THE EMBO JOURNAL

SARS-CoV-2 nucleocapsid suppresses host pyroptosis through blocking GSDMD cleavage

Juan Ma, Fangrui Zhu, Min Zhao, Fei Shao, Dou Yu, Jiangwen Ma, Xusheng Zhang, Weitao Li, Yan Qian, Yan Zhang, Dong Jiang, Shuo Wang, and Pengyan Xia
DOI: 10.15252/embj.2021108249

Corresponding author(s): Pengyan Xia (xiap@pku.edu.cn) , Shuo Wang (wangshuo@im.ac.cn)

Review Timeline:

Submission Date:	12th Mar 21
Editorial Decision:	27th Apr 21
Revision Received:	7th Jun 21
Editorial Decision:	6th Jul 21
Revision Received:	7th Jul 21
Accepted:	13th Jul 21

Editor: Karin Dumstrei

Transaction Report:

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

1st Editorial Decision 27th Apr 2021

Dear Prof. Xia,

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 from the comments, the referees find the analysis interesting and insightful. However, they also indicate that some further experiments are needed in order to solidify the conclusions. I think the points raised should be fairly straightforward to address and I would like to invite you submit a revised version.

I think it would be helpful to the raised points further and we can do so either 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

We generally allow three months as standard revision time. 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. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 26th Jul 2021.

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

Referee #1:

In this study, Ma and colleagues report that SARS-COV-2 antagonizes pyroptosis activation by inhibiting the cleavage of GSDMD. They start off by showing that SARS-COV-2 infection of human monocytes does not trigger much IL-1b release in comparison to a bona fide NLRP3 agonist (LPS+Nigericin). Moreover, SARS-COV-2 infection blocks NLRP3 activation as readout by IL-1b secretion and lytic cell death induction. Interestingly, despite this lack in IL-1b release and pyroptosis, SARS-COV-2 infected cells still display ASC oligomerization, indicative of inflammasome activation. They then go on to express various ORFs of SARS-COV-2 to explore whether these actively repress inflammasome activation. Doing so, they find that nuclecocapsid overexpression (but not any other ORFs) indeed represses pyroptosis, but not caspase-1 or IL-1b processing. Binding studies reveal that nucleocapsid indeed binds to GSDMD and that this binding blocks GSDMD processing by caspase-1. Mutagenesis studies of the GSDMD linker region further imply that nucleocapsid-dependent inhibition of GSDMD processing can be attributed to this region. Indeed, expressing a variant GSDMD molecule, in which this linker region is permutated, rescues inflammasome activation in the presence of nucleocapsid.

Altogether, the authors provide a compelling concept of how SARS-COV-2 inhibits pyroptosis. The data are in most part conclusive and well controlled. Nevertheless, I have a few questions regarding

some of the experiments:

- The SARS and the SARS-COV-2 nucleocapsid proteins are quite similar (90% identity, 94% similarity). As such, it is quite surprising that the authors don't see an interaction of the SARS nucleocapsid with GSDMD. Is it possible that a more sensitive assay would indeed show a functional interaction? Have the authors tried to express the SARS nucleocapsid in THP1 cells to measure its putative inhibitory activity on GSDMD maturation?

- The nucleocapsid truncations (Fig. 3H) are quite coarse and given the structural information on this protein, it is conceivable that these variants are not properly folded. Have the authors tested the individual domains of the nucleocapsid for their inhibitory activity? This would be the NTD and the CTD (compare: doi.org/10.15252/embj.2020105938).

- In a number of assays, the authors describe the transfection of human primary monocytes with plasmid constructs (e.g. Fig. 2G, 3G). Can the authors explain how this transfection does not result in monocyte activation in the first place? For example, numerous studies have shown that human monocytes engage the cGAS-STING axis upon DNA transfection.

- A number of groups have conducted interactome studies on SARS-COV-2 proteins (e.g. doi.org/10.1038/s41586-020-2286-9). For example, in this latter study, a number of interaction partners have been found for nucleocapsid, while GSDMD was not among them. This discrepancy could be due to differences in cell types or in techniques used. However, did the authors identify any interactors in their Y2H screen that have previously been reported? It would be great to see what to ther other hits were.

- The results on the specific role of the GSDMD linker region are quite compelling. The authors suggest that the nucleocapsid specifically interacts with this linker region, which results in the inhibitory effect. The IP studies, however, do not show much reduced binding of the linker substituted GSDMD variant (Fig. 5I). Have the authors tried binding assays with recombinant proteins? In addition, conceptually it is surprising that this linker region has this critical importance, even though human and murine GSDMD are quite divergent in this region (mouse GSDMD was used in the Y2H screen to identify this interaction).

- A recent study suggest that SARS-COV-2 infection indeed does trigger pyroptosis and IL-1b release in human monocytes (doi.org/10.1038/s41420-021-00428-w). Can the authors briefly discuss how these discrepant results can be obtained?

Referee #2:

Ma et al study the immunological consequences of SARS-CoV-2 exposure on human monocytes. Using a monocyte-like cell line (THP-1) and primary monocytes, they show that SARS-CoV-2 triggers inflammasome activation. However, the nucleoprotein (N) protein blocks IL-1β secretion and pyroptosis, both upon SARS-CoV-2 challenge or after a conventional inflammasome activation signal (LPS+Nigericin). They further demonstrate that this inhibition depends on the interact of N with gasdermin D (GSDMD). GSDMD cleavage at the linker region by p20/p10 is inhibited by N, which prevents the formation of the lytic pore and release of cleaved IL-1β. Primary monocytes still respond to SARS-CoV-2 exposition by releasing other pro-inflammatory cytokines. The question the authors address is of importance. COVID-19 is characterized by a cytokine storm but its origin

is not well understood. The role of myeloid cells in SARS-CoV-2 sensing is understudied. Most of the results are convincing and well presented. Some experiments are over-interpreted and some controls are lacking.

Major points:
1. The reanalysis of the scRNA-seq data raises some concerns:
• Were several patients pooled in each category (if yes, how many?) or was one representative donor chosen?
• In Figure 1B, the increase in non-classical monocytes is weak and probably not significant. If several patients were included, a statistical analysis should be performed.
• "Moreover, IL1B was wildly expressed in mild/moderate COVID-19 patient monocytes": visually, it seems that it is expressed at the same level than in healthy donors (Figure 1C).
• "suggesting an induction of this cytokine in the early stage of SARS-CoV-2 infection": disease severity should not be mistaken with temporal evolution of the disease.
• "Correspondingly, IL1B showed a strong co-expression propensity with the NOD-like receptor NLRP3": are non-canonical monocytes co-expressing IL1B and NLRP3? IL1B and NLRP3 expression levels could be correlated in cells expressing one and/or the other.
• Why not looking at NLRP4 and NLRP9b in Figure S1B?
• Overall, a quantification is missing, and the analysis is almost exclusively based on visual examination of tSNE plots.

2. The manuscript states that monocytes (primary or THP-1) are infected with SARS-CoV-2. This raises some concerns:
• The methods do not state what was the primary source of the virus, the strain, the titration method and the infection protocol. More specifically, which MOI was used for infection, was the virus washed and how long were cells exposed to the virus?
• Figure 1D shows viral RNA in SARS-CoV-2-exposed monocytes. However, this could result from capture of incoming viral material. The authors should check whether there is productive infection by measuring for instance viral RNA levels in the supernatant by qRT-PCR over time. Presence of viral material is not sufficient demonstrate a productive infection. The viral RNA detected is not necessarily inside cells and could correspond to extracellular retention. Describing whether monocytes are productively infected or only capture incoming virions is important.
• Were mock-infected cells treated with supernatants from uninfected Vero cells? Indeed, Vero cells produce some cytokines at the basal level which can impact immunological readouts, especially if the inoculum is not removed prior to other analyses. This should be clarified, and the infection protocol thoroughly described.

3. The pseudo-SARS-CoV-2 (Spike-pseudotyped HIV NL4-3) experiments are misleading. They seem to indicate that viral material induces inflammasome inflammation. It is probably the lentivirus that is sensed, and not the Spike protein. Similar results would likely be obtained with a VSV-pseudotyped HIV. Thus, it is just another way to induce inflammasome and pyroptosis in monocytes, not related to SARS-CoV-2 and not different from LPS+Nigericin. The pseudotype results should be removed or completed with VSV-G bearing pseudotypes

4. In the yeast two-hybrid screening, 14/24 interactants are related to murine GSDMD. What about the other 10? This information should be added. Moreover, choosing a mouse cDNA library to look for SARS-CoV-2 N human interactants is intriguing. What is the basis of this choice? Could more interactants be identified if a human library was used?

5. Figure 3E: colocalization between N and GSDMD is not convincing. A co-localization index should

be measured and compared to an irrelevant protein to asess the specificity of the interaction detected by confocal microscopy.

6. In SARS-CoV-2-exposed monocytes or THP-1, is there an association between endogenous GSDMD and viral N?

7. Figure 3I: the fact that almost all 7 fragments of N interact with the 4 fragments pf GSDMD is surprising. Is this relatively low level of specificity and "multiple interfaces" common features in protein-protein interactions?

8. Figure 4E-F: "Nucleocapsid overexpression had little effect on IL-1β secretion or cell viability in GSDMD knockout cells". This is because in GSDMD-/- cells, there is no activation of the inflammasome at all, even upon LPS+Nigericin treatment. So logically, N does not impact inflammasome activation. These data do not show that N acts via GSDMD but that GSDMD is essential for pyroptosis, which is already known.

9. Figure 5A: a control without N should be added as saturating levels of N may already be reached. This could reveal differences in p20/p10 and GSDMD interaction in the presence of lower levels of N.

Minor points:

1. "Previous studies showed up-regulated IL1B expression levels in COVID-19 patient peripheral blood mononuclear cells (PBMCs)": this was also shown in monocytes (Wauters et al., 2021).
2. "Health donors" should be "Healthy donors".
3. The legends do not clearly state if the bar graphs represent the mean{plus minus}SD of independent experiments or technical replicates. In Figure 3 legend, there should be a description of *, not **.
4. In Figure 1F-H, statistical analyses comparing mock- and SARS-CoV-2-exposed monocytes could be added to document the effect of SARS-CoV-2 on inflammasome activation and pyroptosis.
5. Figure S2G: a kb ladder marker should be added.
6. Data in U937, THP-1-derived and mouse peritoneal macrophages are interesting and should be added as supplemental material rather than not shown.
7. Figure S3H should be added as a principal, side by side with Figure 3GH.
8. Figure 4A: if GSDMD is cleaved, shouldn't we detect a smaller band in the full GSDMD blot?
9. Figure S5B: GSDMDΔ350-368 alone also loses its ability to interact with p20/p10.
10. Figure 5B: in the blot image, it seems that there is less N in the input with GSDMDΔ265-284, which could be responsible for the differences observed after GST pulldown. One would expect a stronger effect.
11. Some language mistakes are present throughout the manuscript.

Referee #3:

The manuscript by Ma and colleagues investigates NLRP3 inflammasome signalling upon SARS-CoV-2 infection. They report that while this virus triggers activation of the NLRP3 inflammasome

pathway, the viral nucleocapsid protein binds to the pyroptosis effector GSDMD, and blocks its cleavage by caspase-1. In doing so, the viral nucleocapsid protein suppresses NLRP3-mediated cell death. The authors speculate (but do not show) that in suppressing pyroptosis-mediated viral release from monocytes, the virus has time to productively infect these cells.

This study is timely and interesting, and some of the data is quite compelling. It is extremely difficult to work with SARS-COV-2 (e.g. requires PC3 containment), so the authors are to be commended for this ambitious project. The manuscript, however, suffers from a lack of clarity in communicating precisely how experiments were performed throughout the entire study - this makes it a difficult manuscript to assess, as it is often unclear whether the experiments were performed appropriately. There are also several places in the study where the manuscript conclusions are not strongly supported by the data presented. For this study to be published in an excellent journal such as EMBO, the manuscript requires major revision to improve the clarity and precision of the written English, as well as major experimental revisions as detailed below.

Major general concerns:
1. The manuscript is very hard to understand, and would benefit from heavy editing from a native English speaker to improve clarity.
2. The major message of the manuscript is that SARS-CoV-2 deploys its nucleocapsid protein to prevent NLRP3/GSDMD-induced pyroptosis in infected cells (presumably, to give the virus time to replicate). Many studies indicate that when GSDMD function is blocked (e.g. by knockout) in NLRP3-signalling cells, other (slower) cell death pathways take over (e.g. apoptosis, necroptosis - see "PAN-optosis" literature). A simple experiment that was not done, but should be, is to infect human monocytes with SARS-CoV-2, and monitor cell viability/death, IL-1b release, ASC specks and release of productive virus over time (e.g. 2, 4, 8, 24, 72h). If the author's model is correct, then ASC specks will form quickly after viral infection (e.g. 2-4h), but IL-1b release, cell death and virus release will occur concomitantly at a much later time point (e.g. 24-72h). Demonstration of this would really elevate the impact and rigour of the current study. It is possible that the authors model is incorrect, and they will instead see IL-1b release and cell death occurring at earlier time points without virus release (e.g. 4-8h), reflecting NLRP3-dependent but GSDMD-independent forms of cell death (e.g. apoptosis). If this happens, will the virus have time to replicate?
3. In general there is a lack of clarity on how experiments were performed in the text and figure legends. For example, what exactly is the 'SARS-CoV-2 pseudovirus' - is it a virus-like particle expressing the SARS-CoV-2 Spike protein? In experiments in which cells are infected with SARS-CoV-2 or pseudovirus, how long were cells infected and with what MOI/pfu? In experiments where virus-infected cells were stimulated with LPS+nigericin, how long were the cells infected before adding LPS?
4. None of the experiments with "SARS-CoV-2 pseudovirus" have accompanying samples infected with the pseudovirus without SARS-CoV-2 spike protein. Thus, it is unclear whether it is the pseudovirus itself, or the SARS-CoV-2 spike protein, that activates inflammasome signalling. Please include all appropriate pseudovirus controls.
5. For experiments using THP1 stable cell lines: It is not always clear from the figure legends, methods or manuscript results text which experiments use parental THP1 cells, or THP1 cells stably expressing ectopic proteins. This should be clarified throughout, and it should also be clear exactly how these cells were generated (using lentivirus, transfected plasmids? How were cells selected? Does the study use single clones or pooled stables? Were multiple independent stable cell lines used or not?). Myeloid cell lines including THP1s are inherently clonal - single or pooled clones of even parental THP1 cells can show very different signalling profiles. How was this controlled for in the current study?
6. All western blots should show protein markers and the full blot (not highly cropped regions) so

that the reader can assess full length and cleaved proteins. In some figures, it is unclear whether the data was generated from cell lysates, cell supernatants, or both. Please be explicit. This is particularly important because suppressing GSDMD-dependent cell death should lead to the accumulation of cleavage products (IL-1 p17, CASP1 p20, CASP1 p10) in the cell extracts (as they are usually released into the supernatant by cells dying by pyroptosis). This does not seem to be the case in many of the experiments shown throughout the manuscript - why?
7. All immunoprecipitation experiments should show isotype control antibody pulldowns to ensure co-immunoprecipitation is specific.
8. For many experiments it is unclear whether cells were transfected with recombinant nucleocapsid protein (to get it into the cytosol) or whether they were transfected with nucleocapsid-encoding plasmids. Please ensure this is clear throughout.
9. Please indicate the purity of the CD14+ monocyte preparations used for experiments.

Major concerns for each figure:

Figure 1 and Fig S1
• It is unclear from the results text and figure caption that Fig 1A-C represents published data that has been reanalysed for this study. Please clarify in both of these places. Fig 1A wishes to highlight non-classical monocytes (orange), but there is another orange cell population so it is difficult to discriminate between these cells. Suggest changing the non-classical monocytes population to black (or another easily distinguishable colour).
• Fig 1A shows that the abundance of non-classical monocytes decreases dramatically in severe COVID19 patients. Why would this be, if the virus prevents pyroptosis as the authors propose?
• The authors assert that Fig 1A, 1C, and S1 data suggest that monocytes might be a cellular source of inflammasome cytokines in COVID19 patients. While this is reasonable, the data indicate that CD16+ neutrophils are likely to be a much more prominent cellular source. Why focus on monocytes rather than neutrophils?
• It is unclear but I think Fig 1D (also S2A) is supposed to indicate that the virus was taken up by cells. Is it not possible that the virus was attached to the cell surface rather than being taken up? Immunofluorescence detection within cells would support this point more robustly.
• Fig 1E ASC crosslinking data looks strange - why are ASC oligomers present in untreated cells? A better way to study this would be to detect ASC specks by microscopy (and indeed, better still to show this in conjunction with virus staining, so that the reader can see that it is the infected cells that assemble ASC specks).
• The experimental set-up for studies of virus-infected cells stimulated with LPS+nigericin is extremely unclear in the results text. Please clarify how this was done.
• "Further treatment of SARS-CoV-2-infected monocytes with LPS and nigericin increased definite IL-1b section (Fig. 1F)" - the figure indicates a marginal (and non-significant) change in IL-1b secretion when infected cells were stimulated with LPS+nigericin.

Figure 2 and S2
• In figure S2D, it appears there is still some NLRP3 expression in the "NLRP3-/-" cells?
• What are the "vector only" controls in Figs 2 and S2? Does the vector here refer to the empty vector corresponding to the nucleocapsid-containing vector? Or is this the empty vector corresponding to the SARS-CoV-2 spike? Please clarify
• In Figs 2G-I with primary monocytes, the results text suggested (although it wasn't clear) that the recombinant nucleocapsid protein was transfected into the cells, but the figures seem to have "vector" controls instead of cells transfected without nucleocapsid - please clarify. It would be extremely surprising if the authors had managed to ectopically express proteins within primary monocytes using vectors.

Figure 3 and S3
• All the studies leading up to figure 3 were in human cells. So why did the authors use a murine bone marrow cDNA library here?
• Figure 3E is lacking staining controls (e.g. cells not transfected with/expressing nucleocapsid to control for nucleocapsid staining, and their THP-1 GSDMD-/- to control for staining specificity of GSDMD). This is important because endogenous GSDMD is notoriously difficult to image (available antibodies are poor for this purpose).
• Experiments probing nucleocapsid interaction with GSDMD-C and GSDMD-N should be done side-by-side in a single experiments (not separate experiments in Fig 3B and S3C).
• The authors indicate that nucleocapsid-GSDMD interaction is promoted with inflammasome signalling - this is marginal at best. e.g. Fig 3F slight elevation likely reflects the increased nucleocapsid input.
• "nucleocapsid lacking aa 70-160 and 290-360 had a weaker association with GSDMD than full length nucleocapsid (Fig 3J)" - the difference here is marginal at best. Suggest changing to "slightly/marginally weaker".
• Fig 3K-L experiments are missing controls showing WT versus mutant nucleocapsid are present in similar amounts.
• Fig S3E figure labelling is extremely unclear. Is this SARS-COV-1? Experiments comparing SARS-COV-1 and -2 should be performed and analysed side-by-side in the same experiments to allow proper comparison.

Figure 5 and S5:
• Fig 5A should include a no-nucleocapsid control
• Fig 5D and 5I: GSDMD mutation-induced decrease in nucleocapsid binding is marginal at best
• In figure 5H, shouldn't FLAG-tagged full length GSDMD versus cherry-GSDMD-C be different sizes? These look to be exactly the same size.
• Fig S5B is missing a WT GSDMD control.

Minor and text issues:
1. Some of the introductory material is not quite correct. Inflammasomes such as NLRP3, AIM2 and Pyrin consist of the "inflammasome nucleator"(not "caspase-1 assembler" - this is not a term used in the field, and is misleading) + ASC + caspase-1 (not just nucleator + caspase-1 as suggested in paragraph 2).
2. "There are five functional NLRs" is incorrect - there is a whole family of NLRs, most of which have well-established functions. Perhaps the intention here is to say that five NLRs can nucleate inflammasomes.
3. The introduction repeatedly indicates that p20/p10 is the active form of Casp1 that cleaves GSDMD. It is true that recombinant p20/p10 can cleave GSDMD but this is not the form of caspase-1 that cleaves IL-1 or GSDMD in cells (see PMID: 29432122)
4. "wildly" seems to be the wrong word in the sentence "Moreover, IL-1b was wildly expressed ....". Moderately or strongly might be a better word here.

Point-by-point response to the reviewers

Referee #1

- The SARS and the SARS-COV-2 nucleocapsid proteins are quite similar (90% identity, 94% similarity). As such, it is quite surprising that the authors don't see an interaction of the SARS nucleocapsid with GSDMD. Is it possible that a more sensitive assay would indeed show a functional interaction? Have the authors tried to express the SARS nucleocapsid in THP1 cells to measure its putative inhibitory activity on GSDMD maturation?

Answer: This is a really good suggestion. We firstly repeated the co-immunoprecipitation assay using SARS-CoV-1 nucleocapsid and GSDMD in HEK293T cells. We found that SARS-CoV-1 nucleocapsid does not precipitate GSDMD while SARS-CoV-2 nucleocapsid does (new Fig. 3A). Secondly, we generated THP-1 cells stably expressing SARS-CoV-1 nucleocapsid and checked the NLRP3 inflammasome activation status in these cells post LPS and nigericin stimulation. The protein levels of caspase-1 p20 and GSDMD cleaved fragment were comparable between SARS-CoV-1 nucleocapsid expressing cells and control ones, further suggesting that SARS-CoV-1 nucleocapsid does not inhibit GSDMD maturation (new Fig. S4A).

- The nucleocapsid truncations (Fig. 3H) are quite coarse and given the structural information on this protein, it is conceivable that these variants are not properly folded. Have the authors tested the individual domains of the nucleocapsid for their inhibitory activity? This would be the NTD and the CTD (compare: doi.org/10.15252/embj.2020105938).

Answer: This is a very good point. We generated THP-1 cells stably expressing nucleocapsid NTD or CTD domain and tested their impacts on the IL-1β secretion and cell viability. We found that neither NTD or CTD alone possessed the ability to suppress IL-1β secretion or pyroptosis (new Fig. S3H-J). Together with our truncation assay using nucleocapsid Δ70-160;290-360, we proposed that multiple regions on nucleocapsid are required for binding to and inhibiting GSDMD.

- In a number of assays, the authors describe the transfection of human primary monocytes with plasmid constructs (e.g. Fig. 2G, 3G). Can the authors explain how this transfection does not result in monocyte activation in the first place? For example, numerous studies have shown that human monocytes engage the cGAS-STING axis upon DNA transfection.

Answer: We used a Human Monocyte Nucleofector Kit (Lonza) to electroporate plasmids into human primary monocytes. Indeed, DNA transfection activates the cGAS-STING axis in monocytes shortly after transfection (Mankan AK, EMBO J, 2014, PMID: 25425575). However, in our study, cells were cultured for 36 h post transfection, in which case the activation of the cGAS-STING has been alleviated as demonstrated by the minimal IL-1β expression in these cells (new Fig. 2G). We added this procedure in the methods section.

- A number of groups have conducted interactome studies on SARS-COV-2 proteins (e.g. doi.org/10.1038/s41586-020-2286-9). For example, in this latter study, a number of interaction partners have been found for nucleocapsid, while GSDMD was not among them. This discrepancy could be due to differences in cell types or in techniques used. However, did the authors identify any interactors in their Y2H screen that have previously been reported? It would be great to see what to ther other hits were.

Answer: This is a very good suggestion. Besides GSDMD, four DDX21, three C1orf122, two GBP4 and one G3BP1 clones appeared in our yeast-two hybrid screening. DDX21 and G3BP1 were previously described nucleocapsid interactors (Gordon DE, Nature, 2020). We added this information in our revised manuscript.

- The results on the specific role of the GSDMD linker region are quite compelling. The authors suggest that the nucleocapsid specifically interacts with this linker region, which results in the inhibitory effect. The IP studies, however, do not show much reduced binding of the linker substituted GSDMD variant (Fig. 5I). Have the authors tried binding assays with recombinant proteins? In addition, conceptually it is surprising that this linker region has this critical importance, even though human and murine GSDMD are quite divergent in this region (mouse GSDMD was used in the Y2H screen to identify this interaction).

Answer: We performed binding assays using recombinant GSDMD variants (new Fig. S5G). Similar results were obtained. GSDMD with the linker region substituted still interacts with the nucleocapsid protein, albeit the binding affinity is somewhat lower. This is consistent with our co-immunoprecipitation results showing that nucleocapsid also interacts with GSDMD C terminus (new Fig. S3C). Indeed, this linker region (270-280) binds nucleocapsid directly (Fig. 5E). Whether similar spatial structures within this GSDMD linker region exist among different species needs further investigation.

- A recent study suggest that SARS-COV-2 infection indeed does trigger pyroptosis and IL-1b release in human monocytes (doi.org/10.1038/s41420-021-00428-w). Can the authors briefly discuss how these discrepant results can be obtained?

Answer: This is a very good question. We checked the inflammasome status 3 h post LPS stimulation or 4 h post SARS-CoV-2 administration, while Ferreira et al stimulated monocytes for a longer time (23 h for LPS and 24 h for SARS-CoV-2). We discussed this discrepancy in our revised manuscript.

Referee #2

Major points:

1. The reanalysis of the scRNA-seq data raises some concerns:

• Were several patients pooled in each category (if yes, how many?) or was one representative donor chosen?

Answer: This is a very good point. There were 20 healthy controls, 9 mild/moderate and 7 severe patients in the analysis. We presented this sample information in the revised figures (new Fig. 1B, C and new Fig. S1) and added details in our revised manuscript.

• In Figure 1B, the increase in non-classical monocytes is weak and probably not significant. If several patients were included, a statistical analysis should be performed.

Answer: We added statistical analysis in new Fig. 1B.

• "Moreover, IL1B was wildly expressed in mild/moderate COVID-19 patient monocytes": visually, it seems that it is expressed at the same level than in healthy donors (Figure 1C).

Answer: We calculated the relative expression level of IL1B among healthy controls and patients. We found that IL1B expression is significantly elevated in mild/moderate patients compared to healthy controls (new Fig. 1C). We reworded our description in the revised manuscript.

• "suggesting an induction of this cytokine in the early stage of SARS-CoV-2 infection": disease severity should not be mistaken with temporal evolution of the disease.

Answer: We reworded our description in the revised manuscript.

• "Correspondingly, IL1B showed a strong co-expression propensity with the NOD-like receptor NLRP3": are non-canonical monocytes co-expressing IL1B and NLRP3? IL1B and NLRP3 expression levels could be correlated in cells expressing one and/or the other.

Answer: We reworded our description in the revised manuscript to "Correspondingly, the NOD-like receptor NLRP3 showed a similar rising trend in mild/moderate non-classical monocytes as IL1B did, while other NLRs such as NLRP1, NLRP4, NLRP6 or NLRP9 did not".

• Why not looking at NLRP4 and NLRP9b in Figure S1B?

Answer: We added these data in the new Fig. S1C.

• Overall, a quantification is missing, and the analysis is almost exclusively based on visual examination of tSNE plots.

Answer: We analyzed the sequencing data and presented statistical analyses in new Fig. 1B, C and new Fig. S1B-D.

2. The manuscript states that monocytes (primary or THP-1) are infected with SARS-CoV-2. This raises some concerns:

• The methods do not state what was the primary source of the virus, the strain, the titration method and the infection protocol. More specifically, which MOI was used for infection, was the virus washed and how long were cells exposed to the virus?

Answer: We added this information in our revised manuscript.

• Figure 1D shows viral RNA in SARS-CoV-2-exposed monocytes. However, this could result from capture of incoming viral material. The authors should check whether there is productive infection by measuring for instance viral RNA levels in the supernatant by qRT-PCR over time. Presence of viral material is not sufficient demonstrate a productive infection. The viral RNA detected is not necessarily inside cells and could correspond to

extracellular retention. Describing whether monocytes are productively infected or only capture incoming virions is important.

Answer: This is a very good suggestion. We monitored the productive viruses in monocyte supernatant through RT-PCR analysis of viral RNAs (new Fig. 1D). We found that supernatant viral RNAs were expanded post viral infection, suggesting that SARS-CoV-2 does infect and expand in human monocytes.

• Were mock-infected cells treated with supernatants from uninfected Vero cells? Indeed, Vero cells produce some cytokines at the basal level which can impact immunological readouts, especially if the inoculum is not removed prior to other analyses. This should be clarified, and the infection protocol thoroughly described.

Answer: This is a very good question. We took use of supernatants from uninfected Vero cells as controls. Moreover, these supernatants were ultra-filtered and washed with fresh medium, as the same processing procedure as the viral supernatants. We added this information in our revised manuscript.

3. The pseudo-SARS-CoV-2 (Spike-pseudotyped HIV NL4-3) experiments are misleading. They seem to indicate that viral material induces inflammasome inflammation. It is probably the lentivirus that is sensed, and not the Spike protein. Similar results would likely be obtained with a VSV-pseudotyped HIV. Thus, it is just another way to induce inflammasome and pyroptosis in monocytes, not related to SARS-CoV-2 and not different from LPS+Nigericin. The pseudotype results should be removed or completed with VSV-G bearing pseudotypes

Answer: This is a very good suggestion. We removed results using pseudo-SARS-CoV-2 and performed experiments either using SARS-CoV-2 viruses (new Fig. 2A) or LPS+Nigericin stimulation (new Fig. 2B, C).

4. In the yeast two-hybrid screening, 14/24 interactants are related to murine GSDMD. What about the other 10? This information should be added. Moreover, choosing a mouse cDNA library to look for SARS-CoV-2 N human interactants is intriguing. What is the basis of this choice? Could more interactants be identified if a human library was used?

Answer: These are very good questions. Besides GSDMD, four DDX21, three C1orf122, two GBP4 and one G3BP1 clones appeared in our yeast-two hybrid screening. We added this information in our revised manuscript. At the beginning of our yeast two-hybrid assay, the observation of inflammasome activation post SARS-CoV-2 infection prompted us to choose a myeloid-derived library for screening nucleocapsid binding partners which might be involved in inflammasome signaling. However, there was no human myeloid cell library provided by our Y2H system supplier Clontech. Therefore, we chose a mouse bone marrow-derived Y2H prey library for the screening. Whether new interactants are identified using a human myeloid library will deserve further investigation.

5. Figure 3E: colocalization between N and GSDMD is not convincing. A co-localization index should be measured and compared to an irrelevant protein to asess the specificity of the interaction detected by confocal microscopy.

Answer: This is a very good suggestion. We repeated this experiment and took use of COXIV as an irrelevant control. We calculated the co-localization index and found that nucleocapsid co-localized well with GSDMD but not with COXIV (new Fig. S3E).

6. In SARS-CoV-2-exposed monocytes or THP-1, is there an association between endogenous GSDMD and viral N?

Answer: We challenged human monocytes with SARS-CoV-2 and found SARS-CoV-2 encoded nucleocapsid associated with endogenous GSDMD (new Fig. S3F).

7. Figure 3I: the fact that almost all 7 fragments of N interact with the 4 fragments pf GSDMD is surprising. Is this relatively low level of specificity and "multiple interfaces" common features in protein-protein interactions?

Answer: This is the case. In fact, multiple interfaces between protein-protein interactions exist in the literature (Zhang J, Cell Rep, 2017, PMID: 29141207).

8. Figure 4E-F: "Nucleocapsid overexpression had little effect on IL-1β secretion or cell viability in GSDMD knockout cells". This is because in GSDMD-/- cells, there is no activation of the inflammasome at all, even upon LPS+Nigericin treatment. So logically, N does not impact inflammasome activation. These data do not show that N acts via GSDMD but that GSDMD is essential for pyroptosis, which is already known.

Answer: We reworded our description to "Nucleocapsid overexpression had almost the same effects on IL-1β secretion or cell viability as GSDMD knockout did (Fig. 4E, F), suggesting that nucleocapsid effectively targets GSDMD for inhibiting IL-1β secretion and pyroptosis inside cells".

9. Figure 5A: a control without N should be added as saturating levels of N may already be reached. This could reveal differences in p20/p10 and GSDMD interaction in the presence of lower levels of N.

Answer: We added a control without nucleocapsid in new Fig. 5A.

Minor points:

1. "Previous studies showed up-regulated IL1B expression levels in COVID-19 patient peripheral blood mononuclear cells (PBMCs)": this was also shown in monocytes (Wauters et al., 2021).

Answer: We reworded our description in the revised manuscript to "Previous studies showed up-regulated IL1B expression levels in monocytes of COVID-19 patients".

2. "Health donors" should be "Healthy donors".

Answer: We reworded our description in the revised manuscript as suggested.

3. The legends do not clearly state if the bar graphs represent the mean{plus minus}SD of independent experiments or technical replicates. In Figure 3 legend, there should be a description of *, not **.

Answer: We added detailed description in the revised manuscript. We revised Figure 3 legend as suggested.

4. In Figure 1F-H, statistical analyses comparing mock- and SARS-CoV-2-exposed monocytes could be added to document the effect of SARS-CoV-2 on inflammasome activation and pyroptosis.

Answer: We added statistical analyses comparing mock- and SARS-CoV-2-exposed monocytes in new Fig. 1H-J.

5. Figure S2G: a kb ladder marker should be added.

Answer: We added markers in new Fig. S2A.

6. Data in U937, THP-1-derived and mouse peritoneal macrophages are interesting and should be added as supplemental material rather than not shown.

Answer: We added these data (new Fig. S2B-G).

7. Figure S3H should be added as a principal, side by side with Figure 3GH.

Answer: These two truncation schemes were shown together in new Fig. 3G.

8. Figure 4A: if GSDMD is cleaved, shouldn't we detect a smaller band in the full GSDMD blot?

Answer: We repeated these experiments and showed the full blots. The cleaved fragments were only a small part of total GSDMD, probably explaining why we were not able to detect reduction in full GSDMD.

9. Figure S5B: GSDMD∆350-368 alone also loses its ability to interact with p20/p10.

Answer: We repeated this experiment and found that GSDMD ∆350-368 still possessed the ability to interact with p20/p10 (new Fig. S5B).

10. Figure 5B: in the blot image, it seems that there is less N in the input with GSDMD∆265-284, which could be responsible for the differences observed after GST pulldown. One would expect a stronger effect.

Answer: We repeated this experiment and presented a better one (new Fig. 5D).

11. Some language mistakes are present throughout the manuscript.

Answer: We carefully revised our manuscript and corrected inaccuracies.

Referee #3

Major general concerns:

1. The manuscript is very hard to understand, and would benefit from heavy editing from a native English speaker to improve clarity.

Answer: We carefully reworded our text and corrected inaccurate statements.

2. The major message of the manuscript is that SARS-CoV-2 deploys its nucleocapsid protein to prevent NLRP3/GSDMD-induced pyroptosis in infected cells (presumably, to give the virus time to replicate). Many studies indicate that when GSDMD function is blocked (e.g. by knockout) in NLRP3-signalling cells, other (slower) cell death pathways take over (e.g. apoptosis, necroptosis - see "PAN-optosis" literature). A simple experiment that was not done, but should be, is to infect human monocytes with SARS-CoV-2, and monitor cell viability/death, IL-1b release, ASC specks and release of productive virus over time (e.g. 2, 4, 8, 24, 72h). If the author's model is correct, then ASC specks will form quickly after viral infection (e.g. 2-4h), but IL-1b release, cell death and virus release will occur concomitantly at a much later time point (e.g. 24-72h). Demonstration of this would really elevate the impact and rigour of the current study. It is possible that the authors model is incorrect, and they will instead see IL-1b release and cell death occurring at earlier time points without virus release (e.g. 4-8h), reflecting NLRP3-dependent but GSDMD-independent forms of cell death (e.g. apoptosis). If this happens, will the virus have time to replicate?

Answer: This is a very good suggestion. We infected human monocytes with SARS-CoV-2 and examined the inflammasome status in these cells. ASC specks appeared in virus infected cells as early as 2 h post infection (new Fig. 1G), suggesting an induction of inflammasomes by SARS-CoV-2 infection. However, virus RNAs or secreted IL-1β were hardly detected in supernatant at the early times post infection (new Fig. 1D, E). Moreover, cell death was not prevalent in SARS-CoV-2 infected cells until 24 h post infection (new Fig. 1F). Indeed, virus and IL-1β were released at a later time accompanied by cell death (new Fig. 1D-F). These data strengthen our model that SARS-CoV-2 nucleocapsid inhibits host pyroptosis to favor its replication at earlier time points without virus release (e.g. 4-8h). As to how the cell death occurred 24 h post infection, it is possible that other (slower) cell death pathways take over, the mechanism of which needs further investigation.

3. In general there is a lack of clarity on how experiments were performed in the text and figure legends. For example, what exactly is the 'SARS-CoV-2 pseudovirus' - is it a virus-like particle expressing the SARS-CoV-2 Spike protein? In experiments in which cells are infected with SARS-CoV-2 or pseudovirus, how long were cells infected and with what MOI/pfu? In experiments where virus-infected cells were stimulated with LPS+nigericin, how long were the cells infected before adding LPS?

Answer: These are very good questions. The pseudo-viruses we previously used were virus-like particles expressing the SARS-CoV-2 Spike protein. To investigate the physiological role of SARS-CoV-2 in inflammasome activation, we removed experiments using pseudo-viruses as suggested by Reviewer #2 and repeated key experiments using SARS-CoV-2 viruses (new Fig.2A, new Fig. S3F). We added detailed descriptions on virus infection procedures and LPS+nigericin treatments in our revised manuscript.

4. None of the experiments with "SARS-CoV-2 pseudovirus" have accompanying samples infected with the pseudovirus without SARS-CoV-2 spike protein. Thus, it is unclear whether it is the pseudovirus itself, or the SARS-CoV-2 spike protein, that activates

inflammasome signalling. Please include all appropriate pseudovirus controls.

Answer: We replaced experiments involving pseudo-viruses with new ones using SARS-CoV-2 virus (new Fig.2A, new Fig. S3F).

5. For experiments using THP1 stable cell lines: It is not always clear from the figure legends, methods or manuscript results text which experiments use parental THP1 cells, or THP1 cells stably expressing ectopic proteins. This should be clarified throughout, and it should also be clear exactly how these cells were generated (using lentivirus, transfected plasmids? How were cells selected? Does the study use single clones or pooled stables? Were multiple independent stable cell lines used or not?). Myeloid cell lines including THP1s are inherently clonal - single or pooled clones of even parental THP1 cells can show very different signalling profiles. How was this controlled for in the current study?

Answer: THP1 stable cell lines were generated through lentiviral infection and selected by puromycin. Pooled stables were used and experiments were repeated using different batches of stables. We added these details in the revised methods.

6. All western blots should show protein markers and the full blot (not highly cropped regions) so that the reader can assess full length and cleaved proteins. In some figures, it is unclear whether the data was generated from cell lysates, cell supernatants, or both. Please be explicit. This is particularly important because suppressing GSDMD-dependent cell death should lead to the accumulation of cleavage products (IL-1 p17, CASP1 p20, CASP1 p10) in the cell extracts (as they are usually released into the supernatant by cells dying by pyroptosis). This does not seem to be the case in many of the experiments shown throughout the manuscript - why?

Answer: We repeated these experiments and showed full blots in our revised figures (new Fig.2A, D, G, new Fig. 4A, C, H and new Fig. 6A, G). Indeed, inhibiting GSDMD cleavage leads to accumulated cleavage products of caspase-1 and IL-1β in cell extracts (new Fig. 2D, G).

7. All immunoprecipitation experiments should show isotype control antibody pulldowns to ensure co-immunoprecipitation is specific.

Answer: We repeated these experiments using isotype IgG as controls (new Fig. 3A, B, E, F and new Fig. S3B, C, F).

8. For many experiments it is unclear whether cells were transfected with recombinant nucleocapsid protein (to get it into the cytosol) or whether they were transfected with nucleocapsid-encoding plasmids. Please ensure this is clear throughout.

Answer: We transfected plasmids encoding nucleocapsid into cells. We added these details in our revised manuscript.

9. Please indicate the purity of the CD14+ monocyte preparations used for experiments.

Answer: Purified monocytes were examined through FACS. The purity was above 95%. We added these details in our revised text.

Major concerns for each figure:

Figure 1 and Fig S1

• It is unclear from the results text and figure caption that Fig 1A-C represents published data that has been reanalysed for this study. Please clarify in both of these places. Fig 1A wishes to highlight non-classical monocytes (orange), but there is another orange cell population so it is difficult to discriminate between these cells. Suggest changing the non-classical monocytes population to black (or another easily distinguishable colour).

Answer: These are very good suggestions. We emphasized the reanalysis of published sequencing data in our revised text and figure caption. We also adjusted the colors in new Fig. 1A to make them easier to distinguish.

• Fig 1A shows that the abundance of non-classical monocytes decreases dramatically in severe COVID19 patients. Why would this be, if the virus prevents pyroptosis as the authors propose?

Answer: This is a very good question. When we looked at the expression levels of inflammasome related genes, we found that GSDMD was significantly down-regulated in non-classical monocytes of severe patients (new Fig. S1D). GSDMD down-regulation may lead to other forms of cell death (Zheng M, J Biol Chem, 2020, PMID: 32763970), which might contribute to the observed decreases of non-classical monocytes in severe patients. The exact mechanism of this decrease is worth further investigation.

• The authors assert that Fig 1A, 1C, and S1 data suggest that monocytes might be a cellular source of inflammasome cytokines in COVID19 patients. While this is reasonable, the data indicate that CD16+ neutrophils are likely to be a much more prominent cellular source. Why focus on monocytes rather than neutrophils?

Answer: This is a very good point. Besides monocytes, neutrophils express IL1B as well. However, when we calculated the expression level of IL1B in neutrophils of different groups, we found that IL1B expression level is not varied among healthy donors and COVID-19 patients (new Fig. S1B). We added this description in our revised manuscript.

• It is unclear but I think Fig 1D (also S2A) is supposed to indicate that the virus was taken up by cells. Is it not possible that the virus was attached to the cell surface rather than being taken up? Immunofluorescence detection within cells would support this point more robustly.

Answer: This is a very good suggestion. We incubated human monocytes with SARS-CoV-2 for 1 h and then washed away extracellular viruses. Cells were further cultured for 1 h. We then stained nucleocapsid protein in these cells. We found that nucleocapsid protein is present in infected monocytes, suggesting that SARS-CoV-2 viruses enter human monocytes (new Fig. 1G).

• Fig 1E ASC crosslinking data looks strange - why are ASC oligomers present in untreated cells? A better way to study this would be to detect ASC specks by microscopy (and indeed, better still to show this in conjunction with virus staining, so that the reader

can see that it is the infected cells that assemble ASC specks).

Answer: This is a very good suggestion. We incubated human monocytes with SARS-CoV-2 for 1 h and then washed away extracellular viruses. Cells were further cultured for 1 h. We then stained ASC protein in the infected cells. We found that ASC speck appears post infection suggesting that SARS-CoV-2 viruses induce inflammasome activation in monocytes (new Fig. 1G).

• The experimental set-up for studies of virus-infected cells stimulated with LPS+nigericin is extremely unclear in the results text. Please clarify how this was done.

Answer: We added these details in the revised text.

• "Further treatment of SARS-CoV-2-infected monocytes with LPS and nigericin increased definite IL-1b section (Fig. 1F)" - the figure indicates a marginal (and non-significant) change in IL-1b secretion when infected cells were stimulated with LPS+nigericin.

Answer: We changed this description to "Treatment of SARS-CoV-2 infected monocytes with LPS and nigericin did not further increase IL-1β secretion".

Figure 2 and S2

• In figure S2D, it appears there is still some NLRP3 expression in the "NLRP3-/-" cells?

Answer: We repeated this experiment with another batch of NLRP3 knockout stables and obtained similar results (new Fig. 2A).

• What are the "vector only" controls in Figs 2 and S2? Does the vector here refer to the empty vector corresponding to the nucleocapsid-containing vector? Or is this the empty vector corresponding to the SARS-CoV-2 spike? Please clarify

Answer: We used empty vector corresponding to the nucleocapsid-containing vector in these experiments. We added these details in our revised text.

• In Figs 2G-I with primary monocytes, the results text suggested (although it wasn't clear) that the recombinant nucleocapsid protein was transfected into the cells, but the figures seem to have "vector" controls instead of cells transfected without nucleocapsid - please clarify. It would be extremely surprising if the authors had managed to ectopically express proteins within primary monocytes using vectors.

Answer: We used a Human Monocyte Nucleofector Kit (Lonza) to electroporate plasmids into human primary monocytes. We added the methods in our revised text.

Figure 3 and S3

• All the studies leading up to figure 3 were in human cells. So why did the authors use a murine bone marrow cDNA library here?

Answer: This is a very good question. At the beginning of our yeast two-hybrid assay, the observation of inflammasome activation post SARS-CoV-2 infection prompted us to choose a myeloid-derived library for screening nucleocapsid binding partners which might be involved in inflammasome signaling. However, there was no human myeloid cell library provided by our Y2H system supplier Clontech. Therefore, we chose a mouse bone

marrow-derived Y2H prey library for the screening. Whether new interactants are identified using a human myeloid library will deserve further investigation.

• Figure 3E is lacking staining controls (e.g. cells not transfected with/expressing nucleocapsid to control for nucleocapsid staining, and their THP-1 GSDMD-/- to control for staining specificity of GSDMD). This is important because endogenous GSDMD is notoriously difficult to image (available antibodies are poor for this purpose).

Answer: This is a very good suggestion. We repeated this experiment using GSDMD knockout THP-1 cells as controls. We used anti-GSDMD antibody (CST#93709) for staining endogenous GSDMD. There were anti-GSDMD signals in WT cells but not in GSDMD knockout ones (new Fig. 3D), suggesting the specificity of the antibody we used.

• Experiments probing nucleocapsid interaction with GSDMD-C and GSDMD-N should be done side-by-side in a single experiments (not separate experiments in Fig 3B and S3C).

Answer: We repeated this experiment and put them together (new Fig. S3C).

• The authors indicate that nucleocapsid-GSDMD interaction is promoted with inflammasome signalling - this is marginal at best. e.g. Fig 3F slight elevation likely reflects the increased nucleocapsid input.

Answer: We repeated this experiment and presented better ones (new Fig. 3E, F).

• "nucleocapsid lacking aa 70-160 and 290-360 had a weaker association with GSDMD than full length nucleocapsid (Fig 3J)" - the difference here is marginal at best. Suggest changing to "slightly/marginally weaker".

Answer: We changed the description as suggested.

• Fig 3K-L experiments are missing controls showing WT versus mutant nucleocapsid are present in similar amounts.

Answer: We added WB controls for these experiments (new Fig. S3G).

• Fig S3E figure labelling is extremely unclear. Is this SARS-COV-1? Experiments comparing SARS-COV-1 and -2 should be performed and analysed side-by-side in the same experiments to allow proper comparison.

Answer: We listed the full names of nucleocapsid and repeated these experiments side-by-side (new Fig. 3A).

Figure 5 and S5:

• Fig 5A should include a no-nucleocapsid control

Answer: We added no-nucleocapsid control as suggested in new Fig. 5A.

• Fig 5D and 5I: GSDMD mutation-induced decrease in nucleocapsid binding is marginal at best

Answer: We reworded our description in the revised text.

• In figure 5H, shouldn't FLAG-tagged full length GSDMD versus cherry-GSDMD-C be different sizes? These look to be exactly the same size.

Answer: This is a very good question. Actually, FLAG-tagged full length GSDMD was 520 aa (molecular weight: 56.9 kD) and FLAG-mCherry-GSDMD-C was 492 aa (molecular weight: 54.3 kD). They had almost the same molecular weight and were not distinguishable in the gel we used.

• Fig S5B is missing a WT GSDMD control.

Answer: We repeated this experiment and added WT GSDMD control as suggested (new Fig. S5B).

Minor and text issues:

1. Some of the introductory material is not quite correct. Inflammasomes such as NLRP3, AIM2 and Pyrin consist of the "inflammasome nucleator"(not "caspase-1 assembler" - this is not a term used in the field, and is misleading) + ASC + caspase-1 (not just nucleator + caspase-1 as suggested in paragraph 2).

Answer: We changed this sentence to "Inflammasomes are a macromolecular machinery consisting of pro-caspase-1, ASC and inflammasome nucleators like NOD-like receptors (NLRs), AIM2 and pyrin".

2. "There are five functional NLRs" is incorrect - there is a whole family of NLRs, most of which have well-established functions. Perhaps the intention here is to say that five NLRs can nucleate inflammasomes.

Answer: We reworded this sentence to "To date, there are five inflammasome nucleating NLRs comprising NLRP1, NLRP3, NLRC4, NLRP6 and NLRP9b".

3. The introduction repeatedly indicates that p20/p10 is the active form of Casp1 that cleaves GSDMD. It is true that recombinant p20/p10 can cleave GSDMD but this is not the form of caspase-1 that cleaves IL-1 or GSDMD in cells (see PMID: 29432122)

Answer: We reworded our descriptions about the active caspase-1 dimers.

4. "wildly" seems to be the wrong word in the sentence "Moreover, IL-1b was wildly expressed ....". Moderately or strongly might be a better word here.

Answer: We changed our descriptions as suggested.

1st Revision - Editorial Decision 6th Jul 2021

Dear Pengyan,

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been seen the original referees. As you can see below, the referees appreciate very much the revisions and support publication here. Congratulations on a nice study!

Before sending you the formal acceptance letter, there are just a few formatting issues to resolve:

- please go over text carefully once more to ensure that the experimental procedures and take-home messages are clearly described (referee #3) .

- Did you have approval for working with SARS-CoV2 - if so please state this is Materials and Methods.

- Competing Interests" should be corrected Conflict of Interest

- Italicise journal names in reference list.

- Please make sure to enter funding information in online submission system as well

- The appendix file needs a ToC. The nomenclature of figures should be 'Appendix Figures S#'. Please also correct callouts in text.

- We need a Data Availability section. This is the place to enter accession numbers etc. If there is no data generated that needs to be deposited in a database then state: This study includes no data deposited in external repositories. The section should be placed after the Materials and methods and before Acknowledgements

- Our publisher has 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.

- "Experimental Procedures" should be corrected to 'Materials and Methods'.

That should be all let me know if you need further input from me.

With best wishes

Karin

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 4th Oct 2021.

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

Referee #1:

The authors now present a thoroughly revised version of their manuscript, in which all critical points have been addressed.

Referee #2:

the authors have addressed my concerns

Referee #3:

This manuscript is enormously improved with revision. Experiments now have appropriate controls and the data clearly demonstrate the authors' proposed mechanism; that is, that the SARS-CoV2 nucleocapsid binds to full length GSDMD to protect it from cleavage, thereby subverting inflammasome-dependent cell death and IL-1b release during the early (e.g. 2-8h) infection. The inclusion of new data panels 1D-G provides excellent evidence for this and substantially strengthens the key message of the paper. My congratulations to the authors on their study, particularly given the difficulties of working with SARS-CoV2!

My only remaining suggestion is for some further text editing to increase the clarity of experimental procedures and take-home messages of the manuscript.

2nd Revision - Editorial Decision 13th Jul 2021

Dear Pengyan,

Thanks 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. Congratulations on a nice study!

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The 'Page Charges Authorization Form' is available here:
https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

** Click here to be directed to your login page: https://emboj.msubmit.net

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Pengyan Xia
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2021-108249

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to predetermine sample size. Experiments were independently repeated at least three times to achieve statistical significance.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization or blinding procedures was used in this study.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No randomization or blinding procedures was used in this study.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	YES
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data with normal distribution determined by Shapiro–Wilk normality test were statistically analyzed by two tailed Student's t tests if not specified.
Is there an estimate of variation within each group of data?	Yes, P-values below 0.05 were termed as significant

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes, Batlett's test was used.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes, we provided catalog number of these reagents.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	THP-1 (TIB-202) and U937 (CRL-1593.2) cells were purchased from ATCC.

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

D- Animal Models

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

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Yes, our study was licensed by the Ethics Committee of Institute of Microbiology, Chinese Academy of Sciences.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes, informed consents were obtained from all subjects and experiments conformed to related principles.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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