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Secretion of mitochondrial DNA via exosomes promotes inflammation in Behçet's syndrome

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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.)

1st Editorial Decision **6th Oct 2022**

Dear Hyota,

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

As you can see, the referees find the analysis interesting and are supportive for consideration here. They raise constructive comments that I would like to ask you to address in a revised version.

I am happy to discuss the raised points further and it would be helpful to do so via video call. Let me know when works best.

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

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

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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- I have attached a guide with helpful tips on how to prepare the revised version.

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- 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 (4th Jan 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

- As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study.

- Use the link below to submit your revision:

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Referee #1:

The manuscript by Konaka et al. is interesting. They found that in patients with Behçet syndrome, pyroptotic cells secrete mtDNA encapsulated in exosomes, which triggers NLRP3- and TLR9-dependent inflammatory responses and promotes the progression of Behçet syndrome. The authors have presented reasonable and solid data to address the hypothesis. However, there are still some concerns that should be addressed before considering the publication of this manuscript.

Major points:
1. The major flaw in the study is the lack of evidence that the DNA in the Evs is actually mtDNA and not nuclear DNA.
2. In addition, the authors need further experimental evidence to confirm that the EVs are exosomes and not other microvesicles that exit directly from the plasma membrane. What is the ratio (mtDNA/nDNA), because it is very likely that the released DNA in the cytocol exits extracellularly from the plasma membrane via microvesicles.
3. "Extracellular mtDNA released from THP-1 cells by necrosis or secondary necrosis could be digested by DNase I. Conversely,

mtDNA released by apoptosis or pyroptosis could not be digested with DNase I unless samples were treated with Triton-X100 (Fig. 1D), suggesting that mtDNA is entrapped in EVs and released outside the cell when monocytes undergo apoptosis or pyroptosis."
--Are DNase I treatment assays commonly used to show whether certain things are entrapped in EVs? If so, please cite the original article. If not, the conclusion that mtDNA is included in EVs is a priori and inappropriate here. I suggest that you draw this conclusion after showing the data from TEM below. (page 10-11)
4. Whether the mtDNA that is included in EVs is outside the mitochondria? This can be demonstrated by observing co-localization between mtDNA and mitochondria in addition to detecting co-localization between mtDNA and TSG101 (Figure 3A and C).
5. "First, 100K-EVs isolated from WT and mtDNA-defective ρ0 THP-1 cells were injected into the peritoneum or ankle of C57BL6/J mice." Did the authors confirm whether mtDNA depletion was successful in 100k EVs? (page 15)
6. In the top panel of Fig. 4H, the values seem to be the same in each group. Please check the source data.
7. in Fig. 5A, serum mtDNA levels in patients with RA and SLE do not appear to be higher than those in healthy controls. In contrast, the authors found that mtDNA levels in 16k EVs increase in patients with SLE (Fig. 5F). In other studies, mtDNA levels are increased in these two groups or mouse models (PMID: 34916301, PMID: 12932286, and PMID: 31857488). Thus, this should be discussed.
8. "Liposomes containing BS 100K-EV-derived DNA induced IL -1β and IL -23 production via NLRP3 and TLR9, respectively (Fig. 7F, G)." Nuclear DNA is also released into the cytosol under stress (PMID: 34715021), and cells can secrete different types of DNA via exosomes (PMID: 28508895). Did the authors detect nuclear DNA in EVs?
9. As we know, cytosolic DNA is also recognized by AIM2 and cGAS. How can the authors exclude them and focus on NLRP3 and TLR9?
10. "We observed reduced cytoplasmic mtDNA leakage in Gsdmd- KO THP -1 cells, suggesting that gasdermin-D may form pores in the mitochondrial membrane and promote mtDNA leakage."
- Indeed, as the authors noted, previous studies have reported that activated GSDMs, including GSDME-NT and GSDMD-NT, translocate to the mitochondrial membrane and release mitochondrial contents (PMID: 30976076). However, we should consider that activated GSDMs cause permeabilization of the plasma membrane, which contributes to impaired mitochondrial homeostasis and release of mtDNA, apart from directly forming the pores on the mitochondrial membrane (PMID: 34233045). I therefore suggest that the authors modify the statements or perform further tests to confirm this, such as demonstrating translocalization of GSDMD-NT from the cytosol to the mitochondria, as Rogers et al. did in Fig. 4b (PMID: 30976076). (page 24)

Minor points:
1. Which EVs are confirmed by the data, 16k or 100k? (Figure EV1B-C).
2. "Tsg101 punctate formation was observed in pyroptotic THP-1 cells but less so in apoptotic cells." Please check the wording. Should "punctures" be changed to "punta"? (page 13)
3. In Fig. EV2 J, the Casp 1 band in the third lane is too weak.
4. in Fig. 4B, the Ctrl groups of p0 and WT should be listed.
5. in the sixth line from the bottom on page 15 of the manuscript, the protocol: mtDNA purification from mitochondria is mentioned. The scientific validity of this method for mtDNA purification and encapsulation needs to be further verified (checking the composition of the isolates?).
6. In Fig. 4I, the ankles of the mice were swollen due to the injection of MSU, but it was not clearly highlighted in the figure, so it is suggested to add a label.
7. In Fig. 6H,I, both the nongroup and the ATP+LPS group should be supplemented with the Ctrl group of WT with the Ctrl group of NLRP3 (in agreement with Fig. 6G lane) to illustrate the efficacy of NLRP3 supplementation.
8. in Fig. 7A, the authors enumerate the induction of changes in the levels of IL -1β, IL -23, and TNF-α by BS 100K-EVs, why was IL -23 removed from the detection of pro-inflammatory factors by BS 16K-EVs in Fig. 7B?

Referee #2:

In this study Konaka et al., report that mitochondrial (mt) DNA secreted in exosomes from the pyroptotic cells has inflammatory properties. The authors propose that activated caspase-1 induces GsdmD-dependent mtDNA leakage into the cytoplasm and that caspase-1 is required for the intraluminal membrane vesicle (IMV) formation. They found, that exosomal mtDNA promotes a strong inflammatory response. Finally they show that monocytes from Behçet's syndrome (BS) patients exhibit increased mtDNA secretion via exosomes, causing the disease pathology.
I find the data presented by the authors very interesting as they emphasize the importance of exosomes in human diseases. The paper is well written and the most of the conclusions are supported by the data presented. The major concern is regarding the claim about caspase-1 requirement for IMV formation. The authors did not follow their initial finding that caspase-1 KO THP-1 cells lacked formation of Tsg101 puncta upon LPS+ATP stimulation. Would this finding mean that in the absence of caspase-1 there's no exosome formation upon LPS+ATP treatment? The data presented in Fig. 3A suggest that only some fraction of Tsg101 puncta co-localizes with mtDNA. In addition, the authors did not specify how caspase-1 is involved in the IMV formation (e.g. does it interact with other proteins? Or some other mechanism(s)?). I also think the study would benefit if the authors

characterize better the exosomes (proteins) involved in mtDNA encapsulation, e.g. by performing mass spec on isolated EVs. Below are some specific minor concerns, which should be addressed:
1. The authors stated that they "determined the increased mtDNA levels in the culture supernatant (CS) of LPS-induced human PBMCs via quantitative PCR (Fig EV1A)". Please clarify if you observed the mtDNA in the supernatants just after LPS stimulation? Without signal 2?
2. Please provide the Western blot panel for THP-1 cells and CD14 monocytes (whole cell lysates and supernatants) unstimulated and stimulated with LPS+ATP, including caspase-1, GsdmD, and IL-1β. Please show whole (uncut) blots for all these proteins.
3. Is Caspase 8 activated in these cells? Caspase-8 was recently reported to be activated in response to LPS+ATP and found in the protein complex within exosomes (Bulek et al, 2020).
4. In addition to mtDNA quantification (e.g. Fig 2E, 2G and other) the authors should provide exosomes quantification (e.g. using ZetaView or other nanoparticles analyzer which can provide both quantity and the sizes of the exosomes).
5. There's no information on how the authors quantified the exosomes for the in vivo studies and how many were injected. Please add these information.
6. There are some discrepancy between text and figure labeling. For example, in the text the authors stated that "the increased secretion of mtDNA in 100K-EVs and reduced MMP induced by LPS and ATP stimulation was abrogated by the Ac-YVAD-cmk (Fig 6E)", while in the Fig 6E the authors show only single treatments. The same apply to Fig 6D. Please clarify this issue.
7. Please show the whole uncut Western blots for Fig 6C and 6G, including whole cell lysates. In addition, for panel 6G include caspase-1 and IL-1β, and for 6C include GsdmD.
8. For proving/disapproving that mtDNA signals via TLR9, TLR9 KO should be used (e.g. in monocytes) instead of an overexpression system in THP-1 cells.

Referee #3:

The study by Konaka et al, identifies the role of inflammasome/pyroptosis in the loading of mt-DNA into exosomes in monocytes, and characterize the kinetics and key factors regulating this process in numerous in vitro and in vivo experiments. Moreover, authors associate this process as a unique mechanism contributing to Behcet´s Syndrome compared to other autoimmune disorders. The data are certainly interesting and of a potential therapeutic impact. However, some issues regarding the mechanisms, the pyroptosis versus inflammasome involvement, and the possible involvement of other inflammasome sensors remain to be addressed.

MAJOR COMMENTS
-Some of the microscopy images do not seem representative from quantified effects (green mt DNA non-associated with mitochondria (Figure 2A) and the single zoomed image from figure 3B (in this case there is no quantification).
-The presence of exosomal Mit-DNA has been previously reported and the sensing of mitochondrial DNA loaded into exosomes by dendritic cells has been linked to the cGAS-STING pathway (Torralba et al., Nat Commun 2018). This should be properly discussed in the manuscript. Authors should assess pyroptosis induced mt-DNA sensing in the absence of the cGAS-STING sensor.
-Gasdermin KO cells do not leak mt-DNA (similarly to Caspase 1 KO cells) to the cytoplasm. However, Gasdermin-D did not affect the formation of TSG101 vesicles. Are these vesicles not loaded with mt-DNA? (as suggested by zoomed image in figure 3F), and if so, could authors provide an explanation for this? Otherwise, it may seem that inflammasome activation rather than pyroptosis may account for the mt-DNA release.
-The mt-DNA analysis in the serum of Beckett Syndrome patients compared to plasma from other different autoimmune disorders is quite interesting (Figure 5). In some of these disorders (for example RA and SjS), the inflammasome has been involved. Therefore, could authors correlate levels of active IL1Beta in plasma from these individuals with mt-dsDNA content (as they did for the reporter experiments in Figure 7) to rule out that the patient cohorts other than BS are not exhibiting lower levels of inflammation due to treatment initiation?
-In figure 6H, authors claim that Mo from BS secrete higher levels of mtDNA due to a mutation in NLRP3 associated with the disease, however, the impact of this mutation compared to wt is quite limited and partial, although significant. Have authors considered other contributing factors?
-Authors should rule out the involvement of other inflammasome sensors with known activity in detection of intracellular DNA such as AIM2 or IFI16.

MINOR COMMENTS:

-In the in vivo ankle swelling experiments (Figure 4E-4G), authors inject mt-DNA encapsulated into liposomes, which induced slightly significant higher levels of joint thickness and they try to address the inflammasome involvement using the caspase and DMA inhibitors, but this drug could be affecting more than exosome vesicle sensing. Authors should determine the effect of these inhibitors by injecting exosomes derived from LPS-ATP stimulated monocytes containing the mtDNA to confirm their data in more physiological conditions, as they did in figure 8.
- Data on caspase 1-KO shown in Fig. 7D-E should be described in the text under Results, page 20.

Point-by-Point Response to Referees

We would like to thank the referees for providing comments and suggestions. We have modified the manuscript in accordance with your feedback. We have provided a point-by-point response to the reviewers' comments below.

Response to Referee #1

The manuscript by Konaka et al. is interesting. They found that in patients with Behçet syndrome, pyroptotic cells secrete mtDNA encapsulated in exosomes, which triggers NLRP3- and TLR9-dependent inflammatory responses and promotes the progression of Behçet syndrome. The authors have presented reasonable and solid data to address the hypothesis. However, there are still some concerns that should be addressed before considering the publication of this manuscript.

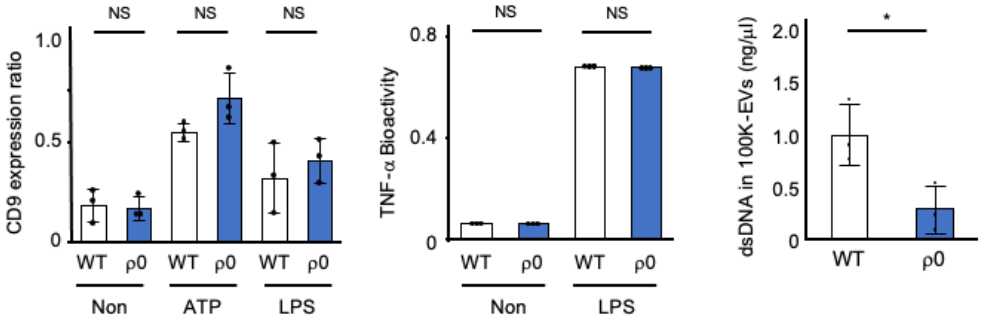
Thank you for your valuable comments and for appreciating that our findings are reasonable and solid. We have responded to your specific comments below.

Major points:

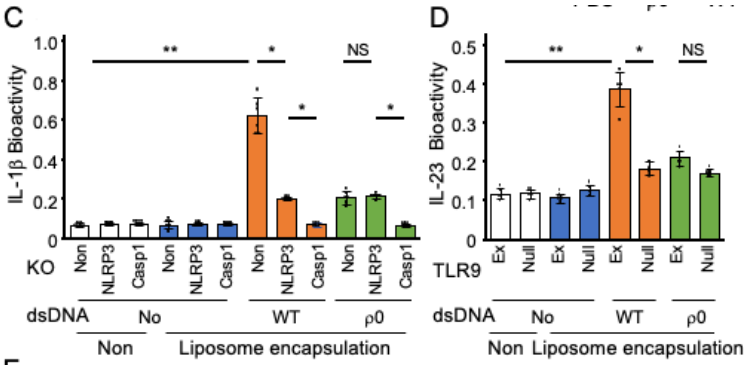
1. The major flaw in the study is the lack of evidence that the DNA in the Evs is actually mtDNA and not nuclear DNA.

To distinguish between nuclear DNA (nDNA) and mtDNA in EVs, some previous studies have attempted to evaluate them by NGS (***Nat Commun.*** Doi: 10.1038/ncomms15287). However, we were unable to perform NGS due to financial and technical reasons. Therefore, we attempted to answer the referee's concerns in two ways.

First, we compared the amount of dsDNA in 100K-EVs of WT and ρ0-THP1 cells. dsDNA was purified from 100K-EVs isolated from the culture supernatant of WT and ρ0 THP1 cells, then the amount of dsDNA was quantified using Quant-iT dsDNA assay kit, which fluoresces when intercalated with dsDNA. The dsDNA level in 100K-EVs were increased in WT THP1 compared to ρ0 THP1 cells although the level of 100K-EVs was comparable (**Fig EV2H–J**).



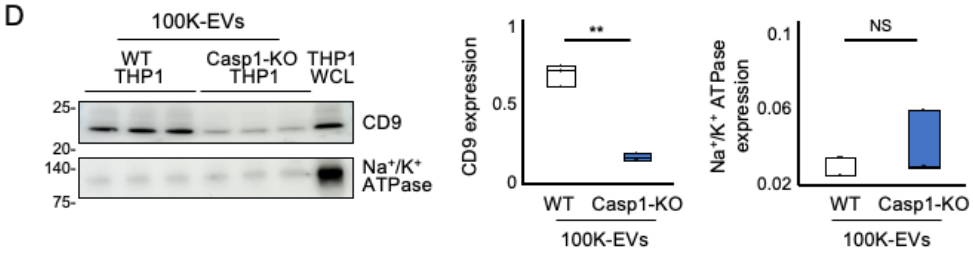
Second, dsDNA isolated from WT-THP1 cells and ρ0-THP1 cells were encapsulated in liposomes and the level of cytokine production by these liposomes was compared. WT-derived dsDNA, which consists of both nDNA and mtDNA, could induce IL-1β and IL-23 production, but ρ0 THP1-derived dsDNA, which consists only of nDNA, failed to induce IL-1β and IL-23 production in an NLRP3 and TLR9 dependent manner, respectively.



These results indicate that mtDNA in 100K-EVs is more responsible for the induction of IL-1β and IL-23 production rather than nDNA. Indeed, dsDNA from ρ0 cells could induce IL-1β and small amount of IL-23 in an NLRP3- and TLR9-independent manner, respectively (**Fig 4C, D**). This may probably be mediated by AIM2 and cGAS. However, the difference in dsDNA levels between WT and ρ0 100K-EVs is likely due to the presence or absence of mtDNA, and the contamination of nDNA is small compared to the amount of mtDNA. Therefore, even if nDNA is present in 100K-EVs, the contribution of inflammation by 100K-EVs is likely to be small. We have presented these data and have discussed it in the revised manuscript.

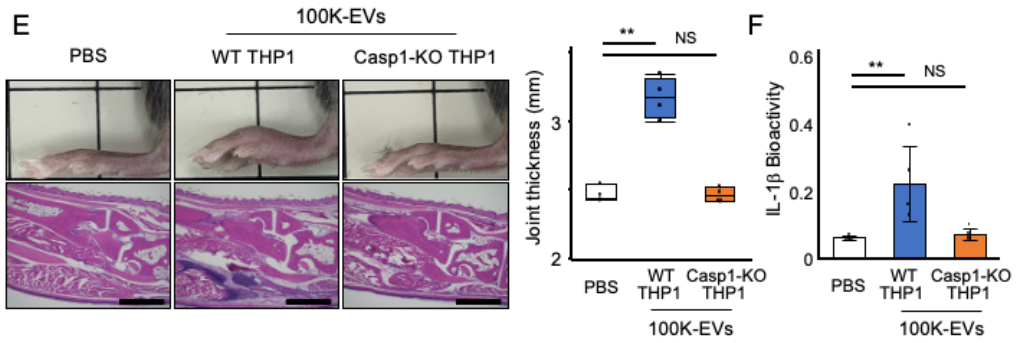
2. In addition, the authors need further experimental evidence to confirm that the EVs are exosomes and not other microvesicles that exit directly from the plasma membrane. What is the ratio (mtDNA/nDNA), because it is very likely that the released DNA in the cytocol exits extracellularly from the plasma membrane via microvesicles.

In accordance with the definition of the Exosome Committee, 100K-EVs were defined as exosomes with the specific marker and size by western blotting, electron microscopy and nanoparticle tracking analysis (NTA) (**Fig EV1B–D, Fig 3C, Fig EV4J–L, Fig 6F**). However, distinguishing exosomes from the same size of microvesicles torn off the plasma membrane is difficult due to the lack of appropriate markers. However, to distinguish between exosomes and microvesicles, we first examined the expression level of CD9 and Na^+/K^+ ATPase, which are thought to be specifically expressed at the exosome and plasma membrane, respectively, in 100K-EVs of WT and Casp1-KO THP1 cells. Na^+/K^+ ATPase were detected in 100K-EVs from both WT and Casp1-KO THP1 cells, while CD9 was rarely detected in 100K-EVs from Casp1-KO THP1 cells (**Fig EV3D**), suggesting that Na^+/K^+ ATPase seems to be a marker of microvesicles in 100K-EVs and that CD9-positive exosomes were rarely generated in the absence of caspase-1.



From this observation, the possibility that the 100K-EVs isolated from WT THP1 cells are contaminated with microvesicles cannot be ruled out.

Therefore, we assessed whether microvesicles could induce inflammation by evaluating the cytokine production and arthritis induced by 100K-EVs isolated from Casp1-KO THP1 cells, in which exosomes are rarely included. Na^+/K^+ ATPase expression of 100K-EVs in CS from WT and Casp1-KO THP1 cells were comparable, but 100K-EVs from WT THP1 cells induced more ankle thickness and IL-1β production than 100K-EVs from Casp1-KO THP1 cells (**Fig EV3E, F**).



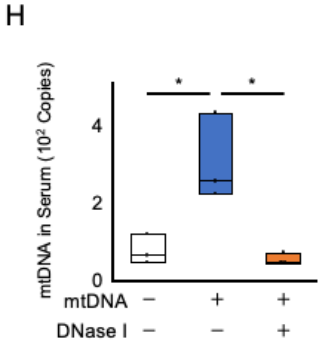
Based on the following results; 1. 100K-EVs predominantly contain mtDNA, 2. mtDNA induces IL-1β and IL-23 production via NLRP3 and TLR9, respectively, 3. mtDNA-defective dsDNA induces lower IL-1β and IL-23 production, 4. Microvesicles induce inflammation with little, it is interpreted that mtDNA contained within exosomes is mainly responsible for 100K-EV-induced inflammation. We added these data and discussed it in the discussion part of the revised manuscript.

3. "Extracellular mtDNA released from THP-1 cells by necrosis or secondary necrosis could be digested by DNase I. Conversely, mtDNA released by apoptosis or pyroptosis could not be digested with DNase I unless samples were treated with Triton-X100 (Fig. 1D), suggesting that mtDNA is entrapped in EVs and released outside the cell when monocytes undergo apoptosis or pyroptosis."

--Are DNase I treatment assays commonly used to show whether certain things are entrapped in EVs? If so, please cite the original article. If not, the conclusion that mtDNA is included in EVs is a priori and inappropriate here. I suggest that you draw this conclusion after showing the data from TEM below. (page 10-11)

We have showed in a previous paper (***Ann Rheum Dis.*** 77, 1507-1515, 2018) that dsDNA spiked into serum is degraded by DNase, but dsDNA in the membrane fraction is not degraded by DNase without prior detergent disruption of the membrane vesicles. Also, in a paper by Valadi et al. (***Nat Cell Biol.*** 9, 654-659, 2007) RNA in exosomes was not degraded by RNase. Therefore, we focused directly on membrane vesicles. However, as mentioned by the referee, it is too early at this stage to conclude that mtDNA is present in membrane vesicles, so we have revised "suggest" to "hypothesize" in the revised manuscript.

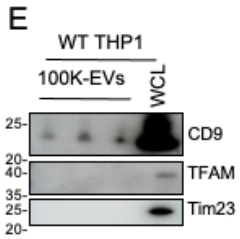
We conducted additional experiments to determine whether mtDNA spiked into BS serum could be digested by DNase I. Spiked mtDNA was found to be completely digested by DNase I



treatment (**Fig EV4H**).

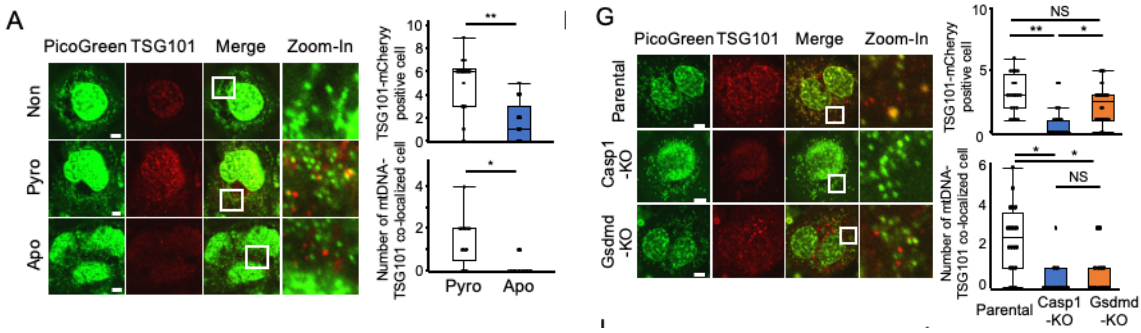
4. Whether the mtDNA that is included in EVs is outside the mitochondria? This can be demonstrated by observing co-localization between mtDNA and mitochondria in addition to detecting co-localization between mtDNA and TSG101 (Figure 3A and C).

The quantification of cytoplasmic mtDNA by qPCR in Figure 2B was from lysate of cytoplasmic fractions, excluding the mitochondrial and nuclear fractions. In addition, we conducted western blotting to determine whether mitochondrial proteins such as Tim23 and TFAM were detected in the 100K-EVs isolated from WT THP1 cells; neither Tim23 nor TFAM were detected in the 100K-EVs (**Fig 3E**). These results suggest that mtDNA in EVs are outside



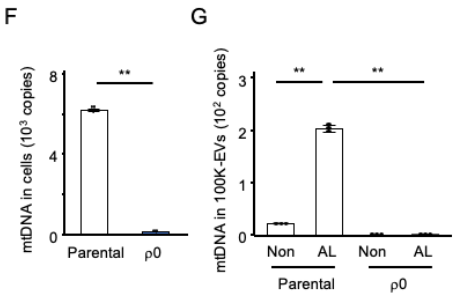
the mitochondria.

Following the referee's suggestion, the co-localization of mtDNA and TSG101, and the corresponding quantitative data are presented in Figures 3A and 3G.



5. "First, 100K-EVs isolated from WT and mtDNA-defective ρ0 THP-1 cells were injected into the peritoneum or ankle of C57BL6/J mice." Did the authors confirm whether mtDNA depletion was successful in 100k EVs? (page 15)

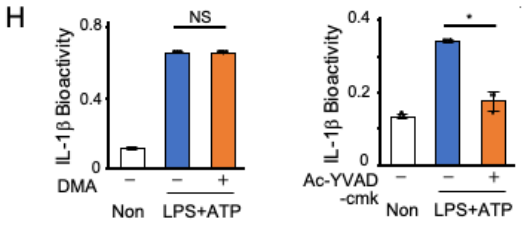
We have confirmed it in Fig EV2B in the original version. In the revised manuscript, the results



are shown in **Figure EV1F, G**.

6. In the top panel of Fig. 4H, the values seem to be the same in each group. Please check the source data.

Unlike caspase-1 inhibitor (Ac-YVAD-cmk), DMA does not affect IL-1β production, so the top row of Figure 4H is correct.

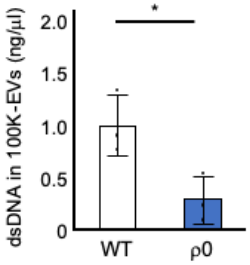


7. in Fig. 5A, serum mtDNA levels in patients with RA and SLE do not appear to be higher than those in healthy controls. In contrast, the authors found that mtDNA levels in 16k EVs increase in patients with SLE (Fig. 5F). In other studies, mtDNA levels are increased in these two groups or mouse models (PMID: 34916301, PMID: 12932286, and PMID: 31857488). Thus, this should be discussed.

As noted by the referee, mtDNA has been reported to be elevated in the serum of patients with autoimmune diseases such as RA and SLE. Although we did not discuss the comparison between RA and SLE and healthy controls in the original submission, the serum mtDNA levels in RA and SLE were significantly elevated compared to healthy controls. More than 90% of the patients with BS were outpatients, whereas almost all RA and SLE patients were with advanced disease requiring hospitalization and had serum samples taken prior to treatment in this study. Nevertheless, serum mtDNA levels in BS were significantly higher than RA and SLE. We have discussed the significance of mtDNA in BS and other autoimmune diseases in the revised manuscript. Additionally, we evaluated the serum mtDNA level by directly purified dsDNA from serum and measured mtDNA by qPCR in Figure 5A. Whereas in Figures 5F and 5G, we investigated which membrane fraction of serum contains mtDNA and isolated dsDNA from 16K-EVs and 100K-EVs isolated from large amount of serum because of the loss of a large amount of mtDNA without being recovered in the washing process. Therefore, it is impossible to compare the level of mtDNA in Figure 5A with those in Figures 5F and 5G.

8. "Liposomes containing BS 100K-EV-derived DNA induced IL -1β and IL -23 production via NLRP3 and TLR9, respectively (Fig. 7F, G)." Nuclear DNA is also released into the cytosol under stress (PMID: 34715021), and cells can secrete different types of DNA via exosomes (PMID: 28508895). Did the authors detect nuclear DNA in EVs?

In Figure 7F and 7G, dsDNA extracted from 100K-EVs was repackaged into liposomes for administration. Therefore, as reviewer pointed out, we cannot rule out the possibility that both nDNA and mtDNA were present. In our response to Major concern 1, we measured the amount of dsDNA using the Quant-iT dsDNA assay kit after extraction of DNA from 100K-EVs, and compared them between WT-100K-EVs (both mtDNA and nDNA are included) and ρ0-100K-EVs (only nDNA is included). The level of dsDNA was significantly lower in ρ0-100K-EVs than the WT-100K-EVs (**Fig EV1)**.

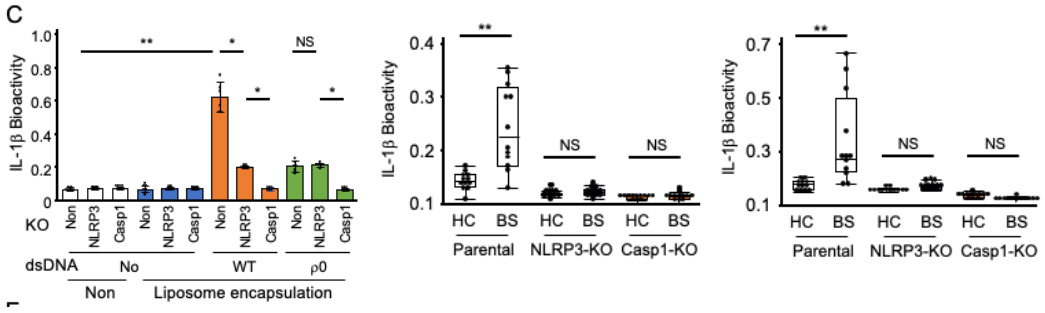


Since we did not analyze dsDNA sequences by NGS, we do not know whether the dsDNA, mtDNA, or nDNA, is more abundant in WT-100K-EVs, but the difference in dsDNA in the 100K-EVs of WT and ρ0 cells is likely due to the difference in mtDNA content; so the contamination of nDNA in the 100K-EVs is likely to be small. Furthermore, as shown in **Figure 4C, D**, dsDNA extracted from ρ0 THP1 cells, which are expected to be only nDNA, failed to induce cytokine production via NLRP3 or TLR9. These results suggest that even when nDNA was present, the inflammation caused by 100K-EVs was mediated by mtDNA, and not nDNA.

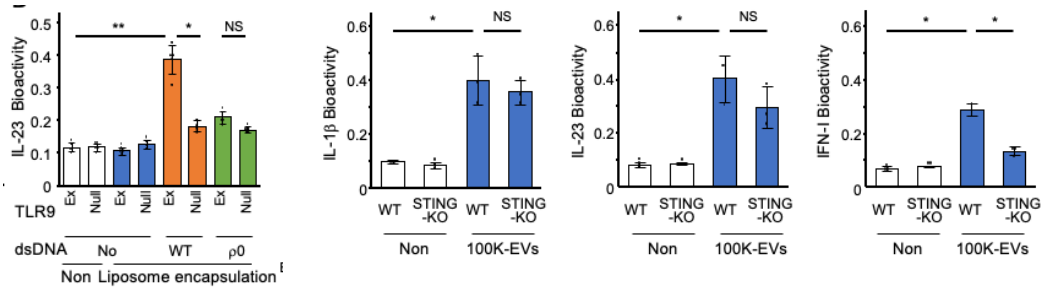
9. As we know, cytosolic DNA is also recognized by AIM2 and cGAS. How can the authors exclude them and focus on NLRP3 and TLR9?

We tried to create AIM2-KO THP1 cells but were not able to establish it. Instead, NLRP3-dependent or -independent and STING-dependent or -independent cytokine production by dsDNA were evaluated. dsDNA isolated from WT or ρ0 THP1 cells was encapsulated in liposomes and evaluated for IL-1β production by adding to WT, NLRP3-KO or Casp1-KO THP1 cells, and for IL-23 production by addition to TLR9-OE or TLR9-null THP1 cells, respectively. mtDNA-defective dsDNA from ρ0 cells could not induce IL-1β production via NLRP3, but slightly induced IL-1β in Casp-1-KO THP1 cells (**Fig 4C**). The difference in IL-1β production between NLRP3-KO-THP1 and Casp1-KO-THP1 cells is probably caused by an NLRP3-independent mechanism such as that through AIM2. Indeed, dsDNA other than mtDNA was contaminated in 100K-EVs and certainly recognized by independent of NLRP3, NLRP3 is essential for sensing mtDNA to IL-1β production. On the other hand, shown in **Figure 7D, F**, IL-1β production by dsDNA extracted from BS 100K-EVs was diminished in NLRP3-KO

THP1 cells as the almost same level as that in Casp1-KO THP1 cells. Thus, we thought the contribution of AIM2 for IL-1β production by BS 100K-EVs may not be so significant.



Additionally, compared to the dsDNA isolated from WT THP1 cells, dsDNA from ρ0 cells could induce very small amount of IL-23 via TLR9 (**Fig 4D**). For cGAS, we evaluated the involvement of the cGAS–STING pathway in cytokine production by adding WT 100K-EV to WT or STING-KO-THP1-ISG reporter cells established in a previous paper (***Ann Rheum Dis.*** 77, 1507-1515, 2018). IL-1β and IL-23 production did not differ much between WT THP1 and STING-KO THP1 cells, but type I IFN (IFN-I) production was reduced in STING-KO THP1 cells compared to WT THP1 cells (**Fig EV3C**).



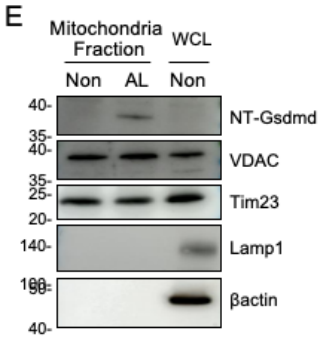
The very slight increase in IL-23 in TLR9-OE-THP1 cells, although not significant, was probably induced via the cGAS-STING pathway, as STING-KO had mildly decreased IL-23 production (**Fig EV3C**).

Indeed, WT 100K-EVs can induce IFN-I production via the cGAS–STING pathway. Activation of caspase-1 has been reported to suppress cGAS–STING signaling by cleaving cGAS (***Immunity*** 46, 393-404, 2017, ***Immunity*** 49, 413-426 e5, 2018). In addition, few reports have implicated IFN-I in the pathogenesis of BS. Therefore, we believe that the cGAS–STING pathway is not so important in a state of caspase-1 hyperactivation such as BS.

10. "We observed reduced cytoplasmic mtDNA leakage in Gsdmd-KO THP-1 cells, suggesting that gasdermin-D may form pores in the mitochondrial membrane and promote mtDNA leakage."

- Indeed, as the authors noted, previous studies have reported that activated GSDMs, including GSDME-NT and GSDMD-NT, translocate to the mitochondrial membrane and release mitochondrial contents (PMID: 30976076). However, we should consider that activated GSDMs cause permeabilization of the plasma membrane, which contributes to impaired mitochondrial homeostasis and release of mtDNA, apart from directly forming the pores on the mitochondrial membrane (PMID: 34233045). I therefore suggest that the authors modify the statements or perform further tests to confirm this, such as demonstrating translocalization of GSDMD-NT from the cytosol to the mitochondria, as Rogers et al. did in Fig. 4b (PMID: 30976076). (page 24)

As the referee suggested, we examined whether GSDMD-NT could be detected by isolating the mitochondria fraction like in a previous literature (***Cell***, doi: 10.1016/j.cell.2022.06.038). Mitochondrial fractions were purified from THP1 cells stimulated with LPS + ATP for 10 min and subjected to western blotting with anti-GSDMD antibody. GSDMD-NT was detected in the mitochondria fraction upon LPS + ATP stimulation (**Fig 2E**).

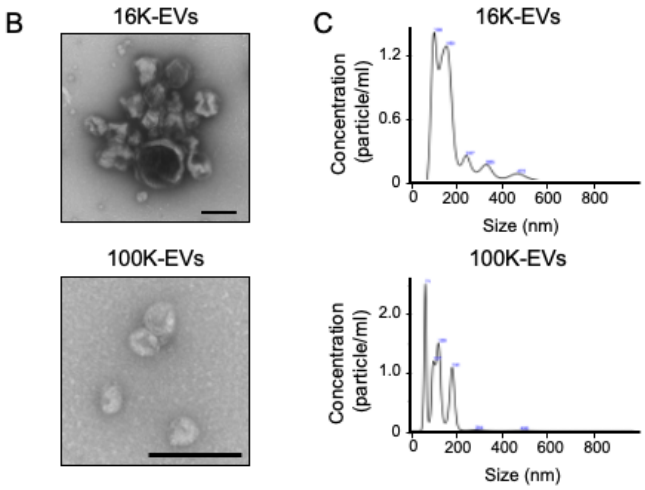


As the referee points out, the possibility that the involvement of permeabilization of mitochondria membrane due to the positive feedback via potassium efflux cannot be ruled out. However, the translocation of GSDMD-NT to mitochondria occurred at approximately 10 min after LPS + ATP stimulation, and leakage of mtDNA into the cytoplasm was subsequently observed at approximately 20 min after LPS + ATP stimulation. Therefore, we believe that mtDNA leakage upon inflammasome activation was mediated by the translocation of GADMD-NT to mitochondria.

Minor points:

1. Which EVs are confirmed by the data, 16k or 100k? (Figure EV1B-C).

Data for 100K-EVs are displayed in Fig EV1B-C. We also added data for the 16K-EVs.

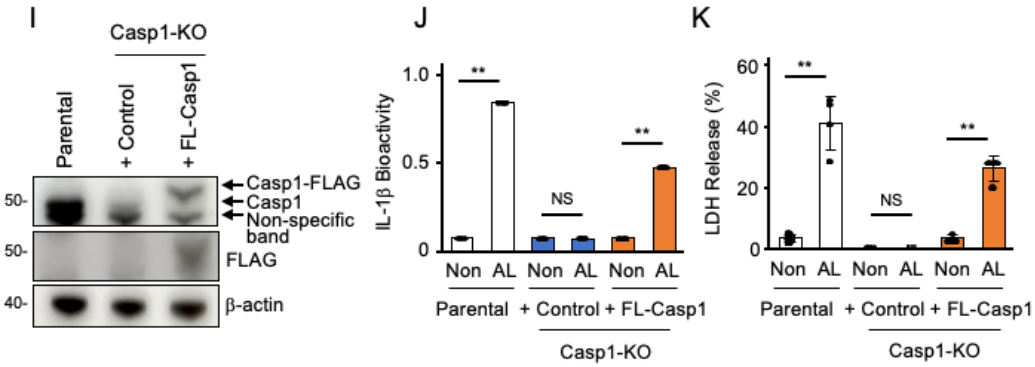


2. "Tsg101 punctate formation was observed in pyroptotic THP-1 cells but less so in apoptotic cells." Please check the wording. Should "punctures" be changed to "punta"? (page 13)

Thank you for pointing out our error. We have changed the wording.

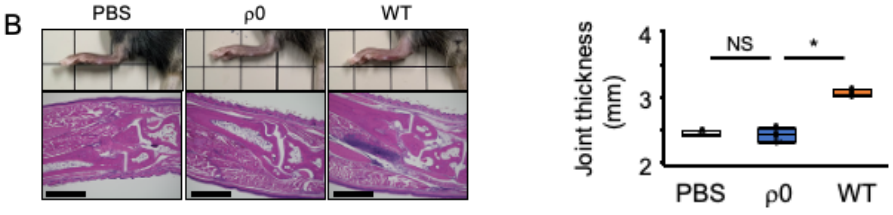
3. In Fig. EV2 J, the Casp 1 band in the third lane is too weak.

We examined it and have presented it in **Fig EV2I**.



4. in Fig. 4B, the Ctrl groups of p0 and WT should be listed.

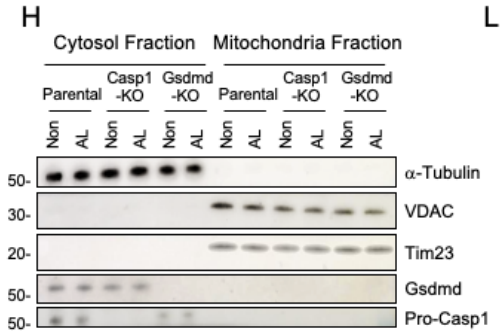
We added the Control data in Fig 4B.



5. in the sixth line from the bottom on page 15 of the manuscript, the protocol: mtDNA purification from mitochondria is mentioned. The scientific

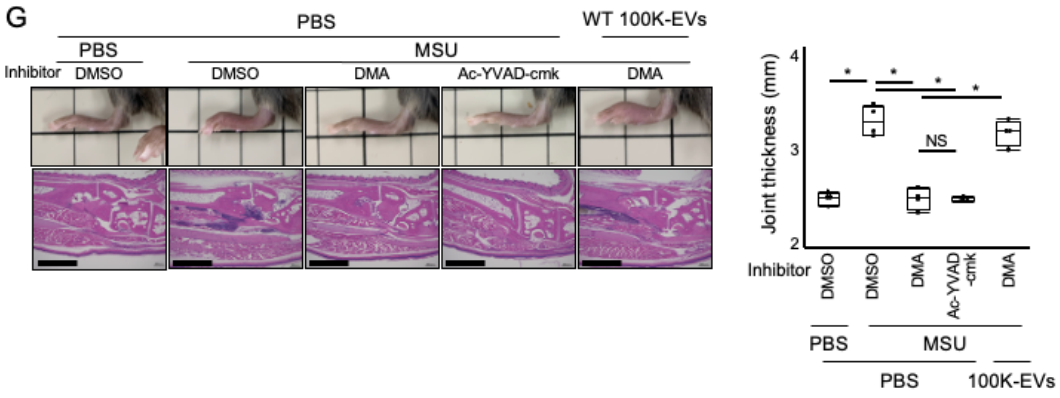
validity of this method for mtDNA purification and encapsulation needs to be further verified (checking the composition of the isolates?).

As shown in **Fig EV2H** by western blotting, mitochondria were clearly isolated.



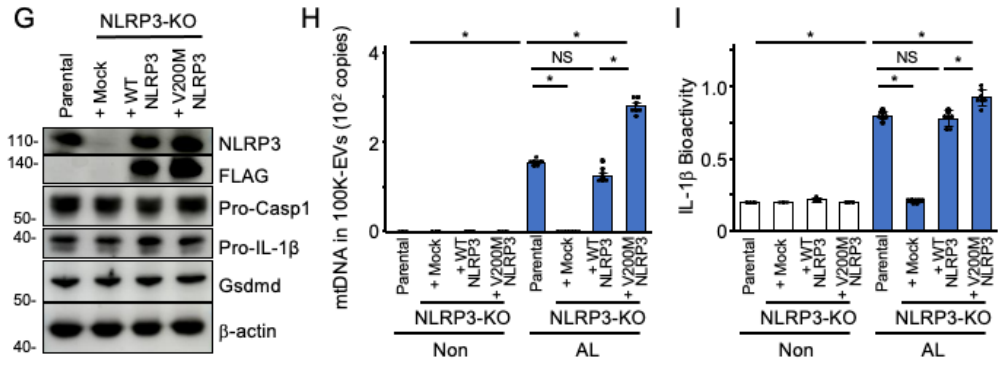
6. In Fig. 4I, the ankles of the mice were swollen due to the injection of MSU, but it was not clearly highlighted in the figure, so it is suggested to add a label.

We added the label of MSU in the revised manuscript of **Fig 4G**.



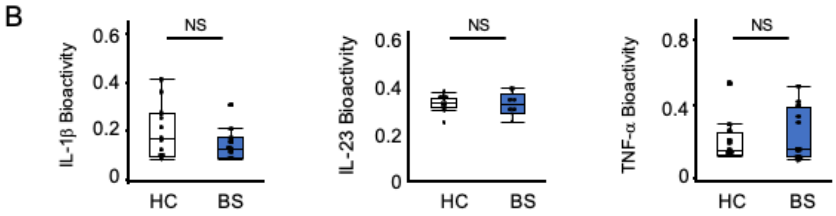
7. In Fig. 6H,I, both the nongroup and the ATP+LPS group should be supplemented with the Ctrl group of WT with the Ctrl group of NLRP3 (in agreement with Fig. 6G lane) to illustrate the efficacy of NLRP3 supplementation.

We added Control (Mock) group in **Fig 6G–I**.



8. in Fig. 7A, the authors enumerate the induction of changes in the levels of IL -1β, IL -23, and TNF-α by BS 100K-EVs, why was IL -23 removed from the detection of pro-inflammatory factors by BS 16K-EVs in Fig. 7B?

There was no intention to hide the IL-23 data. We added the IL-23 data from 16K-EV to **Figure 7B**.



Response to Referee #2

In this study Konaka et al., report that mitochondrial (mt) DNA secreted in exosomes from the pyroptotic cells has inflammatory properties. The authors propose that activated caspase-1 induces GsdmD-dependent mtDNA leakage into the cytoplasm and that caspase-1 is required for the intraluminal membrane vesicle (IMV) formation. They found, that exosomal mtDNA promotes a strong inflammatory response. Finally they show that monocytes from Behçet's syndrome (BS) patients exhibit increased mtDNA secretion via exosomes, causing the disease pathology.

I find the data presented by the authors very interesting as they emphasize the importance of exosomes in human diseases. The paper is well written and the most of the conclusions are supported by the data presented. The major concern is regarding the claim about caspase-1 requirement for IMV formation. The authors did not follow their initial finding that caspase-1 KO THP-1 cells lacked formation of Tsg101 puncta upon LPS+ATP stimulation. Would this finding mean that in the absence of caspase-1 there's no exosome formation upon LPS+ATP treatment? The data presented in Fig. 3A suggest that only some fraction of Tsg101 puncta co-localizes with mtDNA. In addition, the authors did not specify how caspase-1 is involved in the IMV formation (e.g. does it interact with other proteins? Or some other mechanism(s)?). I also think the study would benefit if the authors characterize better the exosomes (proteins) involved in mtDNA encapsulation, e.g. by performing mass spec on isolated EVs.

Thank you for your valuable comments and appreciating that our findings are interesting. We have responded to your concerns below:

Major concerns:

1. The authors did not follow their initial finding that caspase-1 KO THP-1 cells lacked formation of Tsg101 puncta upon LPS+ATP stimulation. Would this finding mean that in the absence of caspase-1 there's no exosome formation upon LPS+ATP treatment?

Yes. We observed the Tsg101 puncta formation and CD9/CD63-positive 100K-EVs in CS upon LPS+ATP stimulation were quite reduced in Casp1-KO-THP1 cells, suggesting that the exosome formation upon LPS + ATP stimulation was quite impaired in the absence of caspase-1.

2. The data presented in Fig. 3A suggest that only some fraction of Tsg101 puncta co-localizes with mtDNA. In addition, the authors did not specify how caspase-1 is involved in the IMV formation (e.g. does it interact with other proteins? Or some other mechanism(s)?).

As the referee pointed out, we are also interested in the role of caspase-1 in triggering ILV formation. We have begun to establish a screening system using methods that can label proteins in close proximity in vitro to identify the target protein of caspase-1. However, the road to success is long and we are yet to obtain data. Therefore, we cannot answer these concerns about

the mechanistical relationship between caspase-1 and ILV formation.

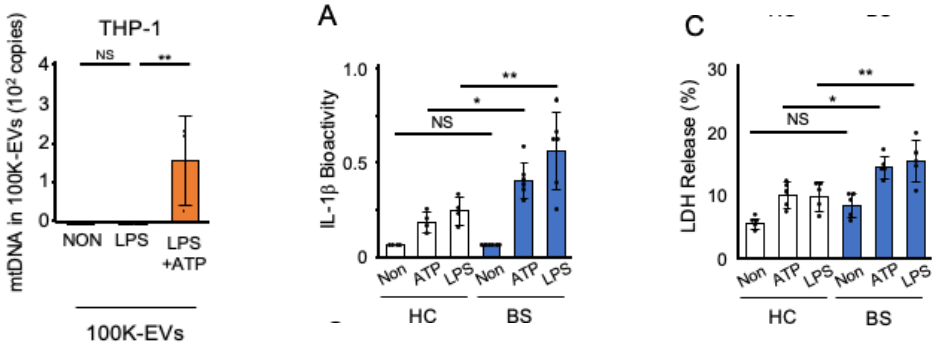
3. I also think the study would benefit if the authors characterize better the exosomes (proteins) involved in mtDNA encapsulation, e.g. by performing mass spec on isolated EVs.

We had investigated the characteristics of BS 100K-EVs by mass spec, but because of the small sample volume, mass spec data was less reproducible and we had to stop the experiment.

Minor concerns:

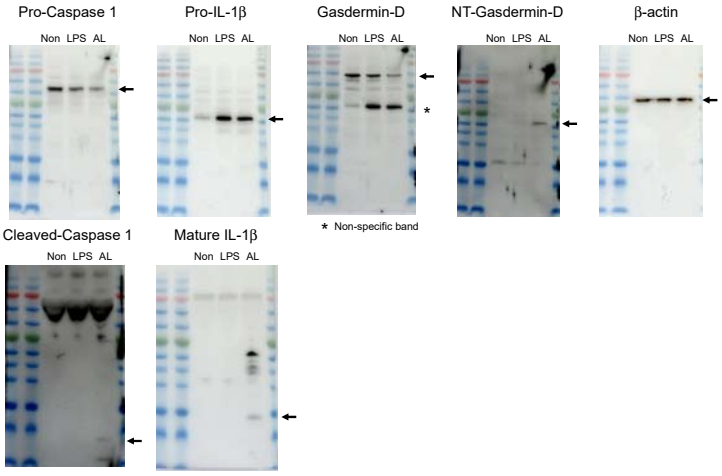
1. The authors stated that they "determined the increased mtDNA levels in the culture supernatant (CS) of LPS-induced human PBMCs via quantitative PCR (Fig EV1A)". Please clarify if you observed the mtDNA in the supernatants just after LPS stimulation? Without signal 2?

For THP1 cells, mtDNA was released only when THP1 cells were stimulated with LPS + ATP, as determined by comparing the amount of mtDNA in 100K-EVs released from THP1 cells stimulated by LPS alone or LPS + ATP (**Fig 1G**). In human $CD14^+$ monocytes, inflammasome activation and following pyroptosis occur by stimulation by LPS alone (**Fig 6A, C**).



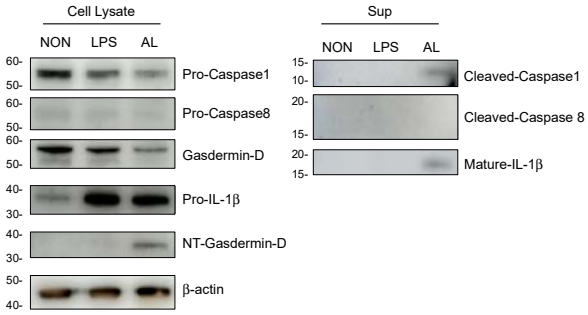
2. Please provide the Western blot panel for THP-1 cells and CD14 monocytes (whole cell lysates and supernatants) unstimulated and stimulated with LPS+ATP, including caspase-1, GsdmD, and IL-1β. Please show whole (uncut) blots for all these proteins.

We have presented the raw western blotting data of capsase-1, IL-1β, and GsdmD in THP1 cells after LPS + ATP stimulation.



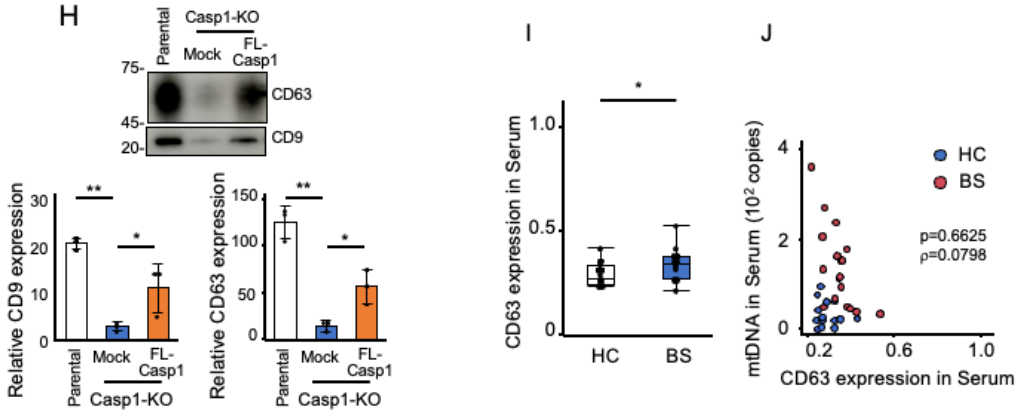
3. Is Caspase 8 activated in these cells? Caspase-8 was recently reported to be activated in response to LPS+ATP and found in the protein complex within exosomes (Bulek et al, 2020).

We checked caspase-8 activation in THP1 cells. In contrast to the colon epithelial cells in the suggested paper (Bulek et al. ***J Clin Invest***. 130:4218–4234, 2020), caspase-8 activation upon LPS + ATP stimulation was not observed in THP1 cells. Therefore, it would be possible that the activation of caspase-8 is not required for exosome-mediated mtDNA secretion.



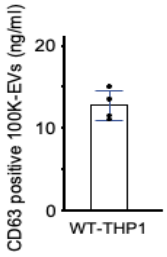
4. In addition to mtDNA quantification (e.g. Fig 2E, 2G and other) the authors should provide exosomes quantification (e.g. using ZetaView or other nanoparticles analyzer which can provide both quantity and the sizes of the exosomes).

We do not have ZetaView or similar equipment. Therefore, we quantified exosome levels by western blotting using antibodies for CD63 and CD9. The secreted level of CD9/CD63-positive 100K-EVs by LPS + ATP was reduced in Casp1-KO but not in WT and GsdmD-KO THP1 cells (**Fig 3H**). Additionally, 100K-EV levels in HC and BS sera were quantified by ELISA for CD63; CD63-positive 100K-EV levels were statistically but not significantly different between HC and BS (**Fig 5I**). Of note, mtDNA levels, rather than CD63-positive 100K-EV levels, were significantly different between HC and BS (**Fig 5J**).



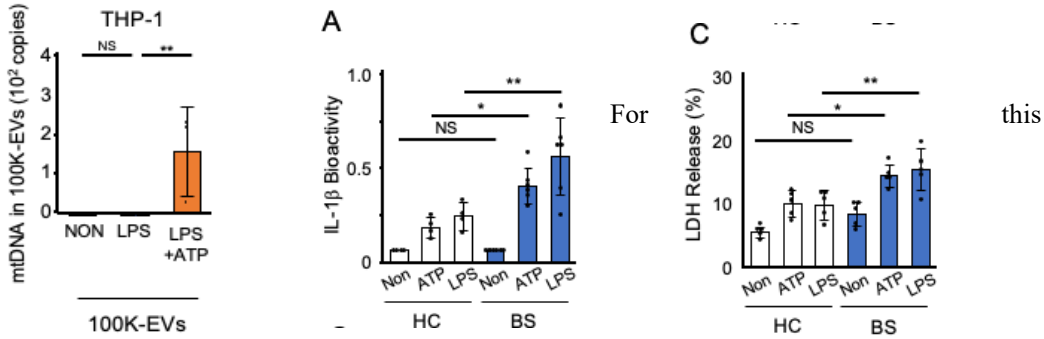
5. There's no information on how the authors quantified the exosomes for the in vivo studies and how many were injected. Please add these information.

For the *in vivo* administration of 100K-EVs isolated from WT THP1 cells, PMA-treated THP1 cells were cultured in the 10 cm dish and stimulated with LPS + ATP. 100K-EVs were isolated from the culture supernatant and administered to each mouse. Since it was difficult to count the 100K-EVs, we quantified the level of CD63 in 100K-EVs from WT THP1 cells by ELISA. Approximately 10 ng/ml of CD63-positive 100K-EVs were administrated into each mouse.



6. There are some discrepancies between text and figure labeling. For example, in the text the authors stated that "the increased secretion of mtDNA in 100K-EVs and reduced MMP induced by LPS and ATP stimulation was abrogated by the Ac-YVAD-cmk (Fig 6E)", while in the Fig 6E the authors show only single treatments. The same apply to Fig 6D. Please clarify this issue.

In THP1 cells, the inflammasome cannot be fully activated without LPS as a first signal (**Fig 1G**). In contrast, in human CD14^{+} monocytes inflammasome activation can be attained without LPS, using ATP or Nigericin, or by LPS alone (**Fig 6A, C**).



For this

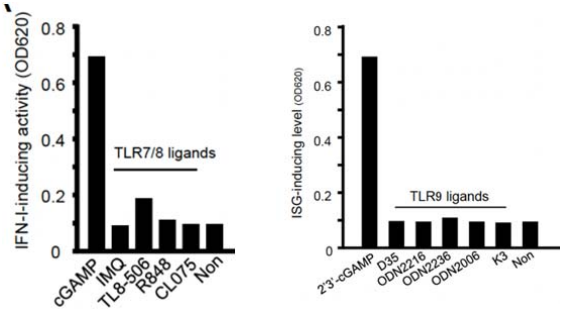
reason, we stimulated human CD14^{+} monocytes with Nigericin or ATP alone for inflammasome activation.

7. Please show the whole uncut Western blots for Fig 6C and 6G, including whole cell lysates. In addition, for panel 6G include caspase-1 and IL-1β, and for 6C include GsdmD.

In Figure 6B, the membrane was cut to the desired band size prior to blotting with the 1st antibody because the sample volume was small. Therefore, the uncut Western blotting data could not be displayed. Additionally, we could not obtain PBMCs from BS patients because no active patients were admitted to our hospital during this period. Therefore, it was difficult to show by western blotting whether gasdermin-D was active in BS CD14^{+} monocytes. However, as shown in Fig. 6C, secreted LDH levels from CD14^{+} monocytes were higher in BS than in HC, suggesting that pyroptosis is also enhanced in BS monocytes.

8. For proving/disapproving that mtDNA signals via TLR9, TLR9 KO should be used (e.g. in monocytes) instead of an overexpression system in THP-1 cells.

The THP1 cells that we used could not respond to any of the ligands of TLR7, TLR8, or TLR9 (data from the previous our paper: ***Ann Rheum Dis.*** 77, 1507-1515, 2018), suggesting that THP1 cells are likely to be TLR7/8/9-null cells. Therefore, we introduced TLR9 into THP1 cells and investigated the involvement of TLR9.



Referee #3

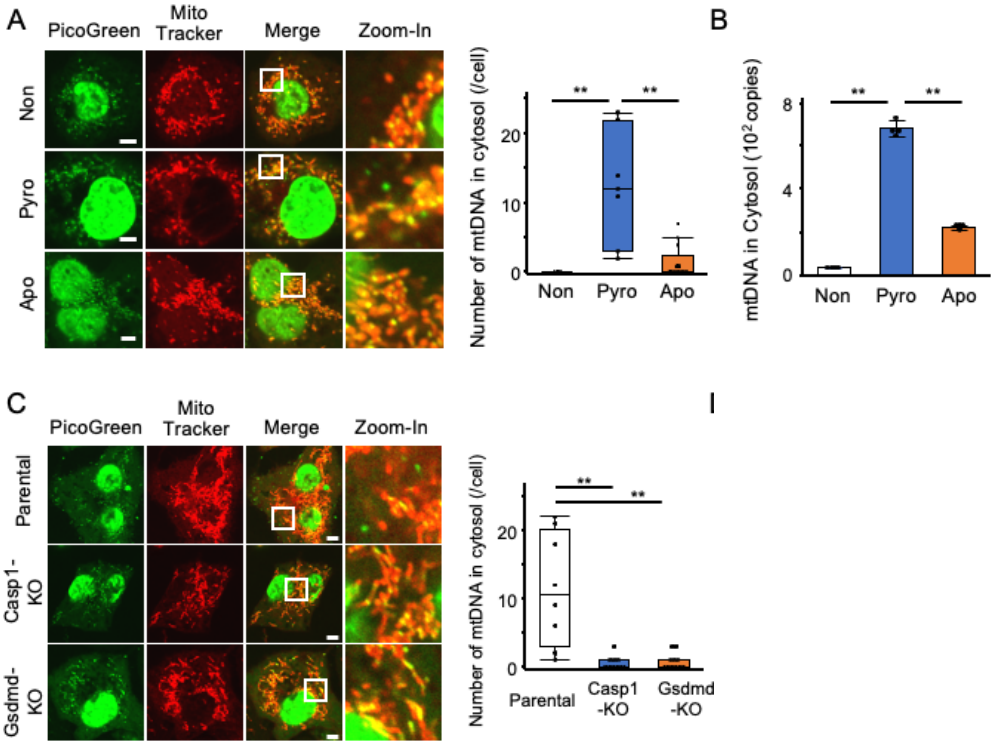
The study by Konaka et al, identifies the role of inflammasome/pyroptosis in the loading of mt-DNA into exosomes in monocytes, and characterize the kinetics and key factors regulating this process in numerous in vitro and in vivo experiments. Moreover, authors associate this process as a unique mechanism contributing to Behcet´s Syndrome compared to other autoimmune disorders. The data are certainly interesting and of a potential therapeutic impact. However, some issues regarding the mechanisms, the pyroptosis versus inflammasome involvement, and the possible involvement of other inflammasome sensors remain to be addressed.

Thank you for your valuable comments and appreciating that our findings are interesting. We have responded to your concerns below:

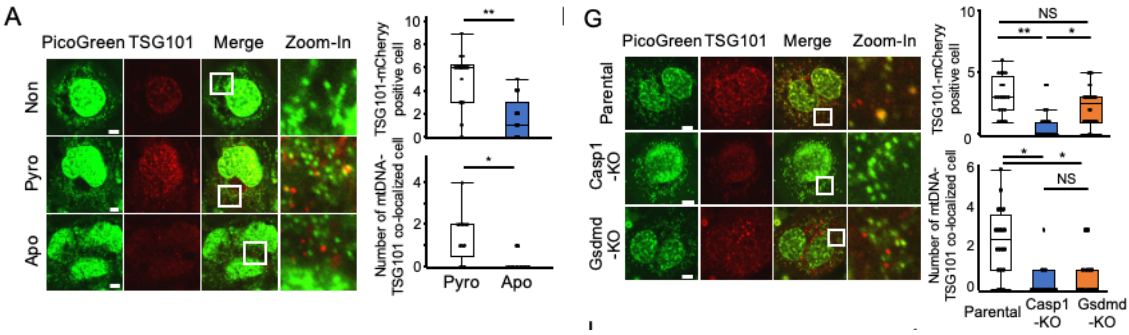
MAJOR COMMENTS

1. Some of the microscopy images do not seem representative from quantified effects (green mt DNA non-associated with mitochondria (Figure 2A) and the single zoomed image from figure 3B (in this case there is no quantification).

We added the quantitative data from confocal images in **Fig 2A** and **Fig 2C**. Also, to avoid evaluation bias in Figure 2A, the levels of leaked cytoplasmic mtDNA were quantified by qPCR after removal of mitochondria fraction in **Fig 2B**.



For **Fig 3A, G**, there was no difference in the co-localization rate of mtDNA and tsg101 puncta because mtDNA and tsg101 puncta tended to reduce simultaneously. Therefore, the number of cells in which mtDNA and tsg101 puncta were co-localized were evaluated.

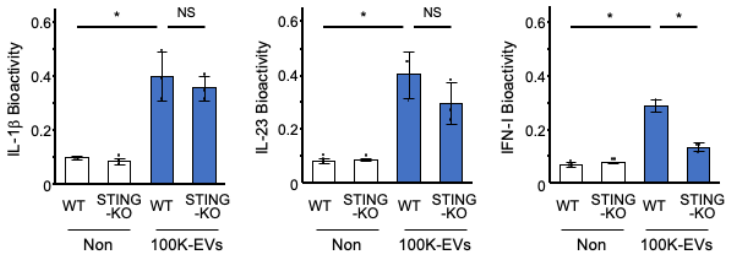


2. The presence of exosomal Mit-DNA has been previously reported and the sensing of mitochondrial DNA loaded into exosomes by dendritic cells has been linked to the cGAS-STING pathway (Torralba et al., Nat Commun 2018). This should be properly discussed in the manuscript. Authors should assess pyroptosis induced mt-DNA sensing in the absence of the cGAS-STING sensor.

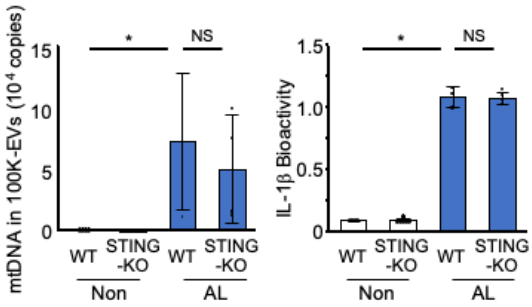
For mtDNA-induced cytokine production via cGAS, we added WT 100K-EV to WT or STING-KO-THP1-ISG reporter cells established in a previous paper (***Ann Rheum Dis.*** 77,

1507-1515, 2018).

IL-1β and IL-23 production did not differ much between WT THP1 and STING-KO THP1 cells, but type I IFN (IFN-I) production by WT 100K-EVs was reduced in STING-KO THP1 cells compared to WT THP1 cells.



Additionally, STING-KO THP1 cells stimulated with LPS and ATP secreted IL-1β and 100K-EVs containing mtDNA to the same extent as WT THP1 cells, suggesting that the cGAS-STING pathway is not involved in inflammasome activation or pyroptosis in this experimental setting.

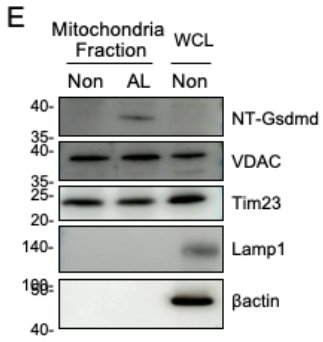


It has been reported that cGAS–STING signaling is suppressed because cGAS and STING are cleaved by caspase-1 activation (***Immunity*** 46, 393-404, 2017, ***Immunity*** 49, 413-426 e5, 2018). Therefore, we believe that cGAS–STING pathway would not be important when cells exposed to noxious stimuli drive inflammasome activation and pyroptosis induction, although we could not rule out the involvement of the cGAS-STING pathway in mtDNA-containing EV secretion in certain circumstances.

3. Gasdermin KO cells do not leak mt-DNA (similarly to Caspase 1 KO cells) to the cytoplasm. However, Gasdermin-D did not affect the formation of TSG101 vesicles. Are these vesicles not loaded with mt-DNA? (as suggested by zoomed image in figure 3F), and if so, could authors provide an explanation for this?

Otherwise, it may seem that inflammasome activation rather than pyroptosis may account for the mt-DNA release.

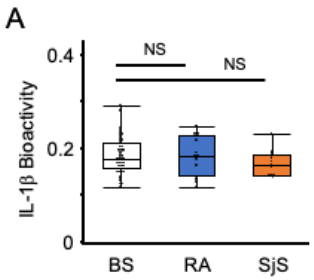
In GSDMD-KO THP1 cells, leakage of mtDNA from mitochondria to the cytoplasm was reduced, resulting in reduced release of mtDNA via exosomes to the extracellular space, although ILV formation was maintained. Additionally, we observed that GSDMD-NT translocated to the mitochondria approximately 10 min after LPS + ATP stimulation.



Therefore, we believed that mtDNA was not loaded into ILVs due to a lack of cytoplasmic mtDNA in the absence of gasdermin-D.

4. The mt-DNA analysis in the serum of Beckett Syndrome patients compared to plasma from other different autoimmune disorders is quite interesting (Figure 5). In some of these disorders (for example RA and SjS), the inflammasome has been involved. Therefore, could authors correlate levels of active IL1Beta in plasma from these individuals with mt-dsDNA content (as they did for the reporter experiments in Figure 7) to rule out that the patient cohorts other than BS are not exhibiting lower levels of inflammation due to treatment initiation?

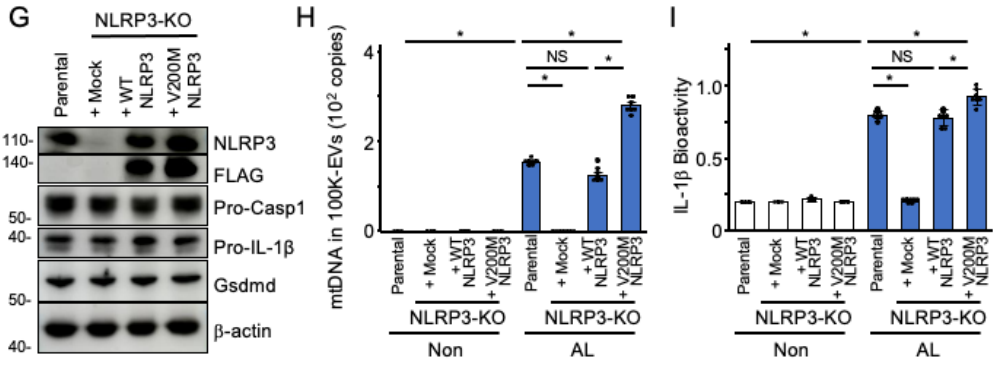
Serum IL-1β activity was measured in RA and SjS patients and compared to that in BS patients; similar IL-1β activity levels were observed in these diseases (**Fig EV4A**) although almost all RA and SjS patients were active patients who required hospitalization and had serum samples taken prior to treatment, whereas more than 90% of the patients with BS were outpatients. Nevertheless, serum mtDNA levels in BS were significantly higher than in RA and SjS.



We also evaluated the relationship between serum IL-1β activity and mtDNA levels in these diseases, but there was no correlation among them in these diseases (data not shown).

5. In figure 6H, authors claim that Mo from BS secrete higher levels of mtDNA due to a mutation in NLRP3 associated with the disease, however, the impact of this mutation compared to wt is quite limited and partial, although significant. Have authors considered other contributing factors?

We again examined the mtDNA secretion via 100K-EVs and IL-1β production in parental THP1 cells, WT-NLRP3-restored-NLRP3-KO THP1 cells, and V200M-NLRP3-restored NLRP3-KO THP1 cells. In V200M-NLRP3-restored-NLRP3-KO THP1 cells, differences in IL-1β production were less pronounced, but both 100K-EV-mediated mtDNA secretion and IL-1β production were increased (**Fig 6G–I**).

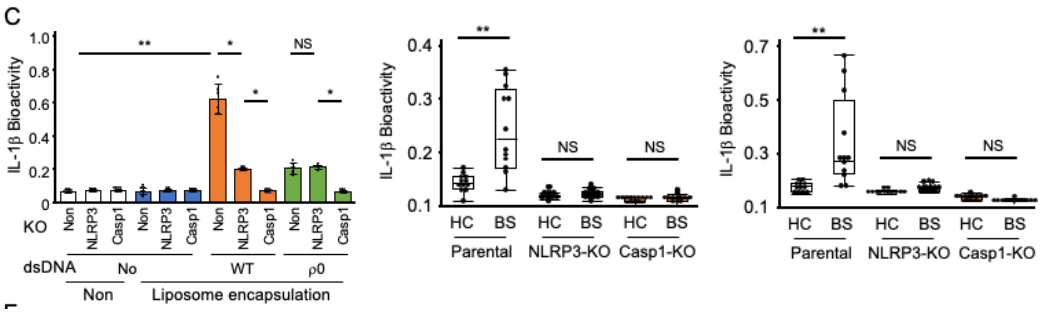


The V200M mutation in NLRP3 is known to have milder clinical symptoms and a weaker degree of inflammasome activation than the NLRP3 mutations found in Cryopyrin-associated periodic syndrome; CAPS (T350M, E569, etc.). Hence, it appeared that IL-1β production was not increased as much in V200M-NLRP3. It would be interesting to see how it would be in CAPS, but we are unable to evaluate it because we do not have serum from CAPS patients. It is known that NLRP3 activation is regulated by various post-transcriptional modifications, NLRP3 expression is not limited to within myeloid cells, and that clinical symptoms and disease

severity vary depending on whether the mutation is a germline or mosaic mutation of NLRP3. Thus, NLRP3 mutations may affect more than just IL-1β and exosome-mediated mtDNA secretion in human diseases, such as BS and CAPS. However, we do not know what factors other than mtDNA and IL-1β cause the disease through this mutation.

6. Authors should rule out the involvement of other inflammasome sensors with known activity in detection of intracellular DNA such as AIM2 or IFI16.

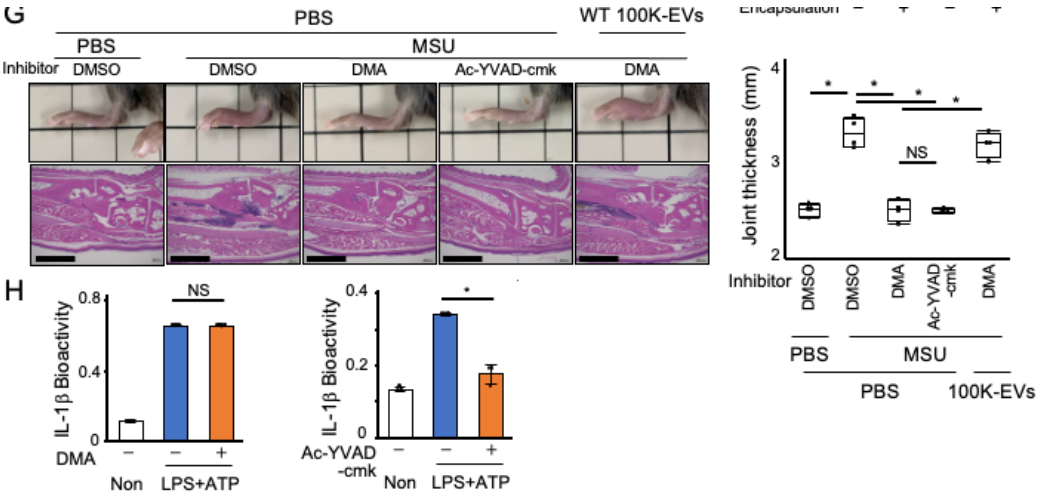
As noted by the referee, AIM2 and IFI16 may be involved in the recognition of dsDNA, including mtDNA. Therefore, we attempted to create AIM2-KO THP1 cells and IFI16-KO THP1 cells, but were unable to establish them. It was also reported that IFI16 interacts with STING to induce ISG after recognizing dsDNA (***Nat Commun.*** 8, 14392, 2017), suggesting that it is difficult to strictly distinguish the involvement of IFI16 and cGAS. Instead of the generation of AIM2-KO and IFI16-KO THP1 cells, dsDNA isolated from WT or ρ0 THP1 cells was encapsulated in liposomes and evaluated for IL-1β production by adding to WT, NLRP3-KO or Casp1-KO THP1 cells and for IL-23 production by addition to TLR9-OE or TLR9-null THP1 cells, respectively. mtDNA-defective dsDNA from ρ0 cells could not induce IL-1β production via NLRP3, but slightly induced IL-1β in Casp-1-KO THP1 cells (**Fig 4C**). The difference in IL-1β production between NLRP3-KO THP1 and Casp1-KO THP1 cells is probably caused by an NLRP3-independent mechanism such as that through AIM2. Indeed, dsDNA in addition to mtDNA might be present in the 100K-EVs and certainly might be recognized independent of NLRP3, but NLRP3 is essential for sensing mtDNA to IL-1β production. On the other hand, as shown in **Figure 7D, F**, BS 100K-EVs induced IL-1β and IL-23 production in WT THP-1 cells, but IL-1β production was completely abolished in NLRP3-KO, almost to the same extent as that in Casp1-KO THP1 cells, and IL-23 production was abolished in TLR9-null THP-1 cells. We also showed that the dsDNA of 100K-EVs is modified with 8-OHdG, which is known to be recognized via NLRP3 (**Fig 3D, 5D, 6F**). Thus, we thought the contribution of AIM2 for IL-1β production by BS 100K-EVs may not be so significant.



MINOR COMMENTS:

1. In the in vivo ankle swelling experiments (Figure 4E-4G), authors inject mt-DNA encapsulated into liposomes, which induced slightly significant higher levels of joint thickness and they try to address the inflammasome involvement using the caspase and DMA inhibitors, but this drug could be affecting more than exosome vesicle sensing. Authors should determine the effect of these inhibitors by injecting exosomes derived from LPS-ATP stimulated monocytes containing the mtDNA to confirm their data in more physiological conditions, as they did in figure 8.

We assumed that the referee was asking for a rescue experiment to see if additional doses of mtDNA-containing exosomes in DMA-treated mice would restore inflammation. To this end, arthritis was induced with MSU in DMA-treated mice and 100K-EVs isolated from WT THP1 cells were administrated. Inhibition of exosome secretion by DMA suppressed MSU-induced arthritis to the same extent as caspase-1 inhibition, and addition of mtDNA-containing exosomes to DMA-treated mice restored ankle thickness and infiltration of inflammatory cells to the same extent as in mice treated with DMSO (**Fig 4G**). Notably, DMA treatment did not impair inflammasome activation, unlike caspase-1 inhibition (**Fig 4H**).



Therefore, these results suggest that exosomes facilitate the MSU-induced arthritis by encapsulating mtDNA to transmit the alert information to the adjacent cells.

2. Data on caspase 1-KO shown in Fig. 7D-E should be described in the text under Results, page 20.

This information is described in the revised manuscript.

Hyota

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Dear Hyota,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the referees and their comments are provided below. They find that the revised version has clarified many of the raised issues. However, there are several issues that should be sorted out in a final revision. Let me know if we need to discuss anything further.

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

- Please check the funding information and that it matches between the online submission system and MS (J2000705023 vs. in eJP: J200705023)

- we need 3-5 keywords

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

- Please relabel COI as Disclosure and competing interests statement

- 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')

- Figure Callouts: Table S1 and Supplementary Table 2 are called out, but there is only one suppl. Table.

- The supple Table should be renamed to Appendix Table S1. The appendix should have a ToC with page numbers

- My colleaugue Daniele had contacted you back in October regarding needed source data. I have attached the files again. Please submit source data with the next revision.

- Please upload a a synopsis image size should be 550 wide by [200-400]

- We need a summary statement plus 3-5 bullet points describing the key findings of the MS.

- The Casp1 blot in 3E is very tightly cropped please show more of the blot. Please also check the other blots.

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

When you submit the revised version please include a point-by-point response also to the editorial points.

Let me know if we need to discuss any of the remaining comments raised by the referees.

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

The authors performed further experiments and adequately addressed my concerns in the revised manuscript. The conclusions that secretion of mtDNA via gasdermin D-dependent mitochondrial permeabilization and exosomes promote the inflammatory process in BS syndrome are better supported by more reliable data. However, other parts of the manuscript, such as the methods section, need to be updated if you have added some experiments that were not shown in the original submission.
1. The information on some antibodies needs to be added to the methods section.
2. Please check the wording "Regents" in the last sentence on page 33. I think it should be "Reagents".

Referee #2:

2nd round of the review - comments to the authors:

Dear Authors,

1. It is unclear what changes were made by the authors to the manuscript as a response to the comments (vs which information is included only in the point-by-point response to the reviewers). Please clarify for each response what changes/modifications/data additions were included in the revised manuscript.
2. RESPONSE to comment #4: I think it is still important to quantify the exosomes at least for the few initial panels - it can be done in collaboration with the lab who has access to the mentioned equipment. What is the system used in Fig. EV1C? - It seems like some kind of quantification method.
In addition, the provided CD63 blots are overexposed and therefore not suitable for the quantifications and further calculations.
3. RESPONSE to comment #5: The authors still did not provide the amount of 100K-EVs administered to the animals - only the concentration is provided.
4. RESPONSE to comment #8: If THP1 line is TLR7/8/9-null cells then it is not suitable for the study of TLR9 signaling. The experiments should be done in the cells with TLR9 expression, where TLR9 knockout would be possible. If THP-1 (or human monocytes) don't express TLR9, how in BS patients, the released mtDNA can trigger TLR9-dependent inflammatory response? Please clarify this issue.

Referee #3:

Authors have properly answered most of the concerns raised in my previous review with àddition of new experimentation and/or explanations.
There are some minor remaining that should be addressed:

a) Fig. 3E: the absence of TFAM signal on the WB in 100K-EVs could be well due to the low sensitivity, as shown by the weak TFAM band identified in WCL (please compared with CD9 relative signal in both EV and WCL). TFAM as well as other mitDNA-associated proteins have been described to be present in EVs from T lymphoblasts (Torralba et al., Nat Commun 2018). This statement should be discussed in the context of other studies.
b) Page 21, there is not correspondence between text and the quotation of Figures in this paragraph. Fig EV6 should be properly cited in this part of the text. This should be corrected

2nd point by point replay

Thank you for providing additional comments and suggestions. We are pleased that the authors' concerns have been addressed by the additional experiments.

Referee #1:

The authors performed further experiments and adequately addressed my concerns in the revised manuscript. The conclusions that secretion of mtDNA via gasdermin D-dependent mitochondrial permeabilization and exosomes promote the inflammatory process in BS syndrome are better supported by more reliable data.

However, other parts of the manuscript, such as the methods section, need to be updated if you have added some experiments that were not shown in the original submission.

1. The information on some antibodies needs to be added to the methods section.

Yes, new antibodies have been added to the Reagent Tables.

2. Please check the wording "Regents" in the last sentence on page 33. I think it should be "Reagents".

Thank you for correcting the error. We have corrected it.

Referee #2:

2nd round of the review - comments to the authors:

Dear Authors,

1. It is unclear what changes were made by the authors to the manuscript as a response to the comments (vs which information is included only in the point-by-point response to the reviewers). Please clarify for each response what changes/modifications/data additions were included in the revised manuscript.

In response to the referee's concerns, we apologize for not indicating where in the revised manuscript the text addressing individual points is located. We recognize that this paper was somewhat redundant in that it needed to address a variety of possibilities. As such, we were concerned that our response to the referee's concerns would be even more redundant in the manuscript. In addition, since our correspondence with the referee will be opened in the EMBO J, we wanted to keep the reflection in the text to a minimum, and some of the points raised were indeed not mentioned in the text. However, we felt it necessary to consider the overall points raised by the referee and have included these sentences in red in the revised manuscript.

We have described the following sentences in the revised manuscript (p30, line 1-6) in response to

the major concerns of referee 2.

We demonstrated that caspase-1 plays an essential role in the ILV-mediated uptake of leaked cytoplasmic mtDNA by showing impaired Tsg101 puncta formation and CD9-positive exosome secretion in Casp1-KO cells. Therefore, caspase-1 may be involved in the initiation of ILV formation; however, we have not been able to determine its substrates so far. Therefore, future studies are expected to elucidate the mechanisms of exosome biosynthesis and spatiotemporal regulation, especially those mediated by caspase-1.

2. RESPONSE to comment #4: I think it is still important to quantify the exosomes at least for the few initial panels - it can be done in collaboration with the lab who has access to the mentioned equipment. What is the system used in Fig. EV1C? - It seems like some kind of quantification method. In addition, the provided CD63 blots are overexposed and therefore not suitable for the quantifications and further calculations.

3. RESPONSE to comment #5: The authors still did not provide the amount of 100K-EVs administered to the animals - only the concentration is provided.

EV particle concentrations were quantified and the results are shown below.

(10^{10} particles/ml)	CS of THP1	Serum from HC	Serum from BS
16K-EVs	9.95	40	32.8
100K-EVs	0.63	0.74	0.44

The peritonitis model was administered 100 µl of this 100K-EV, and the arthritis model was administered 10 µl of this 100K-EV. Thus, the following amounts of 100K-EV were administered in the *in vivo* experiments.

(10^{8} particles)	100K-EVs of THP1	100K-EVs of HC	100K-EVs of BS
Peritonitis model	6.3	7.4	4.4
Arthritis model	0.63	0.74	0,44

The purpose of the quantification of CD63 in the Western blotting in Figures 3H and 3J was to show that caspase-1 deficiency and DMA treatment reduced CD63 expression. Thus, adequate exposure was necessary. On the other hand, Figures EV1D and EV1E are separate experiments, with EV1E quantifying CD63 levels by ELISA.

4. RESPONSE to comment #8: If THP1 line is TLR7/8/9-null cells then it is not suitable for the study of TLR9 signaling. The experiments should be done in the cells with TLR9 expression, where TLR9 knockout would be possible. If THP-1 (or human monocytes) don't express TLR9, how in BS patients, the released

mtDNA can trigger TLR9-dependent inflammatory response? Please clarify this issue.

The THP1 cells used did not respond to TLR7/8/9 ligands, but did respond to TLR2 and TLR4 ligands, indicating that the cells are equipped with Myd88 downstream signals. Therefore, expression of TLR9 would specifically react to TLR9 ligand, thus proving the presence of TLR9 ligand. On the other hand, primary human CD14-monocytes express TLR9 and can respond to TLR9 ligands. Thus, they can produce inflammatory cytokines in response to mtDNA via TLR9.

Referee #3:

Authors have properly answered most of the concerns raised in my previous review with addition of new experimentation and/or explanations.

There are some minor remaining that should be addressed:

a) Fig. 3E: the absence of TFAM signal on the WB in 100K-EVs could be well due to the low sensitivity, as shown by the weak TFAM band identified in WCL (please compared with CD9 relative signal in both EV and WCL). TFAM as well as other mitDNA-associated proteins have been described to be present in EVs from T lymphoblasts (Torralba et al., Nat Commun 2018). This statement should be discussed in the context of other studies.

The text in this section has been revised as follows (p15, line 15- p16, line 8)

It has been reported that mtDNA is packed with TFAM (Alam *et al*, 2003) and released out of mitochondria by herniation of the mitochondrial membrane via BAK/BIX-macropore (McArthur *et al*, 2018). It has also been shown that mitochondrial proteins including TFAM are detected in 100K-EVs isolated from T cell culture supernatants (Torralba *et al*, 2018). Therefore, we examined whether mitochondrial proteins such as Tim23 and TFAM could be detected in purified 100K-EVs. However, these proteins were not detected (**Fig 3E**). Therefore, it could not be completely determined whether mitochondrial proteins are present in 100K-EVs from pyroptotic cells; since there is a possibility that mtDNA is trapped in 100K-EVs that do not contain mitochondrial proteins, or that mtDNA and mitochondrial proteins are trapped simultaneously in 100K-EVs but mitochondrial proteins are not detected, perhaps due to insufficient antibody affinity.

b) Page 21, there is not correspondence between text and the quotation of Figures in this paragraph. Fig EV6 should be properly cited in this part of the text. This should be corrected.

We corrected the text and the quotation of Figures.

2nd Revision - Editorial Decision **7th Aug 2023**

Dear Prof. Takamatsu,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good!

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

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

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^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;
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Material and Methods section

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

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Material and Methods, Reagents and Tools Table

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Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Material and Methods section

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If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Supplementary Table

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

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

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Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Material and Methods section
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure ledgend

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure ledgend
In the figure legends: define whether data describe **technical or biological replicates**.	Not Applicable	

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Material and Methods section
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes	Material and Methods section
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and Methods section
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If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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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.	Not Applicable	
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Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
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If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	