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Prohibitin 1 regulates mtDNA release and downstream inflammatory responses

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Dear Prof. Feng,

Thank you for submitting your manuscript for consideration by the EMBO Journal.
Your manuscript has now been seen by three referees and their comments are provided below.

As you can see from these comments, the referees find the analysis interesting but also that further work is needed to consider publication here. The Referees make the very good point that it could be that NLRP3 inflammasome activation is mediated via mitochondrial dysfunction and not directly by mtDNA release (ref #1 and 3). I think this is an important point and I would like to encourage you to explore this possibility.

I think it would be helpful to discuss the raised points further and I am available to do so via email or video.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to discussing the revisions further with you.

best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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I have attached a PDF with helpful tips on how to prepare the revision.

Please also note the figure format should be portrait and not landscape

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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Referee #1:

In this study, Liu and colleagues build from an initial observation that PHB1 deficiency promotes inflammation (specifically IL-1 release) both in vitro and in vivo and may have relevance for sepsis since low PHB1 levels can be detected. Investigating its mechanistic basis they propose that PHB1 affects mitochondrial permeability transition pore (MPTP) activity, such that loss of PHB1 leads to mtDNA release dependent activation of inflammasome activity. They then describe that loss of PHB1 causes MPTP leading to mtDNA release (through various approaches - including SPG7,AFG3L2 kd, VDAC inhibition).

Overall the study rigorously demonstrates that loss of PHB1 causes inflammation in an mtDNA dependent manner through regulation of MPTP. As the authors highlight, various studies have shown MPTP regulation of mtDNA release but (in my opinion) not to the same depth of examination here, secondly the link with PHB1 activity and regulation of mtDNA release has not been shown (to the best of my knowledge), the physiological relevance of this is maybe relevant in sepsis patients, as the authors

investigate in Figure 1. The data largely support the authors' conclusions however I think there are some outstanding questions to be resolved.

- how do the authors propose that mtDNA activates (or is required) for canonical inflammasome activity ? Presumably a DNA-sensor (e.g. AIM2) is required but there is no discussion, investigation of this key point. The depletion expts. (etbr generation of Rho zero cells) are supportive of a key role for mtDNA but an important control would be to determine whether (in these cells) loss of mtDNA doesn't impact a canonical inducer of inflammasome activity (e.g. for instance uric acid) - could simply be that mitochondrial function contributes to inflammasome activity (as opposed to mtDNA release per se).

- do cells (e.g. BMDMs) lacking PHB1 undergo cell death more readily in response to inflammasome activators ?- this would potentially supporting release of biologically active IL-1 in PHB1 deficient cells (through enhanced secondary lysis of the cells).

Other points:

Definition of MIMP is lacking in the abstract (it won't be apparent to most readers), and there is some confusion between the role of VDAC and BAX/BAK in MPTP in the intro (not in the discussion). BAX/BAK have little (if any role) in regulating MPTP.

Referee #2:

Review on „PHB1 regulates mtDNA release and modulates oxidized mtDNA-dependent inflammatory responses" by Hao Liu et al.

Hao Liu et al. show that reduced protein levels of PHB-1 leads to release of DNA from mitochondria, which translates into increased NLRP3 inflammasome activity and IL-1beta production. Loss of PHB1 is known to have broad consequences for mitochondrial function (citations 24-28). In the present work, the role of PHB1 in mtDNA release is connected to an effect on mPTP/MIMP, a process/concept still controversially debated in the field, as dicussed by the authors. Mechanistically, they suggest that PHB1 does bind to both AFG3L2 and SPG7, but, rather than to promote their interaction, this is supposed to prevent the latter, while absence of PHB1 would promote functional interaction of AFG3L2 and SPG7, and mPTP opening. The authors do not address whether they believe this is the primary function of PHB1 and if the other effects of PHB1 on mitochondrial function previously observed would be consequences of that.
The manuscript would profit from language editing, especially since the meaning is sometimes unclear.

Specific comments:

Line 61, "The mitochondrion is the central hub of immunity," is an overstatement.

Line 71, meaning and wording unclear. "receptors of the inflammasome" would mean something that senses inflammasomes, not something that forms an inflammasome.

Line 48, mPTP is not introduced

Fig S1A, displaying expected mendelian ratios is superfluous.

Fig S1B does not depict what is mentioned in the text. Were the conditional knockouts generated for this project? This should be mentioned in the main text.

Fig 1C, the legend should mention what method was used, including day of culture in MCSF. This applies to figure legends in general: legend text should not interpret the data but explain what was done. Deletion of Phb1 should also be confirmed by Western blot.

Fig 1A, the authors seem to detect spontaneous IL-1beta in control mice of around 100 pg/ml. This should lead to disease. We would expect that IL-1beta levels in healthy mice should be below the detection limit of the method. Was the method correctly performed? If yes, the authors should discuss this and cite the literature concerning whether it is known that mice have spontaneous IL-1beta levels of this magnitude in the serum.

Fig 1B, is this also observed on the level of caspase-1?

Fig 1D, E, is this new? If not, literature should be cited and discussed, that these are control read-outs for F and G. I would consider making D-G one panel since these are measurements from the same samples.

Fig 1I-L, why was ATP used here? ATP is an NLRP3 inflammasome activator (signal 2) and should not influence expression (signal 1). The data presented supports this. I would recommend removing the ATP condition from this panel. Does IL-1beta,

also triggering MyD88-signalling, have the same effect as LPS on Phb1 expression? Is this also seen in mouse cells or primary human PBMCs?

Line 160-1, please check if heading is correct. Is it not rather hat loss of PHB1 leads to increased permeability (spontaneous mPTP opening)?

Line 167 "monoclonal shRNA-expressing" - check wording and use of "monoclonal" in this context.

Line 168-9, in what way is mitochondrial morphology impaired. Please outline the changes observed.

Line 176, please explain the rational for using CsA here and the interpretation of the results better.

Fig S2D is inconsistent with Fig 2C. The loss in Phb1 knockdown is much weaker as in the main figure. Why? The rescue is therefore minimal.

Line 168 "demonstrate whether", please check wording

Line 193, check "retention", is it not rather "uptake"? The same applies to line 227. Conceptually, this is an important difference. If a statement on retention is important to the authors conclusion here, a different analysis is required to come to this.

Line 194-5 "These observations indicate that Phb1 knockdown causes the opening of mPTP." I do not think that the data presented supports this notion. Either explain better based on the literature why the data suggests this as compared to other possible explanations that should be mentioned, or conduct additional experiments.

Fig S2E, this data does not sufficiently support the statement connected to it. Since apoptosis is not the focus here, it could simply be removed from the manuscript.

Line 203, delete "including"

Line 204, why is mtDNA still observed as puncta when outside mitochondria? How do you know Fig 3 A shows mitochondrial DNA, when it is not in mitochondria?

Line 205-6, check wording. Phb1 is not mentioned in the cited study.

Line 209, check wording "marrow bones". Also, the method of BMDM generation is not properly represented in the wording of this manuscript.

Line 208 talks about in vivo, yet BMDMs are used, which is in vitro.

Line 239, please outline the results in a bit more detail.

Line 241, what does Ifnb1 and Isg15 have to do with NLRP3 priming or activation? The impression is that the authors are mixing up the STING and NLRP3 pathways. The only conclusion possible with the data presented concerning the cGAS/STING pathway is that there is no spontaneous activity.

Fig 3M, this data needs some kind of quantification. In how many cells and at what frequency was this observed?

Fig 3N, the knock-down is underwhelming. Why not do MCC950 on BMDMs from conditional knockout mice?

Line 266-9, I don't see specks in these images (4A, B). With a distributed cytoplasmic stain of NLRP3 and no obvious enrichment with the areas of the green signal, it is very difficult to draw any conclusion from these two panels.

Figure 4C, how much mtDNA is bound to NLRP3 under activation conditions (e.g. LPS + nigericin or LPS + ATP) in these cells as well as BMDMs as compared to absence of PHB1?

Fig 4D, oxmtDNA seems to be everywhere in these cells and colocalization therefore seems random. Why do the authors stain for NLRP3 in A and B but for ASC in panel D?

Fig 4E, F, to my understanding, EtBr treatment leads not only depletion of mtDNA, but subsequently of mitochondria themselves. Yet in F, mitochondria are stained. Please explain.

Line 404, how does PHB1 in the plasma membrane relate to the present story?

Referee #3:

This paper has made a useful advance in understanding of the role of PHB1 in limiting the opening of the mitochondrial pore transition complex (mPTC) that allows dissipation of ion gradients and release of mitochondrial DNA. They show that PHB1 limits association of SPG7 and AFG3L2. When PHB1 is reduced, e.g. by hydrogen peroxide treatment, LPS treatment or gene knockdown, the association of SPG7 and AFG3L2 then seems to promote or correlate with mPTC formation. This is an original and interesting contribution to the literature on this topic. The authors show nicely that reduction of PHB1 leads to mitochondrial permeability, loss of membrane potential, increased ROS and release of DNA. However, the quality of the paper is reduced by the assumption that prior published work suggesting that mtDNA directly activates the NLRP3 inflammasome is correct. In its current form I think this paper will perpetuate ideas in the literature regarding mtDNA and NLRP3 that are misleading. I think most of the work here is good quality and could be published with a focus on the role of PHB1 on mPTC, mitochondrial dysfunction and DNA release, with inflammasome activation a likely result of mitochondrial dysfunction including possibly ROS production, without necessarily invoking a role for DNA in that. The published work on mtDNA activating NLRP3 is prominent but controversial, as discussed below. NLRP3 is frequently reported to be activated by mitochondrial dysfunction, and in the PHB1 knockout cells the mitochondria have loss of membrane potential and increased ROS and/or metabolic changes could promote NLRP3 activation. The original contribution of this work is really the role of PHB1 in restraining SPG7 and AFG3L2 association and mPTC formation. The concepts of DNA being released by the mPTC and NLRP3 being activated by mitochondrial dysfunction are not novel and so are not the major contribution here. And the concept of mtDNA activating NLRP3 is not novel, and I would contend that it is wrong.

Specific comments

1. There is no methods section in this paper!

2. Lines 77-78 states that "it is widely known that mtDNA release is required for NLRP3 inflammasome formation (reference 7)". Many papers seem to accept this as fact, but from what I see the evidence is poor. Reference 7 itself had a number of deficiencies; they did not use available gene knockout strains to show whether DNA release lay upstream (as they claimed) or downstream of NLRP3 inflammasome. Furthermore, the use of EtBr to deplete mtDNA is not conclusive proof as discussed below. An earlier paper (Nakahira reference 34) showed that transfection of mtDNA elicited little or no response in AIM2-/- macrophages, suggesting it is not an efficient stimulus of NLRP3. Reference 34 actually concluded that in their systems mitochondrial DNA release was predominantly downstream of NLRP3 inflammasome activation. Although they concluded that mtDNA could enhance NLRP3 activation by other stimuli, as they showed that a 6 hour transfection of DNA enhanced the response to LPS+ATP somewhat in AIM2-/- cells, they neglected possible transcriptional priming effects through TLR9 and cGAS. In addition, colocalisation of DNA with NLRP3 is not proof of an involvement in its activation. There is no clear reason why mtDNA should activate NLRP3 rather than AIM2. AIM2 just recognises the phosphate backbone, and should not be perturbed by DNA oxidation. The requirement for mtDNA as an activator of NLRP3 is controversial as acknowledged in your reference 3 and (PMID: 32055400), but not noted in your manuscript. If you know of papers with better proof of the role of mtDNA in activating NLRP3, please cite them and state directly what the experimental evidence is. Otherwise, consider changing the focus of your paper away from DNA activation of NLRP3. It is clear that NLRP3 is activated, but it is not clear how it is activated.

3. Ethidium bromide does a lot more than just reduce DNA levels in mitochondria. For example, EtBr is reported to reduce oxygen consumption and increase glycolytic activity, reduce mitochondrial creatine kinase mRNA and phosphocreatine (PMID: 29287112). To quote another paper: "In order to gain insight into cellular responses to mitochondrial stress, we treated human diploid fibroblasts with ethidium bromide at concentrations that induced loss of mitochondrial DNA over a period of 7 days. The decrease in mitochondrial DNA was accompanied by a reduction in steady state levels of the mitochondrial DNA binding protein, TFAM, a reduction in several electron transport chain protein levels, increased mitochondrial and total cellular ROS, and activation of p38 MAPK. However, there was an increase in mitochondrial mass and voltage dependent anion channel levels. In addition, mechanistic target of rapamycin (mTOR) activity, as judged by p70S6K targets, was decreased while steady state levels of p62/SQSTM1 and Parkin were increased." (PMID: 25104948). In contrast Nakahira showed that EtBr treatment of J774 gave lower mito-ROS. Also, levels of any proteins encoded in mtDNA may be increased, and they noted this was the case for cytochrome c oxidase 1. In short, EtBr will have profound effects on mitochondrial function and could affect NLRP3 activation through multiple routes. Consequently I question the value or interpretation of Fig 4 E-G.

4. For all the reasons given above, I think you should omit Figure 4. Focussing on proposed DNA activation of NLRP3 distracts from the useful message of your paper that is about the mPTC formation. The localisation of NLRP3 and DNA is not convincing - In some samples NLRP3 is overexposed and when it is nearly everywhere in the cell it will certainly appear to colocalise. In other samples it is more convincing, but in two J774 samples in panel B I think you are looking at micronuclei. The text states that Figure 4 A-B show "specks" but you are not staining for ASC there, so that is not correct to say. There is no clear "speck" from NLRP3 here. Regardless, even with some colocalisation, this does not prove that it is a ligand for NLRP3. ASC colocalisation with 8-OH-dG is similarly unconvincing as there is a lot of 8-OH-dG staining. EtBr data cannot be used to definitively prove and involvement of DNA in the NLRP3 activation for reasons given above.

5. Obviously your story would become more simple if you could show the activation of cGAS by released DNA, as that is an

unambiguous DNA response. You show that knockdown of PHB1 in J774 does not lead to spontaneous cGAS response in J774 (Fig S5A). If these cells are competent for the cGAS pathway (no positive control is shown), I can only assume that the amount of DNA released from mitochondria is just too low to activate. Indeed, Figure 3C and 3K show 2-fold increase in DNA or less in the knockdown cells. This seems to be many-fold lower increase than reference 32, where a cGAS response was seen. Have you looked for cGAS activation in the PHB1 knockout macrophages that give a higher level of cytosolic DNA? If you maintain that you have high levels of DNA release from mitochondria but no cGAS activation, you would have to have some hypothesis on why this is so. Do the same cells not respond to transfected DNA? Or would you conclude that the amount of DNA released is too low? Is there another stimulus you can add that will boost DNA release for cGAS recognition, and then show an enhancing effect of PHB1 loss? If none of this is possible, I think that the data on PHB1, mPTC and DNA release stands by itself, and mitochondrial dysfunction can link to NLRP3 activation through some unknown means.

6. Reference 2 is not appropriate on line 64- it does not describe release of mtDNA to the cytosol. On line 66 - Ref 3 is a review, and you really need primary sources for this statement.

7. Assessment of inflammasome responses are mostly shown by western blot, and that is appropriate, but it then does not show replication or quantification. For the important points it would be good to see quantitative confirmation by IL-1b ELISA combining results of several experiments. For example, Figure 1B and 1N you are making quantitative conclusions on IL-1b production, but we have only a single western blot to go on. Also, number of times western blots were repeated to give the same result is not stated.

8. Figure S2D really does not show good restoration of membrane potential in the knockdown cells by expression of PHB1. Presumably the knockdown is too effective. If you look at the mean fluorescence index that would be more appropriate than the % of cells that fall above an arbritary line. Unless you have a PHB1 gene sequence mutated to avoid knockdown, I think this figure is not useful.

9. It would help understanding of what is known if you can state in the introduction that others have presented data suggesting mPTP is involved in release of DNA (ref 32). It would then be clear that what you are proposing is a mechanism involved in the regulation of pore opening, rather than providing the first description of mPTP in DNA release.

10. Line 238 - NLRP3 is presented as being activated by mtDNA and this is doubtful, as noted above.

11. Figure 3M uses overexpression of ASC and NLRP3 in HeLa cells, and claims on the basis of an image of 1-2 cells to show colocalisation of ASC and NLRP3 enhanced by PHB1 knockdown. Overexpression systems give a lot of spontaneous speck formation, depending on the level of expression. ASC and NLRP3 will tend to cluster together when overexpressed. This data is of no use because it does not quantify an increase in colocalisation. Please delete this figure. However, if you want to focus on inflammasomes, quantification of spontaneous ASC specks in the knockout macrophages would be useful as it would show the frequency of inflammasome formation. Formation of an ASC speck is generally a sign of a pyroptotic cell, and if this was frequent, I would expect your culture to have very low viability.

12. Please make clear for each figure at what level replication is done. For example Figure 1 I to L show either results from separate wells in one experiment, results of separate experiments, or results of four estimates of mRNA levels on a single sample?

Minor Points
1. Line 70-71 - In discussing the NLRP3 inflammasome, there are not several different receptors and "such as" is not appropriate here - NLRP3 is an essential, not an optional component of the NLRP3 inflammasome!

2. Please make sure abbreviations are all defined on first usage. The abstract includes MIMP and mPTP that need definition.

3. Lines 205-206. Here it sounds like ref 32 has already shown results for PHB1 deficiency in J774, making your work not novel. I think that is not what you mean?

4. Line 318. You do not treat HeLa cells with LPS.

5. Line 319-320. Should this actually say "SPG7 and AFG3L2 depend on reduced expression of PHB1 to participate in opening of mPTP, rather than "do not depend"? Also, "reduced" is better than "altered". I am not sure what you are trying to say here.

6. Cyclosporin A can clearly affect mPTC formation. But it is worth bearing in mind that it seems to have unrelated roles in inhibition of the inflammasome. Previous work has confirmed that cyclosporin does inhibit NLRP3 inflammasome activity, but this is not through action on cyclophilin D, as genetic deficiency of cyclophilin D has no effect on inflammasome responses PMID: 24990442

Optional
1. Figure 2A- does this indicate that knockdown of PHB1 promotes mitochondrial fission? Given that LPS causes mitochondrial

fission and also reduction in PHB1, is PHB1 loss causally involved in the LPS-induced fission? This is just curiosity, not expecting any experimental response to this.

2. Would it be helpful to look at the effect of mito-ROS inhibitors? Do they affect the mPTC formation and DNA release when you have low PHB1? If they do not prevent DNA release but do prevent NLRP3 activation that would be useful information.

Point by point responses:

Referee #1:

In this study, Liu and colleagues build from an initial observation that PHB1 deficiency promotes inflammation (specifically IL-1 release) both in vitro and in vivo and may have relevance for sepsis since low PHB1 levels can be detected. Investigating its mechanistic basis they propose that PHB1 affects mitochondrial permeability transition pore (MPTP) activity, such that loss of PHB1 leads to mtDNA release dependent activation of inflammasome activity. They then describe that loss of PHB1 causes MPTP leading to mtDNA release (through various approaches - including SPG7,AFG3L2 kd, VDAC inhibition).

Overall the study rigorously demonstrates that loss of PHB1 causes inflammation in an mtDNA dependent manner through regulation of MPTP. As the authors highlight, various studies have shown MPTP regulation of mtDNA release but (in my opinion) not to the same depth of examination here, secondly the link with PHB1 activity and regulation of mtDNA release has not been shown (to the best of my knowledge), the physiological relevance of this is maybe relevant in sepsis patients, as the authors investigate in Figure 1. The data largely support the authors' conclusions however I think there are some outstanding questions to be resolved.

- how do the authors propose that mtDNA activates (or is required) for canonical inflammasome activity ? Presumably a DNA-sensor (e.g. AIM2) is required but there is no discussion, investigation of this key point. The depletion expts. (etbr generation of Rho zero cells) are supportive of a key role for mtDNA but an important control would be to determine whether (in these cells) loss of mtDNA

doesn't impact a canonical inducer of inflammasome activity (e.g. for instance uric acid) - could simply be that mitochondrial function contributes to inflammasome activity (as opposed to mtDNA release per se).

Response: We greatly appreciate the kind suggestions raised by this reviewer. There are many possible causes of inflammasome activation, and a number of previous studies have reported that defects in mitochondrial function (including loss of membrane potential, increased ROS, and mitochondrial DNA release) are associated with inflammasome activation (Ref. 48 and Ref. 2). Indeed, it is not easy to say that mtDNA is directly correlated with the inflammasome, but we did observe a significant inflammatory inhibition in Rho zero cells (Figure 4F).

As suggested by the reviewer, we first assessed the role of another reported DNA-sensor, AIM2, on the inflammasome, and as demonstrated in Figure S5A-E, no significant changes in AIM2 protein levels (with or without poly(dA:dT) stimulation) were observed in the absence of PHB1 compared to the control (Figure S5A); notably, knockdown of AIM2 inhibit IL-1B production very slightly under LPS+ATP conditions (Figure S5B), but this was not mediated by the binding of released mtDNA to AIM2 , as knocking down PHB1 neither increases the co-localization of AIM2 with mtDNA nor allows AIM2 to bind more mtDNA (Figure S5C-E). Therefore, we think that AIM2 plays a minor role in inflammation caused by reduced PHB1.

Second, we found that using MCC950, an inhibitor of NLRP3, did effectively inhibit IL-1β production in BMDM cells from $Phb1^{MyeKO}$ mice (Figure 3M), and also detected that PHB1 deficiency promoted co-localization and binding of mtDNA and NLRP3 (Figure 4A-E). In a recently published study, Xian et al. also found that oxidized mtDNA released into the cytoplasm via mPTP in response to inflammatory stress activates the formation of NLRP3 inflammasome (Ref. 44), which is also consistent with our study.

In EtBr experiments, we used the classical NLRP3-inflammasome inducer, ATP. In agreement with previous studies (Ref. 2 and Ref. 42), we found that loss of mtDNA inhibits inflammasome activation. Indeed, as mentioned by this reviewer and reported by others in the literature, EtBr treatment can lead to mitochondrial dysfunction, resulting in increased levels of ROS, and glycolysis. In macrophages, these events can promote inflammasome formation (Ref. 47 and Ref. 48). Therefore, to avoid other effects, we treated with only 150 ng/mL of EtBr for 3 days and ensured the clearance of mtDNA under this condition (Figure S6A and B). The use of EtBr, however, significantly inhibited the formation of inflammasome. Based on these results, we believe that the loss of mtDNA probably plays a stronger role. On the other hand, we are not trying to deny that other potential factors are also involved in this process. The field is eager to see the invention of new methods that can explore the role of these factors in mitochondria one by one and thus detect more specifically the effect of mtDNA itself on inflammatory responses. In this regard, we elaborate in the discussion section and de-emphasize the role of mtDNA alone (Line 407-412).

- do cells (e.g. BMDMs) lacking PHB1 undergo cell death more readily in response to inflammasome activators ?- this would potentially supporting release of biologically active IL-1 in PHB1 deficient cells (through enhanced secondary lysis of the cells).

Response: This is a very interesting issue and we extracted BMDM in $Phb1^{F/F}$ and $Phb1^{MyeKO}$ mice respectively and found that PHB1 deficiency did not promote cell death during inflammasome activation (in LPS+ATP condition), with H_2O_2 as positive control (Figure S2C).

Other points:

Definition of MIMP is lacking in the abstract (it won't be apparent to most readers), and there is some confusion between the role of VDAC and BAX/BAK in MPTP in the intro (not in the discussion). BAX/BAK have little (if any role) in regulating MPTP.

Response: We have defined MIMP in the revision and clarified the role of VDAC and BAX/BAK in MPTP.

Referee #2:

Review on „PHB1 regulates mtDNA release and modulates oxidized mtDNA-dependent inflammatory responses" by Hao Liu et al.

Hao Liu et al. show that reduced protein levels of PHB-1 leads to release of DNA from mitochondria, which translates into increased NLRP3 inflammasome activity and IL-1beta production. Loss of PHB1 is known to have broad consequences for mitochondrial function (citations 24-28). In the present work, the role of PHB1 in mtDNA release is connected to an effect on mPTP/MIMP, a process/concept still controversially debated in the field, as dicussed by the authors. Mechanistically, they suggest that PHB1 does bind to both AFG3L2 and SPG7, but, rather than to promote their interaction, this is supposed to prevent the latter, while absence of PHB1 would promote functional interaction of AFG3L2 and SPG7, and mPTP opening. The authors do not address whether they believe this is the primary function of PHB1 and if the other effects of PHB1 on mitochondrial function previously observed would be consequences of that.

The manuscript would profit from language editing, especially since the meaning is sometimes unclear.

Response: Indeed, we were not able to clearly distinguish whether abnormalities in other mitochondrial functions lead to mPTP opening and mtDNA release, or whether in turn mPTP opening and mtDNA release precede other mitochondrial dysfunctions. However, given that PHB1-PHB2 as heterodimers, PHB1 KO could promote mtDNA release and inflammasome formation, while knockdown of PHB2 did not cause this event (Figure 1B and 1J-K), indicating the specificity of PHB1. Meanwhile, the mtDNA release promoted by PHB1 deficiency can be inhibited by mPTP inhibitor (Figure 3I), and blocking the release of mtDNA using CsA, an inhibitor of mPTP, can restore the membrane potential and mitochondrial calcium transient disruption caused

by PHB1 knockdown to some extent (Figure 2C and 2F); knockdown of SPG7, the regulator of mPTP, or depletion of mtDNA can inhibit IL-1β maturation promoted by PHB1 (Figure 4F and 6D-E), all these results imply that mPTP opening does occur after knockdown of PHB1, but before mtDNA release, suggesting that PHB1 plays an important role in the process of mtDNA release and inflammatory response. However, we observed that ROS clearance can also partially inhibit the maturation of inflammatory factors (Figure 4G), so there may be several factors involved in the activation of the inflammasome.

We would also like to thank you for your suggestions on the language editing of the manuscript. We have asked Dr. Isabel Hanson to assist us in improving the language editing of the manuscript before submission, as we have actually mentioned in “The section of acknowledgement” in the previous manuscript.

Specific comments:

Line 61, “The mitochondrion is the central hub of immunity,” is an overstatement.

Response: We have changed the wording and tuned down the statement (Line 58).

Line 71, meaning and wording unclear. “receptors of the inflammasome” would mean something that senses inflammasomes, not something that forms an inflammasome.

Response: We have clarified the wording (Line 70).

Line 48, mPTP is not introduced

Response: We have introduced mPTP in the revised text (line 48-49).

Fig S1A, displaying expected mendelian ratios is superfluous.

Response: We have deleted the mendelian ratios (Figure S1A).

Fig S1B does not depict what is mentioned in the text. Were the conditional knockouts generated for this project? This should be mentioned in the main text.

Response: We redrew the cartoon (Figure S1B).

Fig 1C, the legend should mention what method was used, including day of culture in MCSF. This applies to figure legends in general: legend text should not interpret the data but explain what was done. Deletion of Phb1 should also be confirmed by Western blot.

Response: We checked all the legends and stated the day of culture in culture medium of L929 cells for differentiation of BMDMs. Also, we confirmed that all the legends stated the methods. We performed the WB assays to confirm the Phb1 deletion (Figure S1D).

Fig 1A, the authors seem to detect spontaneous IL-1beta in control mice of around 100 pg/ml. This should lead to disease. We would expect that IL-1beta levels in healthy mice should be below the detection limit of the method. Was the method correctly performed? If yes, the authors should discuss this and cite the literature concerning whether it is known that mice have spontaneous IL-1beta levels of this magnitude in the serum.

Response: We are grateful to the reviewers for the careful reading. We followed the ELISA experiments strictly as described in the kit instructions. The reason why the IL-1beta in control mice in this study was around 100 pg/mL was because two of them had higher values (individual variation of animals) and we included them together for analysis in a realistic way. If the data from these 2 mice were removed, the values returned to normal. In addition, we also noted that some studies in which control mice had higher serum IL-1beta (Ref. 42 and

Ref. 59), and we cited them and elaborated on them in the ‘Discussion section’ (Lines 463-466), although some individuals had higher basal inflammation, any of their behavioral manifestations were normal.

The following are the specific values of this test.

IL-1β (pg/ml)	Ctrl	LPS
Phb1$^{F/F}$	220.638	328.581
	57.03544	319.5495
	188.0004	452.5784
	91.49037	408.5357
	74.44956	347.6988
	67.66382	630.4856
	-	481.4604
	-	507.3853
	-	509.4649
	-	643.2807
Phb1^{MyeKO}	238.6622	2198.364
	193.0731	644.3484
	448.0594	548.0909
	258.3751	561.7314
	405.2095	550.187
	213.4633	1497.358
	-	1594.93
	-	519.8758
	-	2313.851
	-	558.5804
	-	677.5591
	-	1391.806

Fig 1B, is this also observed on the level of caspase-1?

Response: In the inflammasome activation pathway, maturation of IL-1 beta requires activated caspase-1 cleavage for completion. We have previously measured caspase-1 levels in supernatants, but because cell death is not the emphasis of this manuscript, we did not list this data in the previous manuscript and have added it in the revised manuscript as requested by the reviewer (Figure 1B)

Fig 1D, E, is this new? If not, literature should be cited and discussed, that these are control read-outs for F and G. I would consider making D-G one panel since these are measurements from the same samples.

Response: A study in 2020 reported that PHB1 downregulation was associated with sepsis, and we cited this article in the new manuscript (Ref. 27, Line 147-148 and Line 454-455). Also, we integrated the original Figure 1D-G into one panel as suggested by the reviewer (Figure 1E).

Line 160-1, please check if heading is correct. Is it not rather that loss of PHB1 leads to increased permeability (spontaneous mPTP opening)?

Response: We have modified the heading (Line 168-169).

Line 167 "monoclonal shRNA-expressing" - check wording and use of "monoclonal" in this context.

Response: We have modified the wording (Line 174-175).

Line 168-9, in what way is mitochondrial morphology impaired. Please outline the changes observed.

Response: We have added some specific descriptions of mitochondrial morphology (Line 176-179).

Line 176, please explain the rational for using CsA here and the interpretation of the results better.

Response: We explain the reasons for using CsA (Lines 183-186).

Fig S2D is inconsistent with Fig 2C. The loss in Phb1 knockdown is much weaker as in the main figure. Why? The rescue is therefore minimal.

Response: To avoid misunderstandings, we have removed this result.

Line 168 "demonstrate whether", please check wording

Response: We have changed the wording (Line 198-199).

Line 193, check "retention", is it not rather "uptake"? The same applies to line 227. Conceptually, this is an important difference. If a statement on retention is important to the authors conclusion here, a different analysis is required to come to this.

Response: We changed "retention" to "uptake" or "transient" throughout this manuscript according to the report on this calcium probe.

Line 194-5 "These observations indicate that Phb1 knockdown causes the opening of mPTP." I do not think that the data presented supports this notion. Either explain better based on the literature why the data suggests this as compared to other possible explanations that should be mentioned, or conduct additional experiments.

Response: We thank the reviewers for their suggestions. Because CsA is not thought to affect Ca^{2+} uptake (Ref. 33), also we found that the fluorescence intensity of Ca^{2+} indicator in mitochondria was higher with the use of CsA, while the knockdown of PHB1 and hydrogen peroxide caused a decrease in the fluorescence intensity of Ca^{2+} indicator. Meanwhile, CsA treatment could inhibit the decrease of fluorescence intensity caused by PHB1 knockdown. Therefore,

we speculate that the effects of CsA, PHB1 knockdown, and hydrogen peroxide on Ca^{2+} transient are achieved by regulating mPTP opening. In the revised manuscript, we retained an objective description of the data without any inferential description (Line 200-210).

Fig S2E, this data does not sufficiently support the statement connected to it. Since apoptosis is not the focus here, it could simply be removed from the manuscript.

Response: As this reviewer said, apoptosis is not the focus of this article. Therefore, the original image has been removed. However, reviewer 1 wanted to know how cell death under inflammatory conditions after knocking out PHB1, so we detected cell death using PI only without distinguishing exactly which type of death (Figure S2C).

Line 203, delete "including"

Response: We have deleted the word (Line 218).

Line 204, why is mtDNA still observed as puncta when outside mitochondria? How do you know Fig 3 A shows mitochondrial DNA, when it is not in mitochondria?

Response: A large number of proteins, such as TFAM, are bound to mtDNA, and after they are released, it is likely that some of them are still present as aggregates, so that mtDNA still shows a dot-like pattern when stained with dyes. We referred to a large number of previous studies (Ref. 9, 10 and 12) and found that the DNA in the cytoplasm could be visualized in dot form by dsDNA probes/antibodies, and also by comparing with the DNA co-localized with mitochondria in the Control group, as a way to determine that the DNA not co-localized with mitochondria in the knockdown/treatment group was the DNA released from mitochondria. More importantly, we designed specific mtDNA

probes and found that the mtDNA released into the cytosol was indeed increased under these stress conditions. Meanwhile we performed statistical analysis to evaluate this event more objectively (Figure 3B).

Line 205-6, check wording. Phb1 is not mentioned in the cited study.

Response: We have modified the text (Line 218-220).

Line 209, check wording "marrow bones". Also, the method of BMDM generation is not properly represented in the wording of this manuscript.

Response: We have modified the text (Line 224).

Line 208 talks about in vivo, yet BMDMs are used, which is in vitro.

Response: We have modified the text (Line 223).

Line 239, please outline the results in a bit more detail.

Response: We complemented the experiments with cGAS-STING in PHB1 KO BMDMs and developed a description (Line 209-305).

Line 241, what does Ifnb1 and Isg15 have to do with NLRP3 priming or activation? The impression is that the authors are mixing up the STING and NLRP3 pathways. The only conclusion possible with the data presented concerning the cGAS/STING pathway is that there is no spontaneous activity.

Response：Because Ifnb1 and Isg15 are downstream of cGAS-STING, our original intention was to use this index to see if cGAS-STING can also be activated under LPS+ATP conditions. Probably because the given stimulation conditions were not strong enough and the PHB1 knockdown was not effective, the previous results showed little effect on the cGAS-STING pathway. In the new manuscript, we deleted this result and, as suggested by reviewer 3, we switched to a more intense stimulation of H2O2 and repeated the experiment

again in PHB1 KO cells, where the new conditions triggered more mitochondrial DNA release, and we observed activation of genes downstream of cGAS-STING (Figure S7A and B).

Fig 3M, this data needs some kind of quantification. In how many cells and at what frequency was this observed?

Response: This experiment was done in HeLa cells, and the localization of ASC and NLRP3 and the speck were obtained by exogenous overexpression of the results, which may not truly reflect the real situation in inflammatory cells. According to reviewer 3, this experiment will bring unnecessary misunderstanding, so we deleted this data.

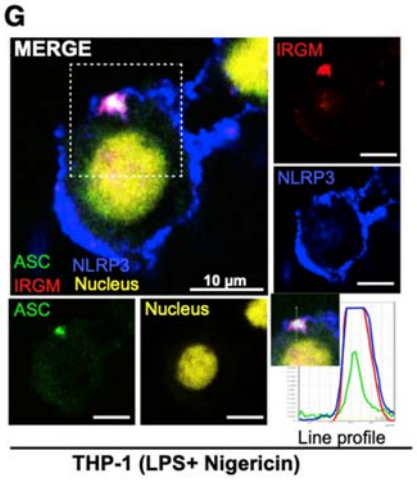
Fig 3N, the knock-down is underwhelming. Why not do MCC950 on BMDMs from conditional knockout mice?

Response：In the revised manuscript, we used BMDMs from PHB1 conditional knockout mice and found that the results were reproducible as PHB1 knockdown, where PHB1 KO exacerbated inflammation, while MCC950 suppressed inflammation (Figure 3M).

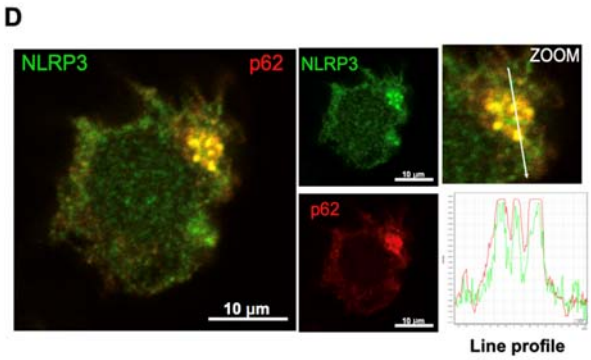
Line 266-9, I don't see specks in these images (4A, B). With a distributed cytoplasmic stain of NLRP3 and no obvious enrichment with the areas of the green signal, it is very difficult to draw any conclusion from these two panels.

Response: We refer to articles using the same antibodies as in our experiments, where the NLRP3 signals are distributed throughout the cytoplasm (Mehto S, et al. Mol Cell. 2019, PMID: 30612879). In our results, co-localization of NLRP3 with mtDNA was indeed observed and statistical analysis was done, but we no longer mention specks; while Figure 4A-4E also shows that NLRP3 does bind more mtDNA regardless of whether it is the case of PHB KD or KO.

In the figure below, the NLRP3 signal is also distributed throughout the cytoplasm:



Most of the NLRP3 is diffusely distributed in the lower panel, and only a small portion of the signal is locally enhanced:



Figures came from the article below “Mehto S, Jena KK, Nath P, et al. The Crohn's Disease Risk Factor IRGM Limits NLRP3 Inflammasome Activation by Impeding Its Assembly and by Mediating Its Selective Autophagy. Mol Cell. 2019;73(3):429-445 e427”

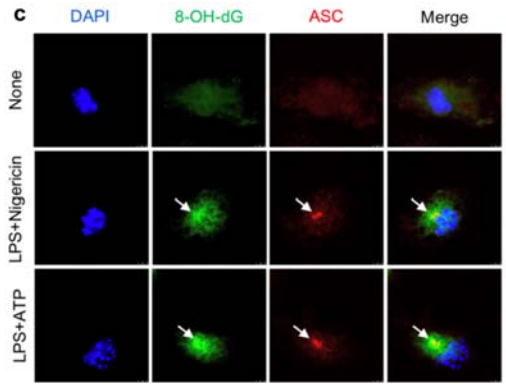
Figure 4C, how much mtDNA is bound to NLRP3 under activation conditions (e.g. LPS + nigericin or LPS + ATP) in these cells as well as BMDMs as compared to absence of PHB1?

Response: We found in BMDMs that NLRP3 was able to bind part of the mtDNA under LPS + ATP inflammatory conditions, whereas in PHB1KO cells, under the same conditions, the bound mtDNA was significantly increased (Figure 4E).

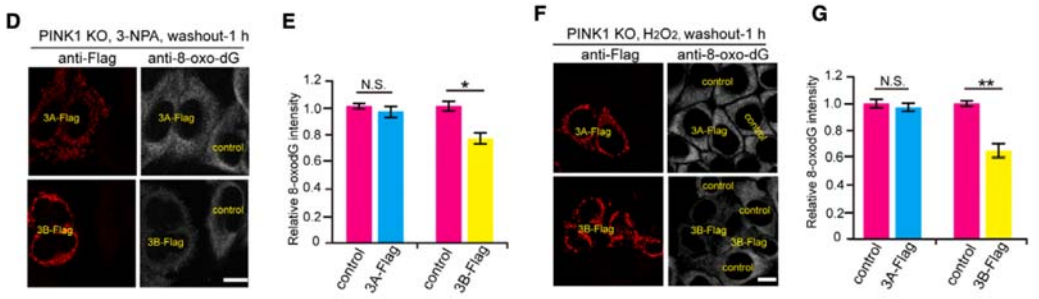
Fig 4D, oxmtDNA seems to be everywhere in these cells and colocalization therefore seems random. Why do the authors stain for NLRP3 in A and B but for ASC in panel D?

Response: We have consulted several important papers and found that the fluorescence staining of ox-mtDNA shows a diffusely distributed phenotype within the cytoplasm (Zhong Z, et al. Nature. 2018, PMID: 30046112; Shu L, et al. EMBO J. 2021, PMID: 33665835). In our results, the fluorescence of ox-mtDNA does also show a diffuse distribution, which may be related to the principle of the method itself, where all oxidized DNA in the cell is stained, which causes a large amount of background fluorescence. A more convincing method to solve this difficulty has not been developed in this field either. To avoid unnecessary misunderstandings, we have removed the ox-mtDNA-related data. Also, in the new manuscript, we uniformly detect its co-localization with mtDNA by staining with NLRP3.

The following two figures you may refer to:



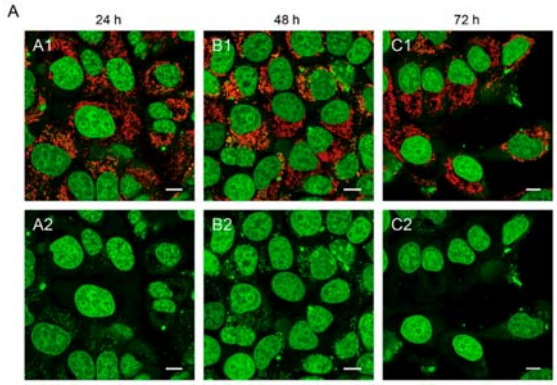
Zhong Z, Liang S, Sanchez-Lopez E, et al. New mitochondrial DNA synthesis enables NLRP3 inflammasome activation. Nature. 2018;560(7717):198-203.



Shu L, Hu C, Xu M, et al. ATAD3B is a mitophagy receptor mediating clearance of oxidative stress-induced damaged mitochondrial DNA. EMBO J. 2021;40(8):e106283.

Fig 4E, F, to my understanding, EtBr treatment leads not only depletion of mtDNA, but subsequently of mitochondria themselves. Yet in F, mitochondria are stained. Please explain.

Response: First, we reviewed the literature using this method, although it is true that the membrane potential can be partially reduced using EtBr (Hung KM, Calkins MJ. Mitochondrion. 2016, PMID: 27581214). However, data published by others showed that even after 72 h of EtBr treatment, a number of mitochondria could still be stained with MitoTracker (Schubert S, et al. International journal of molecular sciences. 2015, PMID: 25941929). At the same time, we repeated the experiment and found that there were still many mitochondria left in the cells, although the mitochondrial morphology changed from network-like to fragmented, at this time both qPCR and fluorescent staining evidence showed that mtDNA was indeed at a significantly lower level (Figure S6 A-B). We have consulted the literature and there is no direct evidence that mitochondria are completely lost under EB treatment. The following figure you may refer to:



Schubert S, Heller S, Loffler B, et al. Generation of Rho Zero Cells: Visualization and Quantification of the mtDNA Depletion Process. International journal of molecular sciences. 2015;16(5):9850-9865

Line 404, how does PHB1 in the plasma membrane relate to the present story?

Response: Some previous studies have reported that PHB1 is localized at the plasma membrane in immune cells, whereas our staining revealed that even in immune cells (BMDM, J774A.1), PHB1 is mainly localized at the mitochondria (at least in the cells used in this paper), and we just wanted to make a verification for other readers (Figure S2A).

Referee #3:

This paper has made a useful advance in understanding of the role of PHB1 in limiting the opening of the mitochondrial pore transition complex (mPTC) that allows dissipation of ion gradients and release of mitochondrial DNA. They show that PHB1 limits association of SPG7 and AFG3L2. When PHB1 is reduced, e.g. by hydrogen peroxide treatment, LPS treatment or gene knockdown, the association of SPG7 and AFG3L2 then seems to promote or correlate with mPTC formation. This is an original and interesting contribution to the literature on this topic. The authors show nicely that reduction of PHB1 leads to mitochondrial permeability, loss of membrane potential, increased ROS and release of DNA. However, the quality of the paper is reduced by the assumption that prior published work suggesting that mtDNA directly activates

the NLRP3 inflammasome is correct. In its current form I think this paper will perpetuate ideas in the literature regarding mtDNA and NLRP3 that are misleading. I think most of the work here is good quality and could be published with a focus on the role of PHB1 on mPTC, mitochondrial dysfunction and DNA release, with inflammasome activation a likely result of mitochondrial dysfunction including possibly ROS production, without necessarily invoking a role for DNA in that. The published work on mtDNA activating NLRP3 is prominent but controversial, as discussed below. NLRP3 is frequently reported to be activated by mitochondrial dysfunction, and in the PHB1 knockout cells the mitochondria have loss of membrane potential and increased ROS and/or metabolic changes could promote NLRP3 activation. The original contribution of this work is really the role of PHB1 in restraining SPG7 and AFG3L2 association and mPTC formation. The concepts of DNA being released by the mPTC and NLRP3 being activated by mitochondrial dysfunction are not novel and so are not the major contribution here. And the concept of mtDNA activating NLRP3 is not novel, and I would contend that it is wrong.

Specific comments

1. There is no methods section in this paper!

Response: We are very sorry for missing the upload of the methodology section, and in the revised version, we have re-listed the methods section.

2. Lines 77-78 states that "it is widely known that mtDNA release is required for NLRP3 inflammasome formation (reference 7)". Many papers seem to accept this as fact, but from what I see the evidence is poor. Reference 7 itself had a number of deficiencies; they did not use available gene knockout strains to show whether DNA release lay upstream (as they claimed) or downstream of NLRP3 inflammasome. Furthermore, the use of EtBr to deplete mtDNA is not conclusive proof as discussed below. An earlier paper (Nakahira reference 34)

showed that transfection of mtDNA elicited little or no response in AIM2-/- macrophages, suggesting it is not an efficient stimulus of NLRP3. Reference 34 actually concluded that in their systems mitochondrial DNA release was predominantly downstream of NLRP3 inflammasome activation. Although they concluded that mtDNA could enhance NLRP3 activation by other stimuli, as they showed that a 6 hour transfection of DNA enhanced the response to LPS+ATP somewhat in AIM2-/- cells, they neglected possible transcriptional priming effects through TLR9 and cGAS. In addition, colocalisation of DNA with NLRP3 is not proof of an involvement in its activation. There is no clear reason why mtDNA should activate NLRP3 rather than AIM2. AIM2 just recognises the phosphate backbone, and should not be perturbed by DNA oxidation. The requirement for mtDNA as an activator of NLRP3 is controversial as acknowledged in your reference 3 and (PMID: 32055400), but not noted in your manuscript. If you know of papers with better proof of the role of mtDNA in activating NLRP3, please cite them and state directly what the experimental evidence is. Otherwise, consider changing the focus of your paper away from DNA activation of NLRP3. It is clear that NLRP3 is activated, but it is not clear how it is activated.

Response: As this reviewer stated, there is no doubt that mtDNA can induce an inflammatory response and that NLRP3 is activated after mitochondrial damage, but whether this is mediated through mtDNA or through other signals (e.g. ROS, etc.), whether it is upstream or downstream, or direct or indirect effects remains unclear, and this was one of the original reasons for initiating this study. However, it has been shown that IL-1beta production by AIM$^{-/-}$ macrophages can be activated with mtDNA (Fig S9B in Ref. 2). In addition, recently published studies have also shown that ox-mtDNA is released and activates NLRP3-inflammasome formation and cGAS-STING pathways via mPTP and VDAC in the presence of NLRP3 activators (Xian H, et al. Immunity. 2022, PMID: 35835107, Ref. 44), which seems to support mtDNA's more upstream position

in the inflammatory response. In general, whenever the literature deals with the role of mtDNA, EtBr depletion of mtDNA is almost always used to demonstrate its role in the inflammasome pathway (Ref. 2 and Ref. 12), but we agree that this is not conclusive evidence. Therefore, we believe that whether mtDNA release is currently upstream or downstream of the inflammasomes pathway remains to be clarified.

To prove this adequately, on the one hand, we need to use a variety of research methods based on different principles, and on the other hand, we urgently need to invent new methods for mtDNA staining, clearance, etc. In the revised manuscript, we have removed the description "mtDNA directly activates NLRP3" (Line 396-397). We would like to clarify to this reviewer that although co-localization of mtDNA and NLRP3 cannot be considered as direct evidence of NLRP3 inflammasomes activation (Figure 4A-D), we found that antibodies to NLRP3 can directly immunoprecipitate mtDNA (Figure 4E). We have also added new experimental results regarding AIM2 (Figure S5A-E) and cGAS and discussed that although AIM2 does not increase binding to mtDNA in the presence of reduced PHB1, knocking down AIM2 does also somewhat reduce IL1-Beta release in the presence of PHB1 knockdown. Although in the previous manuscript, the stimulation conditions were probably not strong enough and the effect of PHB1 knockdown was not good, we did not detect significant activation of the cGAS pathway, but as suggested by this reviewer, we changed to stronger hydrogen peroxide as a stimulus and used PHB1 KO cells, and in this case the cGAS pathway was found to be activated (Figure S7A-B). Therefore, in the new manuscript, we modified some descriptions to weaken the absolute role of mtDNA.

Modified content such as: The cytosolic mtDNA is recognized by NLRP3 and is required for IL-1β maturation and release upon Phb1 depletion’ changed to '

The cytosolic mtDNA and ROS promoted by Phb1-depletion contributes to the inflammatory responses' (Line 265-266).

In discussion section: we replaced the expression 'directly trigger' with 'get involved in/be associated with' (Line 402-403).

3. Ethidium bromide does a lot more than just reduce DNA levels in mitochondria. For example, EtBr is reported to reduce oxygen consumption and increase glycolytic activity, reduce mitochondrial creatine kinase mRNA and phosphocreatine (PMID: 29287112). To quote another paper: "In order to gain insight into cellular responses to mitochondrial stress, we treated human diploid fibroblasts with ethidium bromide at concentrations that induced loss of mitochondrial DNA over a period of 7 days. The decrease in mitochondrial DNA was accompanied by a reduction in steady state levels of the mitochondrial DNA binding protein, TFAM, a reduction in several electron transport chain protein levels, increased mitochondrial and total cellular ROS, and activation of p38 MAPK. However, there was an increase in mitochondrial mass and voltage dependent anion channel levels. In addition, mechanistic target of rapamycin (mTOR) activity, as judged by p70S6K targets, was decreased while steady state levels of p62/SQSTM1 and Parkin were increased." (PMID: 25104948). In contrast Nakahira showed that EtBr treatment of J774 gave lower mito-ROS. Also, levels of any proteins encoded in mtDNA may be increased, and they noted this was the case for cytochrome c oxidase 1. In short, EtBr will have profound effects on mitochondrial function and could affect NLRP3 activation through multiple routes. Consequently I question the value or interpretation of Fig 4 E-G.

Response: Indeed, as this reviewer said, EtBr can have a very large impact on many mitochondria-related functions. We cited and elaborated on the literature you provided in the discussion section of the manuscript (PMID: 25104948 was

ctied as Ref. 45; PMID: 29287112 was cited as Ref. 46). As described in these papers, EtBr treatment affects TFAM, ETC stabilization, leads to elevated ROS, activation of the P38 MAPK pathway, and inhibition of autophagy levels (Ref. 45), while affecting mitochondrial metabolism and promoting glycolysis levels (Ref. 46). These events are all closely associated with activation of inflammasome in macrophages (Ref. 47 and Ref. 48). Other effects of EtBr, such as mtDNA depletion, can inhibit the activation of the inflammasomes (Ref. 42). In the present study, we treated cells with only 150 ng/ml for 3 days in order to reduce the confounding effect of EtBr and determined the effect of mtDNA clearance (Figure S6A). We found, in agreement with several studies, that EtBr does inhibit IL-1β production (Ref. 2, Ref. 40. And Ref. 42). We acknowledge the methodological limitations of EtBr, but it is indeed a classical and commonly used method in the current academic community, and there is no better alternative for the time being. Therefore, on the one hand, our results suggest that the loss of mtDNA does play a strong inflammatory inhibition role, and on the other hand, we do not deny that there are other potential factors (like ROS) involved in this process (Figure4G and S6C). Therefore, in our new manuscript, we describe our data more directly and without further inference and discuss them more objectively in the Discussion section (Lines 409-419).

4. For all the reasons given above, I think you should omit Figure 4. Focussing on proposed DNA activation of NLRP3 distracts from the useful message of your paper that is about the mPTC formation. The localisation of NLRP3 and DNA is not convincing - In some samples NLRP3 is overexposed and when it is nearly everywhere in the cell it will certainly appear to colocalise. In other samples it is more convincing, but in two J774 samples in panel B I think you are looking at micronuclei. The text states that Figure 4 A-B show "specks" but you are not staining for ASC there, so that is not correct to say. There is no clear "speck" from NLRP3 here. Regardless, even with some colocalisation,

this does not prove that it is a ligand for NLRP3. ASC colocalisation with 8-OH-dG is similarly unconvincing as there is a lot of 8-OH-dG staining. EtBr data cannot be used to definitively prove and involvement of DNA in the NLRP3 activation for reasons given above.

Response: Many thanks to this reviewer for the suggestions. In the revised manuscript, we only go through the NLRP3-related data objectively and do not infer conclusions in the results. In addition to NLRP3 co-localization experiments with mtDNA, we also designed probes for mtDNA-specific genes after NLRP3 immunoprecipitation, and using qPCR we were able to detect a large increase in mtDNA bound to it after PHB1 knockdown. Also, to avoid confusion, we replaced the previous image that might be a micronucleus (Figure 4C). We really should not assume that the immunofluorescence data shows speck and therefore modified the relevant expression. We acknowledge the limitations of the 8-OH-dG data because other available literature shows plots that, although they also stain ox-mtDNA with 8-OH-dG, in my opinion, are not very specific (Zhong Z, et al. Nature. 2018, PMID: 30046112; Shu L, et al. EMBO J . 2021, PMID: 33665835). We believe that this method does not reflect the true intracellular levels of oxidised mtDNA, and therefore, data related to oxidised mtDNA will no longer be presented in the new manuscript. Furthermore, we also agree with the limitations of EtBr use in NLRP3 activation experiments, and only describe our data more directly and discuss this more objectively in the Discussion section as replied above. Finally, because we re-evaluated the role of the cGAS-STING pathway, we were able to observe activation of the STING pathway when replacing the original PHB1-KD cells with PHB1 knockout primary cells and stimulating mPTP opening with harsher hydrogen peroxide. Therefore, we weakened the statement that mtDNA is activated only through NLRP3 inflammasomes and changed it to that ‘mtDNA acts through both NLRP3 and STING pathways’.

5. Obviously your story would become more simple if you could show the activation of cGAS by released DNA, as that is an unambiguous DNA response. You show that knockdown of PHB1 in J774 does not lead to spontaneous cGAS response in J774 (Fig S5A). If these cells are competent for the cGAS pathway (no positive control is shown), I can only assume that the amount of DNA released from mitochondria is just too low to activate. Indeed, Figure 3C and 3K show 2-fold increase in DNA or less in the knockdown cells. This seems to be many-fold lower increase than reference 32, where a cGAS response was seen. Have you looked for cGAS activation in the PHB1 knockout macrophages that give a higher level of cytosolic DNA? If you maintain that you have high levels of DNA release from mitochondria but no cGAS activation, you would have to have some hypothesis on why this is so. Do the same cells not respond to transfected DNA? Or would you conclude that the amount of DNA released is too low? Is there another stimulus you can add that will boost DNA release for cGAS recognition, and then show an enhancing effect of PHB1 loss? If none of this is possible, I think that the data on PHB1, mPTC and DNA release stands by itself, and mitochondrial dysfunction can link to NLRP3 activation through some unknown means.

Response: Thank you very much for your suggestion. In the original Figure S5, the possible reasons for cGAS-STING not being fully activated are: 1, the sensitivity of the cell line we originally used may not be as high as that of the primary cells. 2, PHB1 knockdown was not efficient enough. Therefore, in the revised manuscript, we used primary cells from knockout mice and found that the cGAS-STING pathway could be activated in the primary cells with PHB1 deletion. Given that cGAS-STING is a classical DNA binding protein, this is therefore another indication that PHB1 deletion can indeed induce mtDNA release. We also used a more harsh mPTP open stimulation reagent, H_2O_2, and found that it induced the activation of cGAS-STING downstream signals in PHB KO cells (Figure S7A-B).

6. Reference 2 is not appropriate on line 64- it does not describe release of mtDNA to the cytosol. On line 66 - Ref 3 is a review, and you really need primary sources for this statement.

Response: We replaced it for a more appropriate literature.

7. Assessment of inflammasome responses are mostly shown by western blot, and that is appropriate, but it then does not show replication or quantification. For the important points it would be good to see quantitative confirmation by IL-1b ELISA combining results of several experiments. For example, Figure 1B and 1N you are making quantitative conclusions on IL-1b production, but we have only a single western blot to go on. Also, number of times western blots were repeated to give the same result is not stated.

Response: We added ELISA assays for some of the experiments described by the reviewers (Figure 1C, 1K and Figure 6D).

8. Figure S2D really does not show good restoration of membrane potential in the knockdown cells by expression of PHB1. Presumably the knockdown is too effective. If you look at the mean fluorescence index that would be more appropriate than the % of cells that fall above an arbritary line. Unless you have a PHB1 gene sequence mutated to avoid knockdown, I think this figure is not useful.

Response: Thanks to this reviewer's suggestion, we deleted this data in the revised manuscript.

9. It would help understanding of what is known if you can state in the introduction that others have presented data suggesting mPTP is involved in release of DNA (ref 32). It would then be clear that what you are proposing is a mechanism involved in the regulation of pore opening, rather than providing the

first description of mPTP in DNA release.

Response: We state in the introduction section that mPTP can be involved in the release of DNA (Line 88-96).

10. Line 238 - NLRP3 is presented as being activated by mtDNA and this is doubtful, as noted above.

Response: We acknowledge this and state it in the discussion section (Line 395-400).

11. Figure 3M uses overexpression of ASC and NLRP3 in HeLa cells, and claims on the basis of an image of 1-2 cells to show colocalisation of ASC and NLRP3 enhanced by PHB1 knockdown. Overexpression systems give a lot of spontaneous speck formation, depending on the level of expression. ASC and NLRP3 will tend to cluster together when overexpressed. This data is of no use because it does not quantify an increase in colocalisation. Please delete this figure. However, if you want to focus on inflammasomes, quantification of spontaneous ASC specks in the knockout macrophages would be useful as it would show the frequency of inflammasome formation. Formation of an ASC speck is generally a sign of a pyroptotic cell, and if this was frequent, I would expect your culture to have very low viability.

Response: We initially referenced the study by Mehto et al. (Mehto S, et al. Mol Cell. 2019, PMID: 30612879), but we also acknowledge that the results were obtained from overexpressed cells and have limitations, so as per your suggestion, we have removed the data.

12. Please make clear for each figure at what level replication is done. For example Figure 1 I to L show either results from separate wells in one experiment, results of separate experiments, or results of four estimates of mRNA levels on a single sample?

Response: We have merged these images into the same panel (Figure 1G) and we are testing these metrics in the same batch of samples. The information about the samples and the number of repetitions are described in the legend.

Minor Points

1. Line 70-71 - In discussing the NLRP3 inflammasome, there are not several different receptors and "such as" is not appropriate here - NLRP3 is an essential, not an optional component of the NLRP3 inflammasome!

Response: We have changed the wording (Line 68-72).

2. Please make sure abbreviations are all defined on first usage. The abstract includes MIMP and mPTP that need definition.

Response: We have checked all abbreviations and added some definitions in appropriate palces.

3. Lines 205-206. Here it sounds like ref 32 has already shown results for PHB1 deficiency in J774, making your work not novel. I think that is not what you mean?

Response: This was a mistake on our part, we intended to express that we refer to the assay of Ref. 32, which does not mention PHB1, and this has been corrected in the new manuscript.

4. Line 318. You do not treat HeLa cells with LPS.

Response: We have modified this (Line 338-341).

5. Line 319-320. Should this actually say "SPG7 and AFG3L2 depend on reduced expression of PHB1 to participate in opening of mPTP, rather than "do not depend"? Also, "reduced" is better than "altered". I am not sure what you are trying to say here.

Response: This was a clerical error on our part. We intended to say "SPG7 and AFG3L2 do not depend on their altered expression to participate in the opening of mPTP". In the new manuscript, we have revised it (Line 341).

6. Cyclosporin A can clearly affect mPTC formation. But it is worth bearing in mind that it seems to have unrelated roles in inhibition of the inflammasome. Previous work has confirmed that cyclosporin does inhibit NLRP3 inflammasome activity, but this is not through action on cyclophilin D, as genetic deficiency of cyclophilin D has no effect on inflammasome responses PMID: 24990442

Response: We have carefully read the literature you recommended and discussed it in the manuscript (Line 450-452).

Optional

1. Figure 2A- does this indicate that knockdown of PHB1 promotes mitochondrial fission? Given that LPS causes mitochondrial fission and also reduction in PHB1, is PHB1 loss causally involved in the LPS-induced fission? This is just curiosity, not expecting any experimental response to this.

Response: Actually, we did not detect other indicators of mitochondrial fission (e.g. the Ser616 phosphorylation of DPR1) during PHB1 KD, so could not make the conclusion from this data. Indeed, we confirmed PHB1 KD caused the fragmentation of mitochondria. The association between PHB1 loss and LPS-induced fission is worthly investigating further.

2. Would it be helpful to look at the effect of mito-ROS inhibitors? Do they affect the mPTC formation and DNA release when you have low PHB1? If they do not prevent DNA release but do prevent NLRP3 activation that would be useful information.

Response: We treated BMDMs lacking PHB1 with NAC and found that cleaning ROS decreased the production of IL-1beta promoted by PHB1 lacking in macrophages (Figure 4G), indicating that ROS indeed has some role in NLRP3 activation.

Dear Du Feng,

Thank you for submitting the revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referees #1 and 2. As you can see from the comments below, the referees appreciate the introduced changes and support publication here.

Referee #2 is a bit hesitant regarding the link between PHB1-deficiency and mPTP opening, but also recognise the current issues in the field regrading mtDNA in NLRP3 activation and supports publication here. Can you take a careful look at the text and make sure that you have a balanced discussion regarding this issue.

Once I get the manuscript back in then I will accept the MS for publication here.

When you submit the revised manuscript will you also take care of the following points?

- You are missing a Data Availability section. This is the place to enter accession numbers etc. If no data is generated that needs to be deposited in a databasethen please state: Data Availability: This study includes no data deposited in external repositories.

- you can only have max 5 keywords

- The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y'). Please remove the author contribution section.

- Please check the annotation 12 on the title paper (These authors contributed equally to this work on the title page). I think only one author with 12 marked.

- Please re-label Conflict of interest to Disclosure and Competing Interests Statement

- Please upload single figure files

- The appendix needs a ToC

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

That should be all!

best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have addressed my points adequately, an obvious outstanding question is how mtDNA is activating the inflammasome, be it direct or indirect, the authors discuss this in the revised ms.

Referee #2:

Hao Liu have substantially revised and improved the manuscript in many aspects. I am convinced by their evidence that PHB1 loss leads to mitochondrial perturbation and thereby NLRP3 inflammasome activation. Also, their explanation for mitochondrial damage that involves interaction with and separation of AFG3L2 and SPG7 seems novel and is convincing to me. However, I am not convinced about the roles of mPTP and oxmtDNA. If the authors are absolutely convinced that what they see in PHB1-deficiency is mPTP opening, I am willing to accept that, although I personally am not. However, in our hands, neither MCU inhibition nor CsA block NLPR3 induction by standard riggers. Furthermore, I agree the reviewer #3's standpoint on the questionable role of oxmtDNA in NLRP3 activation. I also question the existing literature on this and do not understand why the authors attachment to this concept when it does not add to the novelty of their story. It is sufficient to say that loss of PHB1 damages mitochondria (by mechanism involving AFG3L2 and SPG7) and that activates NLRP3. However, this is their choice and, in the face of a recent Nature paper again perpetuating the ideas of mPTP and oxmtDNA in NLRP3 activation, I would understand the frustration of the authors if they now get held to a higher standard. I therefore recommend publication on the basis of the mentioned merits.

Point-by-point response

Thank you for submitting the revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referees #1 and 2. As you can see from the comments below, the referees appreciate the introduced changes and support publication here.

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Response: We have discussed this issue in the discussion section in the revised version of the MS (lines 396-401; lines 406-412).

Once I get the manuscript back in then I will accept the MS for publication here.

When you submit the revised manuscript will you also take care of the following points?

- You are missing a Data Availability section. This is the place to enter accession numbers etc. If no data is generated that needs to be deposited in a database then please state: Data Availability: This study includes no data deposited in external repositories.
Response: We have added this section to the text (lines 827-828).

- you can only have max 5 keywords
Response: We have deleted superfluous keywords (line 56).

- The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript

submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y'). Please remove the author contribution section.

Response: We have removed the 'Author Contributions' section and specified them in the journal submission system.

- Please check the annotation 12 on the title paper (These authors contributed equally to this work on the title page). I think only one author with 12 marked.

Response: Thank you for pointing out this. We have marked all co-first authors.

- Please re-label Conflict of interest to Disclosure and Competing Interests Statement

Response: We have changed this in the text (lines 845-846).

- Please upload single figure files

Response: We have uploaded single figure files as requested.

- The appendix needs a ToC

Response: We have provide a ToC for the appendix.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

Response: We have responded to the comments and provided the information needed.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

Response:

Summary statement:

Under physiological conditions, AFG3L2 and SPG7 are bound to PHB1, which maintains mitochondrial membrane integrity and retains mtDNA in the matrix. Upon various insults (e.g., induced by LPS or H_2O_2) or knockdown of PHB1, downregulated PHB1 levels enhance the interaction between AFG3L2 and SPG7, which promotes the formation of mPTP. This leads to the release of mtDNA and downstream inflammatory responses.

Bullet points:

- Inflammatory stresses downregulate PHB1 levels in macrophages, promoting inflammatory responses.
- PHB1 deficiency promotes multiple perturbations of mitochondrial homeostasis, including mPTP opening and mtDNA release.
- The interaction between SPG7 and AFG3L2 is enhanced by PHB1 deficiency, leading to mPTP opening, mtDNA release, and downstream inflammatory responses.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.
Response: We have provided figure 7 for the synopsis.

That should be all!

best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

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Response: We appreciated the comments from this reviewer. In fact, we have deleted 'oxmtDNA in NLRP3 activation' part in the last revised version. Since they are still in rigorous debate, we have discussed more in the upcoming revised version.

We added the sentence 'Although NLRP3, recognizing PAMPs and DAMPs, seems to sense oxidated mtDNA and lead to the activation of NLRP3-inflammasomes2,7,38,39, whether mtDNA directly triggers this event remains controversial8,43. Nevertheless, other factors involving defects in mitochondrial function (including loss of membrane potential, increased ROS, or perturbations in mitochondrial electron transport chain) are also associated with inflammasome activation42,44,45'(lines 396-401). And the sentence 'However, the results from our laboratory (not shown) and other laboratories show the limitations of using 8- OH -dG to stain ox-mtDNA. They are not very specific and the fluorescence of ox-mtDNA shows a diffuse distribution, which could be related to the principle of the method itself, in which all oxidized DNA in the cell is stained, causing a large amount of background fluorescence. A more convincing method to solve this difficulty remains to be developed in this field47,48'(lines 406-412).

2nd Revision - Editorial Decision **14th Sep 2022**

Dear Du,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now looked at the introduced changes and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods, Figure legends
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

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Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgement

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Figure legends
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Figure legends
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods, Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Materials and Methods
Studies involving **human participants**: 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.	Yes	Materials and Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Yes	Materials and Methods

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Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	