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Caspase-8-driven apoptotic and pyroptotic crosstalk causes cell death and IL-1β release in X-linked inhibitor of apoptosis (XIAP) deficiency

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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. 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. Vince,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see from the comments below, the referees find the analysis potentially interesting but also find that significant revisions are needed for consideration here. Their concerns are clearly raised below. Should you be able to address the raised concerns then I would like to invite you to submit a revised version.

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

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

I thank you for the opportunity to consider your work for publication. I am looking forward to discussing your revisions further.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a PDF with helpful tips on how to submit the revised version.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (19th Apr 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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

Referee #1:

Manuscript by Hughes and colleagues describes the possible signaling in XIAP deficiency. The authors show that IBD patients with XIAP deficiency show higher levels of caspase-8 and IL-1b processing and Gasdermin D (GSDMDM) activity. Using XIAP knockout mice, the authors show that effects of caspase-8 activity can be rescued by combined deletion of GSDMD, caspase-3 and caspase-7. However, pannexin-1 or GSDMD were not required for NLRP3 activation downstream of caspase-8 claim that. Overall, the authors claim that XIAP deficiency activates multiple cell death pathways, mainly apoptosis and pyroptosis.

Although the authors tried to examine the consequences of XIAP deficiency and elevated caspase-8 activity in a systematic manner, they frequently miss some critical controls and overinterpret their results. For example, the authors model XIAP deficiency by using smac mimetic Compound A. However, Compound A also targets cIAP1 and cIAP2 and thus it does not simply model XIAP deficiency but lack of or inhibition of all these 3 IAPs. For that reason, the authors should check cIAP1 and cIAP2 levels whenever they use Compound A (figures 2, 3, 4, 5, 6, and 7) and use cells and mice deficient in cIAP1 and cIAP2 for proper comparison. Right now, any data produced with Compound A should not be interpreted as XIAP deficiency but XIAP/cIAP1/cIAP2 deficiency and/or inhibition.
Similarly, the authors should examine and show the levels and processing of caspase-3 and caspase-7 in figures 2, 3, 4, 5 and 7. Given that caspases 3 and 7 are major substrates of caspase-8, are inhibitable by XIAP and are implicated in these cell death pathways, it is critical to show how their levels and processing are affecting by various treatments and genotypes (data in fig S4 are not sufficient as they just confirm the knockouts).
For experiments using TAK1 inhibitor the authors should show what is the consequence of TAK1 inhibition on LPS induced

signaling by showing phospho -TAK1, -p38 and/or -MK2. Importantly, the authors should explain how is TAK1 related to XIAP to warrant heavy use of TAK1 inhibitor throughout the study.

Specific points
1. The authors should provide data on the validation of cleaved GSDMD and cleaved caspase-8 antibodies for IHC applications.
2. In figure 2, please indicate LPS treatment (maybe instead of or next to Time?).
3. What is the explanation for the huge band for p30 of GSDMD in lane 6? It has a higher intensity than all FL bands combined in this blot.
4. The authors claim that Compound A causes similar cleavage of caspase-8 in WT and GSDMD deficient cells (page 8, fig 3D) but that is not true. Please compare lanes 2 and 6 in 3D - it is clear that there is much more cleavage of caspase-8 in GSDMD -/- cells.
5. In figure 4B the authors should run a separate gel for GSDMD as it is not clear which band corresponds to GSDMD and which to background from IL-1b.
6. In figure 5 the authors should include a western blot for pannexin-1 and check the processing of caspase-9.
7. The authors should use caspase-3/caspase-7 double knockout cells for proper comparison to GSDMD knockouts and cells with compound knockouts to assess the relevance of caspase-3 and caspase-7 for XIAP deficiency more directly (figures 3 and 7).

Referee #2:

The overall conclusion in the submitted manuscript by Vince and colleagues is that active caspase-8 drives cell death and bioactive IL-1β release upon XIAP inhibition, and that caspase-3, -7, and GSDMD acted redundantly to cause both cell death and IL-1β release function downstream of Caspase-8. Although this conclusion is not that unexpected, there are quite a bit interesting and valuable observations in this study. For example, the authors first show that two independent patients with early onset IBD have genetic mutation in XIAP; PBMC as well as colonic epithelial cells from these patients have a much elevated level of caspase-8 activation. The authors then model this phenomenon in mouse BMDMs using chemical inhibitors for IAP, which mimics the TAK1 inhibitor and causes caspase-8 activation, GSDMD activation, and IL-1 β release independently of NLRP3 and caspase-1. However, the authors further show that macrophages lacking TAK1 and XIAP activity differ in the requirement of Gsdmd for efficient release of mature IL-1 β. In both cases, GSDMD is dispensable for cell death but double deletion of Gsdmd and Gsdme blocks the pyroptosis. Finally, the authors provide conclusive evidences that XIAP inhibition-induced pyroptosis is independent of the NLPR3-caspase-1 canonical inflammasome, thus excluding the involvement of pannexin-1, a controversial topic in the field. In general, the work is a nice and valuable addition to this important topic in the field, which not only enriches our understanding of the molecular pathway, but also provide perspective into the related subset of IBD. There are several issues that need to be addressed before consideration of the manuscript for publication in the EMBO Journal.

Major comments:

1. How does active caspase-8 cleave IL-1β, and where is its specific cleavage site in IL-1β? The authors should demonstrate that IL-1β could indeed be cleaved by active aspase-8 in vitro, which is needed to rule out the involvement of other caspase activations in this process.
2. The authors need to use caspase-8 KO cells as a control to prove that caspase-8 indeed cleaves IL-1β in cells.
3. In Figure 2, why caspase-1 exposure rendered appearance of the pro-IL-1β band? Stripping and re-probing the Western Blot is not recommended here, since the bands of these proteins overlap, leading to erroneous interpretations. Supplemental figure 2 and Figure 4C have the same issue.
4. The data quality of Figure 3A，3B and 3C is not sufficient and the within-group variation is too large to make a strong conclusion. Besides, cell viability determined by PI staining and flow cytometry is also not sufficient as they cannot indicate the exact type of cell death after stimulation. The authors should use multiple methods to show the specific type of death after Cp. A stimulation (such as LDH assay, photograph, and movies).
5. In Figure 3B, the release of IL-1β was not affected by Gsdmd deficiency but was reduced by MCC950 treatment triggered by Cp. A. This is difficult to understand because the authors show that the Cp. A-induced cell death and IL-1β release were independent of caspase-1 and NLRP3 in Figure 1. How to explain this discrepancy?
6. In Figure 3D, how to explain the cleavage of caspase-1 was reduced in Gsdmd-/- and Gsdmd-/-Gsdme-/- BMDMs after Cp. A stimulation? Similar questions also apply to Figure 4B.
7. In Figure 4C, the authors show that ASC oligomer profiling in Gsdmd deficient cells was not affected after Cp. A or TAKi stimulation. Then, why the cleavage of caspase-1 was greatly reduced in Gsdmd deficient cells? Besides, how is NLRP3 inflammasome assembled after Cp. A or TAKi stimulation? Is it caspase-8 dependent or GSDME dependent?
8. Data quality of ASC-oligomerization in Supplemental Figure 3 is insufficient. The authors should prove that the BAX/BAK-mediated, activation-induced ASC-oligomerization is indeed NLRP3 dependent.
9. In Figure 5, the authors should use NLRP3 KO BMDMs as a control to clarify that ASC-oligomerization is indeed caused by NLRP3 activation.
10. It is highly risky to suggest caspase-8-induced cell death by using multiple genes KO iBMDMs generated by CRISPR/Cas9.

This is because after multiple rounds of gene editing, the KO cells were already different from WT cells. In our own experience, we have found that the property of Casp3-/-Casp7-/- iBMDMs changed quite a bit, compared to normal iBMDMs. Furthermore, the authors need to complement caspase-8 in the KO iBMDMs to demonstrate that this phenotype is indeed caspase-8 dependent.

11. The authors suggested that in the absence of caspase-1, -3, and -7, activation of GSDMD causes cell death upon XIAP antagonism. To draw a firm conclusion, the authors need to complement caspase-1, -3, -7, or GSDMD in the KO iBMDMs to support the conclusion. Similar experiments are also in need for Supplementary Figure 7.

We would like to thank the reviewer's and the editor for their guidance and input into our manuscript, which have considerably improved the quality of our data and, we believe, have increased the impact of our findings.

Our revised manuscript contains 17 panels of new data spread across 7 main figures, 5 Expanded View Figures and 4 Appendix Figures, and includes the addition of new clinical data showing that the detection of elevated cleaved caspase-8 and GSDMD in XIAP deficient patients suffering from inflammatory bowel disease is reduced following treatment. We would like to take this opportunity to highlight the novel and impactful findings or our study:

1. We show for the first time that XIAP deficient patients contain elevated levels of activated GSDMD and caspase-8 in inflamed colonic mucosae and LPS treated PBMCs, and that the activation of these key cell death molecules is reduced following therapy (New data, Figure 1D and 1E of the revised manuscript).

2. We show for the first time that XIAP targeting with IAP antagonists can still cause efficient cell death and inflammatory IL-1β activation even upon the combined genetic deletion of key apoptotic (BID, caspase-3, -7), and pyroptotic (caspase-1, -11) effectors, and this occurs as a consequence of caspase-8 cleavage of GSDMD. This redefines how caspase-8 can signal cell death in innate immune cells. These findings have relevance beyond IAP deficiency, as genetic mutations in A20 and the LUBAC machinery, which results in auto-inflammation in humans, can also pre-dispose to heightened caspase-8 and inflammasome responses.

3. We discover that extrinsic apoptotic activation of the NLRP3 inflammasome, as observed upon IAP loss or TAK1 inhibition, occurs via a distinct mechanism to intrinsic apoptotic activation of NLRP3. This is a remarkable finding as both these apoptotic pathways converge on caspase-3 and -7 activation. However, our experiments show that only intrinsic (and NOT extrinsic) apoptotic signalling to caspase-3 and -7 and their processing of Pannexin-1 contributes to NLRP3 inflammasome assembly. These conclusions are supported by our analysis of Pannexin-1 deficient mice in addition to our novel caspase-3 and -7 non-cleavable Pannexin-1 point mutant mouse.

4. Our genetic and biochemical data using our two distinct Pannexin-1 mutant strains of mice refute a main conclusion of a recently published EMBO Journal study that has received significant attention (PMID: 30902848). We do not wish to imply that the authors of this previous study have done anything wrong other than come to conclusions that cannot be supported in the light of our work and, as such, believe that the publication of our data in the same forum is important for scientific transparency.

Text changes are highlighted in yellow in our revised manuscript, while we have addressed all the reviewer comments, point-by-point, below.

Referee #1:

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claim that. Overall, the authors claim that XIAP deficiency activates multiple cell death pathways, mainly apoptosis and pyroptosis.

Although the authors tried to examine the consequences of XIAP deficiency and elevated caspase-8 activity in a systematic manner, they frequently miss some critical controls and overinterpret their results. For example, the authors model XIAP deficiency by using smac mimetic Compound A. However, Compound A also targets cIAP1 and cIAP2 and thus it does not simply model XIAP deficiency but lack of or inhibition of all these 3 IAPs. For that reason, the authors should check cIAP1 and cIAP2 levels whenever they use Compound A (figures 2, 3, 4, 5, 6, and 7) and

use cells and mice deficient in cIAP1 and cIAP2 for proper comparison. Right now, any data produced with Compound A should not be interpreted as XIAP deficiency but XIAP/cIAP1/cIAP2 deficiency and/or inhibition.

Author Response 1

Compound A targeting of XIAP and cIAPs

The reviewer correctly notes that the smac-mimetic Compound A targets XIAP and cIAPs. We clearly state this in our manuscript when we first use Compound A; "XIAP deficiency can be modelled in cells by treatment with the IAP antagonist smac-mimetic Compound A (Cp. A), which targets XIAP and cIAPs". For additional clarity, we have changed most references to "XIAP inhibition" in the revised manuscript to "IAP inhibition" to better reflect Cp. A targeting. However, it is worth noting that the inflammatory and cell death phenotype of XIAP deficient cells is critically dependent on Toll-like Receptor (TLR) and TNF Receptor 2 (TNFR2)-mediated degradation of cIAP1 (PMID: 28723569). In this publication we defined these requirements both chemically with smac-mimetics, and genetically through the generation of gene targeted mice e.g., co-deletion of XIAP and TNFR2 completely prevents TLR-induced cIAP1 degradation and the excess cell death and inflammasome activation resulting from XIAP deficiency. Importantly, in macrophages derived from XIAP and TNFR2 double knockout mice, cell death and inflammasome responses are fully restored by smac-mimetic targeting of cIAPs (PMID: 28723569). Therefore, in essence, XIAP deletion results in both XIAP/cIAP loss upon pathogen sensing, which is accurately mirrored by Compound A treatment as these proteins, XIAP/cIAPs, are the specific targets of Compound A.

Use of cells and mice deficient in cIAP1 and cIAP2 for comparison

We previously generated and examined mice deficient in cIAP1 and cIAP2 (cIAP1/2), and also utilized cIAP1/2 specific smac-mimetics, in order to directly compare the removal of cIAPs with XIAP loss (PMID: 25693118 and PMID: 22365665). These studies showed that cIAP1/2 targeting alone does not recapitulate either XIAP loss or Compound A treatment; in terms of both sensitizing to TLR- and TNF-mediated macrophage death and inflammasome responses.

Examining cIAP1 and cIAP2 levels whenever using Compound A

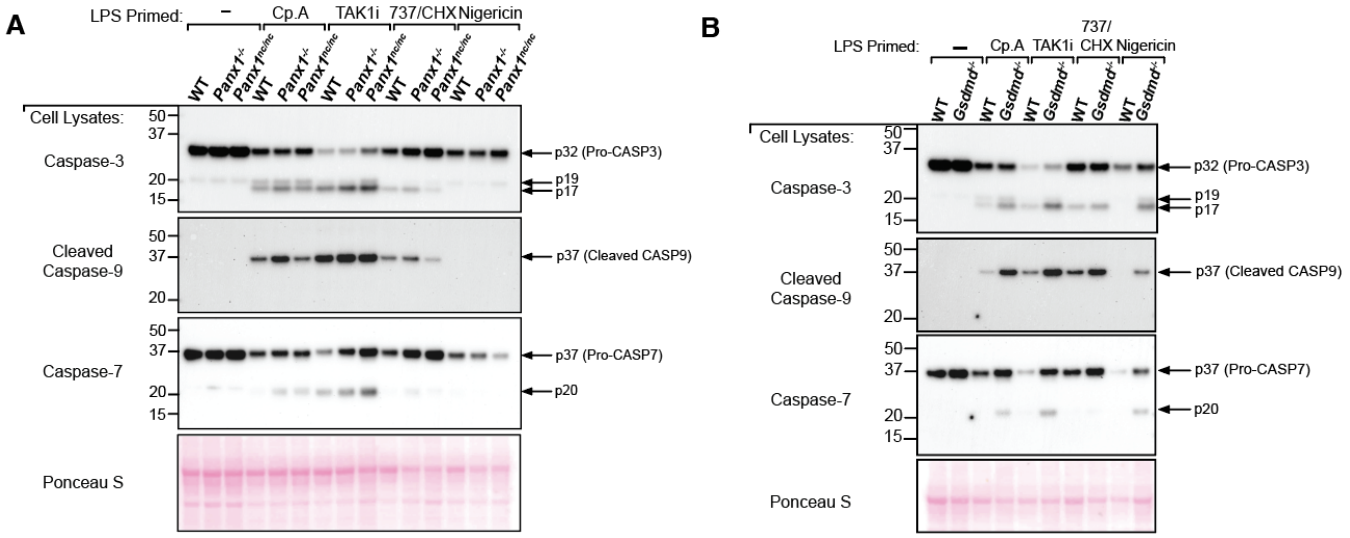
Examining cIAP levels upon Compound A treatment is one way to measure its bioactivity, as we and others have previously documented (e.g. PMID: 22365665). However, Compound A-induced degradation of cIAPs does not reflect its inhibitory activity against XIAP (which is not degraded) and, in fact, some smac-mimetics (e.g. birinapant) whilst efficient at targeting and degrading cIAPs are poor inhibitors of XIAP, as we have reported (PMID: 25693118, PMID: 24684347). A more robust measure of Compound A-mediated inhibition of XIAP, and as we use throughout our manuscript, is its ability to cause macrophage cell death and/or activate IL-1β, both cellular outcomes we have rigorously documented to result from XIAP targeting in previous studies (PMID: 25693118, PMID: 22365665, PMID: 28723569). All our assays contain control wildtype cell measurements to ensure that, on the day of the experiment, the Compound A we used was functionally active against XIAP, and all experiments independently repeated (the exact number of independent experiments conducted is denoted in each figure legend).

Similarly, the authors should examine and show the levels and processing of caspase-3 and caspase-7 in figures 2, 3, 4, 5 and 7. Given that caspases 3 and 7 are major substrates of caspase-8, are inhibitable by XIAP and are implicated in these cell death pathways, it is critical to show how their levels and processing are affecting by various treatments and genotypes (data in fig S4 are not sufficient as they just confirm the knockouts).

Author Response 2

Unfortunately, we do not have all the experimental samples shown in figures 2, 3, 4, 5 and 7 as the data for this study was collected over several years and/or the samples have all been used in the western blots presented. However, in our revised manuscript we have performed additional experiments which demonstrate the processing of caspase-3 and -7 in response to Compound A treatment in both wildtype (WT), *Gsdmd*$^{-/-}$, *Panx1*$^{-/-}$ and *Panx1*$^{nc/nc}$ BMDMs, which are presented as new data in Figure EV3A, EV3B, reproduced below for convenience. These results show that the inhibition of IAPs or TAK1 results in caspase-3 and caspase-7 processing, as expected, and that the deletion of Pannexin-1 does not impact these events. On the other hand, GSDMD deletion moderately increases

intracellular levels of cleaved caspase-3, -7 and -9, despite GSDMD loss not accelerating cell death (Figure 3A, 4A and new data Figure EV2), which one might predict to occur upon the detection of increased cleaved caspases. This data is consistent with the idea that processed caspases, including caspase-1, caspase-3 and caspase-7 can be released through GSDMD pores, and the deletion of GSDMD thereby moderately increases intracellular levels of these proteins (please also see **Author Response 16**, where we expand on this topic following a query by Reviewer 2 on why GSDMD loss reduces processed caspase-1 p20 release).



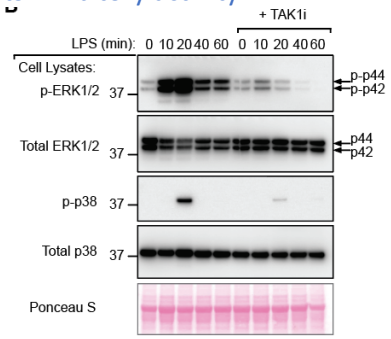
New data, Figure EV3A and EV3B. *BMDMs of the indicated genotypes were seeded at a density of 4 x 10^5 cells per well and primed with LPS (100 ng/ml) for three hours before treatment with Cp. A (1 μM) or TAK1i (250 nM) for six hours, ABT-737 (1 μM) and CHX (20 μg/ml) for four hours, or nigericin (10 μM) for 45 minutes. Cell lysates were harvested and analysed by western blot. Data represent three independent experiments.*

For experiments using TAK1 inhibitor the authors should show what is the consequence of TAK1 inhibition on LPS induced signalling by showing phospho -TAK1, -p38 and/or -MK2. Importantly, the authors should explain how is TAK1 related to XIAP to warrant heavy use of TAK1 inhibitor throughout the study.

Author Response 3

Control blots for TAK1 inhibition

In our revised manuscript Figure EV2D, reproduced below for convenience, we provide controls to show the expected impact of TAK1 inhibition on downstream signalling; the phosphorylation of ERK and p38. We would also note that TAK1 inhibition is well established to sensitize macrophages to LPS-mediated cell death, and akin to Compound A treatment, the cell death we measure throughout our study also represents an excellent readout of its inhibitory activity.



New data, Figure EV2D. *BMDMs were seeded at a density of 4 x 10^5 cells per well and pre-treated with TAK1i (250 nM) for 1 hour prior to the addition of LPS (100 ng/ml) for 0, 10, 20, 40 and 60 minutes. Cell lysates were collected and analysed by western blot.*

Rationale for comparing IAP inhibition to TAK1 inhibition

We have explored the impact of TAK1 inhibition on cell death and inflammasome signalling because it is a distinct activator of caspase-8. Therefore, the experiments using the TAK1 inhibitor provide additional insight into whether the cell death and inflammatory responses we uncover are unique to IAP loss, or if other caspase-8 activating agents cause a similar inflammatory cell death. This comparison shows the potential relevance of our findings to the mutations in other genes (e.g. LUBAC, A20) that can also pre-dispose to heightened caspase-8 activation, cell death and multi-organ inflammation.

Specific points

1. The authors should provide data on the validation of cleaved GSDMD and cleaved caspase-8 antibodies for IHC applications.

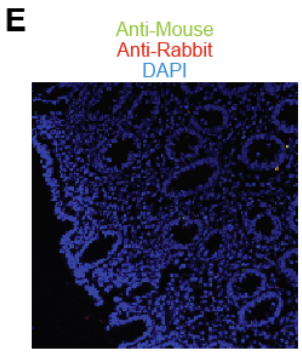
Author Response 4

We believe the IHC cleaved GSDMD and caspase-8 staining to be accurate for the following reasons:

1. In our revised manuscript we provide additional secondary antibody control staining, presented in Figure EV1E and reproduced below for convenience, to document primary antibody signal specificity.
2. Importantly, we show that the detection of cleaved GSDMD and cleaved caspase-8 correlates with IBD in XIAP deficiency as:

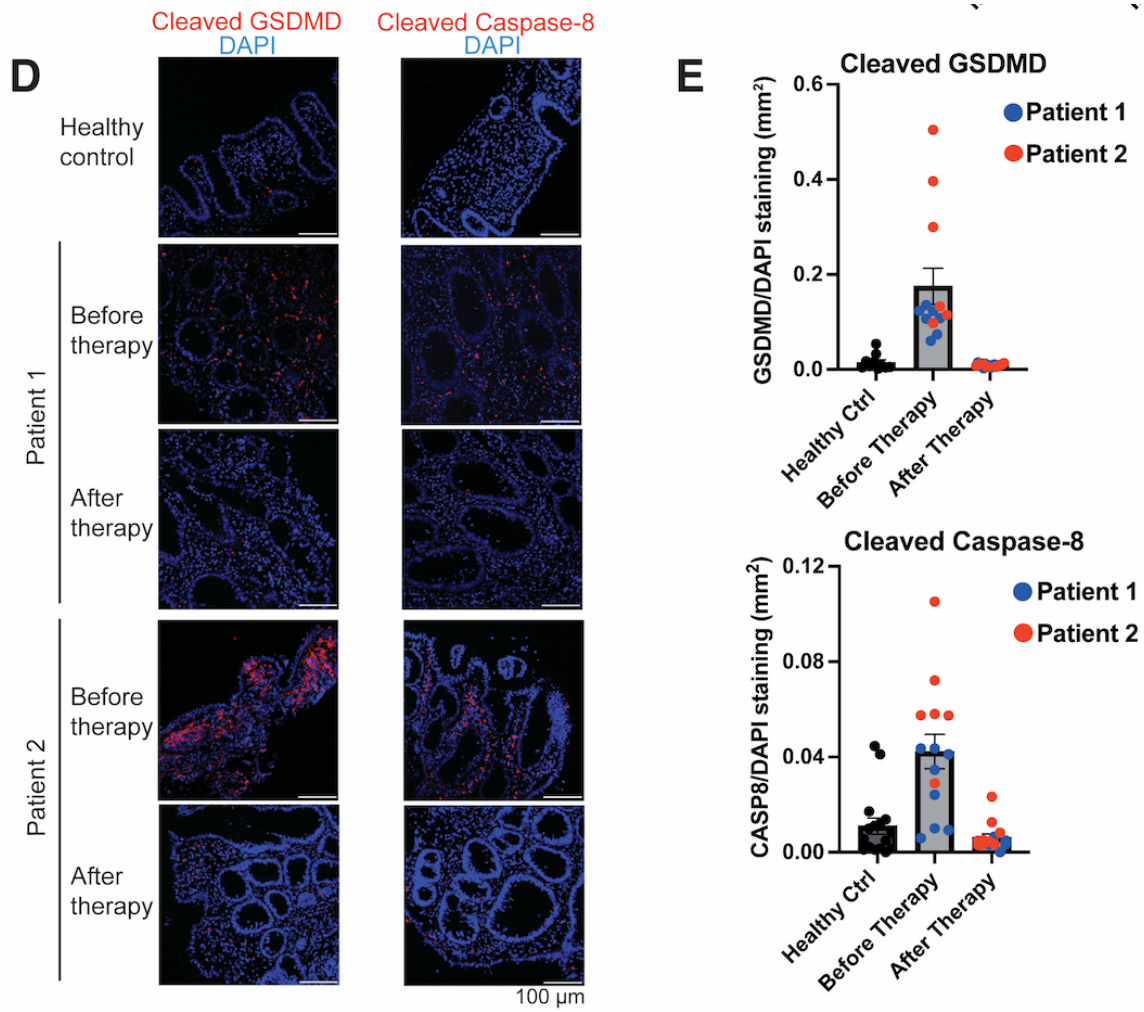
- Our healthy patient control staining shows little detection of cleaved GSDMD or cleaved caspase-8 relative to XIAP loss (Figure 1D and 1E), which is also reflected in our western blot analysis of patient PBMCs (Figure 1B).
- Our new patient data incorporated as Figure 1D and 1E of the revised manuscript, reproduced below for convenience, shows that anti-TNF and immunosuppressive therapy reduces the levels of cleaved caspase-8 and cleaved GSDMD detected in the colonic mucosae of these two XIAP deficent patients.

3. The human specific cleaved GSDMD antibody has been validated by the suppliers (IHC staining and western blot) and has been cited/validated in 31 publications (Cell Signaling, #36425). The cleaved caspase-8 (Asp384) p18 antibody (Affinity, #AF5267) has been similarly validated by both IHC staining and western blot by the supplier and in 9 citing publications.



New data, Supplemental Figure 1E.

Secondary antibody alone control staining of patient colonic biopsies. Additional control images are provided in the raw data files.



New post-therapy data, Figure 1D and 1E. Immunofluorescence staining of the colonic mucosae of XIAP deficient patients pre- and post-immunosuppressive therapy versus healthy controls, including its quantification in panel E (all images used for quantification are supplied in the raw data files associated with this manuscript).

2. In figure 2, please indicate LPS treatment (maybe instead of or next to Time?).

Author Response 5

The labelling "Time" in Figure 2 reflects the time of treatment with Cp. A, TAKi or nigericin, not LPS. We have adjusted Figure 2 to read "Treatment time (hr)", and clearly indicate in the figure legend that all cells in this experiment were LPS primed before being stimulated with the indicated compounds.

3. What is the explanation for the huge band for p30 of GSDMD in lane 6? It has a higher intensity than all FL bands combined in this blot.

Author Response 6

This dominant GSDMD p30 band is a result of treating cells with nigericin, which is a more potent activator of caspase-1 than either Cp. A or TAK1i, and thus it causes more processing of GSDMD to its pore-forming p30 fragment.

4. The authors claim that Compound A causes similar cleavage of caspase-8 in WT and GSDMD deficient cells (page 8, fig 3D) but that is not true. Please compare lanes 2 and 6 in 3D - it is clear that there is much more cleavage of caspase-8 in GSDMD -/- cells.

Author Response 7

We believe that the increased cellular cleaved caspase-8 (at 6 hours) upon GSDMD deficiency results from delayed release of processed caspases, which can exit via GSDMD pores. This also explains the reduced caspase-1 p20 fragment in the cell supernatants in GSDMD deficient macrophages, which could be incorrectly interpreted as reduced inflammasome activation, and is why we performed ASC cross-linking assays as a more direct and accurate readout of NLRP3 inflammasome formation and activity. Please also see **Author Response 2 and 16**, where we provide new relevant data and expand on this topic in more detail.

5. In figure 4B the authors should run a separate gel for GSDMD as it is not clear which band corresponds to GSDMD and which to background from IL-1b.

Author Response 8

The GSDMD antibody probing is used here as a simple control to show that GSDMD deleted macrophages do not express GSDMD, which it and other experiments using these gene targeted mice (e.g. Figure 3D), clearly demonstrates.

6. In figure 5 the authors should include a western blot for pannexin-1 and check the processing of caspase-9.

Author Response 9

In our revised manuscript we demonstrate the detection of pannexin-1 (and its absence in pannexin-1 deleted mice) in Figure 5D. We have also performed new experiments using Compound A, TAK1i and ABT-737/CHX treatments in wildtype and Pannexin-1 mutant mice to examine caspase-9 processing (New data, Figure EV3A and EV3B of the revised manuscript, shown in **Author Response 2, above**, for convenience). We note that we have previously documented robust caspase-9 processing upon ABT-737/CHX treatment to activate BAX/BAK in PMID: 30485804. These new data demonstrate that i) pannexin-1 does not alter caspase-9 activity downstream of caspase-8 or BAX/BAK, activation, and ii) that although caspase-9 processing is detected upon IAP inhibition and caspase-8 activation it is, unlike ABT-737/CHX treatment, dispensable for cell death (as demonstrated by our genetic experiments in macrophages lacking both BAX and BAK, BID and/or caspase-9; Figure 6A, 6B and 6C). However, it is possible that BAX/BAK and caspase-9 activation may contribute, albeit redundantly, to cell death following IAP loss and, accordingly, we have included a new paragraph in the discussion of our revised manuscript to note this possibility:

"Upon IAP inhibition we observed caspase-9 processing; a hallmark of apoptosome formation downstream of BAX/BAK-mediated mitochondrial outer membrane permeabilization. Caspase-8 can contribute to BAX/BAK activation via its transcriptional roles or its cleavage of BID (Li *et al.*, 1998; Simpson *et al*, 2022). Therefore, even though our genetic data show that BAX/BAK deficiency has no impact on LPS killing upon Cp. A treatment, and that the loss of BID did not impede cell death resulting from XIAP targeting, as expected (Jost *et al*, 2009), it remains possible that BAX/BAK act redundantly with caspase-8 triggering of caspase-1, -3, -7 and GSDMD."

7. The authors should use caspase-3/caspase-7 double knockout cells for proper comparison to GSDMD knockouts and cells with compound knockouts to assess the relevance of caspase-3 and caspase-7 for XIAP deficiency more directly (figures 3 and 7).

Author Response 10

Please see **Author Response 19 below** where we use three different cell types (iBMDMS, embryonic fibroblasts and foetal liver derived macrophages) and two genetic targeting strategies (CRISPR/Cas9 and crosses of *caspase-3*$^{+/-}$ and caspase-7$^{+/-}$ mice to generate double knockout fibroblasts and foetal liver macrophages) to show that combined caspase-3 and -7 deletion does not impact cell death and its triggering of IL-1β release upon IAP loss. Further, we demonstrate that the additional deletion of caspase-1 and BID, or caspase-1, -6,-11 and -12, on top of caspase-3 and caspase-7 co-deletion, does not prevent, to any detectable measure, these processes, and that only the co-deletion of GSDMD can abrogate macrophage

death and IL-1β release (Figure 6, Figure 7 and Figure EV4 of the revised manuscript). Therefore, combined with our past work showing that caspase-8 is essential for cell death and IL-1β release upon IAP antagonism (PMID: 28723569), this genetic data demonstrate that downstream caspase-8 apoptotic effectors and GSDMD act redundantly to cause cell death upon XIAP loss, and our revised manuscript title, "Redundancy and crosstalk in apoptotic and pyroptotic signalling drives cell death and IL-1β release in XIAP deficiency" reflects these unexpected findings. Importantly, we show that the activation of caspase-8 and GSDMD are also detected at elevated levels in XIAP deficient patients, and this is reduced upon therapy (Figure 1 of the revised manuscript; includes new post-therapy data).

Given the size of the current manuscript (7 main figures and 9 additional figures), and the significance of the four major findings (outlined at the beginning of this response letter), the in-depth analysis required to further define the mechanisms and genetics of caspase-8 killing and activation of the NLRP3 inflammasome following IAP loss are being planned for a future publication; which involves the re-derivation of our caspase-3 and -7 gene targeted mice (colony shut-down in 2020 due to the COVID-19 pandemic) and crossing with Pannexin-1 and GSDMD mutant animals (please see **Author Response 19 below** for further discussion).

Referee #2:

The overall conclusion in the submitted manuscript by Vince and colleagues is that active caspase-8 drives cell death and bioactive IL-1β release upon XIAP inhibition, and that caspase-3, -7, and GSDMD acted redundantly to cause both cell death and IL-1β release function downstream of Caspase-8. Although this conclusion is not that unexpected, there are quite a bit interesting and valuable observations in this study. For example, the authors first show that two independent patients with early onset IBD have genetic mutation in XIAP; PBMC as well as colonic epithelial cells from these patients have a much elevated level of caspase-8 activation. The authors then model this phenomenon in mouse BMDMs using chemical inhibitors for IAP, which mimics the TAK1 inhibitor and causes caspase-8 activation, GSDMD activation, and IL-1 β release independently of NLRP3 and caspase-1. However, the authors further show that macrophages lacking TAK1 and XIAP activity differ in the requirement of Gsdmd for efficient release of mature IL-1 β. In both cases, GSDMD is dispensable for cell death but double deletion of Gsdmd and Gsdme blocks the pyroptosis. Finally, the authors provide conclusive evidence that XIAP inhibition-induced pyroptosis is independent of the NLPR3-caspase-1 canonical inflammasome, thus excluding the involvement of pannexin-1, a controversial topic in the field. In general, the work is a nice and valuable addition to this important topic in the field, which not only enriches our understanding of the molecular pathway, but also provide perspective into the related subset of IBD. There are several issues that need to be addressed before consideration of the manuscript for publication in the EMBO Journal.

Major comments:

1. How does active caspase-8 cleave IL-1β, and where is its specific cleavage site in IL-1β? The authors should demonstrate that IL-1β could indeed be cleaved by active aspase-8 in vitro, which is needed to rule out the involvement of other caspase activations in this process.

Author Response 11

The *in vitro* cleavage of IL-1β by caspase-8 at the same site cleaved by caspase-1 (IL-1β D117) has previously been demonstrated by our laboratory and other groups (e.g. PMID: 18725521, PMID: 22365665, PMID: 33976225). In Figure 7 of our manuscript, we also delete several caspases at the same time (caspase-1, -3, -7 and -9, or caspase-1, -3, -7, -11 and -12) to show that IAP targeting still allows IL-1β cleavage in these cells, and that it is only upon casapase-8 co-deletion that IL-1β processing is completely blocked.

2. The authors need to use caspase-8 KO cells as a control to prove that caspase-8 indeed cleaves IL-1β in cells.

Author Response 12

We have previously shown that caspase-8 cleaves IL-1β, both by using recombinant caspase-8 cleavage assays and by using macrophages derived from caspase-8 deficient mice (PMID: 22365665, PMID: 25693118).

3. In Figure 2, why caspase-1 exposure rendered appearance of the pro-IL-1β band? Stripping and re-probing the Western Blot is not recommended here, since the bands of these proteins overlap, leading to erroneous interpretations. Supplemental figure 2 and Figure 4C have the same issue.

Author Response 13

The membrane in Figure 2 was not stripped but was re-probed with caspase-1 following the IL-1β blot. This is standard practice because the antibody used to detect pro-caspase-1 and cleaved caspase-1 (~45 kDa and ~20 kDa respectively) pick up specific caspase-1 bands (as our use of caspase-1 deficient macrophages show), which are distinct and clearly separated from pro-IL-1β and activated IL-1β (31 kDa and 17 kDa, respectively). This has been well documented in many publications examining inflammasome responses. Please note, Figure 4C and Supplemental Figure 2 of the original manuscript were not probed with anti-IL-1β and so these blots cannot have any residual IL-1β bands from previous probing's.

4. The data quality of Figure 3A, 3B and 3C is not sufficient and the within-group variation is too large to make a strong conclusion. Besides, cell viability determined by PI staining and flow cytometry is also not sufficient as they cannot indicate the exact type of cell death after stimulation. The authors should use multiple methods to show the specific type of death after Cp. A stimulation (such as LDH assay, photograph, and movies).

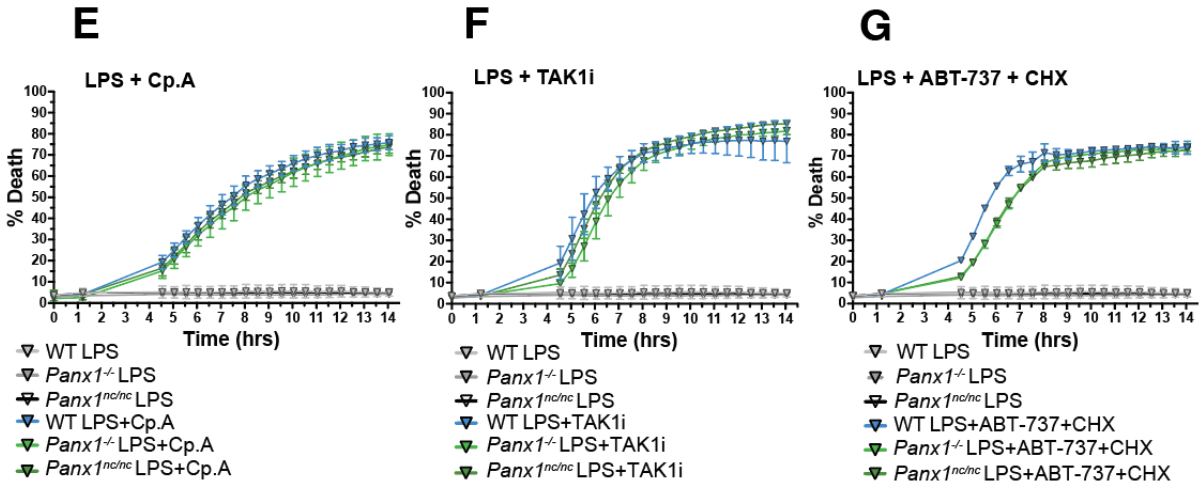
Author Response 14

Figure 3A, 3B and 3C data quality

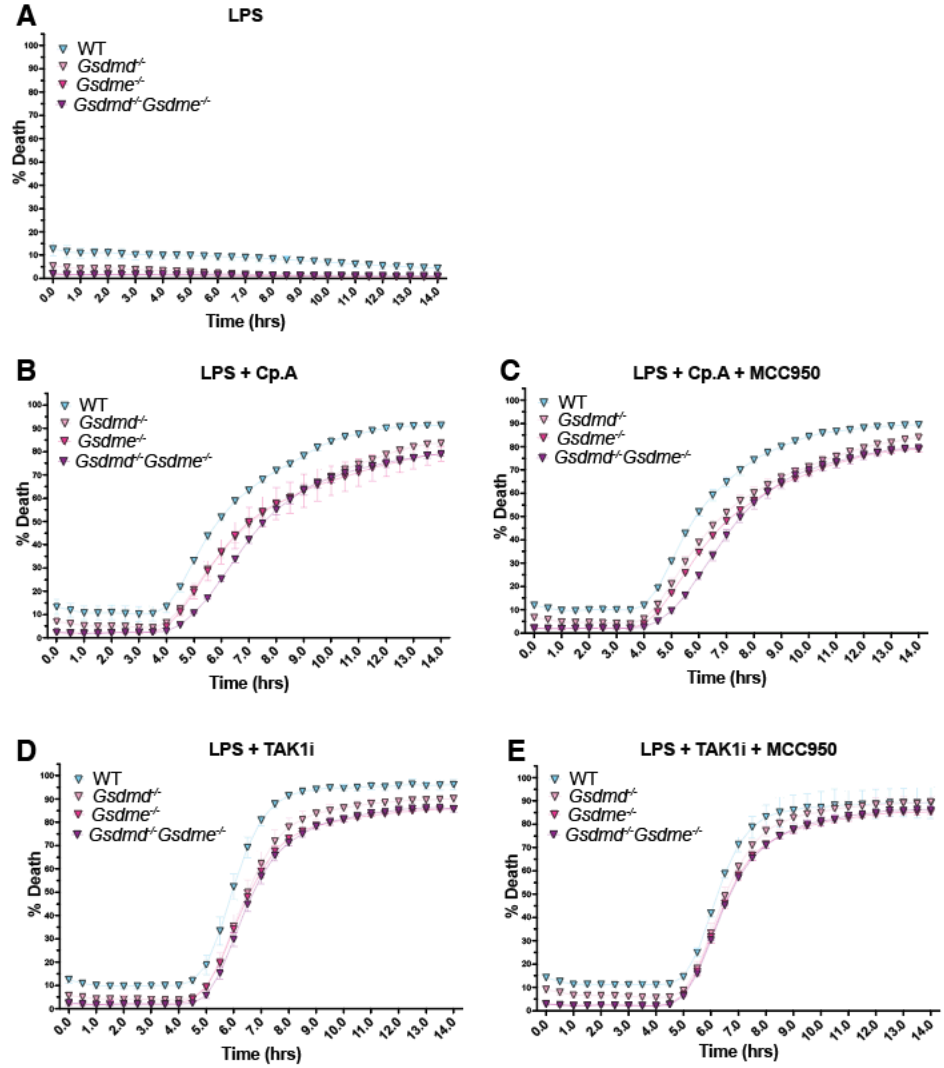
We are unclear why the reviewer feels the data quality in Figure 3A-C is not sufficient to draw conclusions from. Figure 3A measures cell death in bone marrow derived macrophages from the indicated genotypes at different time points following Compound A treatment (4, 8 and 16 hours) and therefore does not represent a single measurement taken at a single point in time. It also contains the appropriate control (nigericin treatment) to demonstrate that the genotypes are behaving as expected. Each symbol in Figure 3A-C represents a separate mouse and a completely independent experiment often performed by different researcher's weeks apart. Therefore, the data displayed in Figure 3 and associated error bars represents true biological variability, not assays performed in triplicate that simply measure pipetting error, as is often the case. Importantly, our collated independent experiments clearly demonstrate that neither Compound A killing, nor IL-1β release, is prevented by Gasdermin D loss, or Gasdermin D and Gasdermin E co-deletion, even when NLRP3-caspase-1 is also inhibited by MCC950.

Cell death measurements

- The type of cell death occurring is best assessed by genetic approaches, as we employ in our manuscript. This is because commonly used cell death markers, such as propidium iodide, Annexin V staining and LDH release do not discriminate as to the type of cell death occurring. For example, apoptosis, necroptosis and pyroptosis all result in PI staining, Annexin V staining and LDH release, and the kinetics of these responses depend on both the stimulus strength and the cell type.
- We use propidium iodide staining and flow cytometry in our manuscript as it is considered the gold standard in precisely measuring cell death. This is because, unlike other assays, it measures cell death at the single cell level (unlike the LDH assay) and allows the precise quantification of the viability of many thousands of cells in any given population, which is more difficult to accurately achieve by other methods.
- In our revised manuscript we provide new data assessing cell death in real time by IncuCyte live cell imaging of SYTOX green uptake in SPY620-DNA positive cells (Figure 5E, 5F, 5G and Figure EV2A, EV2B, EV2C, EV2E, EV2F, reproduced below for convenience). This approach clearly shows that neither the loss of Pannexin-1 or the mutation of the caspase-3/-7 cleavage site in Pannexin-1 significantly impacts cell death kinetics, nor does the sole or combined deletion of GSDMD and GSDME (i.e. the slope of the cell death "line" over time resulting from IAP loss, TAK1 inhibition, or BAX and BAK activation are comparable).



New data, Figure 4E, 4F, 4G. *BMDMs of the indicated genotypes were treated, as indicated, with LPS (100 ng/ml) for three hours before treatment with Cp. A (1 μM), TAK1i (250 nM) or ABT-737 (1 μM) and CHX (20 μg/ml). Cell viability was determined through real time IncuCyte analysis and measured as the proportion of SYTOX Green positive cells vs SPY620-DNA positive cells. Data are presented as n =3 mean+/-SD, and representative of one of three independent experiments.*



New data, Figure EV2. *BMDMs of the indicated genotypes were treated, as indicated, with LPS (100 ng/ml) for three hours before treatment with Cp. A (1 μM), TAK1i (250 nM) or ABT-737 (1 μM) and CHX (20 μg/ml). Cell viability was determined through real time IncuCyte analysis and measured as the proportion of SYTOX Green positive cells vs*

SPY620-DNA positive cells. Data are presented as n =3 mean+/-SD, and representative of one of three independent experiments.

5. In Figure 3B, the release of IL-1β was not affected by Gsdmd deficiency but was reduced by MCC950 treatment triggered by Cp. A. This is difficult to understand because the authors show that the Cp. A-induced cell death and IL-1β release were independent of caspase-1 and NLRP3 in Figure 1. How to explain this discrepancy?

Author Response 15

Previous studies (PMID: 24882010, PMID: 25693118, PMID: 22365665) have demonstrated that, as a result of XIAP deletion or antagonism with Compound A, caspase-8 activates NLRP3-caspase-1 and also processes IL-1β directly (i.e. 30-50% of activated IL-1β comes from NLPR3-caspase-1-mediated processing and the remainder from caspase-8 directly activating IL-1β). Our data using MCC950 to inhibit NLRP3 in Figure 3B is consistent with these studies. However, our experiments now shows that IL-1β release resulting from these IL-1β activating pathways is not impacted by GSDMD loss, likely because it can still exit the cell via caspase-8 triggering of other caspases, such as caspase-3 and -7, that will cause plasma membrane damage independent of GSDMD. This is one of the novel findings of our study. It also provides additional evidence supporting our conclusion that GSDMD pores are not involved in NLRP3 assembly downstream of caspase-8 activation, as otherwise GSDMD deletion should prevent IL-1β release to a similar extent (~30-50%) as NLRP3 loss.

6. In Figure 3D, how to explain the cleavage of caspase-1 was reduced in Gsdmd-/- and Gsdmd-/-Gsdme-/- BMDMs after Cp. A stimulation? Similar questions also apply to Figure 4B.

7. In Figure 4C, the authors show that ASC oligomer profiling in Gsdmd deficient cells was not affected after Cp. A or TAKi stimulation. Then, why the cleavage of caspase-1 was greatly reduced in Gsdmd deficient cells? Besides, how is NLRP3 inflammasome assembled after Cp. A or TAKi stimulation? Is it caspase-8 dependent or GSDME dependent?

Author Response 16 (to reviewer questions 6 and 7)

Reduced caspase-1 p20 release in GSDMD deficient cells

This is an astute observation that also initially puzzled us. Across many independent experiments the reduced caspase-1 p20 fragment release in GSDMD deficient macrophages was consistently observed by multiple researchers in our laboratories. This is the main reason why we sought to measure NLRP3 activity directly via NLRP3-induced ASC cross-linking assays, which clearly showed that GSDMD pores are not responsible for caspase-8 driven NLRP3 responses (Figure 4C and Appendix Figure 1A, 1B). Therefore, the reduced caspase-1 p20 release in GSDMD deficient macrophages reflects a mild reduction in its ability to exit the cell through the GSDMD pore. Indeed, we observe a moderate corresponding accumulation of cell lysate caspase-1 p20 at early time points in Cp. A and TAK1 inhibitor treated *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$*Gsdme*$^{-/-}$ macrophages, and also a similar increase in cell lysate-associated cleaved caspase-8, when compared to wildtype cells (e.g. Figure 3D, 4B and Appendix Figure 1A). Consistent with these observations, our new data also shows that upon IAP or TAK1 inhibition the genetic deletion of GSDMD causes a mild increase in intracellular cleaved caspase-3, -7 and -9 (new data, Figure EV3A and EV3B; please see **Author Response 2**), despite GSDMD deficiency not accelerating cell death (new data, Figure EV2; please see **Author Response 14**).

How NLRP3 is assembled following Compound A or TAK1i treatment: caspase-8 or GSDME dependent?

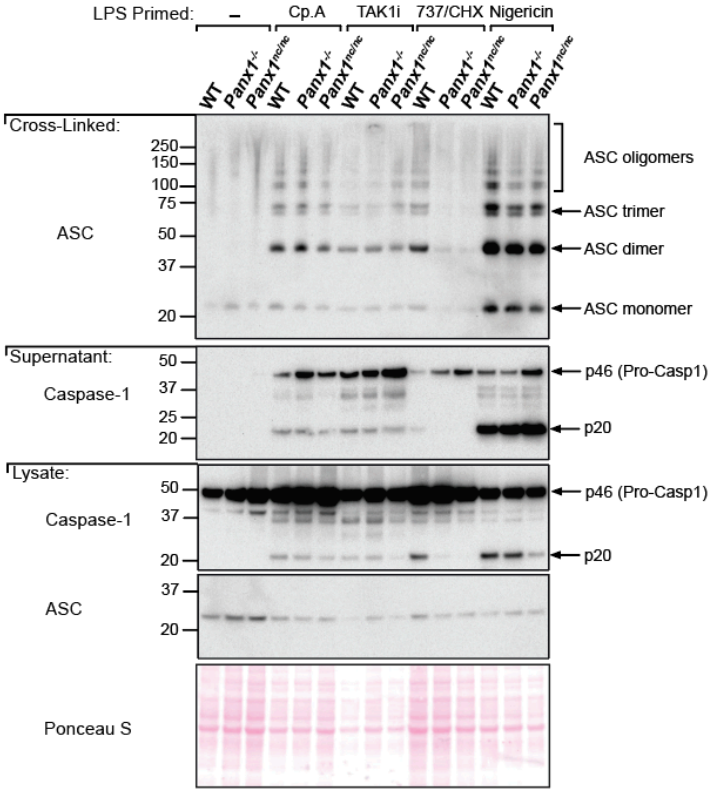
Prior studies (PMID: 24882010, PMID: 25693118, PMID: 22365665) have shown that the deletion of RIPK3-Caspase-8 entirely attenuates NLRP3 inflammasome activation following IAP antagonism or XIAP deletion. Regarding GSDME, our data in Figure 3 and new data in Figure EV2, show that GSDME plays no role in cell death, NLRP3-induced caspase-1 processing, nor IL-1β activation and release, indicating that in the context of IAP inhibition, GSDME is dispensable. Prior studies published in *Science* and the *EMBO J* suggest that caspase-8-mediated GSDMD pore formation (PMID: 30361383) and caspase-3/-7-pannexin-1 channel activation (PMID: 31211242) converge on potassium ion efflux to trigger NLRP3-caspase-1 signalling. However, as our genetic data using *Gsdmd*$^{-/-}$ and *Panx1*$^{-}$

$^{-/-}$ animals and, importantly, our novel caspase-3 and -7 non-cleavable Pannexin-1 mutant mouse, demonstrate, neither of these caspase-8 or caspase-3/-7 substrates are required for NLRP3 inflammasome assembly downstream of IAP loss or TAK1 inhibition. On the other hand, we show that caspase-3 and -7 cleavage of Pannexin-1 is required for efficient BAX/BAK-induced NLRP3 signalling. These findings are important as they genetically prove that i) distinct mechanisms operate by which mitochondrial and extrinsic apoptosis can activate the NLRP3 inflammasome to cause inflammation, and ii) provide strong evidence that calls into question a main conclusion of a recent EMBO Journal study (PMID: 31211242).

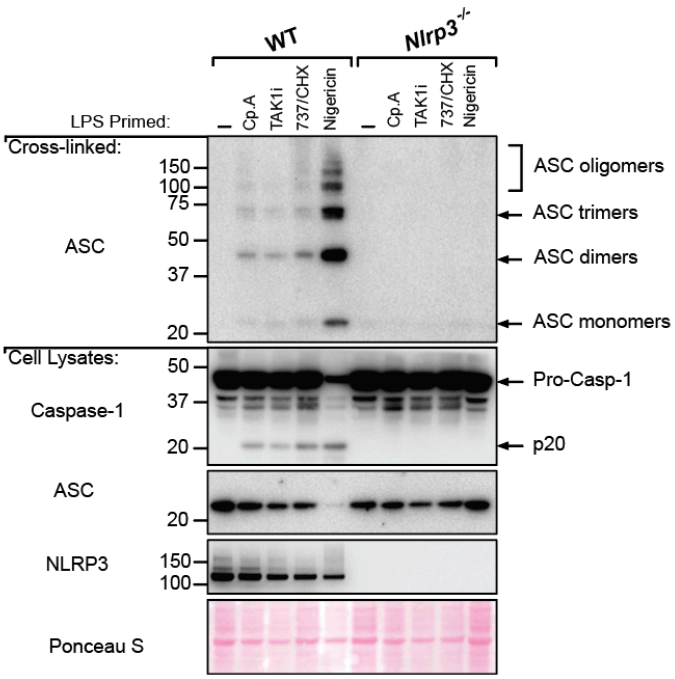
8. Data quality of ASC-oligomerization in Supplemental Figure 3 is insufficient. The authors should prove that the BAX/BAK-mediated, activation-induced ASC-oligomerization is indeed NLRP3 dependent.

Author Response 17

Supplemental Figure 3 of our original manuscript represents an independent repeat of Figure 5A and 5B to show the reproducible nature of our experiments. We have replaced this data set with a third independent repeat experiment (revised manuscript Appendix Figure 2, reproduced below for convenience). We have also performed the requested experiments in NLRP3 deficient mice (Appendix Figure 1B, reproduced below for convenience), which align with our findings using the specific NLRP3 inhibitor MCC950, documenting that Cp. A treatment, TAK1 inhibition, or BAX/BAK induction, all specifically signal NLRP3-driven ASC oligomerization (revised manuscript, Figure 4C and Appendix Figure 1A).



Appendix Figure 2. *BMDMs were treated, as indicated, with LPS (100 ng/ml) for 3 hours before treatment with Cp. A (1 μM), TAK1i (250 nM) for 6 hours, ABT-737 (1 μM) and CHX (20 μg/ml for 4 hours and Nigericin for 45 mins. ASC cross-linking assays were performed to assess inflammasome activation via the formation of ASC dimers, trimers and higher order oligomers.*



New data, Appendix Figure 1B. *BMDMs were treated, as indicated, with LPS (100 ng/ml) for 3 hours before treatment with Cp. A (1 μM), TAK1i (250 nM) for 6 hours, ABT-737 (1 μM) and CHX (20 μg/ml for 4 hours and Nigericin for 45 mins. ASC cross-linking assays were performed to assess inflammasome activation via the formation of ASC dimers, trimers and higher order oligomers.*

9. In Figure 5, the authors should use NLRP3 KO BMDMs as a control to clarify that ASC-oligomerization is indeed caused by NLRP3 activation.

Author Response 18

Please see **Author Response 17** and associated data, above, presented in Appendix Figure 1B of the revised manuscript.

10. It is highly risky to suggest caspase-8-induced cell death by using multiple genes KO iBMDMs generated by CRISPR/Cas9. This is because after multiple rounds of gene editing, the KO cells were already different from WT cells. In our own experience, we have found that the property of Casp3-/-Casp7-/- iBMDMs changed quite a bit, compared to normal iBMDMs. Furthermore, the authors need to complement caspase-8 in the KO iBMDMs to demonstrate that this phenotype is indeed caspase-8 dependent.

Author Response 19

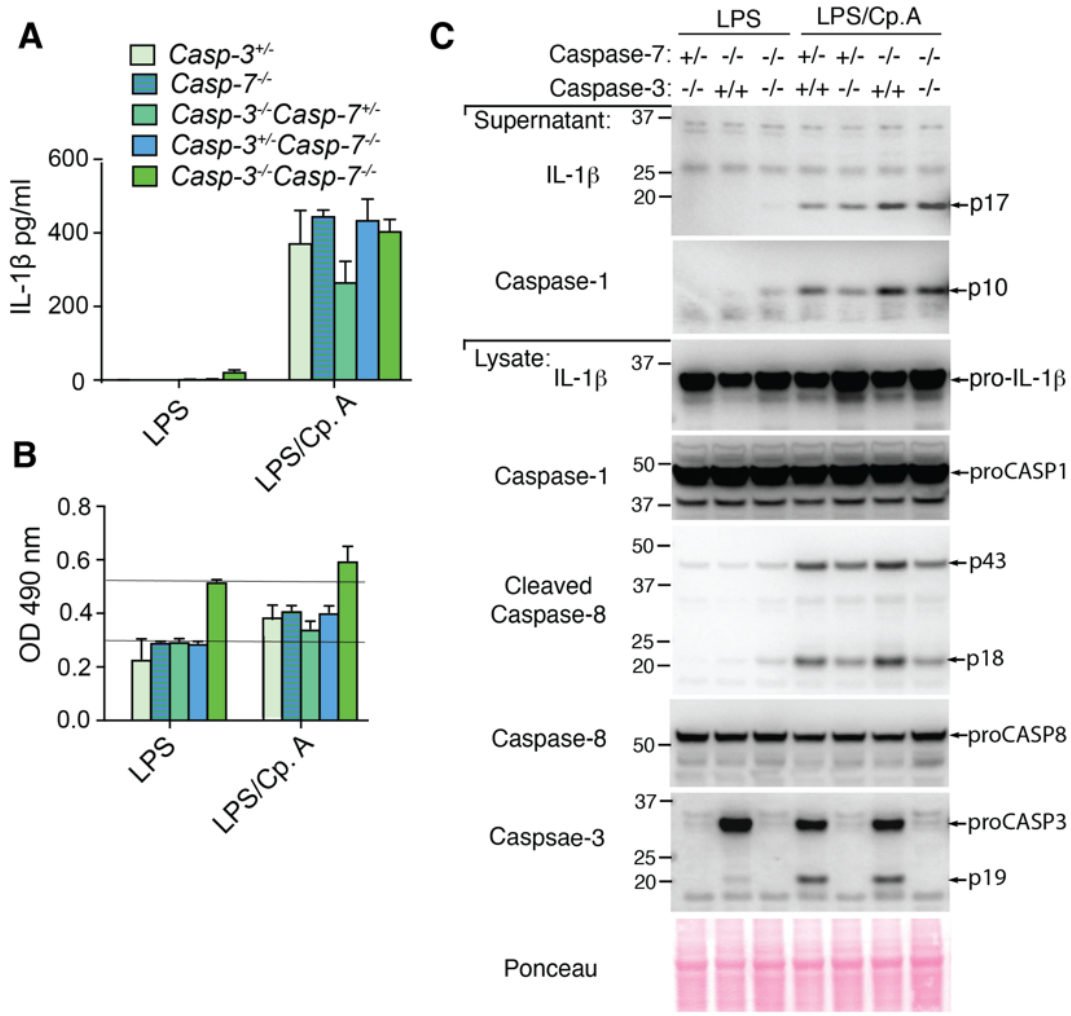
Conclusions on the caspase-3 and -7 phenotype

We agree that it is important that our data be sufficiently strong to support one of our main novel conclusions; that the loss of caspase-3 and caspase-7 does not prevent cell death resulting from IAP deficiency, despite caspase-3 and -7 being required for efficient intrinsic apoptotic killing. This conclusion is drawn from 3 lines of evidence using three different types of cells and two different types of genetic targeting:

1. CRISPR/Cas9 targeting of caspase-3 and -7 in iBMDMs, even in the combined absence of caspase-1,-11 and BID, or caspase-1, -6, -11 and -12, does not limit cell death and cell death-induced IL-1β activation and release resulting from IAP inhibition, despite efficiently blocking BAX/BAK-mediated killing (Figure 6 and 7 and Figure EV4 of the revised manuscript).
2. Fibroblasts derived from caspase-3 and -7 double knockout mice (not generated via CRISPR/Cas9) still die upon IAP antagonist treatment yet, are profoundly resistant to intrinsic apoptotic killing (Figure EV5 of the revised manuscript).

3. Primary foetal liver derived macrophages derived from mice lacking caspase-3 and -7 (not generated via CRISPR/Cas9) respond like control cells to IAP antagonism and undergo cell death-mediated activation and release of IL-1β (Response Figure 1, below). Please note, this data is preliminary and not included in the revised manuscript. It is being built upon for a separate publication which requires us to rederive our caspase-3 and -7 heterozygous animals (colony was shut-down during the 2020 COVID-19 pandemic) and perform extensive crossing (including to our GSDMD and Pannexin-1 mutant mice) and, as such, is beyond the scope and timeframe of the current work.

Importantly, the cumulative evidence we have gathered using both CRISPR/Cas9 targeting and published mice lacking caspase-3 and -7 (PMID: 30485804), as well as three different cell types, all show that caspase-3 and -7 are dispensable for cell death and associated IL-1β activity resulting from IAP loss. This is one of the new (and unexpected) findings of our work that significantly impacts our understanding on how intrinsic and extrinsic cell death is executed.



Response Figure 1. Analysis of foetal liver derived macrophages from caspase-3 and -7 deficient mice. *Macrophages of the indicated genotypes were treated with LPS or with LPS for 2.5 hours followed by Cp. A (1 μM) for 6 hours. Subsequently cell supernatants were analysed for IL-1β release (A), LDH release (B) or both cell supernatants and total cell lysates examined by western blot (C).*

Caspase-8-dependent phenotype

Using primary macrophages derived from caspase-8 deficient mice, we have previously documented that all cell death and inflammasome responses resulting from XIAP antagonism are caspase-8 mediated (PMID: 25693118);

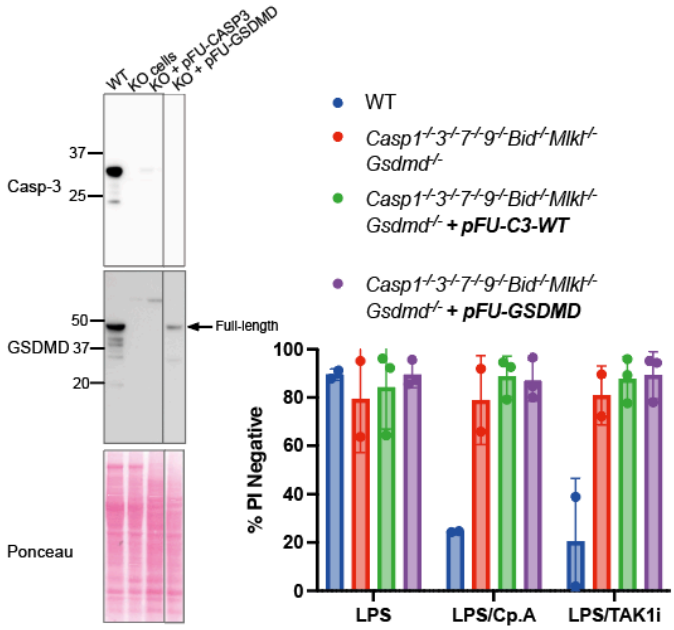
hence, upon IAP loss, caspase-8 is the essential apical caspase from which downstream cell death signalling emanates. This previous work is consistent with our CRISPR/Cas9 gene targeting using iBMDMs in the current manuscript. We first published the reproducible and consistent cell death responses in CRISPR/Cas9 targeted iBMDMs when compared to primary BMDMs two years ago in *Immunity* (PMID 32735843). Even so, in our current manuscript our assays using iBMDMs include appropriate controls to show abrogation of the relevant cell death pathway targeted via CRISPR/Cas9 gene editing including, i) functional cell death pyroptosis assays for caspase-1/-11 deletion, necroptosis assays for RIPK3/MLKL deletion, BAX/BAK apoptosis assays for caspase-3 and -7 deletion, extrinsic apoptosis assays for caspase-8 deletion (Figure 6A, 6B, 6C), ii) immunoblots to show a lack of protein for the relevant gene knocked out (Appendix Figure 3) and iii) Toll-like Receptor stimulations to demonstrate that the inflammasome priming responses are consistent among our CRISPR/Cas9 gene targeted iBMDMs (Appendix Figure 4) and also with those previously reported for primary caspase-3 and -7 (e.g. see PMID: 30485804) and caspase-8 (e.g. see PMID: 24990442) gene targeted macrophages.

11. The authors suggested that in the absence of caspase-1, -3, and -7, activation of GSDMD causes cell death upon XIAP antagonism. To draw a firm conclusion, the authors need to complement caspase-1, -3, -7, or GSDMD in the KO iBMDMs to support the conclusion. Similar experiments are also in need for Supplementary Figure 7.

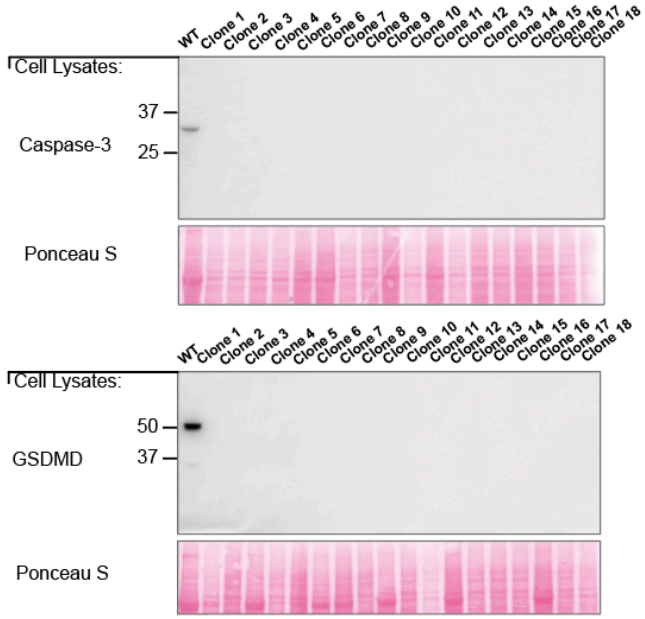
Author Response 20

We have gone to significant lengths in our attempts to complement our iBMDMs with relevant genes. Lentiviral constructs containing caspase-3 and GSDMD were generated which were compatible (e.g. mutation of the sgRNA target site) for expression in our CRISPR/Cas9 targeted iBMDMs. These included caspase-3 (pFU-C3-WT) and GSDMD (pFU-GSDMD). Despite three independent attempts, where cells were successfully infected (with up to three rounds of infection) and selected (using puromycin), we were unable to restore significant expression of caspase-3 or GSDMD in pools of selected cells and, as a consequence, these cells were still resistant to cell death (see representative experiments in Response Figure 2, below). Therefore, to identify cells where caspase-3 and GSDMD expression was restored to wildtype levels we sorted single cells by flow cytometry. However, the analysis of 36 single cell clonal lines failed to identify any clones that restored significant caspase-3 or GSDMD expression (Response Figure 3, below).

We are unsure as to why these experiments were unsuccessful as, using a similar approach, we have previously had no trouble in successfully complementing IL-1$\beta^{-/-}$ and *Ripk3*$^{-/-}$ deficient iBMDMs with IL-1β and RIPK3, respectively (PMID:35800780 and PMID:33976225). As such, we believe that *Caspase-1*$^{-/-}$*3*$^{-/-}$*7*$^{-/-}$*9*$^{-/-}$*Bid*$^{-/-}$*Mlkl*$^{-/-}$*Gsdmd*$^{-/-}$ iBMDMs may have adapted for increased basal caspase-8 activity over time due to the absence of any negative selection (i.e. normally, in presence of the caspase-8 substrates such as caspase-3,-7, BID and GSDMD there will be constant selection to limit basal caspase-8 activity so that cells do not die). As such, in our experiments where we infected these iBMDMs with GSDMD and caspase-3 lentiviral plasmids, these death effectors may be spontaneously activated by caspase-8 to cause macrophage death, thereby ensuring we never recover iBMDMs with adequate GSDMD or caspase-3 expression. Hence, our inability to complement *Caspase-1*$^{-/-}$*3*$^{-/-}$*7*$^{-/-}$*9*$^{-/-}$*Bid*$^{-/-}$*Mlkl*$^{-/-}$*Gsdmd*$^{-/-}$ iBMDMs with significant levels of caspase-3 and GSDMD may indicate that we have restored cell death. Figure EV5D of the revised manuscript further emphasizes how restoring death effector protein levels can be difficult, as this data shows that caspase-8 expression alone causes its autoactivation and subsequent cleavage of GSDMD without any stimulus.



Response Figure 2. *Caspase-1$^{-/-}$3$^{-/-}$7$^{-/-}$9$^{-/-}$Bid$^{-/-}$Mlkl$^{-/-}$Gsdmd$^{-/-}$ (indicated as "KO") iBMDMs stably selected for caspase-3 (CASP3) or GSDMD expression via puromycin treatment were examined by western blot (left panel) and cell death (right panel; symbols indicate independent experiments).) The western blot represents the analysis of one of three independent experimental attempts to restore caspase-3 and GSDMD expression via viral infection and selection. Please note, in the experiment shown all samples were run on the same gel, but irrelevant lanes have been removed for clarity (delineated by a line).*



Response Figure 3. *Caspase-1$^{-/-}$3$^{-/-}$7$^{-/-}$9$^{-/-}$Bid$^{-/-}$Mlkl$^{-/-}$Gsdmd$^{-/-}$ deficient iBMDMs infected and selected for caspase-3 (top panel) or GSDMD (bottom panel) expression by puromycin treatment were single cell sorted and clonal populations expanded and analysed by western blot.*

Dear James,

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original two referees and their comments are provided below.

As you can see from the comments below, referee #1 is not convinced that the revisions go far enough to consider publication here. I have discussed the points further with referee #2 and would like to propose the following revisions:

1) Compound A concern:

KD: Please follow the reviewer's request and blot the expression of cIAP1/2 upon compound A treatment and use cIAP1/2-specific smac mimetic to demonstrate that targeting cIAP1/2/ does not give the same cell death/inflammation phenotype as XIAP inhibition. I recognise that similar data are shown in previous publications, but we also find it of value to show the data in the same experimental setting.

2) Caspase-3 and caspase-7 processing

KD: It would be good to perform the assay under the same condition as those in Fig. 2, 3, 4, 5 and 7. You don't need to show the full set of blots as shown in the original figures.

3) Data on Caspase-3/7 knock out cells:

KD: You don't have to include data on Caspase-3/7 knock out cells in the present study.

Can you please get back to me and discuss the points above with me.

When you submit the revised version will you also take care of the following points:

- You can only have 5 keywords

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

- REFERENCE FORMAT: Citations with more than 10 authors please cut after 10 authors + et al.

- We need ORCID for Lanlan Geng, would also be good to include an institutional email address for L. Geng.

- Please check that the funding matches between online submission and MS file.

- Callouts for Appendix figures should be Appendix Figure S1-S4

- The appendix needs an eTOC

- Author email bounced for Rochelle Tixeira (R.Tixeira@latrobe.edu.au) please fix.

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

As said above, it would be good if you would contact me to discuss the points above.

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

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

Use the link below to submit your revision:

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

Referee #1:

Revised version
In the revised version of their manuscript the authors attempted to address some of reviewers' criticism. However, their response falls short of addressing the critical issues raised by reviewers.

The authors refusal to check the levels of cIAP1/2 proteins when they used Compound A precludes proper interpretation of their results given that cIAP1/2 are the main targets of Compound A. XIAP is affected by Compound A but only partially as it predominantly binds BIR3 domain of XIAP with minimal/no effect on the BIR1, BIR2 or the RING domains. The title and the abstract are misleading as they state XIAP deficiency or XIAP loss when in fact, XIAP deficiency is only examined in figures 1 and 7. The rest of experiments in the majority of figures uses Compound A that greatly and negatively affects cIAP1/2, and likely other IAPs. Thus, it is not correct to call this XIAP deficiency.

Next, the request to show simple processing of caspase-3 and caspase-7, which indicates their activity and/or involvement in examined phenomena is rejected. The authors claim they do not have these samples any more. But the bigger concern surrounding the authors refusal to perform these simple experiments is whether these experiments could be reproduced? Related to caspase-3/7 issues the authors should perform cell death assay on casp3/casp7 single and double knockout cells using PI staining following treatment with LPS +/- Cp A, and include that data along with the data from Response figure 1 in their manuscript.

Appendix figure 1B should be included in the manuscript.

Referee #2:

The authors have performed extensive experiments to address my previous comments. I am OK with the current version although the novelty and significance of the study has decreased.

Dear Editors and Reviewers,

Thanks very much for your careful consideration of our manuscript. We appreciate the proposed revisions, and have now completed the agreed upon new experiments, detailed below. These new data have been incorporated as five new figure panels (Figure 2A, EV Figure 1F, EV Figure 1G, EV Figure 3B and EV Figure 3D) into the revised manuscript (with all changes tracked).

Dear James,

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original two referees and their comments are provided below.

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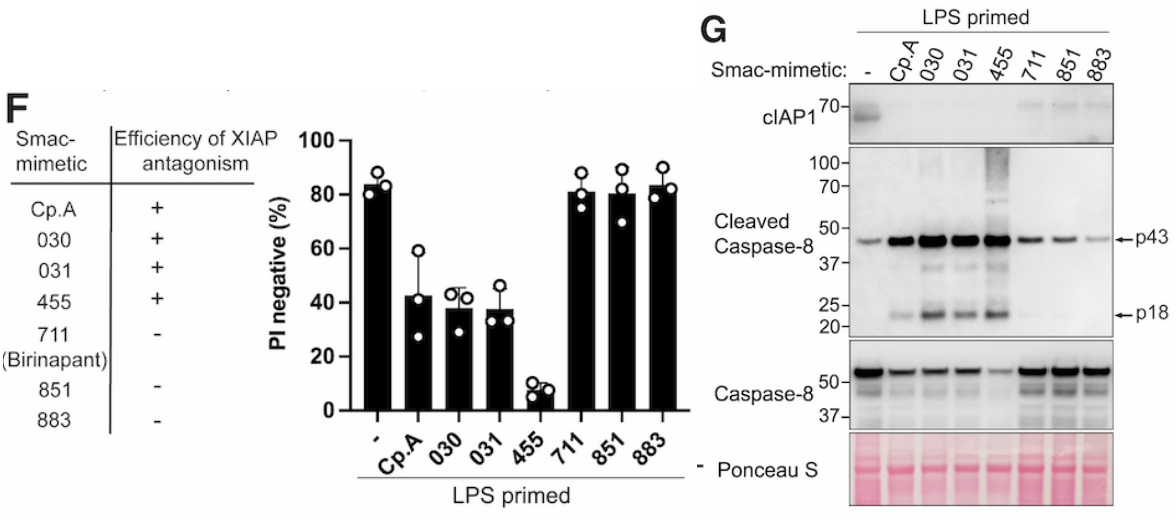
1) Compound A concern:

KD: Please follow the reviewer's request and blot the expression of cIAP1/2 upon compound A treatment and use cIAP1/2-specific smac mimetic to demonstrate that targeting cIAP1/2/ does not give the same cell death/inflammation phenotype as XIAP inhibition. I recognise that similar data are shown in previous publications, but we also find it of value to show the data in the same experimental setting.

We have performed these experiments using a variety of smac-mimetic compounds that have selectivity for the cIAPs, or target both XIAP and cIAPs (new data, revised manuscript Figure 2A, EV Figure 1F, EV Figure 1G, reproduced below for convenience). This demonstrates to readers (without them having to refer to previous studies) that smac-mimetic targeting of XIAP, but not cIAP1/2, is essential for the ensuing cell death and inflammatory phenotype, as noted in our revised manuscript text:

"We next sought to model XIAP deficiency through the use of small molecule IAP antagonist (smac-mimetic) compounds. Although TLR and/or TNF-induced cIAP degradation is required for the IL-1b activation and macrophage death that can result from XIAP loss (Lawlor *et al.*, 2017), the loss of cIAPs alone does not suffice to cause these responses (Lawlor *et al.*, 2015). Consistent with these studies, only IAP antagonists that inhibit XIAP and cIAPs efficiently (Cp.A, 030, 031, 455) caused high levels of macrophage death, caspase-8 processing, and associated caspase-1 and IL-1b activation, while those with selectivity for cIAPs (711 [birinapant], 851 and 883), did not (**Fig. 2A and Fig. EV1F, EV1G**) (Condon *et al*, 2014; Lawlor *et al.*, 2015). Therefore, XIAP deficiency can be modelled in cells by treatment with bivalent IAP antagonists, such as Compound A (Cp. A), which target XIAP and cIAPs (Vince *et al*, 2007)."



New data, EV Figure 1 F and G. BMDMs were seeded at a density of 4 x 10^5 cells per well, primed with 20 ng/ml of LPS for three hours then treated, as indicated, with 500 nM of the indicated smac-mimetic compounds for 20 hours. Cell viability (**F**) was determined by PI staining and flow cytometry and measured as a proportion of PI-negative (live) cells. Data represent the mean of three independent experiments (symbols). Error bars are the mean ± SD. Alternatively, cell lysates were analysed by western blot (**G**). Data representative of two independent experiments.

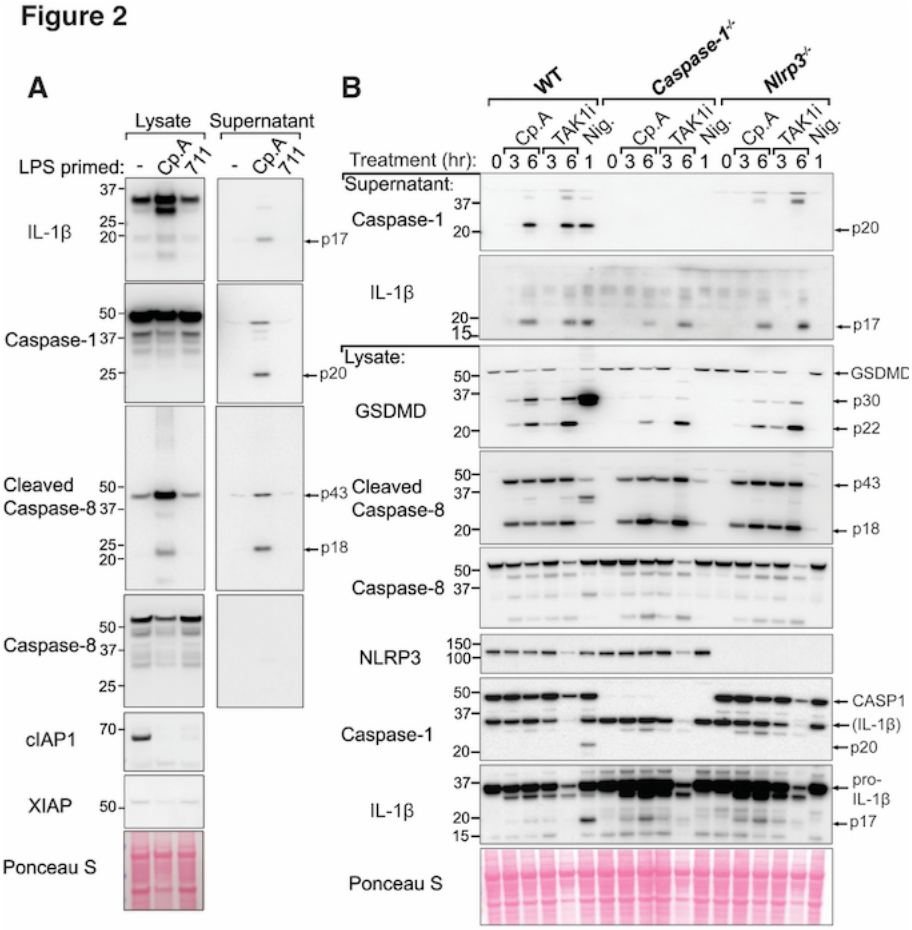


Figure 2. NEW data panel A. BMDMs were seeded at a density of 4 x 10^5 cells per well, primed with 20 ng/ml of LPS for three hours then treated, as indicated, with 500 nM of Cp.

A or 711 (Birinapant) for 20 hours and cell lysates and supernatants were analysed by western blot. Ponceau staining depicts protein loading. Data representative of two independent experiments.

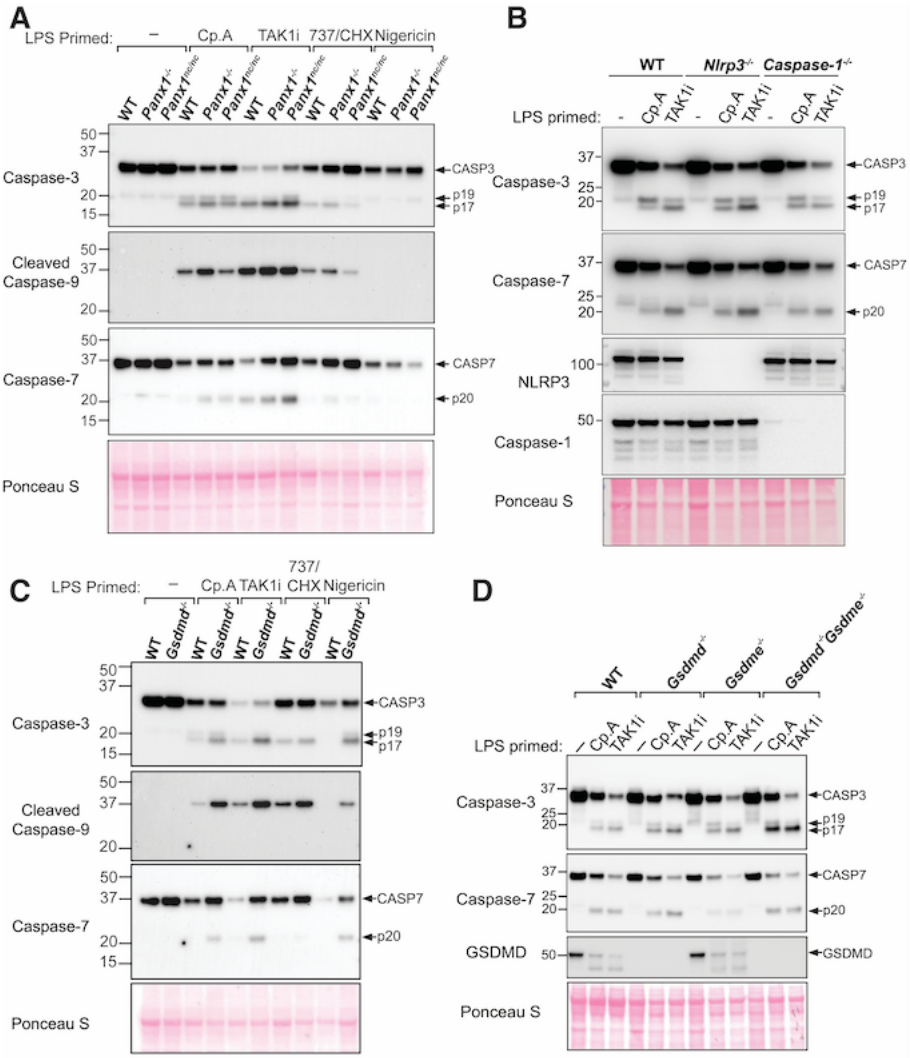
2) Caspase-3 and caspase-7 processing

KD: It would be good to perform the assay under the same condition as those in Fig. 2, 3, 4, 5 and 7. You don't need to show the full set of blots as shown in the original figures.

We included experimental data examining caspase-3 and -7 processing in WT vs GSDMD knockout macrophages (conditions depicted in Figures 3 and 4), and WT vs Panx1 knockout vs Panx1 point mutant macrophages (conditions shown in Figure 5) in our previous revisions (EV Figure 3A and 3B).

We have now performed additional caspase-3 and caspase-7 processing western blots in the additional genotypes depicted in Figure 2 (NLRP3 and caspase-1 deficient macrophages) and Figure 3 (GSDME deficient and GSDMD/GSDME double knockout macrophages). These new data have been incorporated into EV Figure 3B and 3D of the revised manuscript (reproduced below for convenience).

Regarding the reviewers request that we perform these blots under conditions in Figure 7, we would note that this figure examines cell death responses in caspase-3 and -7 double knockout macrophages, and we already show that these cells completely lack caspase-3 and -7, as expected (Appendix Figure 3).



Expanded View Figure 3. Evaluation of Caspase-3, -7 and -9 processing in BMDMs in response to apoptotic and pyroptotic stimuli

A, B (New data), C and D (New data). BMDMs of the indicated genotypes were seeded at a density of 4 x 10^5 cells per well and primed with LPS (100 ng/ml) for three hours before treatment with Cp. A (1 mM) or TAK1i (250 nM) for six hours, ABT-737 (1 mM) and CHX (20 mg/ml) for four hours, or nigericin (10 mM) for 45-60 minutes. Cell lysates were harvested and analysed by western blot. Data represent 3 (**A and C**) or 2 (**B and D**) independent experiments.

3) Data on Caspase-3/7 knock out cells:

KD: You don't have to include data on Caspase-3/7 knock out cells in the present study.

Thank you. This is much appreciated (as we indicated to the reviewer in our response letter, this colony will take almost another year to rederive).

Can you please get back to me and discuss the points above with me.

When you submit the revised version will you also take care of the following points:

- You can only have 5 keywords

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Please note the ORCID number entry box in the online system read “N/A” and did not allow us to enter it here, so the number was addd below this in the “EMBO” entry box in the online system. To clarify, his ORCID number is: 0000-0002-0336-952X

The current email address is the best one for people to contact him on, so we have had to leave this as is.

- Please check that the funding matches between online submission and MS file.

Checked.

- Callouts for Appendix figures should be Appendix Figure S1-S4

Corrected.

- The appendix needs an eTOC

Completed.

- Author email bounced for Rochelle Tixeira (R.Tixeira@latrobe.edu.au) please fix.

We have provided a current email address for Rochelle.

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

the issues.

We adjusted Figure legends as requested (and have responded to comments and tracked changes accordingly in the revised manuscript).

As said above, it would be good if you would contact me to discuss the points above.

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

Revised version
In the revised version of their manuscript the authors attempted to address some of reviewers' criticism. However, their response falls short of addressing the critical issues raised by reviewers.

The authors refusal to check the levels of cIAP1/2 proteins when they used Compound A precludes proper interpretation of their results given that cIAP1/2 are the main targets of Compound A. XIAP is affected by Compound A but only partially as it predominantly binds BIR3 domain of XIAP with minimal/no effect on the BIR1, BIR2 or the RING domains. The title and the abstract are misleading as they state XIAP deficiency or XIAP loss when in fact, XIAP deficiency is only examined in figures 1 and 7. The rest of experiments in the majority of figures uses Compound A that greatly and negatively affects cIAP1/2, and likely other IAPs. Thus, it is not correct to call this XIAP deficiency.

We have performed new experiments showing that only XIAP targeting with smac-mimetics reproduces the XIAP deficient phenotype, while those that selectively target cIAP1/2 do not (see above for new data), as expected. We also addressed this point in our previous response letter where we explained and referenced our and other labs past genetic and biochemical studies examining cIAP1/2 deficient/inhibited macrophages to those lacking

XIAP. Our manuscript examines levels of cIAP1 following IAP targeting in Figure 2A, EV Figure 1G and Figure 7, and this shows the expected degradation of cIAP1.

Our previous reviewer response and revised manuscript also clearly refers to the use of Cp. A to model XIAP loss, and explains how Cp. A targeting of XIAP/cIAPs, mirrors XIAP deletion (as genetic XIAP loss destabilizes cIAPs):

"We next sought to model XIAP deficiency through the use of small molecule IAP antagonist (smac-mimetic) compounds. Although TLR and/or TNF-induced cIAP degradation is required for the IL-1b activation and macrophage death that can result from XIAP loss (Lawlor *et al.*, 2017), the loss of cIAPs alone does not suffice to cause these responses (Lawlor *et al.*, 2015). Consistent with these studies, only IAP antagonists that inhibit XIAP and cIAPs efficiently (Cp.A, 030, 031, 455) caused high levels of macrophage death, caspase-8 processing, and associated caspase-1 and IL-1b activation, while those with selectivity for cIAPs (711 [birinapant], 851 and 883), did not (**Fig. 2A and Fig. EV1F, EV1G**) (Condon *et al*, 2014; Lawlor *et al.*, 2015). Therefore, XIAP deficiency can be modelled in cells by treatment with bivalent IAP antagonists, such as Compound A (Cp. A), which target XIAP and cIAPs (Vince *et al*, 2007)."

We use the term "XIAP deficiency" in our title and abstract, as our manuscript provides first-time clinical data showing that diseased cells/tissue from XIAP deficient humans display features of both apoptotic (as reflected by caspase-8 processing) and pyroptotic (as reflected by GSDMD processing) signalling, which is alleviated following immune suppressive therapy.

Next, the request to show simple processing of caspase-3 and caspase-7, which indicates their activity and/or involvement in examined phenomena is rejected. The authors claim they do not have these samples any more. But the bigger concern surrounding the authors refusal to perform these simple experiments is whether these experiments could be reproduced? Related to caspase-3/7 issues the authors should perform cell death assay on casp3/casp7 single and double knockout cells using PI staining following treatment with LPS +/- Cp A, and include that data along with the data from Response figure 1 in their manuscript.

Caspase-3 and -7 processing and reproducibility

Our revised manuscript (noting that the data was included in the previous reviewer response for ease of reference) examined caspase-3 and -7 processing in WT and GSDMD KO macrophages as well as in WT, Panx1 and Panx1 point mutant macrophages, thus we are perplexed as to why reviewer #1 believes us to have refused to perform these experiments. We have now performed additional caspase-3 and -7 blots for GSDMD and GSDME single and double knockout macrophages and in NLRP3 and Caspase-1 knockout macrophages (New data, EV Figure 3B and 3D, reproduced above).

Our figure legends clearly define how many times each experimental western blot data set ws reproduced, and our cell death graphs always represent combined independent experiments of n = 3-5. We previously mentioned no longer having access to the original

samples to explain why we would conduct these cleavage assays separately to the data that were presented in the original manuscript.

Caspase-3 and -7 double knockout analysis

Our manuscript shows that CRISPR/Cas9 deletion of caspase-3 and -7 in immortalised macrophages, or fibroblasts derived from caspase-3 and -7 gene targeted mice, does not impact cell death responses resulting from IAP targeting. As stated in our previous response letter, Response Figure 1, using foetal macrophages derived from caspase-3 and -7 deficient animals, represents preliminary data acquired prior to the COVID-19 pandemic. Unfortunately, during the pandemic we had to shut down this mouse colony, and to rederive these mice and reproduce these findings would take almost a year.

Appendix figure 1B should be included in the manuscript.

We have incorporated this data as Figure 4D in the revised manuscript.

Referee #2:

The authors have performed extensive experiments to address my previous comments.

We are glad we were able to extensively address the reviewer comments.

I am OK with the current version although the novelty and significance of the study has decreased.

Thank you. We also outlined the novelty and significance of our clinical observations and mechanistic findings at the beginning of our previous response letter, copied below for convenience:

1. We show for the first time that XIAP deficient patients contain elevated levels of activated GSDMD and caspase-8 in inflamed colonic mucosae and LPS treated PBMCs, and that the activation of these key cell death molecules is reduced following therapy.

2. We show for the first time that XIAP targeting with IAP antagonists can still cause efficient cell death and inflammatory IL-1b activation even upon the combined genetic deletion of key apoptotic (BID, caspase-3, -7), and pyroptotic (caspase-1, -11) effectors, and this occurs as a consequence of caspase-8 cleavage of GSDMD. This redefines how caspase-8 can signal cell death in innate immune cells. These findings have relevance beyond IAP deficiency, as genetic mutations in A20 and the LUBAC machinery, which results in auto-inflammation in humans, can also pre-dispose to heightened caspase-8 and inflammasome responses.

3. We discover that extrinsic apoptotic activation of the NLRP3 inflammasome, as observed upon IAP loss or TAK1 inhibition, occurs via a distinct mechanism to intrinsic apoptotic activation of NLRP3. This is a remarkable finding as both these apoptotic pathways converge on caspase-3 and -7 activation. However, our experiments show that only intrinsic

(and NOT extrinsic) apoptotic signalling to caspase-3 and -7 and their processing of Pannexin-1 contributes to NLRP3 inflammasome assembly. These conclusions are supported by our analysis of Pannexin-1 deficient mice in addition to our novel caspase-3 and -7 non-cleavable Pannexin-1 point mutant mouse.

4. Our genetic and biochemical data using our two distinct Pannexin-1 mutant strains of mice refute a main conclusion of a recently published EMBO Journal study that has received significant attention (PMID: 30902848). We do not wish to imply that the authors of this previous study have done anything wrong other than come to conclusions that cannot be supported in the light of our work and, as such, believe that the publication of our data in the same forum is important for scientific transparency.

2nd Revision - Editorial Decision **19th Dec 2022**

Dear James,

Thank you for submitting your revised MS to The EMBO Journal.

I have now had a chance to take a careful look at the revisions and the added data. I appreciate the introduced changes and I am very pleased to accept the manuscript for publication in the EMBO Journal.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
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  - definition of 'center values' as median or average;
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Methods, section "Cell culture"

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