvΔaosR::aosR* in the presence of DETA-NO (Fig S3d).

Comment.

• Fig 7, the graphs shown do not match AT ALL with the raw data provided in the Source data file. This is again very puzzling.

Response:

The source table indicates absolute raw unprocessed values. To make data more comprehensible and normalize with respect to the control, we processed the raw values in following steps:

1. Calculate average of all replicates of control for each strain
2. Divide raw values by average obtained in step 1 for both normal and oxidized conditions (independently for each strain).
3. Calculate average and standard deviation from the values obtained in step 2.

The data processing was described in the legends of the revised manuscript, wherein we stated,

"The absolute values obtained under normal conditions were normalized to 100% for each strain and the relative values were calculated for samples processed from CHP treated cells. The data indicates relative levels of metabolites (mean percent ± SD) of four replicates (n=4)."

However, while addressing the current comments and putting together the source data file, we realized that the labelling in the source data file was swapped for 7b and 7c. We cross checked the data obtained with the original data from mass spec provided by our collaborator. We apologize for this inadvertent error in the labelling in the source data file.

In order to avoid confusion, in addition to the unprocessed raw data values, we have also included processed values presented in the figures in source table.

Comment.

• Fig 7f, the way data is shown is very misleading; instead of "fold change", the raw 390/488 ratio data should be shown, which would inform about the redox status of the cytosol in both strains. The complemented strain should be included here.

Response:

Towards this, a chloramphenicol resistance cassette was inserted in the unique HpaI site of pMV762-Mrx1-roGFP2 plasmid. The plasmid thus generated was electroporated into *Rv*, *RvΔaosR* and *RvΔaosR::aosR* and the redox status of

the cytosol was examined. The data is provided in Fig 7f and indicates that redox status of *RvΔaosR* undergoes a more drastic shift upon exposure to oxidative stress compared with *Rv* and *RvΔaosR::aosR*. Moreover, while *Rv* and *RvΔaosR::aosR* were able to return to their ambient redox states within 60 min, *RvΔaosR* failed to do so. As suggested by the reviewer, we have plotted the data as V500/FITC ratio, rather than fold change.

Comment.

• Fig 7h, a reductive agent other than Cys should be included to formally prove that the observed effect is due to Cys and not to an unspecific redox change.

Response:

The second sample in Fig 7h is *RvΔaosR*+CHP without cysteine. We agree that the addition of CHP+DTT may mitigate each other's effects and can act as a control. However, DTT addition may have unintended consequences as it is a strong reducing agent. An alternate control is to utilize the *Rv* sample without CHP or DTT. We have shown in Fig 3g that the transcriptional changes in all nine genes are dependent on the addition of CHP. Since this aspect has already been established in Fig 3g,we have performed the experiment described in Fig 7h only under oxidative stress conditions.

Comment.

Other points

• Fig 1b, in text lines 80-81, indicate the survival is compromised at day 4 post-infection.

Response:

We have incorporated the change suggested by the reviewer.

Comment.

• Fig 1c is pointless.

Response:

We have removed the panel in line with the suggestion.

Comment.

• Legend to Fig 2e should be changed to CFUs/lung (?)

Response:

We have incorporated the change suggested by the reviewer.

Comment.

• Line 176 - cysO is not shown in the Fig S8b, please fix.

Response:

We apologize for the mistake. Necessary changes have been made.

Comment.

• Fig 5k, why is diamide used here while CHP was used in all other experiments?

Response:

Diamide is an oxidative stress inducer, similar to CHP. Fig 1f suggests AosR assists mycobacterium combat oxidative stress induced by either CHP or diamide. The EMSA experiments were performed with both CHP and diamide. However, the differences in binding to promoter region were more prominent in the case of diamide compared with reduced condition. This is probably due to direct effect of diamide on disulfide bond formation of AosR, which is important for its interaction with SigH (Fig 5f-g, Fig 6g-h) and consequently improved survival during oxidative stress (Fig 1f, Fig S3e).

Comment.

• In the discussion, "redox stress" should rather be discussed as "oxidative stress"

Response:

We have incorporated the change suggested by the reviewer.

• Line 372, ref. 16 & 46 are irrelevant to the claim.

Response:

The study in Ref 16 suggests Tn seq mutants of mec cysO and CysM have compromised survival within host macrophages, and the relevant information was first highlighted by study in Ref 46.

Referee #2:

Comment.

The authors strengthened their manuscript with additional data. However, they still claim, in the summary and throughout the manuscript that Rv1332 (AosR) "facilitates mycobacterial survival in the murine lungs and spleens". This is not supported by the data. The mutant grows more slowly in mouse lungs and spleens, but there is no evidence for a survival defect. This needs to be corrected throughout the manuscript.

Response:

We thank the reviewer of his/her incisive comments. We have incorporated the change suggested by the reviewer. The word "survival" has been replaced by "growth".

Comment.

Given the growth defect observed in mice and survival defect (decline in CFU) in peritoneal macrophages, it seems that peritoneal macrophages are not a good model for the *in vivo* infection. I don't expect the authors to repeat the experiment with other primary cells such as bone marrow derived macrophages, but it would be good to discuss the discrepancy between ex vivo and in vivo phenotype.

Response:

We have modified the discussion to include point suggested by the reviewer.

"In contrary to attenuated growth *in vivo,* the mutant exhibited decline in CFUs in the peritoneal macrophages. This apparent discrepancy in phenotype observed with *ex vivo* and *in vivo* infection experiments might probably be due to the pre-activated status of peritoneal macrophages."

The authors must include the source, gender and age of the IFNg-/- and phox-/- mice in the methods section.

Response:

We have incorporated the relevant information in methods section. "Balb/c, C57BL/6 (B6), and mice with targeted deletions of gp91phox (B6.129S6-

Cybbtm1Din/J; JAX# 002365) or IFN-g (B6.129S7-Ifngtm1Ts/J; JAX#002287) were obtained from The Jackson Laboratory. NADPH Phagocyte oxidase (gp91) breeder pair-homozygous null mice ($gp91^{-/-}$; Jackson Lab) and heterozygous female ($gp91^{+/-}$; Jackson Lab) were procured from Dr. Apurva Sarin's laboratory with due permission from The Jackson Laboratory. Only the homozygous null litters confirmed by PCRs (using primer sets mentioned in Table S12) were used for the experiment. Four-six weeks old mice of either sex were injected with 4% thioglycolate (Hi-Media) into the peritoneum cavity of the mice."

2nd Revision - Editorial Decision 16th Apr 2021

Thank you for submitting a revised version of your manuscript. I sincerely apologise for the delay in processing of your manuscript due to the Easter holidays and the resulting manuscript backlog at our office. We have now performed the final editorial checks, and unfortunately it appears that several editorial issues still need to be addressed before I can officially accept the manuscript.

Please feel free to contact me if have any further questions regarding these points. I look forward to receiving the final revised version!

The auhors performed the requested editorial changes.

3rd Revision - Editorial Decision 20th Apr 2021

Editor accepted the manuscript.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Vinay K. Nandicoori
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-106111R-Q

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?	The sample size was chosen based on surveying relevant literature. All experiment were performed with a minimum of techinical triplicates, repeated atleast twice (biological duplicates).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal infection experiment, each group included a minimum 5 wild type animals of either gender per time point. Animal infection experiments with gp91phox (B6.129S6-Cybbtm1Din/J; JAX#002365) or IFN-g (B6.129S7-Ifngtm1Ts/J; JAX#002287) mice were performed with 4 and 3 animals of either gender per group per time point.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animals were not excluded unless they developed any visible secondary infection. The criteria was pre-established.
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.	The animals of either gender, randomly distrubted in cages by animal facility incharge were used for experiments.
For animal studies, include a statement about randomization even if no randomization was used.	The animals of either gender, randomly distrubted in cages by animal facility incharge were used for experiments.
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.	All the results provided by other investigators were independently evaluated by the first author and the corresponding author. Results provided by the first author were evaluated by the corresponding author.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The animals of either gender, randomly distrubted in cages by animal facility incharge were used for experiments.
5. For every figure, are statistical tests justified as appropriate?	Yes. The statistical method used was chosen based on surveying relevant literature.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA

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

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	NA

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).	Antibodies against various mycobacterial proteins (AosR, CysK1, CysK2, CysM, ClpS, CysO and Rv1333) were raised in mice (Supplementary Figure 13). anti-SigA and anti-GroEL-1 antibodies (used as controls) were raised in the rabbit (Supplementary Figure 13). Monoclonal anti-FLAG M2 antibody was purchased from Sigma (Sigma-F1804).
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. We used primary peritoneal macrophages isolated freshly from mice after thioglycolate injection.

* 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.	Balb/c, C57BL/6 (B6), and mice with targeted deletions of gp91phox (B6.129S6-Cybbtm1Din/J; JAX#002365) or IFN-g (B6.129S7-Ifngtm1Ts/J; JAX#002287) were obtained from The Jackson Laboratory. NADPH Phagocyte oxidase (gp91) breeder pair-homozygous null mice (gp91-/-; Jackson Lab) and heterozygous female (gp91+/-; Jackson Lab) were procured from Dr. Apurva Sarin's laboratory with due permission from The Jackson Laboratory. All animals were housed and bred at the animal facility of the National Institute of Immunology. Infection experiments with live animals were performed in the Tuberculosis Aerosol Challenge Facility, International Centre for Genetic Engineering and Biotechnology. Experiments were performed with 4-6 week old mice of either gender in a randomized manner.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The animal experimental protocol was approved by the Animal Ethics Committee of the National Institute of Immunology, New Delhi, India (IAEC#409/16 & IAEC#462/18) as per the guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA),Government of India.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNA-Seq data is available at the NCBI Gene Expression Omnibus Database, accession number GSE161190.
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).	RNA-Seq data is available at the NCBI Gene Expression Omnibus Database, accession number GSE161190.
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.