

# SNX10-dependent LPS sensing by caspase-5 and resulting Lyn signaling causes gut barrier dysfunction

Xu Wang, Jiahui Ni, Yan You, Guize Feng, Sulin Zhang, Wei-Lian Bao, Hui Hou, Hai-Dong Li, Li-Xin Liu, Mingyue Zheng, Yi-Rui Wang, Hua Zhou, Weixing Shen, and Xiaoyan Shen

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## Transaction Report:

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

Dear Dr. Shen,

Thank you for submitting your manuscript entitled "Liberation and sensing of cytosolic LPS through SNX10 mediates gut barrier dysfunction in colitis" [EMBOJ-2021-108080] to The EMBO Journal. Your study has now been assessed by three reviewers, whose reports are enclosed below.

As you can see, the referees concur with us on the potential interest of your findings. However, they also raise several critical points that need to be addressed before they can support publication here.

Given the overall interest of your study, I am pleased to invite submission of a manuscript revised as indicated in the reports attached herein. I would like to point it out that addressing all referees' points in a conclusive manner, as well as a strong support from the referees, would be essential for publication in The EMBO Journal.

I should also add that it is our policy to allow only a single round of major revision. Therefore, acceptance of your manuscript will depend on the completeness of your responses in this revised version.

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content is published elsewhere.

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 made available online. For more details on our Transparent Editorial Process, please visit our website: [http://emboj.embopress.org/about#Transparent\\_Process](http://emboj.embopress.org/about#Transparent_Process)

Before submitting your revised manuscript, deposit any primary datasets and computer code produced in this study in an appropriate public database (see <http://msb.embopress.org/authorguide#dataavailability>). Please remember to provide a reviewer password, in case such datasets are not yet public. The accession numbers and database names should be listed in a formal "Data Availability" section (placed after Materials & Method). Provide a "Data availability" section even if there are no primary datasets produced in the study.

I thank you again for the opportunity to consider this work for publication and look forward to your revision.

Yours sincerely,

Elisabetta Argenzio, PhD  
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:

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**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

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.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 5th Jul 2021.

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

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Referee #1:

The authors investigate the role of SNX10 in the translocation of LPS from bacterial outer membrane vesicles (OMVs) into the cytosol of host cells, a pathway of potential importance for inflammatory bowel disease. The manuscript reports an essential role of SNX10 in an OMV/LPS-induced pathway involving lyn, PIKfyve, caspase5 and the transcription factors Snail and Slug that results in the downregulation of E-cadherin (protein, not mRNA), leading to a breakdown of epithelial barrier function. The newly identified role for SNX10 in gut homeostasis was confirmed in vivo in mice, using spontaneous gut inflammation in IL10<sup>-/-</sup> mice as a model for IBD. In addition, the authors report that DC-SX029, a newly identified LMW SNX10 inhibitor, ameliorates the effects of OMV in vitro and in vivo.

This is clearly an exciting manuscript that identifies an important role for SNX10 in the control of epithelial homeostasis and that should therefore be of significant interest to the community. The identification of a small molecule inhibitor of SNX10 with anti-inflammatory activity in vivo is also of significant interest.

However, a number of issues should be addressed before publication.

1. Are other cytoplasmic LPS pathways (such as pyroptosis) dependent on SNX10 and are they inhibited by DC-SX029?
2. Fig6J: Casp5 depletion appears to interfere with LPS release into the digitonin fraction, which the authors call 'cytosolic'. If, as proposed by the authors, Casp5 acts as a cytosolic LPS receptor, how would it regulate an upstream process, i.e. entry of LPS into the cytosol?
3. Page8: The authors suggest that SNX10 deficiency might directly block LPS release from early endosomes. Results in Fig5E could also be interpreted as enhanced uptake of LPS in SNX10 def cells. Maybe a pulse chase experiment could clarify the situation?
4. Twice in the manuscript the authors start working on a new entity - PIKfyve (p9) and CD-SX029 (p10). It is entirely unclear how the authors identified CD-SC029 and PIKfyve as entities of interest. Relevant information should be added.
5. The manuscript contains a number of overstatements, which are largely unnecessary and could be avoided if worded more precisely. Examples:
  - i) p5, first paragraph: the authors' conclusion that epithelial cells trigger a pathway different from inflammasome activation and pyroptosis is an overstatement - their cells clearly don't release IL1, but there could be many reasons other than lack of pyroptosis (for example lack of IL1 gene induction despite pyroptosis still be fully functional).
  - ii) p7: The authors claim to have shown that lyn phosphorylation is responsible for OMV-induced downregulation of E-cadherin. Their data are consistent with such scenario but to maintain their claim, lyn would need to be depleted / knocked out.
  - iii) p7: The authors claim that the interaction of Casp5 with SNX10 is essential for OMV induced lyn phosphorylation. While the authors demonstrate an essential role for Casp5 and SNX10 - they do not provide any evidence that the interaction of Casp5 and SNX10 is required.
  - iv) Fig5C, D: effects of SNX10 overexpression are unconvincing, SNX10 complementation is much better. Would consider toning down results of 5c.
  - v) Page8: LPS '...outside early endosomes...' - taken literally that would mean free in the cytosol but LPS could equally well be in a Rab5 negative membrane-surrounded compartment. Would suggest to formulate more precisely.
  - vi) The title should be rephrased: "Liberation and sensing of cytosolic LPS through SNX10..." Liberation of cytosolic LPS makes little sense and no evidence for sensing of LPS through SNX10 is provided at all.
6. Some of the IF images look strange:
  - i) Fig3b: From the description of the experiment I assume the authors detect endogenous lyn. But why is lyn visible in only some cells and why in such a strange pattern? Are these SNX10 induced aggregates?
  - ii) Fig4C: Similar to 3b, the IF looks strange. Is endogenous caspase 5 really a nuclear protein that occurs in distinct dots?

7. Fig3e: The p-lyn blot is an unconvincing example of lyn phosphorylation due to SNX-10 overexpression. The signal looks more like an artificial blob on top of the lyn band.

8. Fig4G: Depletion of Casp5 has strong effects on Snail, Slug, p-lyn and E-cadherin. It would be good to have a negative control other than irrelevant siRNA - ideally Casp4 siRNA. In general, it would be good to have more than one siRNA per gene.

Referee #2:

Bacterial gram-negative outer membrane vesicles are important inducers of inflammation previously shown to activate the cytosolic LPS sensor caspase-4, one of two such sensors present in humans (caspase-5 being the second one). The mechanism by which LPS, derived from endocytosed OMVs, is delivered towards sensing by cytosolic LPS receptors is not well understood. The study by Wang et al. makes the remarkable discovery that the membrane trafficking protein sorting nexin 10 (SNX10) is required for the release of LPS into the host cell cytosol following OMV ingestion by colonic epithelial cells. While this is an important discovery in its own right, the study goes on to demonstrate that OMVs induce a SNX10-dependent signaling cascade requiring the poorly characterized LPS sensor caspase-5 and Lyn kinase. Caspase-5/ Lyn signaling prompts the nuclear translocation of the transcription factors Snail/Slug and consequential downregulation of E-cadherin, an adhesion molecule important for maintaining epithelial barrier functions. Again, this is an exceptionally important discovery which reveals a novel and unique role for caspase-5 as an inducer of a transcriptional response impacting tissue integrity and likely (here I am speculating) increasing accessibility of infection loci for influx of various types of immune cells. The study therefore provides a compelling explanation for the puzzling fact that many human cells express two cytosolic LPS sensor, although caspase-4 had been shown to be essential for noncanonical inflammasome activation. Wang et al. teaches us that caspase-5 engages a distinct caspase-4-independent signaling cascade that likely acts synergistically with caspase-4-mediated responses during infections. However, many inflammatory response can often do more harm than good and are linked to diseases such as ulcerative colitis. Wang et al. show that this is also true for SNX10-mediated inflammation which, they demonstrate, promotes acute and chronic colitis in mouse models of disease. Just to put a cherry on top, the study further demonstrates that a novel SNX10 inhibitor can ameliorate disease in these models. Overall, this is a highly innovative and important study. The data are well analyzed and presented, and the paper is very well written. I have a few very minor comments that the authors may want to consider.

- The MS should provide some information on the approach by which DC-SX029 was identified as a potential SNX10 inhibitor
- The MS should provide details on the production of the SNX10 floxed mice: methodology - e.g. CRISPR + gRNA sequences or homologous recombination; target vector? Which exon is floxed? (maybe provide a map and/or sequence information). Alternatively, if this mouse line has already been published, then please provide a citation
- Lack of IL-1beta secretion does not demonstrate that OMVs don't activate caspase-4 in colonic epithelial cells. Many human epithelial cell lines express little or no caspase-1 and therefore don't process pro-IL1beta. However, in the same cells caspase-4 activation can drive IL-18 secretion in response to infections with gram-negative bacteria. This has been shown in a CACO-1 cell clone (see publication PMID: 25121752 - which is probably worth citing here). If SNX10 promotes LPS accessibility for the cytosolic LPS sensors caspase-5 it is expected to also do the same for caspase-4 (robustly expressed in the cells used in this study - see Fig. 4A). Therefore, the authors maybe want to test whether they observe IL-18 secretion in OMV-treated cells and whether deletion of SNX10 results in diminished IL-18 secretion
- While the pulldown data strongly suggest that caspase-4 plays no role in Lyn signaling, it would further strengthen the MS to demonstrate the caspase-4 KO cells indeed still downregulate E-cadherin when treated with OMVs
- Fig. 3I - is Tolimidone treatment sufficient to downmodulate E-cadherin expression in SNX10 KO cells? Data are only shown for tolimidone treatment in the presence of OMVs
- The colocalization of LPS with Rab5 and the lack of co-localization of LPS with Rab7 and/or Lamp2A is hard to see due to the small size of the images. These data are largely confirmatory of reports by Vanja et al. (2016) and I do not doubt these findings. However, it would be nice to see some enlarged images - maybe some of these data (Rab7, Lamp2a) could be moved into an EV figure to make some space for the enlarged images
- The discussion on the role of IRGs and GBPs in OMV-mediated inflammasome activation would benefit from some editing for accuracy and the inclusion of additional citations: i) there's no evidence that Irgb10 is required for OMV-mediated caspase-4 activation; rather 2 recent papers demonstrated that the immunity related GTPases IRGM2 proteins and Gabarapl2 inhibit caspase-4 (caspase-11 in mouse) activation in response to OMVs (see PMID: 33124769 and PMID: 33124745); this is interesting and worth mentioning considering that the humanIRGM hypomorphic allele is a Crohn's disease risk factor; ii) please, cite the original paper demonstrating the importance of GBPs in caspase-4 (11) activation in response to OMVs - PMID: 28974614

Referee #3:

Intestinal barrier dysfunction induced by alteration of intestinal microbial composition triggers the initiation and recurrence of inflammatory bowel disease (IBD). In this study Wang et al present an interesting model where the internalization of bacterial outer membrane vesicles (OMV) in human intestinal epithelial cells promoted the recruitment of caspase-5 and PIKfyve to

membranes of early endosomes by sorting nexin 10 (SNX10), which subsequently triggered LPS release from OMV into the cytosol for the sensing by caspase-5. The activated caspase-5 resulted in Lyn phosphorylation, which induced the nuclear translocation of Snail/Slug, leading to the downregulation of E-cadherin and intestinal barrier dysfunction. In added significance to this study the authors developed a novel small molecular compound DC-SX029 that blocked SNX10 protein-protein interaction. SNX10 deletion or DC-SX029 treatment could restore OMV-induced intestinal barrier dysfunction by blocking LPS release, sensing and signaling transduction and ameliorate mouse colitis. This is important and well executed study that warrant publication in EMBO journal. However, the model is still unclear and need some further experiment to understand this non-canonical role that caspase-5 play, a model figure would be much appreciated as well.

Major comment:

1. The authors show the role of SNX10 under the condition of OMV internalization. Is this specific phenotype specific to OMV treatment or other vacuolar Gram-negative bacteria infection shows the same effect?
2. It has been reported that GBPs and IRGB10 (expressed in mice only) is essential for LPS release. Does SNX10 affect the expression or recruitment of GBPs and IRGB10? In addition, how does SNX10 can be recruited to OMV?
3. The authors argue that the interaction of SNX10 and caspase-5 is essential for OMV-induced Lyn phosphorylation. However, it's not clear whether the interaction between SNX10 and caspase-5 is important or not. To clarify their opinion, they need to add more evidence such as the reconstitution of mutant SNX which is lack of interaction with caspase-5? Furthermore, it is not clear how caspase-5 works in this context. Does it function as a scaffold? Or does LPS mediated caspase5 activation is necessary for OMV-induced Lyn phosphorylation? (How about to use caspase-5 inhibitor?)
4. Could caspase-5 mediate the interaction between SNX10 and Lyn or PIKfyve and Lyn? Authors need to investigate the role of caspas-5 using caspase-5 knockdown. Also, is the recruitment of caspase-5 dependent on PIKfyve or vice versa? Could knockout, knockdown or treatment of inhibitors for caspase-5 or PIKfyve could block the recruitment of each other?
5. It is pretty curious which protein can phosphorylate Lyn to regulate the expression of E-cadherin in OMV internalization. Like caspase-5, the role of PIKfyve is not clear yet. Could PIKfyve function as a direct kinase for Lyn in this context?
6. The authors showed that targeting SNX10 protects against chronic and acute colitis. They need to show whether SNX10 can regulates the secretion of IL-1b secretion in this condition and whether the regulation of aberrant IL-1b secretion is important for this protection.

Minor point:

Authors present only IL1b data from BMDM, it would be appreciated to show also cytotoxicity data following treatment of BMDM with OMV



Dear Reviewers,

We thank the reviewers for their comments concerning our manuscript entitled “Liberation and sensing of cytosolic LPS through SNX10 mediates gut barrier dysfunction in colitis” (Manuscript Number: EMBOJ-2021-108080). All those comments are valuable and very helpful for improving our manuscript. We have studied these comments carefully and have made correction so that we hope to meet with approval. Revised portions are marked in red in the manuscript. The main corrections in the manuscript and the responses to the reviewers' comments are as follows:

### **Responses to Reviewers:**

#### **Referee #1:**

1. **The reviewer's comment:** Are other cytoplasmic LPS pathways (such as pyroptosis) dependent on SNX10 and are they inhibited by DC-SX029?

**Response:** In our current study, we find that SNX10 deletion or DCSX029 treatment could restore OMV-induced intestinal barrier dysfunction by blocking LPS release, sensing and signaling transduction in intestinal epithelial cells. To explore whether other cytoplasmic LPS pathways (such as pyroptosis) are dependent on SNX10 and they are inhibited by DC-SX029, we detected the effects of SNX10 knockout or DC-SX029 treatment on the cell viability as well as the secretion of IL-1 $\beta$  and IL-18 in BMDMs after OMV (100  $\mu$ g/mL) treatment. We found that either SNX10 knockout or DC-SX029 treatment increased the cell viability and inhibited the secretion of IL-1 $\beta$  and IL-18 in BMDMs (Appendix Fig S1A and B), indicating SNX10 may involve in OMV-induced pyroptosis signaling. We added the results in the revised manuscript (Page 13). The precise role of SNX10 and the detail mechanism in this signaling are being studied in our laboratory now.

2. **The reviewer's comment:** Fig6J: Casp5 depletion appears to interfere with LPS release into the digitonin fraction, which the authors call 'cytosolic'. If, as proposed by the authors, Casp5 acts as a cytosolic LPS receptor, how would it regulate an upstream process, i.e. entry of LPS into the cytosol?

**Response:** In the present study, we found OMV treatment enhanced the interaction and co-localization of caspase-5 and PIKfyve in the Rab5-positive early endosomes, while SNX10 deficiency impaired this co-localization (Fig 6D, E, G and H). Either SNX10 or caspase-5 deficiency could inhibit LPS release into the cytosol (Figs 5B and 6J). These results support that the recruitment of caspase-5 by SNX10 to PIKfyve on the membranes of early endosomes triggered the release of LPS into the cytosol.

3. **The reviewer's comment:** Page8: The authors suggest that SNX10 deficiency might directly block LPS release from early endosomes. Results in Fig5E could also be interpreted as enhanced uptake of LPS in SNX10 def cells. Maybe a pulse chase experiment could clarify the situation?

**Response:** In the present study, the distribution of LPS in the subcellular organelle was visualized by immunostaining with the antibodies against LPS, therefore, it isn't suitable for pulse-chase experiment. We added the detailed method in Materials and Methods of the revised manuscript (Page 21). In order to exclude the possible effect of SNX10 KO on the uptake of LPS, we measured the LPS content in the cell culture supernatants after OMV treatment for the indicated time. As shown in Fig 5G of the revised manuscript, the LPS content in the cell culture supernatants were equally decreased in both WT and SNX10 KO Caco-2 cells, and no significant difference was found between WT and SNX10 KO Caco-2 cells. This result indicates that SNX10 KO did not affect the uptake of LPS.

4. **The reviewer's comment:** Twice in the manuscript the authors start working on a new entity - PIKfyve (p9) and DC-SX029 (p10). It is entirely unclear how the authors identified DC-SC029 and PIKfyve as entities of interest. Relevant information should be added.

**Response:** We have added the relevant information of PIKfyve and DC-SX029 in Page 9 and Page 11 in the revised manuscript respectively. PIKfyve was involved in the endosome maturation (PMID: 21878991) and the cargo exit from early endosomes (PMID: 16954148). To explore the mechanism that SNX10 deficiency inhibited LPS release of OMVs from early endosomes, we screened the interaction proteins of SNX10 and found PIKfyve might involve in this process.



As described in our latest publication (PMID: 34010669), we identified a novel small molecule DC-SX029 as a potential SNX10 inhibitor. Here we use DC-SX029 to confirm the effects of SNX10 KO on intestinal epithelial barrier function as well as the mechanisms.

**5. The reviewer's comment:** The manuscript contains a number of overstatements, which are largely unnecessary and could be avoided if worded more precisely. Examples:

i) p5, first paragraph: the authors' conclusion that epithelial cells trigger a pathway different from inflammasome activation and pyroptosis is an overstatement - their cells clearly don't release IL1, but there could be many reasons other than lack of pyroptosis (for example lack of IL1 gene induction despite pyroptosis still be fully functional).

**Response:** We agree to the reviewer's opinion. Human epithelial cells express little or no caspase-1 and therefore don't process pro-IL1beta. However, caspase-4 activation can drive IL-18 secretion in response to infections with gram-negative bacteria. As shown in the revised Fig EV1F and Appendix Fig S1A, OMV (100 µg/mL) treatment induced IL-18 secretion and reduced the cell viability in BMDMs, however only a slight increase of IL-18 secretion was detected in Caco-2, HT-29 and NCM460 cells. Together with the results that OMV (100 µg/mL) treatment could not significantly induced cell death in these intestinal epithelial cells (Fig EV1A-D), we speculate OMV (100 µg/mL) treatment triggers a pathway different from inflammasome activation and pyroptosis. We revised the sentence to avoid overstatement in Page 5.

ii) p7: The authors claim to have shown that lyn phosphorylation is responsible for OMV-induced downregulation of E-cadherin. Their data are consistent with such scenario but to maintain their claim, lyn would need to be depleted / knocked out.

**Response:** In our original manuscript, we found that SNX10 deficiency maintained the expression of E-cadherin through inhibiting OMV-induced Lyn phosphorylation (Fig 3C-H). Thus, Tolimidone, a Lyn agonist, was used to increase Lyn phosphorylation in order to block the effect of SNX10 deficiency on OMV-induced downregulation of E-cadherin. As shown in the revised Fig 3I, Tolimidone could mimic OMV-induced Lyn phosphorylation and downregulation of E-cadherin in WT cells. While in SNX10 KO cells, Tolimidone could

partially recover the decreased phosphorylation of Lyn and increased E-cadherin caused by SNX10 KO. We revised the sentence to avoid overstatement in Page 7.

iii) p7: The authors claim that the interaction of Casp5 with SNX10 is essential for OMV induced lyn phosphorylation. While the authors demonstrate an essential role for Casp5 and SNX10 - they do not provide any evidence that the interaction of Casp5 and SNX10 is required.

**Response:** In our original manuscript, caspase-5 rather than caspase-4 was pulled down by Flag-tagged SNX10 (Fig 4B). Co-localization of SNX10-Flag and caspase-5 was further confirmed (Fig 4C). Impairment of the interaction between caspase-5 and SNX10 by SNX10 deficiency abolished the interaction and co-localization of caspase-5 and Lyn induced by OMVs (Fig 4D-F). *CASP5* siRNA could also inhibit OMV-induced Lyn phosphorylation (revised Fig 4G). To investigate which region is responsible for the interaction of caspase-5 and SNX10, deletion mutagenesis was done as described in our previous study (PMID: 31208298). As shown in new Fig 4H and 4I of the revised manuscript, deletion of PX domain, but not C or N terminal region of SNX10 abolished the interaction of caspase-5 with SNX10 and inhibited Lyn phosphorylation induced by OMVs. These results support that the interaction of caspase-5 and SNX10 is essential for OMV-induced Lyn phosphorylation.

iv) Fig5C, D: effects of SNX10 overexpression are unconvincing, SNX10 complementation is much better. Would consider toning down results of 5c.

**Response:** We appreciate the helpful suggestion and revised the statement of the results of Fig 5C and D in Page 8.

v) Page8: LPS '...outside early endosomes...' - taken literally that would mean free in the cytosol but LPS could equally well be in a Rab5 negative membrane-surrounded compartment. Would suggest to formulate more precisely.

**Response:** We revised the sentence according to the reviewer's suggestion in Page 8.

vi) The title should be rephrased: "Liberation and sensing of cytosolic LPS through SNX10..." Liberation of cytosolic LPS makes little sense and no evidence for sensing of LPS through SNX10 is provided at all.

**Response:** We revised the title of the manuscript to be more meaningful according to the reviewer's suggestion.

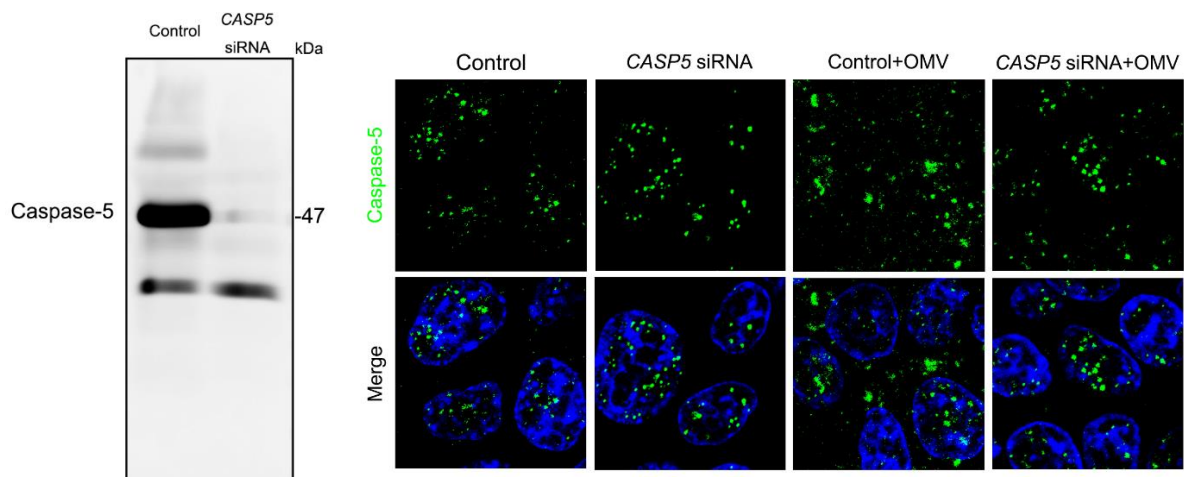
6. **The reviewer's comment:** Some of the IF images look strange:

i) Fig3b: From the description of the experiment I assume the authors detect endogenous lyn. But why is lyn visible in only some cells and why in such a strange pattern? Are these SNX10 induced aggregates?

**Response:** Lyn is a member of Src family tyrosine kinases that are classified as cytosolic enzymes, and can bind to membrane upon phosphorylation and be visible by immunostaining. In Fig 3B, Caco-2 cells were transfected with SNX10-Flag plasmids, and then stained with antibodies against Flag and Lyn. Only a part of cells expressed SNX10-Flag. In these cells, more Lyn might be recruited to the organelle membrane by SNX10-Flag, and thus an increased staining of Lyn particles which was co-localized with SNX10-Flag was observed.

ii) Fig4C: Similar to 3b, the IF looks strange. Is endogenous caspase 5 really a nuclear protein that occurs in distinct dots?

**Response:** We speculate that the nuclear staining may result from the nonspecific binding of the caspase-5 antibody. To confirm it, *CASP5* siRNA followed by Western blotting and immunostaining was used to analyze the specificity of the caspase-5 antibody. As shown in the following figures, a distinct band below the predicted band (47 kDa) of caspase-5 could be detected by the caspase-5 antibody in *CASP5* knockdown Caco-2 cell lysates. Furthermore, OMVs could induce the cytoplasmic staining rather than the nuclear staining of the caspase-5 antibody, and the cytoplasmic staining rather than the nuclear staining was abolished by caspase-5 knockdown. These data support the nuclear staining was caused by the nonspecific binding of the caspase-5 antibody.



7. **The reviewer's comment:** Fig3e: The p-lyn blot is an unconvincing example of lyn phosphorylation due to SNX-10 overexpression. The signal looks more like an artificial blob on top of the lyn band.

**Response:** We repeated the experiments, and the p-Lyn and Lyn blots were replaced in revised Fig 3E and quantified again by ImageJ software (revised Fig 3F).

8. **The reviewer's comment:** Fig4G: Depletion of Casp5 has strong effects on Snail, Slug, p-lyn and E-cadherin. It would be good to have a negative control other than irrelevant siRNA - ideally Casp4 siRNA. In general, it would be good to have more than one siRNA per gene.

**Response:** According to the reviewer's suggestion, *CASP4* siRNA was used as a negative control, and two siRNAs were used to silence per gene. As shown in the revised Fig 4G, interference of caspase-5 rather than caspase-4 could inhibit OMV-induced Lyn phosphorylation, Snail/Slug nuclear localization and E-cadherin reduction.

**Referee #2:**

1. **The reviewer's comment:** The MS should provide some information on the approach by which DC-SX029 was identified as a potential SNX10 inhibitor

**Response:** More information regarding the approach by which DC-SX029 was identified as a potential SNX10 inhibitor can be found in our latest publication (PMID: 34010669). We have added the relevant information in Page 11 in the revised manuscript.

2. **The reviewer's comment:** The MS should provide details on the production of the SNX10 floxed mice: methodology - e.g. CRSISPR + gRNA sequences or homologous recombination; target vector? Which exon is floxed? (maybe provide a map and/or sequence information). Alternatively, if this mouse line has already been published, then please provide a citation

**Response:** *Snx10* floxed mice and *Vill-cre* mice were purchased from Shanghai Research Center for Model Organisms (Shanghai, China). Mice containing the *Snx10*-flox (flanked by loxP) gene were established by inserting a homozygous loxP fragment into exon 4 and exon 5 of the mouse *Snx10* gene (Appendix Fig S5A), and then crossed them with *Vill-cre* mice to obtain *Snx10*-flox homozygous (*Snx10<sup>fl/fl</sup>*) *Vill-cre* positive mice, in which the intestinal epithelium-specific Villin1 (*Vil1*) promoter can control Cre enzyme expression restricted to the intestinal epithelium, resulting in the specific knockout of the *Snx10* gene in the intestinal epithelium.

The *Snx10* gene was modified by flox using homologous recombination in fertilized eggs, and the specific strategy is shown in Appendix Fig S5A. The process was as follows: Cas9 mRNA and gRNA were obtained by in vitro transcription; the homologous recombination vector (donor vector) was constructed by In-Fusion cloning. Cas9 mRNA, gRNA and homologous recombinant vector (shown in Appendix Fig S5B) were microinjected into the fertilized eggs of C57BL/6J mice, and F0 generation mice were obtained by microinjection of fertilized eggs. The genotypes were identified by long fragment PCR and the PCR products were sequenced. F1 generation mice (*Snx10<sup>fl/+</sup>*) were obtained by crossing F0 generation mice with wild-type C57BL/6J mice.

The target gene *Snx10* sequence was obtained from Mouse Genome Informatics (MGI) library, number 1919232; the transcript targeted by the protocol is *Snx10*-001, Ensembl number ENSMUST00000049152; loxP is inserted at both ends of exons 4 and 5.

Intron3 target sequence: ggagggccatgccaccagggctaactgctcattcctgctgcatggcttcttct

The guide RNA (gRNA) target sequences:

Guide #1 GAATGAGCAGTGTTAGCCCT GGG

Guide #2 GAGCAGTGTTAGCCCTGGGT GGG

Intron5 target sequence: gtctcttaagagcacagtagatacagcggcagcatacacatctgaggcaggag

The guide RNA (gRNA) target sequences:

Guide #3 CCTGCCTCAGATGTGTATGC TGG

Guide #4 AGCGCCAGCATAACACATCTG AGG

*Vill-cre* mice can specifically knockout/recombine the target gene contained in the flox fragment by *Vill* promoter-driven expression of Cre recombinase in epithelial cells of the small and large intestine. Crosses between the two mice yielded the intestinal epithelial cell *Snx10* gene conditional knockout mice *Vill-cre*<sup>+</sup>*Snx10*<sup>fl/fl</sup> (SNX10 cKO), and negative control mice *Vill-cre*<sup>-</sup>*Snx10*<sup>fl/fl</sup> (WT).

These details on the production of the *Snx10* floxed mice were added in the Appendix file.

**3. The reviewer's comment:** Lack of IL-1beta secretion does not demonstrate that OMVs don't activate caspase-4 in colonic epithelial cells. Many human epithelial cell lines express little or no caspase-1 and therefore don't process pro-IL1beta. However, in the same cells caspase-4 activation can drive IL-18 secretion in response to infections with gram-negative bacteria . This has been shown in a CACO-1 cell clone (see publication PMID: 25121752 - which is probably worth citing here). If SNX10 promotes LPS accessibility for the cytosolic LPS sensors caspase-5 it is expected to also do the same for caspase-4 (robustly expressed in the cells used in this study - see Fig. 4A). Therefore, the authors maybe want to test whether they observe IL-18 secretion in OMV-treated cells and whether deletion of SNX10 results in diminished IL-18 secretion

**Response:** According to the reviewer's suggestion, the effect of SNX10 deficiency on IL-18 secretion in OMV-treated Caco-2 cells was explored. The results showed that OMV (100 µg/mL) treatment only induced a slight increase of IL-18 secretion in Caco-2 cells compared with that in BMDMs (revised Fig EV1F). The OMV-induced IL-18 secretion in intestinal epithelial cells was in consistent with the results of the previous study (PMID: 25121752), and we also found that SNX10 deficiency prominently inhibited OMV-induced IL-18 secretion in Caco-2 cells (Appendix Fig S2). However, OMV (100 µg/mL) treatment had no effect on the cell viability of Caco-2 cells (Fig EV1B), indicating the lower level of IL-18 couldn't induce cell pyroptosis of Caco-2 cells. The results were added and the publication (PMID: 25121752) was cited in the revised manuscript (Page 5).

4. **The reviewer's comment:** While the pulldown data strongly suggest that caspase-4 plays no role in Lyn signaling, it would further strengthen the MS to demonstrate the caspase-4 KO cells indeed still downregulate E-cadherin when treated with OMVs

**Response:** According to the reviewer's suggestion, *CASP4* siRNA was used to confirm our results. As shown in the revised Fig 4G, caspase-4 knockdown had no effect on OMV-induced Lyn phosphorylation, Snail/Slug nuclear localization and E-cadherin reduction.

5. **The reviewer's comment:** Fig. 3I - is Tolimidone treatment sufficient to downmodulate E-cadherin expression in SNX10 KO cells? Data are only shown for tolimidone treatment in the presence of OMVs

**Response:** According to the reviewer's suggestion, the effect of Tolimidone on E-cadherin expression in SNX10 KO cells was investigated. As shown in the revised Fig 3I, in the absence of OMVs, Tolimidone treatment could induce Lyn phosphorylation, Snail/Slug nuclear localization, and E-cadherin downregulation in SNX10 KO Caco-2 cells.

6. **The reviewer's comment:** The colocalization of LPS with Rab5 and the lack of co-localization of LPS with Rab7 and/or Lamp2A is hard to see due to the small size of the images. These data are largely confirmatory of reports by Vanja et al. (2016) and I do not doubt these findings. However, it would be nice to see some enlarged images - maybe some of these data (Rab7, Lamp2a) could be moved into an EV figure to make some space for the enlarged images

**Response:** Thanks for the suggestion! The lack of co-localization of LPS with Rab7 or LAMP2A was moved to the Appendix file (see Appendix Fig S3A and B). The colocalization of LPS with Rab5 was enlarged in revised Fig 5E.

7. **The reviewer's comment:** The discussion on the role of IRGs and GBPs in OMV-mediated inflammasome activation would benefit from some editing for accuracy and the inclusion of additional citations: i) there's no evidence that Irgb10 is required for

OMV-mediated caspase-4 activation; rather 2 recent papers demonstrated that the immunity related GTPases IRGM2 proteins and GabarapL2 inhibit caspase-4 (caspase-11 in mouse) activation in response to OMVs (see PMID: 33124769 and PMID: 33124745); this is interesting and worth mentioning considering that the human IRGM hypomorphic allele is a Crohn's disease risk factor; ii) please, cite the original paper demonstrating the importance of GBPs in caspase-4 (11) activation in response to OMVs - PMID: 28974614

**Response:** We thank the reviewer for the constructive suggestions! We have revised the discussion and cited the publications mentioned above (PMID: 33124769; PMID: 33124745; PMID: 28974614) in the revised manuscript (Page 14).

**Referee #3:**

This is important and well executed study that warrant publication in EMBO journal. However, the model is still unclear and need some further experiment to understand this non-canonical role that caspase-5 play, a model figure would be much appreciated as well.

**Response:** Thanks for the excellent comment! A model figure was added in new Fig EV5E, and the figure legend was added in the revised manuscript (Page 42).

Major comment:

1. **The reviewer's comment:** The authors show the role of SNX10 under the condition of OMV internalization. Is this specific phenotype specific to OMV treatment or other vacuolar Gram-negative bacteria infection shows the same effect?

**Response:** OMVs produced by Gram-negative bacteria were internalized via endocytosis, and released LPS into the cytosol from early endosomes (PMID: 27156449). Consistently, our present study showed that SNX10 played an essential role in the LPS release from OMVs (derived from E. coli BL21) into the cytosol in the early endosome, however it had no effect on OMV internalization (Fig 5G). The other vacuolar Gram-negative bacteria infection such as Enterohemorrhagic E. coli infection has been reported to share the same mechanism as OMV treatment (PMID: 27156449). Therefore, we believe SNX10 has the same effect on the other vacuolar Gram-negative bacteria infection.



**2. The reviewer's comment:** It has been reported that GBPs and IRGB10 (expressed in mice only) is essential for LPS release. Does SNX10 affect the expression or recruitment of GBPs and IRGB10? In addition, how does SNX10 can be recruited to OMV?

**Response:** According to the reviewer's suggestion, the mRNA expression of GBPs (such as GBP2 and GBP5) and IRGB10 in the colonic epithelium of WT and SNX10 cKO mice was detected. The result showed that SNX10 deficiency had no effect on the expression of GBP2, GBP5 and IRGB10 at the transcriptional level (Appendix Fig S6). Our future study will investigate whether SNX10 involves in the recruitment of GBPs and IRGB10.

SNX10 belongs to the sorting nexin family that contains conserved PX-domain through which SNX10 binds to PtdIns-3-P localized in early endosomes and be recruited to the membranes of early endosomes (PMID: 22193161; PMID: 22168438; PMID: 31692073). In our present study, OMV treatment increased the interaction of SNX10 and PIKfyve that mainly located to the early endosomes (Fig 6C and 6G), playing an essential role in the LPS release from OMVs into the cytosol.

**3. The reviewer's comment:** The authors argue that the interaction of SNX10 and caspase-5 is essential for OMV-induced Lyn phosphorylation. However, it's not clear whether the interaction between SNX10 and caspase-5 is important or not. To clarify their opinion, they need to add more evidence such as the reconstitution of mutant SNX which is lack of interaction with caspase-5? Furthermore, it is not clear how caspase-5 works in this context. Does it function as a scaffold? Or does LPS mediated caspase5 activation is necessary for OMV-induced Lyn phosphorylation? (How about to use caspase-5 inhibitor?)

**Response:** According to the reviewer's suggestion, we further confirmed the role of the interaction of SNX10 and caspase-5 in OMV-induced Lyn phosphorylation. To investigate which region of SNX10 is responsible for its interaction with caspase-5, deletion mutagenesis was done as described in our previous study (PMID: 31208298). As shown in revised Fig 4H, deletion of PX domain, but not C or N terminal region of SNX10 abolished the interaction of SNX10 and caspase-5. Furthermore, overexpression of SNX10 rather than PX domain-deleted SNX10 facilitated Lyn phosphorylation induced by OMVs (revised Fig 4I). These results further confirmed the essential role of the interaction of SNX10 and caspase-5

in OMV-induced Lyn phosphorylation. In our present study, internalization of OMVs in human intestinal epithelial cells promoted the recruitment of caspase-5 and PIKfyve to the membranes of early endosomes by SNX10, which subsequently triggered LPS release from OMVs into the cytosol for the sensing by caspase-5. The activated caspase-5 resulted in Lyn phosphorylation, which induced the nuclear trans-localization of Snail/Slug, leading to the downregulation of E-cadherin and the disruption of intestinal epithelial barrier function. As shown in revised Fig 4G, interference of caspase-5 rather than caspase-4 could inhibit OMV-induced Lyn phosphorylation, indicating that LPS mediated caspase5 activation is necessary for OMV-induced Lyn phosphorylation.

**4. The reviewer's comment:** Could caspase-5 mediate the interaction between SNX10 and Lyn or PIKfyve and Lyn? Authors need to investigate the role of caspas-5 using caspase-5 knockdown. Also, is the recruitment of caspase-5 dependent on PIKfyve or vice versa? Could knockout, knockdown or treatment of inhibitors for caspase-5 or PIKfyve could block the recruitment of each other?

**Response:** In our present study, SNX10 acted as an adaptor protein to assemble a complex containing Lyn, PIKfyve and caspase-5 for LPS release, sensing and signaling transduction. SNX10 KO impaired the formation of this complex (Fig 6C). Caspase-5 interference had no effect on OMV-induced interaction between SNX10 and Lyn or PIKfyve and Lyn (Appendix Fig S4A). In order to investigate whether the recruitment of caspase-5 is dependent on PIKfyve or vice versa, additional experiments has been conducted. We found that PIKfyve knockdown had no effect on OMV-induced recruitment of caspase-5 by SNX10-Flag (Appendix Fig S4B). Similarly, caspase-5 knockdown didn't affect OMV-induced recruitment of PIKfyve by SNX10-Flag (Appendix Fig S4C). These results were added in Page 9 in the revised manuscript.

**5. The reviewer's comment:** It is pretty curious which protein can phosphorylate Lyn to regulate the expression of E-cadherin in OMV internalization. Like caspase-5, the role of PIKfyve is not clear yet. Could PIKfyve function as a direct kinase for Lyn in this context?

**Response:** As reported previously, Lyn is a member of the Src family of protein tyrosine kinases, and the Tyr396 site can easily undergo autophosphorylation (PMID: 9477973; PMID: 7685656). Lyn autophosphorylation is associated with an increase in its kinase activity (PMID: 8530369). In our present study, SNX10 deficiency, Apilimod (a PIKfyve inhibitor) treatment or caspase-5 knockdown could block the release of LPS into the cytosol (Figs 5B and 6I and J) and inhibit OMV-induced Lyn phosphorylation (Figs 3C, 4G and 6F). Thus, we speculate that caspase-5 activation by sensing cytosolic LPS promotes Lyn autophosphorylation.

**6. The reviewer's comment:** The authors showed that targeting SNX10 protects against chronic and acute colitis. They need to show whether SNX10 can regulate the secretion of IL-1 $\beta$  secretion in this condition and whether the regulation of aberrant IL-1 $\beta$  secretion is important for this protection.

**Response:** In consistent with previous report (PMID: 25121752), OMV-induced IL-1 $\beta$  secretion could not be detected in Caco-2, HT-29 or NCM460 cells (Fig EV1E). However, at the animal level, intestinal epithelium-specific knockout of SNX10 (SNX10 cKO) or DC-SX029 (the SNX10 inhibitor) treatment could reduce the serum levels of proinflammatory factors including IL-1 $\beta$  in mice with IL-10 deficiency-induced chronic colitis or DSS-induced acute colitis (Appendix Fig S7A and 7B) and protect the intestinal barrier function in mice with colitis.

Minor point:

**1 The reviewer's comment:** Authors present only IL1 $\beta$  data from BMDM, it would be appreciated to show also cytotoxicity data following treatment of BMDM with OMV

**Response:** We measured the cell viability of BMDMs treated with OMVs according to the reviewer's suggestion, and found that OMVs induced significant cell death in BMDMs. SNX10 knockout or DC-SX029 treatment inhibited OMV-induced cell death in BMDMs (Appendix Fig S1A). The precise role of SNX10 and the detail mechanism in this effect are being studied in our laboratory now.

Dear Prof. Shen,

Thank you for submitting your revised manuscript to The EMBO Journal. Your manuscript has until now been handled by my colleague Elisabetta Argenzio, but as Elisabetta no longer is working for The EMBO Journal, I have stepped in as 2nd editor of your manuscript.

Your manuscript has now been re-reviewed by the three referees and their comments are provided below. As you can see, the referees appreciate the introduced changes and support publication here. They have some remaining comments that I would like to ask you to address in a final version. I like the title suggested by referee #2.

When you submit the revised version will you also make sure to add ORCID ID for Zhou & W Shen.

Also, you have uploaded source data for the different figures. However, in many cases the blots or figure panels shown in the source data figures are the same as shown in the main figures. For the source data we need the full blots.

You can use the link below to upload the revised version.

Let me know if you have any further questions.

With best wishes

Karin

Karin Dumstrei, PhD  
Senior Editor  
The EMBO Journal

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 19th Dec 2021.

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

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Referee #1:

The authors have addressed my comments with new experimental data and insightful discussions. I am therefore happy to recommend their manuscript for publication.

(I appreciate that the authors changed their title in response to my comment. I am not sure, however, that the new title is any better than the old one.)

Referee #2:

The authors have expertly addressed all of my concerns and added substantial new data and information to the revised manuscript. The additional data further support the conclusions of the paper. Importantly, new data presented here solidifies the proposed model, in which LPS-induced caspase-5 activation promotes Lyn signaling. Furthermore, new data incorporated during revisions demonstrates that SNX10 functions upstream of both cytosolic LPS sensors, i.e. caspase-4 and caspase-5, but downstream of Rab5. Overall, I consider this a landmark study in the field. It characterizes SNX10 as a critical regulator of intracellular LPS processing leading to immune activation and inflammation in disease models. Moreover, this study has begun to unravel the enigma of the existence of two distinct cytosolic LPS sensors. Whereas caspase-4 (caspase-11 in rodents) has been extensively studied, the function of caspase-5 has remained a mystery and its existence has typically been chalked up to biological redundancy. Wang et al. demonstrate that caspase-5 is not redundant to caspase-4 but rather fulfills a unique role as a regulator of the Lyn/ slug-snail/ E-cadherin pathway in response to cytosolic LPS. I am certain that this work and all the new questions it raises will be well received in the field and spark many new avenues of research.

I still have some minor suggestions for editing to further improve what is already an outstanding manuscript:

- The title of the manuscript is appropriate. However, I would advise to change the title so that it messages the discovery of a functional role for caspase-5 in immune signaling. One of the most exciting aspects of this study is that it reveals (to my knowledge for the first time) signaling events occurring downstream of Caspase-5 (other than some studies suggesting it may also be a (minor) inducer of pyroptosis functioning in manners similar to caspase-4). I believe a published manuscript would receive more attention, if this important aspect of the paper is also highlighted in the title of this study. E.g. "SNX10-dependent LPS sensing by cytosolic caspase-5 and resulting Lyn signaling causes gut barrier dysfunction"

- Typos / grammar/ etc.: page 13 "may involve" change to "may be involved" or "is involved"; page 7 change "was resulted" to "resulted"; top of page 9 change "wasn't changed as time went on" to "remained unchanged"; page 10 change "didn't" (colloquial) to "did not" or "failed to"; page 9 - the new sentence at the bottom of page 9 ("To explore the mechanism that SNX10 deficiency inhibited LPS release of OMVs from early endosomes, we screened the interaction proteins of SNX10 and found PIKfyve might involve in this process.") needs to be edited for language

- Please, provide vendor and cat number for DC-SX029. I would expect that there will be broad interest in using this small molecule inhibitor by the research community

- The authors only refer to Appendix Fig S6 in the Discussion and not in the Results section.

- I would like to ask the authors to remove the citation of the review article by Rathinam et al. from this sentence in the Discussion: "It was reported guanylate binding proteins (GBPs) [are] involved in governing LPS access to the cytosol (Rathinam et al, 2019) and were essential for caspase-11 (caspase-4 in human) activation in response to OMVs (Finethy et al, 2017)" and instead cite the appropriate research articles making the original discovery: Meunier et al. (PMID: 24739961) and Pilla et al. (PMID: 24715728)

Referee #3:

The author has satisfied my concerns.

Point-by-point response #2

### **Responses to Reviewers:**

#### **Referee #1:**

**The reviewer's comment:** The authors have addressed my comments with new experimental data and insightful discussions. I am therefore happy to recommend their manuscript for publication. (I appreciate that the authors changed their title in response to my comment. I am not sure, however, that the new title is any better than the old one.)

**Response:** We thank the reviewer for the positive comment! We have now changed the title again to make it more meaningful. The revised title was "SNX10-dependent LPS sensing by caspase-5 and resulting Lyn signaling causes gut barrier dysfunction".

#### **Referee #2:**

1. **The reviewer's comment:** The title of the manuscript is appropriate. However, I would advise to change the title so that it messages the discovery of a functional role for caspase-5 in immune signaling. One of the most exciting aspects of this study is that it reveals (to my knowledge for the first time) signaling events occurring downstream of Caspase-5 (other than some studies suggesting it may also be a (minor) inducer of pyroptosis functioning in manners similar to caspase-4). I believe a published manuscript would receive more attention, if this important aspect of the paper is also highlighted in the title of this study. E.g. "SNX10-dependent LPS sensing by cytosolic caspase-5 and resulting Lyn signaling causes gut barrier dysfunction"

**Response:** Thanks for the great comment! According to the reviewer's suggestion, we have now changed the title as "SNX10-dependent LPS sensing by caspase-5 and resulting Lyn signaling causes gut barrier dysfunction".

2. **The reviewer's comment:** Typos / grammar/ etc.: page 13 "may involve" change to "may be involved" or "is involved"; page 7 change "was resulted" to "resulted"; top of page 9 change "wasn't changed as time went on" to "remained unchanged"; page 10 change "didn't" (colloquial) to "did not" or "failed to"; page 9 - the new sentence at the bottom of page 9 ("To explore the mechanism that SNX10 deficiency inhibited LPS release of OMVs from early

endosomes, we screened the interaction proteins of SNX10 and found PIKfyve might involve in this process.") needs to be edited for language

**Response:** We thank the reviewer for the careful reading and helpful comments. We have corrected the spelling and grammatical errors mentioned above by the reviewer. Revised portions are marked in red in the manuscript.

3. **The reviewer's comment:** Please, provide vendor and cat number for DC-SX029. I would expect that there will be broad interest in using this small molecule inhibitor by the research community

**Response:** The novel small molecule DC-SX029 was synthesized in our cooperative laboratory (Drug Discovery and Design Center, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences).

4. **The reviewer's comment:** The authors only refer to Appendix Fig S6 in the Discussion and not in the Results section.

**Response:** We have now referred to Appendix Fig S6 in the Results section in Page 10 of the revised manuscript. Revised portions are marked in red.

5. **The reviewer's comment:** I would like to ask the authors to remove the citation of the review article by Rathinam et al. from this sentence in the Discussion: "It was reported guanylate binding proteins (GBPs) [are] involved in governing LPS access to the cytosol (Rathinam et al, 2019) and were essential for caspase-11 (caspase-4 in human) activation in response to OMVs (Finethy et al, 2017)" and instead cite the appropriate research articles making the original discovery: Meunier et al. (PMID: 24739961) and Pilla et al. (PMID: 24715728)

**Response:** According to the reviewer's suggestion, we have revised the corresponding citations in the Discussion.

**Referee #3:**

**The reviewer's comment:** The author has satisfied my concerns.

**Response:** We thank the reviewer for the supportive comment!

Dear Prof. Shen,

Thank you for submitting your revised manuscript. I have now had a chance to look at the revised version and all looks good. I am therefore very pleased to accept the manuscript for publication in The EMBO Journal.

Yours sincerely,

Karin Dumstrei, PhD  
Senior Editor  
The EMBO Journal

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Corresponding Author Name: Xiaoyan Shen

Journal Submitted to: The EMBO journal

Manuscript Number: EMBOJ-2021-108080R

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

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

#### A- Figures

##### 1. Data

The data shown in figures should satisfy the following conditions:

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

##### 2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple  $\chi^2$  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?	All of our experiments have been conducted in at least three biological replicates. The information is described at the corresponding figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample size for mouse experiments was chosen based on pilot studies of the same type of experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	Mice of specific genotypes were randomly allocated to control/treatment.
For animal studies, include a statement about randomization even if no randomization was used.	In order to eliminate subjective bias, animals were randomly assigned to groups.
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.	All results were assessed blindly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	All results were assessed blindly.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, normal distribution was evaluated using GraphPad Prism (version 9).
Is there an estimate of variation within each group of data?	Yes, variation within each group of data was reported in each group of data as SD.

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Is the variance similar between the groups that are being statistically compared?	Yes.
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### 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 ( <a href="#">see link list at top right</a> ), 1DegreeBio ( <a href="#">see link list at top right</a> ).	Catalog numbers of the antibodies have been provided in Materials and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines used in this study were obtained from indicated sources. Cells were routinely checked and determined as negative for mycoplasma contamination.

\* 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.	We have provided the information for mice used in Materials and Methods.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal handling procedures and protocols were approved by the ethics committee for experimental research, Shanghai Medical College, Fudan University.
10. We recommend consulting the ARRIVE guidelines ( <a href="#">see link list at top right</a> ) (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 ( <a href="#">see link list at top right</a> ) and MRC ( <a href="#">see link list at top right</a> ) recommendations. Please confirm compliance.	NA

### E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
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 ( <a href="#">see link list at top right</a> ) and submit the CONSORT checklist ( <a href="#">see link list at top right</a> ) 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 ( <a href="#">see link list at top right</a> ). 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 ( <a href="#">see link list at top right</a> ) or Figshare ( <a href="#">see link list at top right</a> ).	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 ( <a href="#">see link list at top right</a> ) or EGA ( <a href="#">see link list at top right</a> ).	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 ( <a href="#">see link list at top right</a> ) and deposit their model in a public database such as Biomodels ( <a href="#">see link list at top right</a> ) or JWS Online ( <a href="#">see link list at top right</a> ). 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 ( <a href="#">see link list at top right</a> ) and list of select agents and toxins (APHIS/CDC ( <a href="#">see link list at top right</a> )). According to our biosecurity guidelines, provide a statement only if it could.	NA
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