EMBO reports

Pannexin-1 limits the Production of proinflammatory Cytokines during Necroptosis

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(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 February 2019

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think the manuscript is of interest, but requires major revisions to allow publication here. As the reports are below, I will not detail them here, but most importantly, and required for further consideration here, the physiological relevance of the findings needs to be proven, as indicated by referee #1 (major point 2 of referee #1, and also point 2 of referee #2). I think the experiment suggested by referee #1 (harvest EVs, treat macrophages, and analyse effects on inflammation) would address this. Moreover, we agree with referees #2 and #3 that the use of a KO cell line would significantly strengthen the conclusions (point 1 of referee #1 and of referee #3). I also agree with referee #1 that the manuscript needs significant rewriting, also to describe in a more comprehensive manner the goals and major findings of the study.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact me if a 3-months time frame is not sufficient so that we can discuss the revisions further.

Supplementary/additional data: 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 labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

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When submitting your revised manuscript, we will require:
- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

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

REFEREE REPORTS

Referee #1:

Duoanne etal. Have proposed a role for Pannexin 1 in regulating EV release during necroptosis. They propose it is PANX1 that gives cells their "leakiness" prior to cell death and that Rab27a/b are both important for EV release and PANX1 activity.

Major points:

1) While this paper nicely identifies a role for PANX1 in release of EVs from necroptotic cells, the mechanism and relation to MLKL are unclear from the manuscript. After reading through the manuscript several times I am afraid to admit that I am still uncertain as to what the main point of the manuscript is. Is it about the leakiness, or about panx1 playing a role in EV generation, or is it about EV release being an important aspect of necroptotis that is regulated by panx1? As it is written it comes across more as a series of experiments without a coherent narrative. From the data it seems

that MLKL activation is required for PANX1 activation, but not vice versa. Panx1 activity is required for EV release. Panx1 activation then promotes cleavage of MLKL somehow, probably through it openning, although if this cleavage is important or not is not further pursued, although it is used as a surrogate marker for PANX1 activity.
The authors then appear to say that RAB27 activity is also required for release of EVs and PANX1 activation. This may be supported by PANX1 or Rab27b loss seeming to have the same effect on EV generation, at least in retaining CD63+ structures in perinuclear region (Fig. 5D), while MLKL and rab27a loss seem to have smaller vesicles and secrete less. However the evidence that PANX1 is not activated in the absence of Rab27 is, I think, not presented, other than to use cleavage of MLKL as a marker for example. I am still at a loss however in the model suggested by the reviewers, what PANX1 and Rab27 are doing and how they interact with each other. This is not discussed in any length in the manuscript and make it very difficult to follow what the actual goal of the study is.

2) Secondly it is not clear to what extent this pathway has any biological activity. If the authors wish to make the argument that EVs secreted with the necroptotic stimuli lead to a more potent inflammation (this appears to be their argument in the end of the discussion), then it would be useful to harvest the EVs and then wash them and then add them to macrophages for example to see what inflammatory affect is produced.

Other than the property of allowing TOPRO-3 to enter cell, what mechanistically does this "leakiness" mean. Given this seems to be the main point of the paper, this is not really addressed. Are the authors proposing that it helps to recruit immune cells to sites of necroptotic cell death? This again could be further addressed by the above experiment to look for secretion of chemokines or the authors could perfomr trans-well migration assays comparing PANX1si cells to control si cells for example.

Overall, the manuscript needs significant rewriting to make it clear what the goal of the study is and the relevance this has on EV biology or necroptosis or both.

Specific points:
• There is no mention of Panx1 or EV generation/release in the introduction at all. This seems to be a rather crucial topic to address given it is not likely to be an area of a lot of expertise for the necroptosis field. This topic should therefore be introduced in the intro too.
• It is curious that the authors have chosen to use QVD as the caspase inhibitor to induce necroptosis. I am surprised this is working so well, as generally QVD does not block the caspase-8/cFLIP heterodimers that are required to cleave RIPK1/3, and therefor is a very poor necroptosis inducer. For this reason most stimuli use zVAD.FMK instead. Have the authors tried to use this instead of QVD? It would be interesting to determine if ZVAD can block the cleavage of MLKL and PANX1.
• On Page 4 the authors make the claim that because rab27 silenced cells do not have the leakiness and also cleavage of MLKL, that PANX1 is not activated. As there is no clear reason why these events are happening I do not think this data supports this conclusion. Perhaps I am mistaken but MLKL cleavage is not an accepted measure of PANX1 activity. To make this claim, the authors should look at something known to be directly due to PANX1 activity, such as ATP release.
• I think the authors should make more of a point about MLKL being required for EV release without necroptosis stimuli (see figure 5A-C). This has of course been shown previously (Yoon et.al.), but indicates that MLKL has a more general role in vesicle traffic, which does not appear to be shared by PANX1, which only seems to be playing a role in necroptotic stimuli.
• The PANX1 and Rab27b phenotypes appear strikingly similar to each other, but different to MLKL or rab27a si cells (see figure 5D). Is it known if rab27b and panx1 interact at the PM?
• Figure 5H, the authors claim that loss of PANX1 did not affect volume, just numbers, but the quantification would suggest otherwise.

Referee #2:

The manuscript by Douanne et al. demonstrates the activation of Pannexin-1 downstream of MLKL oligomerization and its consequences. The approach is solid, the experiments are well controlled and most of the data are clean. Thus, I support the publication of this manuscript with several minor

concerns.

1) The most important data are the involvement of Pannexin-1 upon necroptosis. Although the authors have used both siRNA and small chemical compound inhibitor, however, the strength of the data can be further improved by either crispr based knockout or another independent siRNA oligo(es). Also, a pannexin-1 reconstitution should rescue the phenotype of the loss-of-function deficiency (Very critical!!).

2) It is important at least to indicate the probable physiological/biological function of the activation of Pannexin-1 "channel". This would help to generalize the concept and expand the impacts of this article. As Pannexin-1 channel is involved in extracellular vesicle release, which has been associated with IL-1b release/secretion in BMDCs (S Yoon, et al. Immunity, 2017), the author could use the same system (just do siRNA based KD) to test the role of Pannexin-1 in Il-1b production. This is a very simple in vitro assay (fig. 6-7) and can be easily done by most labs. Other equivalent/alternative assays to test the biology of Pannixin-1 during necroptosis are also encouraged.

3) This paper didn't really touch the concept of "channel" (such as what molecule/charged ions passed through during necroptosis), but only mentioned the requirement of Pannexin-1. Thus I would suggest in the title, the authors should remove "channel", which could be misleading. Just state "Pannexin-1 govern the...", which is more precise.

Referee #3:

The manuscript of Douanne et al. reports on a novel link between MLKL, Pannexin-1 channels and extracellular vesicle generation in the context of necroptosis induction. The authors report that MLKL oligomers activate Pannexin-1 channels concomitantly to the loss of phosphatidylserine asymmetry. They also present that Pannexin-1 channels operate downstream of MLKL, to favor necroptosis-driven release of small extracellular vesicles. Overall, this is a good study that would be of interest to the field. Please find below some remarks that should clarify some aspects of the study.

Comments

•Many of the conclusions are drawn based on the use of a single siRNA or a unique pharmacological inhibitor. The authors should generate KO lines for the studied genes by using the CRISP/Cas9 technology, or at least show results obtained with different siRNAs for each gene. Trovafloxacin is used as a Panx-1 pharmacological inhibitor. The specificity of this inhibitor is however not clear. The study would benefit from using an extra Panx-1 inhibitor, i.e. the 10Panx-1 peptide, to strengthen the observations.

•The authors should explain in the introduction the rational for focusing on Pannexin-1 channels, and refer to the literature on the concept of hemichannels. Because hemichannels can also be formed by connexins, it would be of interest to additionally test the effect of inhibiting Connexin hemichannels, e.g. with the Connexin hemichannel blockers L2 or Gap19 (Cx-mimetic peptides for Cx43).

•The authors should be consistent in the study of the two variants of Rab27 (influence of depletion on MLKL oligomerization and the AnnV staining), to be able to draw conclusions on functional differences.

•The basal viability of the cells in the untreated condition is very low in Fig 1C? This figure shows an average of 74% living cells in UT condition (meaning 26% of dead cells). This is not a normal background level of a healthy culture. It would be good to repeat the experiment with healthier cultures.

•Throughout the article, the used term 'small Extracellular Vesicles' could be more precise. The term 'Extracellular Vesicles' is referring to secreted vesicles, and no consensus was reached on terminology. Adding 'small', however, does not provide extra information, and should hence be replaced by the appropriate terminology, e.g. exosomes, microvesicles, ectosomes.

•The authors make the following statement:
"Although less dramatic than during apoptosis [21,25], PANX1 was also cleaved during necroptosis at a site close to the characterized caspase site, as evidenced by a slight decrease in full-length PANX1 combined with the presence of a cleaved fragment in PNGase F-treated samples (Fig. 2A, 2G and S2A)."
How can the authors be sure that the site is 'close to the characterized caspase site'? Would it not be possible to have another site, on the other extremity of the protein, which can also give a similar size?

•The statement regarding the less intense lower band of PANX1 upon Trovafloxacin in necroptosis conditions is speculative. The observation of a reduced cleaved form of PANX1 upon Trovafloxacin in necroptosis (while no modification was observed during apoptosis (Fig S2B and S2C)) has to be put in perspective with Fig 2I, where less cell death is shown. This decrease can also be due to lower cell death levels (reflecting a less intense band).

•The authors should present the uncut blots for MLKL in Fig 5A and 5B. Concerning those same blots, it is very speculative how the authors claim that 'the PANX1 band appeared slightly smaller, suggesting that it may be cleaved PANX1.'

•In Fig 5A-B, what was loaded for the WB on extracellular vesicles: same volume [after purification] or same quantity [protein]? This information should also be added to the Materials & Methods section.

•X-axis ticks are missing in Fig 1K.

•Ref. 13 is not complete.

1st Revision - authors' response 12 June 2019

Response to Reviewer 1

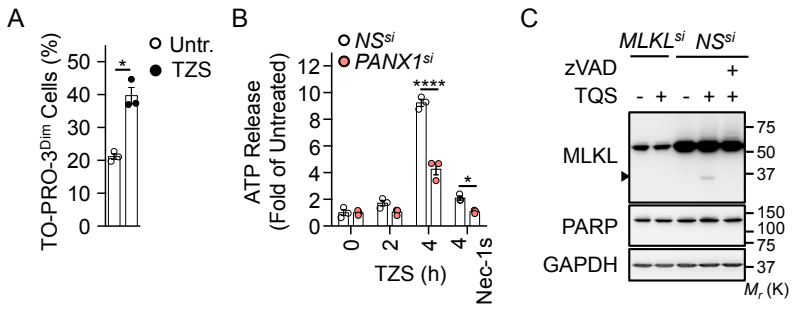
1. After reading through the manuscript several times I am afraid to admit that I am still uncertain as to what the main point of the manuscript is
We thank the Reviewer for his/her honest assessment on our initial manuscript. In order to clarify our message, both the text and the figures of the revised manuscript were significantly rearranged.

2. It is not clear to what extent this pathway has any biological activity. If the authors wish to make the argument that EVs secreted with the necroptotic stimuli lead to a more potent inflammation (…), then it would be useful to harvest the EVs and then wash them and then add them to macrophages for example to see what inflammatory affect is produced. Other than the property of allowing TO-PRO-3 to enter cell, what mechanistically does this "leakiness" mean. Given this seems to be the main point of the paper, this is not really addressed. Are the authors proposing that it helps to recruit immune cells to sites of necroptotic cell death? This again could be further addressed by the above experiment to look for secretion of chemokines or the authors could perform trans-well migration assays comparing *PANX1*^{si} cells to *control*^{si} cells for example.
We carried out several experiments to address these concerns, also raised by Reviewer 2. This involved the use of additional strategies to silence (RNAi, CRISPR/Cas9) or inactivate (chemical inhibition) PANX1. As expected from our initial manuscript, this led to a nearly complete inhibition in the uptake of TO-PRO-3. By contrast, cell death was only modestly affected, reinforcing the idea that “leakiness” of the plasma membrane may be dissociable from cell death (please, see the new Figure 2).
In addition to executing cell death, active MLKL targets intracellular vesicles and was demonstrated to regulate constitutive endosomal trafficking, the synthesis and secretion of small extracellular vesicles (EVs) [1–3]. MLKL oligomers also govern the production of pro-inflammatory chemokines during the early steps of necroptosis [4]. We inferred that PANX1 activation influences these MLKL cell death-independent functions. First, small EVs purified from necroptotic HT-29 cells were mixed

with human monocytes/macrophages, and pro-inflammatory signature was assessed by qPCR [5]. However, these attempts remained unsuccessful and additional work will be required to set up a robust experimental system. In a second series of experiments, we carried out an unbiased antibodies array for the presence of 120 cytokines in the culture media from cells engaged in the necroptotic process. Supporting a recent publication [4], triggering necroptosis promoted the expression and release of a subset of pro-inflammatory cytokines such as IL-8/CXCL8 and CXCL1. This was further enhanced when PANX1 was silenced, suggesting a role for PANX1 in tampering with inflammation. In line with this, qPCR analysis revealed that more IL-8 mRNA was produced without PANX1. This was accompanied by an increase in the abundance in IL-8, as measured by immunoblotting and ELISA. Importantly, the same was true in cells treated with the PANX1 inhibitor CBX. Consistent with the data from the literature [6], most of released IL-8 was soluble whereas only a small amount could be detected within small EVs (our unpublished results). Of note, the presence of the necroptotic stimuli in the supernatants from HT-29 cells did not allow us to perform transwell experiments with confidence. Lastly, we assessed the impact of RAB27 proteins, which allow PANX1-mediated "leakiness" of the plasma membrane, on IL-8 synthesis. Paralleling the situation with PANX1, the silencing of RAB27A or RAB27B significantly increased the production of IL-8. Taken together, our data suggests that MLKL initiates a negative feedback loop via PANX1 to finely tune the production of cytokines during necroptosis. These results are now included in the new Figure 4 of the manuscript.

3. There is no mention of Panx1 or EV generation/release in the introduction at all. This seems to be a rather crucial topic given it is not likely to be an area of a lot of expertise for the necroptosis field. This topic should therefore be introduced in the intro too.
We thank the Reviewer for this suggestion, and amended the text accordingly.

4. It is curious that the authors have chosen to use QVD as the caspase inhibitor to induce necroptosis. I am surprised this is working so well, as generally QVD does not block the caspase-8/cFLIP heterodimers that are required to cleave RIPK1/3, and therefor is a very poor necroptosis inducer. For this reason, most stimuli use zVAD.FMK instead. Have the authors tried to use this instead of QVD? It would be interesting to determine if ZVAD can block the cleavage of MLKL and PANX1.
We agree with the Reviewer that zVAD.fmk is often used to study necroptosis. However, the potent caspase inhibitor QVD is also widely used to efficiently trigger necroptosis [7–13]. Albeit less violent, cell death with QVD was potent and entirely prevented in MLKL-silenced cells, further reinforcing the idea that it is indeed necroptosis. Our data with QVD that cells became "leaky" to TO-PRO-3 nicely fits with similar observations made by Gong *et al* in cells treated with TZS [14]. Accordingly, we observed that the substitution of QVD with zVAD.fmk also led to an increase in the uptake of TO-PRO-3 by cells. This was also accompanied by a release of ATP in the culture media, which was significantly reduced when PANX1 was silenced. Prompted by the Reviewer, we further assessed the impact of zVAD.fmk on MLKL, and found that it prevented its cleavage. zVAD.fmk has been shown to target additional proteases besides of caspases [15–18]. Because silencing PANX1, ITPK1, RAB27 or treatment with NSA and PANX1 inhibitors also prevented MLKL processing, it is tempting to speculate that a non-caspase, zVAD.fmk-sensitive, protease may be involved in this post-signaling event.

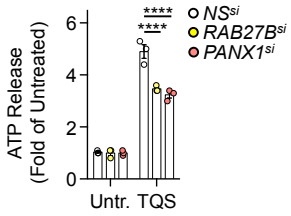


A, *HT-29 cells were pre-treated with 20 mM zVAD (Z) plus 5 mM Birinapant (S), and exposed to 10 ng.mL^{-1} of TNFa (T) for 4 hours. TO-PRO-3 uptake was analyzed by flow cytometry. Data are means ± SEM of three independent experiments. *P<0.1 (t-test).* ***B,*** *HT-29 cells were transfected with the indicated siRNAs for 72 hrs, and treated as in (A). Necrostatin-1 (Nec-1s, 20 mM) was also used. ATP release in cell supernatants was evaluated by CellTiter-Glo (means ± SEM, n=3, *P<0.1, ****P<0.0001, ANOVA).* ***C,*** *HT-29 cells were transfected with the indicated siRNAs for 72 hrs, and*

treated as indicated for 5 hours. Western blotting analysis was performed with the indicated antibodies. Arrowhead, MLKL cleaved fragment. Molecular weight markers (M_r) are shown.

5. On Page 4 the authors make the claim that because Rab27 silenced cells do not have the leakiness and also cleavage of MLKL, that PANX1 is not activated. As there is no clear reason why these events are happening I do not think this data supports this conclusion. Perhaps I am mistaken but MLKL cleavage is not an accepted measure of PANX1 activity. To make this claim, the authors should look at something known to be directly due to PANX1 activity, such as ATP release.

We agree that although MLKL processing does not occur when PANX1 is silenced or inhibited, this does not constitute, *per se*, a marker for PANX1 activation. However, the uptake of TO-PRO-3 is a well-accepted read-out for PANX1 activation [19–24], and our extensive work with siRNA, CRISPR/Cas9 and small inhibitor compounds shows that TO-PRO-3 uptake during the early stages of necroptosis reflects the activation of PANX1. Hence, the decrease in TO-PRO-3 uptake without RAB27 likely reflects a defect in PANX1 activation. This was confirmed by a decrease in the release of ATP in the supernatants of cells silenced for RAB27B or PANX1.



*HT-29 cells were transfected with the indicated siRNAs for 72 hrs, and pre-treated with 10 mM QVD-OPh (Q) together with 5 mM Birinapant (S), prior stimulation with 10 ng.mL^{-1} of TNFa (T) for 6 hours. ATP release in cell supernatants was evaluated by CellTiter-Glo (means ± SEM, n=3, ****P<0.0001, ANOVA).*

6. I think the authors should make more of a point about MLKL being required for EV release without necroptosis stimuli (see figure 5A-C). This has of course been shown previously [1], but indicates that MLKL has a more general role in vesicle traffic, which does not appear to be shared by PANX1, which only seems to be playing a role in necroptotic stimuli.

We agree with the Reviewer, and changed the manuscript accordingly. A simplified Expanded View Figure (Fig. EV5) now deals with this facet of MLKL.

7. The PANX1 and Rab27b phenotypes appear strikingly similar to each other, but different to MLKL or rab27a si cells (see figure 5D). Is it known if rab27b and panx1 interact at the PM?

This is an insightful suggestion, which we tried to address experimentally. However, we failed to detect significant binding of PANX1 to RAB27 (data not shown).

8. Figure 5H, the authors claim that loss of PANX1 did not affect volume, just numbers, but the quantification would suggest otherwise.

The reviewer is right. Thank you for catching this mistake.

Response to Reviewer #2

1. The most important data are the involvement of Pannexin-1 upon necroptosis. Although the authors have used both siRNA and small chemical compound inhibitor, however, the strength of the data can be further improved by either crispr based knockout or another independent siRNA oligo(es). Also, a pannexin-1 reconstitution should rescue the phenotype of the loss-of-function deficiency (Very critical!!).

This is a valid point also raised by Reviewer #3 (please see his/her points 1 and 2), and several lines of experimentations were therefore carried out. First, two additional siRNA sequences, which led to a very efficient knockdown, were used. Both oligoribonucleotides potently suppressed the uptake of TO-PRO-3 and enhanced the production of pro-inflammatory IL-8 during necroptosis (please, see the new Figures 2 and 4). We then deployed a CRISPR/Cas9 strategy to knock out PANX1. Two single-guide RNA (sgRNA) were used to deplete endogenous *PANX1* in HT-29 cells without single-cell cloning. Again, "leakiness" of the plasma membrane was also prevented in these settings (please, see the new Figure 2).

Rescuing those cells was a challenging task we tried to undertake. To this end, PANX1 (Ref. [25]) was retrovirally delivered to *PANX1*^{CRISPR} cells together with GFP. Not only PANX1 expression was

restored, but its abundance surpassed endogenous levels. We found that complementation of *PANX1*^{CRISPR} cells restored the ability to uptake TO-PRO-3 in response to the necroptotic insult (new Figure 2). Of note, similar results were also obtained with an shRNA targeting *PANX1* in the 5'-UTR region (new Fig. EV4). Collectively, these new data reinforce our conclusion on the role of PANX1 during necroptosis.

2. It is important at least to indicate the probable physiological/biological function of the activation of Pannexin-1 "channel". This would help to generalize the concept and expand the impacts of this article. As Pannexin-1 channel is involved in extracellular vesicle release, which has been associated with IL-1b release/secretion in BMDCs [1], the author could use the same system (just do siRNA based KD) to test the role of Pannexin-1 in Il-1b production. This is a very simple in vitro assay (fig. 6-7) and can be easily done by most labs. Other equivalent/alternative assays to test the biology of Pannexin-1 during necroptosis are also encouraged.
This insightful remark was also shared with Reviewer 1, and several aspects are therefore discussed in our response to Reviewer 1, point 2. In brief, we now conclusively show that PANX1 restrains MLKL-mediated production of pro-inflammatory cytokines such as IL-8. These results suggest new ways to modulate some of the MLKL-dependent pro-inflammatory functions associated with necroptosis.

3. This paper didn't really touch the concept of "channel" (such as what molecule/charged ions passed through during necroptosis), but only mentioned the requirement of Pannexin-1. Thus I would suggest in the title, the authors should remove "channel", which could be misleading. Just state "Pannexin-1 govern the...", which is more precise.
We agree and changed the title as suggested.

Response to Reviewer #3

1. Many of the conclusions are drawn based on the use of a single siRNA or a unique pharmacological inhibitor. The authors should generate KO lines for the studied genes by using the CRISP/Cas9 technology, or at least show results obtained with different siRNAs for each gene. Trovafloxacin is used as a Panx-1 pharmacological inhibitor. The specificity of this inhibitor is however not clear. The study would benefit from using an extra Panx-1 inhibitor, i.e. the 10Panx-1 peptide, to strengthen the observations.
Some of these concerns were shared with Reviewer 2 (please, see our response to his/her point 1). In addition to our extensive work on PANX1 silencing, deletion and inhibition (data presented in the new Figure 2), we implemented our revised manuscript with additional siRNA sequences for MLKL, ITPK1 and RAB27B (please, see the new Figures EV1 and EV5). Of note, RAB27A knockdown was obtained with a mixture of siRNA. This is now clearly indicated in the methods section. Prompted by the Reviewer, two well-accepted PANX1 chemical inhibitors, namely CBX and Probenecid, were tested in addition to Trovafloxacin. Both inhibitors potently suppressed the uptake of TO-PRO-3 during necroptosis. However, when incubated with for 24 hours, we noted a potential toxic effect of Probenecid and Trovafloxacin, and therefore focused on CBX. These data, presented Figure 2, reinforce our conclusions on the role of PANX1 during necroptosis.

2. The authors should explain in the introduction the rational for focusing on Pannexin-1 channels, and refer to the literature on the concept of hemichannels. Because hemichannels can also be formed by connexins, it would be of interest to additionally test the effect of inhibiting Connexin hemichannels, e.g. with the Connexin hemichannel blockers L2 or Gap19 (Cx-mimetic peptides for Cx43).
We agree, and changes have been made in the introduction. At the Reviewer's suggestion, HT-29 cells were pretreated with Gap19. This did not alter the uptake of TO-PRO-3 during necroptosis. We also used lanthanum, which was shown to serve as a connexin, and not PANX1, channel blocker (Refs [26–28]), and found no impact on PM "leakiness". These results were included in the new Figure EV4.

3.The authors should be consistent in the study of the two variants of Rab27 (influence of depletion on MLKL oligomerization and the AnnV staining), to be able to draw conclusions on functional differences.

We agree that this was rather confusing, and therefore repeated the key experiments in order to visualize the impact of RAB27A and RAB27B on the same blots and graphs (Figure 3H-K).

4. The basal viability of the cells in the untreated condition is very low in Fig 1C? This figure shows an average of 74% living cells in UT condition (meaning 26% of dead cells). This is not a normal background level of a healthy culture. It would be good to repeat the experiment with healthier cultures.
We thank the Reviewer for this comment. A repeat experiment is now presented on Figure 1C.

5. Throughout the article, the used term 'small Extracellular Vesicles' could be more precise. The term 'Extracellular Vesicles' is referring to secreted vesicles, and no consensus was reached on terminology. Adding 'small', however, does not provide extra information, and should hence be replaced by the appropriate terminology, e.g. exosomes, microvesicles, ectosomes.
Although it is still true that no consensus has yet emerged on specific markers of EV subtypes, such as endosome-origin “exosomes” and plasma membrane-derived “ectosomes”; the International Society of Extracellular Vesicles (ISEV) endorses the generic term of “extracellular vesicles” to define non self-replicating particles delimited by a lipid bilayer and released from cells [29]. In keeping with this idea, EVs can be rather defined based on functional properties, such as their size where “small EVs” (sEVs) range around 100 nm, while “large EVs” are above 200 nm. Alternatively, EVs can be distinguished according to their density, their biochemical composition (eg CD63-positive EV, AnnexinV-positive EV), or referred in a more descriptive way (eg hypoxic EVs, oncosomes, apoptotic bodies). In agreement with the ISEV recommendation and nomenclature, the “small EVs” term was preferred.

6. The authors make the following statement: "Although less dramatic than during apoptosis, PANX1 was also cleaved during necroptosis at a site close to the characterized caspase site, as evidenced by a slight decrease in full-length PANX1 combined with the presence of a cleaved fragment in PNGase F-treated samples (Fig. 2A, 2G and S2A)."
How can the authors be sure that the site is 'close to the characterized caspase site'? Would it not be possible to have another site, on the other extremity of the protein, which can also give a similar size?
We apologize for not explaining better this aspect. The epitope recognized by our antibody to PANX1 is surrounding the Leucine residue in position 17. Hence, a processing at the NH_2-terminal part of the protein will cause a loss of immunoreactivity. The proteolysis we observe is therefore likely to occur on the COOH-terminal side of the protein. A cartoon has been included in the Figure EV4 to improve clarity. It should be stressed that PANX1 remains essentially intact during necroptosis, in contrast to apoptosis, and that this cleavage results from PANX1 activation (please, see below).

7. The statement regarding the less intense lower band of PANX1 upon Trovafloxacin in necroptosis conditions is speculative. The observation of a reduced cleaved form of PANX1 upon Trovafloxacin in necroptosis (while no modification was observed during apoptosis (Fig S2B and S2C)) has to be put in perspective with Fig 2I, where less cell death is shown. This decrease can also be due to lower cell death levels (reflecting a less intense band).
This is an interesting hypothesis, which we experimentally challenged. We found that inhibiting PANX1 with CBX, Probenecid or Trovafloxacin did not improve cell survival upon necroptosis in a standard Celltiter glo assay (new Figure 2). Yet, PANX1 residual processing was reduced whenever its activity was blocked (new Figure EV4N). Combined, these data suggest that PANX1 cleavage occurs subsequently to its activation irrespectively of cell death, and is unlikely to directly control its activation.

8. The authors should present the uncut blots for MLKL in Fig 5A and 5B. Concerning those same blots, it is very speculative how the authors claim that 'the PANX1 band appeared slightly smaller, suggesting that it may be cleaved PANX1.'
This is a valid point. However, at the Reviewer’s 1 request, we tried to simplify our message, and therefore removed this set of data from the revised manuscript.

9. In Fig 5A-B, what was loaded for the WB on extracellular vesicles: same volume [after purification] or same quantity [protein]? This information should also be added to the Materials & Methods section.
As mentioned above, those results were discarded from the revised manuscript for sake of clarity. Nevertheless, same volume of samples was loaded on the blots.

10. X-axis ticks are missing in Fig 1K.
We thank the Reviewer for catching this. Ticks are now back.

11. Ref. 13 is not complete. (Cai Z et al, NCB 2014)
This has been fixed.

2nd Editorial Decision 12 July 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, referees #1 and #2 now support the publication of your manuscript in EMBO reports. Referee #3 has further concerns we ask you to address in a final revised version of your manuscript. Please also address the suggestion of referee #2 regarding the heatmap (Fig. 4A).

Further, I have these editorial requests:

- Please add up to 5 keywords to the title page of the manuscript.

- EV Figures and EV Movies are separate groups of elements. Thus, please label and call out the EV Figures 'Figure EV1', 'Figure EV2' and 'Figure EV3', and the EV Movies 'Movie EV1' and 'Movie EV2'. Please carefully update all the call-outs in the manuscript text, and change the file names of the source data accordingly. Please finally check that figure panels are called out in the manuscript text.

- I seems there is no call-out for Figure 2F. Please check.

- Please provide the legends for the movies as separate text file per movie, and upload these ZIPed together with the movie file. Please remove these legends from the manuscript text file.

- Please combine the source data for one Figure into one pdf.

- Please provide the source data also for any new Western blot data provided in the final revised version (in case).

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

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.

REFEREE REPORTS

Referee #1:

The authors have adequately addressed the reviewer concerns and the manuscript is suitable for publication.

Referee #2:

Please change the color code of the heatmap in Figure 4A. The colors used here are kind of against intuition and very hard to read. I am afraid some of the audience will feel confused. Usually, people use warm color (red) for upregulation and cold color (blue) for downregulation. Most of the background colors (0) could be grey-ish color, for example. Please make this heatmap more readable.

Referee #3:

The authors performed new experiments to address most of my initial comments. I however still have problems with the interpretation of the newly generated results.

The authors present a new Fig.4 entitled "Pannexin-1 restrains the production of Cytokines associated with necroptosis". I have several problems with that figure. First of all, there is absolutely no proof that it is necroptosis, and not just TNF stimulation, that induces the production of cytokines. Second, it is very unclear to me whether Panx1 restrains the production of cytokines "associated with necroptosis". Indeed, Panx1 silencing already upregulates the expression of the cytokines in absence of TQS stimulation (Fig. 4A). It therefore seems crucial that the authors evaluate the effect of Panx1 silencing on the levels of cytokines expression in cells solely stimulated with TNF (and TNF+S?). Also, since the role of Panx1 would be downstream of MLKL, it is important to show that the enhanced cytokine expression caused by Panx1 repression is lost upon additional MLKL silencing. In line with this idea, the effect of CBX in panels 4F-H should also have been evaluated alone, and in association with siMLKL or siPanx1. Finally, it is unclear what the term "relative mRNA levels" means in the RT-PCR graphs. Are all the values relative to untreated NC ? or each TQS value relative to their corresponding untreated value? How do the authors mechanistically explain an effect on gene transcription?

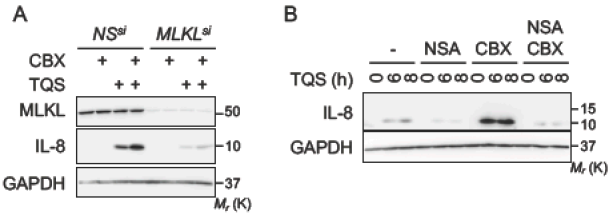
Additional comments:
- Carbenoxolone (CBX) does not only target pannexin-channels, but also gap junctions, hemichannels, Ca2+-channels, P2X7-receptors...
- Fig. 3A - 'NSA prevented MLKL oligomerization' - There is no oligomerization visible on that blot.
- Fig. 3C - p-RIPK1S166 is greatly increased upon ITPK1si, while the authors say it does not overtly change....
- Fig. 3D - Not clear whether ITPK1si affects MLKL oligomerization. The authors state that this small MLKL band (indicated with the arrow) is Panx-1 dependent, but silencing ITPK1 also prevent its appearance.
- Fig. 3G - This does not make sense. The authors state that RAB27a or RAB27b knockdown markedly reduce small EVs release while the graph shows that RAB27asi increases the amount of particles when compared to NSsi in untreated conditions.
- The authors state:" this data suggests that MLKL oligomerization and RAB27-dependent vesicular trafficking control "leakiness" of the plasma membrane. However, it does not seem that MLKL oligomerization is required since RAB27 silencing does not affect oligomerization but reduces TO-PRO-3DIM+ cells.
- What is the role of the "cleaved" fragment of MLKL? Does it have a direct role on TO-PRO-3 uptake?
- If MLKL is upstream of PANX1 and that PANX1 restrict cytokine expression, why don't you also see an increase, and not a decrease, in cytokine expression upon MLKL silencing ?

In conclusion, while it seems clear that PANX1 silencing affects the ability of TO-PRO-3 to enter the cells, the functional meaning of this "leakiness" is not really clear. The authors now propose that it would limit cytokine expression but the presented data are not convincing. The manuscripts ends up with many open questions, and it feels that at least some of these questions should already be answered in this manuscript for considering publication in the journal.

2nd Revision - authors' response 22 July 2019

1. The authors performed new experiments to address most of my initial comments.
We thank Reviewer#3 for this assessment of our revised work.

2. I however still have problems with the interpretation of the newly generated results. The authors present a new Fig.4 entitled "Pannexin-1 restrains the production of Cytokines associated with necroptosis". I have several problems with that figure. First of all, there is absolutely no proof that it is necroptosis, and not just TNF stimulation, that induces the production of cytokines. Second, it is very unclear to me whether Panx1 restrains the production of cytokines "associated with necroptosis". Indeed, Panx1 silencing already upregulates the expression of the cytokines in absence of TQS stimulation (Fig. 4A). It therefore seems crucial that the authors evaluate the effect of Panx1 silencing on the levels of cytokines expression in cells solely stimulated with TNF (and TNF+S?). Also, since the role of Panx1 would be downstream of MLKL, it is important to show that the enhanced cytokine expression caused by Panx1 repression is lost upon additional MLKL silencing. In line with this idea, the effect of CBX in panels 4F-H should also have been evaluated alone, and in association with siMLKL or siPanx1. (...) How do the authors mechanistically explain an effect on gene transcription?
Reviewer#3 wonders whether TNFa could promote IL-8 production in non-necroptotic cells, and whether interfering with MLKL would prevent IL-8 exacerbated induction resulting from PANX1 inactivation. Hints may come from the seminal work from Junying Yuan's group (Zhu K *et al*, Cell Death and Disease 2018), in which it was elegantly demonstrated that IL-8 is induced by TNFa solely in the context of necroptosis and not upon challenge with TNFa alone or combined with Smac mimetics. The production of cytokines associated with necroptosis requires the RIP1/RIP3/MLKL nexus and involves the activation of NF-kB (Zhu K *et al*, Cell Death and Disease 2018). Although we agree that defining how exactly PANX1 fits in this signaling pathway and cooperates with MLKL is interesting, we feel that it falls beyond the scope of the current manuscript. Nevertheless, prompted by the Reviewer, we tested whether interfering with MLKL represses the enhanced IL-8 induction driven by CBX during necroptosis. As shown below, the induction of IL-8 by TQS or by TQS+CBX was inhibited in MLKL-silenced cells (panel A, below). The same was true at the mRNA level (data not shown). In agreement, the treatment with necrosulfonamide (NSA) to inhibit MLKL also efficiently prevented necroptosis-mediated IL-8 expression, both in the presence and in the absence of CBX (panel B, below). Although a slight increase in our cytokines array is observed without PANX1 (Fig. 4A), the transcription of IL-8 and its abundance, as measured by western blotting and ELISA, are not changed at the basal level. The same is true with cells treated with CBX alone (Fig. 4B-H). Combined, this suggests that PANX1 inhibition exacerbates the MLKL-dependent production of IL-8 associated with necroptosis.



A, *HT-29 cells were transfected with a siRNA for MLKL, or scramble non-specific (NS) siRNA for 72 hours. Cells were pre-treated with 10 μM QVD-Oph (Q) plus 5 μM Birinapant (S), 100 μM Carbenoxolone (CBX), and exposed to 10 ng.mL-1 of TNFa (T) for 6 hours. Western blotting analysis with the indicated antibodies. Molecular weight markers (Mr) are shown.*
B, *HT-29 cells were treated as in (A), and 5 μM Necrosulfonamide (NSA) was also used. Western blotting analysis was performed with the indicated antibodies.*

3. Finally, it is unclear what the term "relative mRNA levels" means in the RT-PCR graphs. Are all the values relative to untreated NC ? or each TQS value relative to their corresponding untreated value?
The values presented are relative to untreated normal controls. This is now clearly stated in the revised manuscript.

4. Carbenoxolone (CBX) does not only target pannexin-channels, but also gap junctions, hemichannels, Ca2+-channels, P2X7-receptors...
We agree with the Referee that CBX does not solely targets PANX1. Nevertheless, our

conclusions obtained with CBX were also supported by data with RNAi and CRISPR.

5. Fig. 3A - 'NSA prevented MLKL oligomerization' - There is no oligomerization visible on that blot.
At the Reviewer's suggestion, another exposure was used. The same was done for Fig.3D.

6. Fig. 3C - p-RIPK1S166 is greatly increased upon ITPK1si, while the authors say it does not overtly change....
The text was changed in the light of this comment.

7. Fig. 3D - Not clear whether ITPK1si affects MLKL oligomerization. The authors state that this small MLKL band (indicated with the arrow) is Panx-1 dependent, but silencing ITPK1 also prevent its appearance.
In line with Dovey *et al*, our data show that ITPK1 silencing prevents the oligomerization of MLKL (Dovey CM et al, Mol Cell 2018). For sake of clarity, another exposure for the blot Fig.3D is now presented. Of note, ITPK1 functions upstream of PANX1, and therefore also promotes the appearance of this smaller MLKL band.

8. Fig. 3G - This does not make sense. The authors state that RAB27a or RAB27b knockdown markedly reduce small EVs release while the graph shows that RAB27asi increases the amount of particles when compared to NSsi in untreated conditions.
We apologize for not better explaining this result. Our data show that RAB27 knockdown prevents the increase of small EV release in response to TQS. The text has been amended.

9. The authors state:" this data suggests that MLKL oligomerization and RAB27-dependent vesicular trafficking control "leakiness" of the plasma membrane. However, it does not seem that MLKL oligomerization is required since RAB27 silencing does not affect oligomerization but reduces TO-PRO-3DIM+ cells.
In this manuscript, we conclusively showed that MLKL oligomerization controls the activation of PANX1 in a RAB27-dependent fashion. The fact that MLKL oligomerization normally occurs without RAB27 therefore fits with this idea and does not militate against a role in plasma membrane leakiness.

10. What is the role of the "cleaved" fragment of MLKL? Does it have a direct role on TO-PRO-3 uptake?
As indicated in our initial response and our revised manuscript, the processing of MLKL occurs subsequently to PANX1 activation and is therefore unlikely to participate in the uptake of TO-PRO-3. Nevertheless, we agree that defining the function, if any, of this proteolysis would be of interest in the future.

11. If MLKL is upstream of PANX1 and that PANX1 restrict cytokine expression, why don't you also see an increase, and not a decrease, in cytokine expression upon MLKL silencing?
Please, see our response to point #2.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Nicolas Bidere
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-47840V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was chosen based on previous experiments. The number of independent experiments and the type of statistical analyses are mentioned in the respective figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No in vivo experiments were used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
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5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. ANOVA tests were used.
Is there an estimate of variation within each group of data?	N/A
Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used were described in the Material and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines are specified in the Material and Methods section and are mycoplasma-free.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
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21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A