EMBO reports

Functional Platelet-Derived Mitochondria Induce the Release of Human Neutrophil Microvesicles

Jacob Léger, Marie-France Soucy, Vanessa Veilleux, Robert Foulem, Gilles Robichaud, Marc Surette, Eric Allain, and Luc Boudreau
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Corresponding author(s): Luc Boudreau (luc.boudreau@umoncton.ca)

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Boudreau,

Thank you for the submission of your research manuscript to EMBO reports. I have now received the full set of referee reports that are copied below.

As you will see, all referees #2 and #3 have many major concerns, indicating that the relevance of the findings remains unclear, conclusions of the study are insufficiently supported by the data and in vivo evidence is scarce. They mention also several technical and experimental shortcomings and indicate novelty issues. Referee #1 is overall more positive, but also has concerns.

Given these comments and considering the amount of work required to address them and the fact that we only invite revision of papers that receive enthusiastic support from the referees upon initial assessment, we cannot offer to publish your manuscript.

I am sorry to have to disappoint you this time. I nevertheless hope that the referee comments will be helpful in your continued work in this area, and I thank you once more for your interest in our journal.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

In this manuscript, Leger et al showed that the release of human neutrophil microvesicles (MVs) may be induced by functional mitochondria deriving from activated platelets. This follows important observation of the same group (Boudreau et al, 2014) and of Dache et al (2021) who demonstrated that cell-free respiratory competent mitochondria may circulate in blood. This work provides a great advance in the field by clearly linking platelet/mitochondria/neutrophil/MVs. The observation is clearly supported by relevant and very well executed experiments. The text is concise and the figures and their legend are of high quality. This is a nice piece of work.

The reviewer has a few minor concerns:
1. May be because of the selected format in EMBO reports, the text is short and lacks more information about neutrophil MVs. Authors should better describe microvesicles and in particular what the literature offers on why and how N.MVs increased in inflammatory conditions, and as well some clues on their implications in some diseases. In addition, the observation would be enriched by describing how it could lead to diagnosis or novel therapies or impact on intercellular communication.
2. Is freeMitos a standard abbreviation?
3. There are citation numbers in line 164.
4. Some limitations on mitotracker use should be indicated
5. Higher magnification of a N.MV would be appreciated as well as associated sizing
6. Indication of statistical differences between groups are not always clear since sometimes reference group is not mentioned.
7. No description of the Fig.1 H is in the legend
8. Suppl Fig 2 should be to my opinion in the text.

Referee #2:

In this report Leger and colleagues investigate the effect of mitochondrial addition to PMNs finding that isolated mitochondria induce neutrophil microvesicles. The basis of this work comes from various reports indicating a pro-inflammatory function of free mitochondria in various conditions, including as mediators of sterile sepsis. While the subject is topical and the findings of interest, I consider the novelty of the findings limited and the depth of investigation superficial. There is no mechanistic insight into how mitochondria induced vesicle release or what the biological importance of this may be. I am sorry that I cannot be more supportive at this time, some comments that I hope may help are below.

- with respect to free mitochondria, the authors effectively lyse platelets and extract their mitochondria, its unclear how similar these (extracted) mitochondria are to ones that are released from viable platelets. Some comparison should be made, and controlled for - conventional mitochondrial preps. are usually contaminated with ER for instance, one can imagine that this may be lacking from free mitochondria expelled by platelets. I think this is a key consideration given the authors use this system to model effects of free mitochondria on PMNs.

- figure 1C is very confusing, legend states red is the cell membrane, magenta are free mitos ? - this doesn't look to be the case. With respect to the rest of this figure it would be good to gauge what the extent the PMN mitochondrial content are derived from free mitochondria versus pre-existing mitochondria and whether free mitochondria are indeed functional if taken up into PMNs (difficult to imagine they are functiional if they are phagocytosed)

- in figure 2 the authors describe similarities between DAMP application and free mitochondria on calcium release, calpain activation and ev release - however there is no mechanistic insight (or investigation of its relevance), could it be that lysis of some mitochondria during preparation is phenocopying DAMP activity leading to these effects, and relating back to the first point how much is this representing genuinely free mitochondria released by platelets ?

Referee #3:

In the manuscript entitled "Functional Platelet-Derived Mitochondria Induce the Release of Human Neutrophil Microvesicles" the authors describe that isolated mitochondria from platelets induce the release of neutrophil micro vesicles and induce transcriptional chances in neutrophils.

While this is an interesting observation, it is not completely novel as part of this mechanism was already proposed in a previous paper by the last author (Boudreau et al Blood 2014). The induction of changes in gene-expression in neutrophils by isolated mitochondria from platelets is a novel observation, however the elicited changes in neutrophils did not explain why the neutrophils were more likely to release mitochondria.

The authors investigated the oxidative state of neutrophils after incubation with isolated platelet mitochondria and didn't observe any changes in respiration. Changes in calcium mobilization were observed after incubation with platelet mitochondria in similar fashion as DAMPs, however the underling mechanism of which G-protein coupled receptor was engaged was not investigated. These changes in calcium mobilization resulted in more PMN-derived microvesicles, albeit with very high variation in figure 2 CD.

While the authors show in their isolated platelet mitochondrial system that neutrophils release extracellular vesicles, what is the role on physiological processes of inflammation was not investigated. Nor was there any in vivo evidence for this phenomenon, so it might be an in vitro artifact. The claims should be substantiated with both activated platelet and see if released mitochondria have the same effect on neutrophils.

Ca-mobilization in neutrophils will effect a wide variety of biological neutrophil funcitions as increase in chemotaxis/migration, enhanced ROS production and increased phagocytosis. It is unclear why the authors did not investigate these process and focused on release of microvesicles and not on other better estabilshed neutrophil functions.

Technical comments:
In Figure 1 A-C the authors claim that mitotracker labeled platelets are taken up by neutrophils. However, the dye used to label mitochondria is not covalently attached to mitochondria and based on membrane potential. How can the authors exclude the possibility that it is not dye transfer from labelled platelet mitochondria that label then neutrophil's mitochondria.

Dear Dr. Breiling,

Thank you very much for the fast and efficient review process by your journal. It is very appreciated. After discussing with my fellow co-authors, we were wondering if you would reconsider giving us to the opportunity to submit a revised manuscript within a respectable timeline (3 months).

We strongly believe (a sentiment shared by reviewer #1) that the results presented in this short communication, which were first presented at the 12th World Congress Targeting Mitochondria in late 2021, will be of significant interest to the scientific community. Hence, we reported the first transcriptomic analysis of free functional mitochondria on immune cells (neutrophils). T

Most importantly, the comments/concerns from the 3 reviewers could be addressed with minimal additional experiments in a revised manuscript as I realize that further discussion/clarification could have dissipated any concerns for some technical aspect. I will take the blame on this one as this is my first experience as a young researcher to submit my research as a short communication with this abundance of data. However, this does not take away from the research quality and also from the degree of difficulty from a technical standpoint that was performed in this manuscript (human mitochondria isolated from platelets and subsequent co-incubation with human neutrophils…). This is the reason that we published our rigorous purification methods of platelet-derived mitochondria in manuscript in 2020 (Léger et al., Platelets) and that we were subsequently invited in 2021 for a book chapter on this isolation process in Mitochondria Medicine.

While the horizontal transfer of mitochondria between cells has been demonstrated by our group and others in recent years, we can provide additional data for some mechanistic of the mitochondria internalisation as requested by the reviewers. Therefore, we strongly believe that the concerns/comments by the 3 reviewers are not major concerns, and will be addressed in the revised manuscript.

I sincerely hope that you will reconsider your decision and provide us with the opportunity to submit a revision to your prestigious journal.

Thanks again for your consideration and do not hesitate to contact me in regards to any questions you would have.

Have a nice day

Luc

Dear Dr. Boudreau,

Thank you for your letter. Although it remains unclear what specific experiments or changes to the manuscript you envisage, but if you feel you are able to address the referee concerns and will have data that would considerably strengthen the study (as outlined in their reports), I would reconsider a resubmitted and substantially revised manuscript. Please submit the revised paper as new submission including your detailed point-by-point response and indicate during submission and in your cover letter that this is a re-submission, also mentioning the previous manuscript number and this conversation. Our assistant will then link the new submission to the previous version.

When submitting your revised manuscript, please also review the instructions that follow below to speed up the process.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

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http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in the respective section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

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

Data availability

The datasets produced in this study are available in the following databases:

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

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

Moreover:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our new reference format: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and add a statement declaring your competing interests. Please name that section 'Disclosure and Competing Interests Statement' and add it after the acknowledgements section.

Please let me know if you have further questions.

Best,

Achim Breiling

Point-by-point response to reviewer:

Referee #1:
In this manuscript, Leger et al showed that the release of human neutrophil microvesicles (MVs) may be induced by functional mitochondria deriving from activated platelets. This follows important observation of the same group (Boudreau et al, 2014) and of Dache et al (2021) who demonstrated that cell-free respiratory competent mitochondria may circulate in blood. This work provides a great advance in the field by clearly linking platelet/mitochondria/neutrophil/MVs. The observation is clearly supported by relevant and very well executed experiments. The text is concise and the figures and their legend are of high quality. This is a nice piece of work.

- *We would like to thank the reviewer for these compliments for our work on this manuscript. We also strongly believe that further clarification on the respiration capability of cell-free mitochondria needs to be addressed, as this was a major topic of concern during the 12th World Congress Mitochondria in October 2022. The data presented in this manuscript will clarify some of the important literature gaps in the interaction of freeMitos with human neutrophils.*

The reviewer has a few minor concerns:

1. May be because of the selected format in EMBO reports, the text is short and lacks more information about neutrophil MVs. Authors should better describe microvesicles and in particular what the literature offers on why and how N.MVs increased in inflammatory conditions, and as well some clues on their implications in some diseases. In addition, the observation would be enriched by describing how it could lead to diagnosis or novel therapies or impact on intercellular communication.

- *This is a great comment by the reviewer and needs clarification in our text. We agree that the short format might have limited us on providing more background on neutrophil-derived microvesicles. We have corrected this in the revised manuscript in the introduction section at line 76. While the production of extracellular vesicles has been extensively studied in other cell types such as platelets, erythrocytes and monocytes/macrophages, the role of neutrophil-derived microvesicles and their impact in inflammatory diseases remains poorly understood. However, a recent review by Dow and Ridger ((Dow & Ridger, 2021)) provides a glimpse of the landscape of the importance of neutrophil microvesicles in diseases. We also added text in the Results/discussion section at line 182 to provide more background on the importance of neutrophil microvesicles in health and diseases.*

2. Is freeMitos a standard abbreviation?

- *We first introduced the term freeMitos for extracellular mitochondria from platelet origin in 2014 (Boudreau et al, 2014). Subsequently, other groups have used the same term to describe mitochondria released from platelets (Caicedo et al, 2021), and importantly to distinguish them from mitochondria-containing microparticles (mitoMPs). In this paper, while we isolate mitochondria directly from platelets, we still believe that they should be identified as free extracellular mitochondria, due to the absence of platelet membrane as reported in our isolation paper in 2020* (Léger *et al*, 2020).

3. There are citation numbers in line 164.

- *We thank the reviewer for noticing this omission by our part. We have added the correct references in the revised manuscript at line 169.*

4. Some limitations on mitotracker use should be indicated

- *The reviewer is correct, there are several limitations with the use of MitoTracker as previous studies have pointed out* (Sargiacomo *et al*, 2021). *However, since we can't perform transfections on primary cell types (either platelets or neutrophils in our case), our best option at this time is the use of MitoTracker. Of importance, MitoTracker Deep Red was added to whole platelets before mitochondria isolation. Since the uptake of MitoTracker Deep Red is dependent on the membrane potential of mitochondria, the fact that mitochondria are still labeled with MitoTracker after our isolation process is a good demonstration of the efficiency of our protocol we have in place. Furthermore, Mitotracker labeling was only performed to demonstrate the interaction and co-localization of freeMitos with neutrophils. MitoTracker labeling was not performed for the transcriptomic analysis and any of the subsequent evaluation of neutrophils' phenotype characterization. We have clarified this in the methods section in lines 237 of the revised manuscript.*

5. Higher magnification of a N.MV would be appreciated as well as associated sizing.

- *We thank the reviewer for this comment. What we wanted to do was to give an overall qualitative view of the microvesicles released in the supernatant. In the revised manuscript, we have updated Figure 2 (panel H) and added an additional Figure EV2: Sizing of the vesicles released from* PMN *demonstrating the size of the vesicles of each of our conditions using nanoparticle tracking analyzer (NanoSight). This should provide a*

better quantitative approach for the comparison of the size of each population of microvesicles released from PMN.

6. Indication of statistical differences between groups are not always clear since sometimes reference group is not mentioned.

- *We added some clarification in the methods section at line 362 and in the Figure legends.*

7. No description of the Fig.1 H is in the legend

- We thank the reviewer for noticing this omission by our part. The description of panel H was in the figure legend, however the letter H was missing…..it has been corrected in the revised manuscript in the figure legend.

8. Suppl Fig 2 should be to my opinion in the text.

- *We agree with the reviewer. The flow cytometry gating strategy was included in the original version of our manuscript, but we decided to move to the supplementary section just before the submission. We strongly agree that when using flow cytometry, showing the gating strategy used in the study is essential to demonstrate the rigorous approach by the researchers. The former Supplementary Figure 2 is now included as panel D in Figure 2. The Figure 2 legend has also been modified accordingly.*

Referee #2:

In this report Leger and colleagues investigate the effect of mitochondrial addition to PMNs finding that isolated mitochondria induce neutrophil microvesicles. The basis of this work comes from various reports indicating a pro-inflammatory function of free mitochondria in various conditions, including as mediators of sterile sepsis. While the subject is topical and the findings of interest, I consider the novelty of the findings limited and the depth of investigation superficial. There is no mechanistic insight into how mitochondria induced vesicle release or what the biological importance of this may be. I am sorry that I cannot be more supportive at this time, some comments that I hope may help are below.

- We thank the reviewer for the comments. We strongly believe that the novelty of this work was the ability to show a transcriptomic effect of platelet-derived free mitochondria on human neutrophils. It is important to recognize that we are using mitochondria and neutrophils from the same blood donors. From a technical standpoint, this is a very complex feature given the short lifespan of human neutrophils ex vivo and their low quantity of RNA per cell. While the mechanistic aspect of the paper could be further

investigated, we clearly provide some insight in which the calpain pathway is activated, a necessity in microvesicles formation. We believe that we have demonstrated sufficient mechanistic insight for a short manuscript, as it was initially intended to provide transcriptomic data to other researchers interested in this field.

- with respect to free mitochondria, the authors effectively lyse platelets and extract their mitochondria, its unclear how similar these (extracted) mitochondria are to ones that are released from viable platelets. Some comparison should be made, and controlled for – conventional mitochondrial preps are usually contaminated with ER for instance, one can imagine that this may be lacking from free mitochondria expelled by platelets. I think this is a key consideration given the authors use this System to model effects of free mitochondria on PMNs.

- *The reviewer's comment is relevant. While we agree that it would have been interesting to collect freeMitos released from activated platelets, it brings back the technicality aspect in this paper as pointed out in the previous comment that platelets would first need to be activated (at least one hour) with an agonist. Since we use the same blood donor for both platelet-derived mitochondria and neutrophil isolation mitochondria, and those mitochondria cannot be frozen, we had a very limited timeframe to execute our experimental approach. In addition, different agonists could affect mitochondrial integrity differently, and platelet-derived mitochondria are released from platelets by different physiological agonists (thrombin, collagen, immune complexes, or ADP) at different ratios with other subpopulations of platelet-derived microvesicles*(Boudreau *et al*, 2014). *We agree with the reviewer that isolating freeMitos from platelet activation would be of interest, and to that end our laboratory has been extensively exploring the possibility of isolating each platelet-derived microvesicle subpopulation for some time (cell sorting flow cyto, opti-prep vs beads isolation), but with little success. Also, it is unknown whether free mitochondria released by activated platelets are indeed devoid of other cellular structures such as ER. Our new TEM images of our mitochondria added as Figure EV3 in the revised manuscript show intact mitochondria as confirmed by our previously reported* (Léger *et al*, 2020, 2021) *preparations that retain mitochondrial integrity as measured by cytochrome c assay by high sensitivity respiration assay. Therefore, for these reasons we conclude that isolating mitochondria from platelets was the best approach for this study.*

- figure 1C is very confusing, legend states red is the cell membrane, magenta are free mitos ? - this doesn't look to be the case. With respect to the rest of this figure it would be good to gauge what the extent the PMN mitochondrial content are derived from free mitochondria versus pre-existing mitochondria and whether free mitochondria are indeed functional if taken up into PMNs (difficult to imagine they are functiional if they are phagocytosed)

- *Of importance, we do not state that freeMitos are functional upon interacting with PMN, in fact, we believe the opposite (see respiration results Figure 1F). We agree with the reviewer that phagocytosis could be involved. However, the aim of Figure 1C was to demonstrate and complement our flow cytometry results that demonstrate an association between platelet-derived mitochondria and PMNL. MitoTracker was only added to the initial platelets preparation to confirm exogenous intake of mitochondria by the recipient cell. The red labeling shown in panel C and Video 1 is the membrane of PMN labelled with CellMask Orange as described in our methods section. The red does not represent PMN mitochondria. We have tried several other membrane labeling for PMN, including WGA, PKH67 and anti-CD11b/CD66b labeling. Under our experimental setting, the CellMask labeling was the best combination when evaluating the interaction between extracellular mitochondria and PMN. For the mitochondrial content increase, MitoTracker labelling is controversial and unreliable. This is the reason we proceeded with immunoblotting of mitochondria protein (COX IV) and cellular respiration, which we believe provides a better overall view of the functionality of freeMitos upon their uptake by PMN.*

- in figure 2 the authors describe similarities between DAMP application and free mitochondria on calcium release, calpain activation and ev release - however there is no mechanistic insight (or investigation of its relevance), could it be that lysis of some mitochondria during preparation is phenocopying DAMP activity leading to these effects, and relating back to the first point how much is this representing genuinely free mitochondria released by platelets ?

- *We thank the reviewer for this comment. While we agree that we see some similarities between calcium release, calpain activation and PMN-MVs release, we see a significantly different transcription modulation profile and IL-8 release. We added some clarification in the results/discussion section to enhance our take-home message at line 184. We believe that our take-home message from this manuscript is that freeMitos immediately released from platelets can induce calcium release in immune cells, in this case PMN. FreeMitos have been shown to be located in tissues where PMN are abundant (i.e. synovial fluid of rheumatoid patients). Consequently, PMN activation doesn't require that the integrity of the freeMitos is completely or partially affected to initiate an inflammatory response. Since extracellular mitochondria integrity are extremely susceptible to inflammatory induced enzymes (i.e. sPLA2-IIA)*(Boudreau *et al*, 2014)*, we demonstrate that fully functional extracellular mitochondria can initiate a basal inflammatory response by PMN.*

Referee #3:

In the manuscript entitled "Functional Platelet-Derived Mitochondria Induce the Release of Human Neutrophil Microvesicles" the authors describe that isolated mitochondria from platelets induce the release of neutrophil micro vesicles and induce transcriptional chances in neutrophils.

While this is an interesting observation, it is not completely novel as part of this mechanism was already proposed in a previous paper by the last author (Boudreau et al Blood 2014). The induction of changes in gene-expression in neutrophils by isolated mitochondria from platelets is a novel observation, however the elicited changes in neutrophils did not explain why the neutrophils were more likely to release mitochondria.

- *We thank the reviewer for this comment. We do not state nor demonstrate that platelet-derived free mitochondria induce the release of mitochondria from neutrophils, but rather induce neutrophils to produce microvesicles. In fact, neutrophils do not harbour innate intracellular mitochondria. In addition, the paper cited by the reviewer is different from the current manuscript since that paper investigated sPLA2-IIA treated mitochondria (not freemitos) in the context of inflammation (arthritis) and platelet concentrate bags. In addition, the authors did not investigate the role or function of PMN-derived MVs.*

The authors investigated the oxidative state of neutrophils after incubation with isolated platelet mitochondria and didn't observe any changes in respiration. Changes in calcium mobilization were observed after incubation with platelet mitochondria in similar fashion as DAMPs, however the underling mechanism of which G-protein coupled receptor was engaged was not investigated. These changes in calcium mobilization resulted in more PMN-derived microvesicles, albeit with very high variation in figure 2 CD.

> *Our intention with the evaluation of neutrophil oxidative status post-incubation with platelet-derived mitochondria is to measure any functional benefit at the mitochondrial level in recipient cells. Given the potential contribution of foreign mitochondrial molecular components or function to the recipient cells, we evaluated mitochondrial-dependant activity in the form of overall oxidative respiration rates of recipient neutrophils. On the other hand, the purpose of Ca^{+} mobilization analysis was to first validate the preliminary signaling events (calcium mobilization) required for cells (i.e., PMNs) to produce and shed microvesicles, and secondly, to demonstrate the post-signaling events of DAMP activation in PMNs.*

As for the reviewer's comment on uninvestigated mechanisms involving G-protein coupled receptors, we are unsure of the reviewer's request and intent. We can assume that the comment potentially relates to G-coupled receptors and their role in the internalization process of recipient cells to various types of extracellular vesicles? As you may know, the internalization of various vesicles and/or extracellular cargo is a vast landscape with only a paucity of mechanistic elucidation studies, notably in platelet-derived biomaterial. We would be happy to provide more insight upon the reviewer's specific concern encompassing G-protein coupled receptors in our study. However, the characterization of neutrophil-freeMito interacting mechanisms was not our objective in the current study but rather, the resulting outcome of freeMito activation of PMN inflammatory response, which is highly reminiscent of DAMP signaling processes.

While the authors show in their isolated platelet mitochondrial system that neutrophils release extracellular vesicles, what is the role on physiological processes of inflammation was not investigated. Nor was there any in vivo evidence for this phenomenon, so it might be an in vitro artifact. The claims should be substantiated with both activated platelet and see if released mitochondria have the same effect on neutrophils.

- *It is a great comment by the reviewer. However, as indicated to the previous reviewer, there is an important technically to the isolation of activated platelets mitochondria and subsequent incubation with PMN using the same blood donor. While it would be of interest to further characterize the effects of freeMitos from activated platelets, it brings up an important question, which agonist to be used (i.e Thrombin vs collagen vs immune complex)? As each agonist release specific microvesicles cargo, they could also affect PMN signalling in their own way. Most importantly, since inflammatory enzymes are released from activated platelets and affect mitochondrial integrity* (Boudreau *et al*, 2014)*, we believe that our method is the best approach to obtain mitochondria that are immediately released from platelets.*
  From an in vivo standpoint, freeMitos have been found in several biological fluids, including in RA. Since neutrophil protein markers have been found in vesicles isolated from the synovial fluid of RA patients(Foers *et al*, 2020)*, freeMitos could contribute to the release of PMN-MVs in disease. We added some clarification at line 182.*

Ca-mobilization in neutrophils will effect a wide variety of biological neutrophil functions as increase in chemotaxis/migration, enhanced ROS production and increased phagocytosis. It is unclear why the authors did not investigate these process and focused on release of microvesicles and not on other better established neutrophil functions.

- *We agree with the reviewer that other physiological functions of neutrophils could have been investigated. We focussed on PMN since other labs have previously looked at the effects of DAMPs on PMN (reviewed in (Pittman & Kubes, 2013), but none have ever evaluated the effects on microvesicles release. In addition, for this short study, since our transcriptomic effect both revealed genes that regulate calcium and microvesicles formation, this is the reason we have focused on PMN-MVs. This is the novel findings of our study. We strongly believe that characterization of the other PMN physiological functions by intact freeMitos is of great interest but is out of the scope of the current study.*

Technical comments:

In Figure 1 A-C the authors claim that mitotracker labeled platelets are taken up by neutrophils. However, the dye used to label mitochondria is not covalently attached to mitochondria and based on membrane potential. How can the authors exclude the possibility that it is not dye transfer from labelled platelet mitochondria that label then neutrophil's mitochondria.

- *We agree with the reviewer that the MitoTracker dyes have some limitation (see response to reviewer #1). We now mention this in the revised manuscript at line 237. As stated, we add the MitoTracker dye (100 nM, lower end of the concentration usually used with this type of dye in immune cells) to the whole platelet before the mitochondria isolation* (Léger *et al*, 2021, 2020). *Cells are washed, then mitochondria are isolated and co-incubated with PMN. While highly unlikely given our experimental setting, we agree with the reviewer that we cannot exclude a possible transfer to the recipient PMN mitochondria. However, this would confirm an interaction between platelet-derived mitochondria and PMN, which is the main objective of the first part of the study.*

References:

Boudreau LH, Duchez A-C, Cloutier N, Soulet D, Martin N, Bollinger J, Paré A, Rousseau M, Naika GS, Lévesque T, *et al* (2014) Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood* 124: 2173–2183

Caicedo A, Zambrano K, Sanon S, Luis Vélez J, Montalvo M, Jara F, Moscoso SA, Vélez P, Maldonado A & Velarde G (2021) The diversity and coexistence of extracellular mitochondria in circulation: A friend or foe of the immune system. *Mitochondrion* 58: 270–284

Dow R & Ridger V (2021) Neutrophil microvesicles and their role in disease. *Int J Biochem Cell Biol* 141: 106097

Foers AD, Dagley LF, Chatfield S, Webb AI, Cheng L, Hill AF, Wicks IP & Pang KC (2020) Proteomic analysis of extracellular vesicles reveals an immunogenic cargo in rheumatoid arthritis synovial fluid. *Clin Transl Immunology* 9: e1185

Léger JL, Jougleux J-L, Savadogo F, Pichaud N & Boudreau LH (2020) Rapid isolation and purification of functional platelet mitochondria using a discontinuous Percoll gradient. *Platelets* 31: 258–264

Léger JL, Pichaud N & Boudreau LH (2021) Purification of Functional Platelet Mitochondria Using a Discontinuous Percoll Gradient. *Methods Mol Biol* 2276: 57–66

Pittman K & Kubes P (2013) Damage-associated molecular patterns control neutrophil recruitment. *J Innate Immun* 5: 315–323

Sargiacomo C, Stonehouse S, Moftakhar Z, Sotgia F & Lisanti MP (2021) MitoTracker Deep Red (MTDR) Is a Metabolic Inhibitor for Targeting Mitochondria and Eradicating Cancer Stem Cells (CSCs), With Anti-Tumor and Anti-Metastatic Activity In Vivo. *Front Oncol* 11: 678343

Dear Dr. Boudreau,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. Original referee #1 was not responsive to my invitations to re-assess the study. However, reading your point-by-point response, I consider his/her points as adequately addressed. As you will see, referee #2 now supports the publication of your study, but suggests textual changes to the discussion. In contrast, referee #3 does still not support publication of the study.

Considering that original referee #1 already supported publication of the previous version of the study after minor revisions (and his/her points have been addressed in a satisfactory manner during revision) and that referee #2 is now also positive (and indicated during cross commenting that further in vivo data is outside the scope of the current study), I decided to proceed with the manuscript. I thus ask you to address the remaining point of referee #2 in a final revised manuscript. Please also provide a final point-by-point response addressing the remaining issues by both referees. I would also suggest discussing limitations further in the final revised manuscript (i.e. the future need of in vivo validation), also strengthening your arguments that the findings likely have in vivo relevance (and are not an in vitro artefact).

Moreover, I have these editorial requests I also ask you to address:

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- Also in the authors checklist, please provide details on the entry in row 49 ('Short novel DNA or RNA including primers'). Please fill in column E.

- Please order the manuscript sections like this (using these names):
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- Please add a title page with a table of contents to the Appendix file. Please fit Appendix Table S2 onto one page. Please add all methods (and related references) to the main text of the manuscript and remove the methods information from the Appendix file.

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I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

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Best,

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Senior Editor
EMBO Reports

Referee #2:

The authors have satisfactorily addressed my comments through textual rebuttal. I appreciate the technical difficulties in various aspects of the work - not least isolation of released mitochondria from activated platelets - nonetheless would suggest that some commentary is added to the discussion noting the potential caveats of assaying mitochondria from experimentally lysed platelets vs. mitochondria released from platelets.

Referee #3:

I do appreciate the efforts of the authors to address my concerns, however even after this round of revision the author fail to explain how (molecular mechanism) the changes in gene-expression in neutrophils by isolated mitochondria from platelets, explain why the neutrophils were more likely to release microvesicles.

They also did not perform additional experiments to show that this is not an in vitro artifact and that release mitochondria from activated platelets could elicit the release of microvescicles. Nor did they provide any in vivo evidence this phenotype exists in animal models.

This paper is of interest however it needs additional work to make it suitable for publication in EMBO reports.

Referee #2:

The authors have satisfactorily addressed my comments through textual rebuttal. I appreciate the technical difficulties in various aspects of the work - not least isolation of released mitochondria from activated platelets - nonetheless would suggest that some commentary is added to the discussion noting the potential caveats of assaying mitochondria from experimentally lysed platelets vs. mitochondria released from platelets.

- *We are very pleased that we have addressed the reviewer's concerns in our rebuttal. We thank the reviewer for their great comment. We agree with the reviewer that subsequent studies will be needed to further explore the integrity of extracellular mitochondria from platelet origin that are obtained from lysed platelets versus those generated from platelet activation. Since we previously demonstrate that the subpopulation of platelet-derived extracellular mitochondria is dependent on the type of agonist used (thrombin, collagen, ADP or immune complexes), it would be a stand- study alone (but a very interesting one!) to determine the integrity and content (RNA, protein, etc.) of extracellular mitochondria. We have added additional comments on this in the discussion at line 188.*

Referee #3:

I do appreciate the efforts of the authors to address my concerns, however even after this round of revision the author fail to explain how (molecular mechanism) the changes in gene-expression in neutrophils by isolated mitochondria from platelets, explain why the neutrophils were more likely to release microvesicles.

- *We thank the reviewer for the constructive comment that helped us strengthen the manuscript. We agree that subsequent studies should investigate the molecular mechanism that affects the gene expression in neutrophils. For the current study, however, we strongly believe that we have shown both strong transcriptomic and protein data (calpain's pathway) to support our findings.*

They also did not perform additional experiments to show that this is not an in vitro artifact and that release mitochondria from activated platelets could elicit the release of microvescicles. Nor did they provide any in vivo evidence this phenotype exists in animal models.

- *This is a great comment by the reviewer; however, in vivo studies are not in the current scope of the study. The fact that sterile inflammation causes the release of PMV-MVs is of great significance to the field of extracellular vesicles. Before moving to in vivo models, we need further ex vivo and in vitro studies to better characterized freeMitos released from activated platelets.*

This paper is of interest however it needs additional work to make it suitable for publication in EMBO reports.

Dr. Luc Boudreau
Université de Moncton
Department of Chemistry and Biochemistry
Canada

Dear Dr. Boudreau,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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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 replicates.
- 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 Presentation.

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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.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
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