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The phagosomal solute transporter SLC15A4 favors inflammasome activity via mTORC1 signaling and autophagy restraint in dendritic cells

Cynthia Lopez-Haber, Daniel Netting, Zachary Hutchins, Xianghui Ma, Kathryn Hamilton, and Adriana Mantegazza
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Dear Adriana,

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

As you can see the referees #1 and 2 find the analysis interesting while referee #3 is more hesitant if the insights provided are sufficient to consider publication here. Having looked at the MS and referee reports I do find the analysis interesting, and I would like to invite a revised manuscript should you be able to extend the analysis along the lines suggested by the referees.

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

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

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 discuss your revisions further with you.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

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

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (20th Jul 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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

Referee #1:

In this study by Mantegazza et al, the authors report the interesting finding that an SLC transporter regulates inflammasome activities in dendritic cells. This conclusion is supported by complementary in vitro and in vivo datasets. While there are several questions of underlying mechanisms that can be offered, the conclusion that SLC15A4 regulates inflammasome activities via mTORC1 is supported by the data provided. Future studies are probably necessary in order to understand how SLC15A4 is controlled, in terms of localization and activity, in dendritic cells.

Referee #2:

Review of the study entitled "SLC15A4 favors inflammasome function via mTORC1 signaling and autophagy restraint in dendritic cells" from Lopez-Haber et al.,

Here the authors study the role of the SLC15A4 carrier transporter in murine dendritic cells as a controller of optimal inflammasome response.
Specifically the authors could demonstrate that:

- SLC15A4 is recruited to dendritic cell phagosomes
- SLC15A4 slightly contributes to inflammasome response upon Salmonella infection as well as sterile inflammasome activator exposure (MSU, Alum).
- Dendritic cells from feeble mice show decreased release of IL1beta in models of DSS-driven inflammation
- SLC15A4-controled Autophagy fluxes participate in inflammasome regulation
- SLC15A4 is important for efficient inflammasome positioning away from autophagic membranes

This is an interesting study that addresses the molecular and cellular links between autophagy and inflammasome activation through the prism of the SLC15A4 solute carrier. Two missing points rely on a/ the critical mechanistic mechanisms by which all those processes articulate between each other and 2/ whether the author' findings can be universally applicable to inflammasome studies or are specific of certain inflammasome and/or type of ligand delivery.
Below, there are some suggestions in order to address those missing points:

Major points:
- SLC15A4 regulates Salmonella-induced NLRC4 and Alum/MSU-induced NLRP3 inflammasome response. Could the authors also determine if the murine AIM2 (DNA) inflammasome, the PYRIN inflammasome (TcdB toxin or other) or the non-canonical Caspase-11 inflammasome (LPS sensing) are also regulated similarlyhttps://www.sciencedirect.com/science/article/pii/S0165247821000523?
- Similarly, if SLC15A4 regulates phagocytosis-dependent inflammasome positioning and activation, could the authors test WT and SLC15A4 -/- dendritic cells for inflammasome response to inflammasome ligand electroporation (direct introduction of ligand into the host cell cytosol e.g. Flagellin electroporation, LPS electroporation, DNA electroporation?)
- Are dendritic cells dying of inflammasome stimulation? Or is there a similar mechanism of Gasdermin D-driven IL1beta secretion but not cell shrinkage as previously observed by Kagan' group (and others) https://www.cell.com/cell-reports/pdf/S2211-1247(20)31370-X.pdf? Indeed, dendritic cells are extremely important for antigen presentation, hence a guess would be that if they do express inflammasome signaling, there might be some protective mechanisms to avoid cell death and further antigenic presentation to adaptive immune cells?
- SLC15A4 is also expressed in macrophages. Do SLC15A4-/- macrophages respond similarly than dendritic cells to inflammasome stimulation (at least to Salmonella or MSU or Alum?)
- What about the response of human dendritic cells in which SLC15A4 has been invalidated or targeted (siRNA?).

Minor:
It would be intersting that the authors discuss the recent concepts of dendritic cell hyperactivation in their discussion part as when it is about dendritic cells, there is still some intensive discussions about their ability of going into pyroptosis or not and the purpose of such skill, given their important function as antigen-presenting cells.

Referee #3:

In this study, Haber et al., described that the lysosomal amino-acid transporter, SLC15A4, promoted the activation of NLRC4 inflammasome and the secretion of downstream cytokines induced by Salmonella Typhimurium infection. They also showed that the loss of function of SLC15A4 in mice caused less severe gut inflammation in DSS-induced mouse model. Mechanismly, they suggested that SLC15A4 sustains NLRC4 inflammasome assembly in the perinuclear region through regulation of the mTOR pathway and autophagy. Although this study might be interesting to specialists in the field, the conceptual advance provided by this study is limited. The detailed mechanism by which SLC15A4 regulates autophagy activity through mTOR also needs to be further clarified.
Major concerns:
1. In Fig.1E, the cleaved Casp1 should be detected in the supernatants of the treated cells. In Fig.1I, the increased level of LC3-II may be due to the inhibition of autophagic flux rather than enhanced autophagy activity, please confirm it. The figure legend was confusing.
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3. In Fig. 5, how does SLC15A4 regulate autophagy activity through mTOR? Through phosphorylation of ULK1? Or TFEB-dependent autolysosome-associated gene transcription?
4. Why the position of inflammasome assembly regulates the activation of the inflammasomes? No data or cited references can prove it.
Minor:
1. The scale bar was missing in In Fig. 2D. The significance analysis was missing in Fig. EV2A.

Referee #1:

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We are pleased to hear that the reviewer finds our findings of interest. We agree that future studies will be necessary to address SLC15A4 dynamics in dendritic cells.

Referee #2:

Review of the study entitled "SLC15A4 favors inflammasome function via mTORC1 signaling and autophagy restraint in dendritic cells" from Lopez-Haber et al.,

Here the authors study the role of the SLC15A4 carrier transporter in murine dendritic cells as a controller of optimal inflammasome response.
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- SLC15A4 regulates Salmonella-induced NLRC4 and Alum/MSU-induced NLRP3 inflammasome response. Could the authors also determine if the murine AIM2 (DNA) inflammasome, the PYRIN inflammasome (TcdB toxin or other) or the non-canonical Caspase-11 inflammasome (LPS sensing) are also regulated similarly
https://www.sciencedirect.com/science/article/pii/S0165247821000523?

We agree with the reviewer that it would be interesting to know if our findings on the NLRP3 and NLRC4 inflammasomes upon particulate stimuli also apply to other inflammasomes and/or types of ligands. We have now investigated the production of IL-1β as a readout for inflammasome activation, upon stimulation of the AIM2 inflammasome – using double-stranded DNA (dsDNA) complexed with lipids (lyovec) – and non-canonical caspase-11 dependent inflammasome – using *Salmonella* typhimurium (STm) grown to stationary phase, in conditions that prevent the expression of flagellin and SPI-1 type III secretion system (T3SS) and therefore NLRC4 stimulation, or STm lacking both flagellin genes (ΔfliCΔfljB) (Wynosky-Dolfi M., J. Exp. Med. 2014).
We found that the absence of SLC15A4 did not impair AIM2 activity upon stimulation with dsDNA [poly(dA:dT); Fig. EV2C)]. Given that dsDNA was directly delivered into the cytosol, this may explain why a phagolysosomal transporter does not play a role in inflammasome activity in this context (see comment below). Further studies will be required to assess the role of SLC15A4 in AIM2 activity after bacterial infection, in the absence of NLRC4 and NLRP3 activities. However, we believe these studies are beyond the scope of the present manuscript.
In the case of the non-canonical inflammasome, STm that do not inject flagellin (either ΔfliCΔfljB mutant or WT STm grown to stationary phase) cause caspase-11-dependent cell death (Broz P., Nature 2012; Kayagaki N., Nature 2011; Wynosky-Dolfi M., J. Exp. Med. 2014). We found that cell death – measured by LDH release – was similar between WT and SLC15A4^{feeble} BMDCs (Fig. EV3A). Similarly, in these conditions, caspase-11 and its substrate gasdermin D cleavage were not affected in SLC15A4 ^{feeble} DCs (Fig. EV3B, C). This suggests that the regulation of caspase-11 activity is not dependent on SLC15A4. Interestingly, we do not detect significant LC3-II induction after stimulation with mutant STm (Fig. EV3B, D), suggesting that caspase-11 activity is not modulated by autophagy, in contrast to our observations with canonical inflammasomes (Fig. 1G, I).

- Similarly, if SLC15A4 regulates phagocytosis-dependent inflammasome positioning and activation, could the authors test WT and SLC15A4 -/- dendritic cells for inflammasome response to inflammasome ligand electroporation (direct introduction of ligand into the host cell cytosol e.g. Flagellin electroporation, LPS electroporation, DNA electroporation?)

Following the reviewer's suggestions, we tested IL-1β production in response to the T3SS inner rod protein fused to the amino-terminal domain of anthrax toxin's lethal factor, co-administered with anthrax toxin's protective antigen (Rod-Tox) – which stimulates the NLRC4 inflammasome –, *Listeria monocytogenes* listeriolysin O (LLO) – which stimulates the NLRP3 inflammasome – and dsDNA in lipid complexes – which stimulates the AIM2 inflammasome as mentioned above (Reyes Ruiz V., PNAS 2017; Rathinam V., Nature Immunol. 2010; Mantegazza A., PLoS Pathogens 2017). In all these cases, IL-1β production was comparable between WT and SLC15A4^{feeble} BMDCs (Fig. EV2C), suggesting that SLC15A4 does not affect inflammasome activity if ligands are delivered directly into the cytosol after plasma membrane damage or via lipid complexes. In contrast, our data show that SLC15A4 plays a role in inflammasome activity after phagocytosis.

- Are dendritic cells dying of inflammasome stimulation? Or is there a similar mechanism of Gasdermin D-driven IL1beta secretion but not cell shrinkage as previously observed by Kagan'

group (and others) https://www.cell.com/cell-reports/pdf/S2211-1247(20)31370-X.pdf? Indeed, dendritic cells are extremely important for antigen presentation, hence a guess would be that if they do express inflammasome signaling, there might be some protective mechanisms to avoid cell death and further antigenic presentation to adaptive immune cells?

As the reviewer points out, Kagan's group and others showed that some oxidized phospholipids trigger IL-1β secretion from live cells. However, the same group and others showed that different NLRP3 and NLRC4 stimuli such as alum and flagellin-expressing STm induce cell death. STm ΔfliCΔfljB mutant or WT STm grown to stationary phase causes delayed cell death compared to flagellin-expressing STm (Wynosky-Dolfi M., J. Exp. Med. 2014) as mentioned above and also shown in Fig. EV3A. The state of hyperactivation appears to be dependent on the inflammasome ligand – mutant STm could be considered "weaker" stimuli – and on the duration of the stimuli, and seems to be cell type-specific – as shown between murine DCs and macrophages (Zanoni I. et al., Immunity 2017), and also in human DC subsets by Dudziak's group (Hatscher L. et al., Sci. Signal. 2021). We have included these considerations in the Discussion.

- SLC15A4 is also expressed in macrophages. Do SLC15A4-/- macrophages respond similarly than dendritic cells to inflammasome stimulation (at least to Salmonella or MSU or Alum?)

We found that macrophages stimulated with STm activate the NLRC4 inflammasome similarly comparing WT to SLC15A4^{feeble} bone marrow-derived macrophages (BMMΦs) (Fig. EV3E, F). Interestingly, the kinetics of autophagy induction, measured by LC3-II lipidation, appears to be different between BMDCs and BMΦs, as we showed before upon STm and alum stimulation (Mantegazza A., PLoS Pathogens 2017), and now in Fig. EV3F, G. Considering phagosomal pH is decreased in BMΦs compared to BMDCs (Lukacs G., JBC 1991; Savina A., Cell 2006), as well as in human cells (Mantegazza A., Blood 2008), we speculate that increased V-ATPase activity – which is known to promote autophagy – in BMMΦs favors the increased initial autophagy induction upon STm stimulation. This inherent increased phagosomal acidification in BMMΦs may hamper the detection of differences between WT and SLC15A4^{feeble} cells.

- What about the response of human dendritic cells in which SLC15A4 has been invalidated or targeted (siRNA?).

We believe that the study of human dendritic cells is beyond the focus of the present manuscript. We hope the reviewer will agree.

Minor:
It would be interesting that the authors discuss the recent concepts of dendritic cell hyperactivation in their discussion part as when it is about dendritic cells, there is still some intensive discussions about their ability of going into pyroptosis or not and the purpose of such skill, given their important function as antigen-presenting cells.

We now added a brief reference to DC hyperactivation in the Discussion section, in the context of cell-intrinsic and ligand-dependent responses, given that although

hyperactivation is an intriguing concept that warrants future investigation, it is not the focus of the present manuscript. We hope the reviewer will find this addition satisfying.

Referee #3:

In this study, Haber et al., described that the lysosomal amino-acid transporter, SLC15A4, promoted the activation of NLRC4 inflammasome and the secretion of downstream cytokines induced by Salmonella Typhimurium infection. They also showed that the loss of function of SLC15A4 in mice caused less severe gut inflammation in DSS-induced mouse model. Mechanismly, they suggested that SLC15A4 sustains NLRC4 inflammasome assembly in the perinuclear region through regulation of the mTOR pathway and autophagy. Although this study might be interesting to specialists in the field, the conceptual advance provided by this study is limited. The detailed mechanism by which SLC15A4 regulates autophagy activity through mTOR also needs to be further clarified.

We have addressed the reviewer's comments below, and we think our new data further support our initial observations and clarify SLC15A4 regulation of autophagy via mTORC1 signaling. We believe that our findings that mTORC1 regulates both inflammasome positioning and activity are novel and represent a significant conceptual advance in understanding inflammation. We hope the reviewer will now agree.

Major concerns:
1. In Fig.1E, the cleaved Casp1 should be detected in the supernatants of the treated cells. In Fig.1I, the increased level of LC3-II may be due to the inhibition of autophagic flux rather than enhanced autophagy activity, please confirm it. The figure legend was confusing.

As the reviewer points out, caspase-1 may be detected in cell lysates and supernatants. We consider it more appropriate to show its presence and cleavage in cell lysates, to detect pro- and cleaved forms, and because caspase-1 performs its function intracellularly. We now also include the detection of caspase-1 p20 in cell supernatants, in the source data corresponding to the new Fig. 5G. Caspase-11 p30 was only detected in cell supernatants, as shown in Fig. EV3C, and as previously reported (Broz P., Nature 2014; Moretti J., Nature Immunology 2022).

We agree with the reviewer that increased levels of LC3-II in SLC15A4^{feeble} DCs could be due to either enhanced autophagy induction or decreased autophagic clearance. We hypothesized that autophagy initiation increases due to reduced mTOR signaling in SLC15A4^{feeble} DCs. We now performed additional experiments treating WT and SLC15A4^{feeble} DCs with chloroquine, which alkalinizes lysosomes and prevents autophagic degradation (Klionsky D., Autophagy 2021). In this scenario, we replicate the observation that LC3-II levels are higher in SLC15A4^{feeble} DCs upon STm stimulation, suggesting that autophagy induction is indeed increased in the absence of SLC15A4 (Fig. EV1C, D). We also show that knock-down of ATG5 or ATG7 rescues defective inflammasome activity in SLC15A4^{feeble} DCs (see response below).

2. The number and the size of ASC speck was compatible between SLC15A4feeble and wild-

type cells. There was no obvious colocalization between ASC and p62 or LC3 even in wild-type cells indicating that the effect was not because of autophagic degradation (Figs 4 and EV3). Whether the effect was dependent on classical autophagy machinery? Could you observe the consistent phenomenon in ATG5/ATG7 knockout cells. The production of IL-1B should be detected in SLC15A4feeble DC cells treated with autophagy inhibitor, such as Bafliomycin or chloroquine.

As the reviewer summarizes, the kinetics of ASC speck formation, and the size of ASC specks are similar between WT and SLC15A4^{feeble} DCs. In contrast, we detect that ASC specks appear increasingly surrounded by LC3 or p62 puncta in SLC15A4^{feeble} DCs, in which specks form away from the nucleus. We previously showed by flow cytometry that ASC specks seem sequestered by autophagic membranes when they are formed peripherally (Mantegazza A., PLoS Pathogens 2017). We hypothesize that sequestration or isolation of ASC specks by autophagic membranes reduces inflammasome activity, measured by caspase-1 and GSDMD cleavage, as well as IL-1β secretion.
We now show that knocking-down ATG5 or ATG7 rescues the defect in inflammasome activity in SLC15A4^{feeble} DCs, supporting that increased autophagy is downregulating inflammasome activity (Fig. 5F, G), likely via sequestration of ASC specks.

3. In Fig. 5, how does SLC15A4 regulate autophagy activity through mTOR? Through phosphorylation of ULK1? Or TFEB-dependent autolysosome-associated gene transcription?

We hypothesized that the impairment in nutrient sensing caused by the absence of SLC15A4 would negatively impact mTOR activation, similarly to what was described for the arginine transporter SLC38A9 (Rebsamen M., Nature 2015; Wang S., Science 2015), promoting autophagy. We show that mTOR phosphorylation and downstream mTOR signaling (p70 and S6) are reduced in SLC15A4^{feeble} DCs. We thank the reviewer for pointing out ULK. We investigated ULK1 phosphorylation and now show that it is also decreased in SLC15A4^{feeble} DCs upon STm stimulation, which likely explains the increased autophagy induction in these cells (two sets of experiments shown in Fig. 5A and EV5A). Given the short time of STm stimulation, the observed autophagy induction appears incompatible with TFEB transcriptional regulation.

4. Why the position of inflammasome assembly regulates the activation of the inflammasomes? No data or cited references can prove it.

Our lab and others' observations (Martin B., Nature Commun 2014), suggest that inflammasomes are more active in the perinuclear region. We hypothesize that NLRC4 ASC specks are protected from autophagic sequestration in this location. We show that LC3 puncta are less detected in the perinuclear area, and that restoring perinuclear positioning by expressing SLC15A4 or constitutively active mTORC1 in SLC15A4^{feeble} DCs correlates with increased inflammasome activity. We have modified the Discussion to reflect that the relationship between inflammasome activity and perinuclear positioning has not been completely elucidated but nonetheless suggested.

Minor:
1. The scale bar was missing in In Fig. 2D. The significance analysis was missing in Fig. EV2A.

We thank the reviewer for pointing these out. Scale bars were hardly seen in Fig. 2D. We have added the corresponding values in this Figure and Figure legend and the significance analysis in Fig. EV2A.

Dear Adriana,

Thank you for submitting your revised manuscript. Your study has now been seen by referees #2 and 3. As you can see below, the referees appreciate the introduced changes. Referee #3 still has two issues that I would like to respond to in a final revision. Let me know if we need to discuss the points further.

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

- COI needs to be re-labeled as Disclosure statement and competing interests

- I think callouts to Fig EV4 panels are missing. Please check.

- The movies need to be ZIPed with their respective legends. The legends should be removed from the Article file.

- Scale bars are missing from Figure 1B

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

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

So almost there!

Let me know if we need to discuss anything further

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Use the link below to submit your revision:

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

Referee #2:

Here the authors fairly adressed all my questions either experimentally or by discussing it.
I support and congratulate the authors for their experimental and scientific novelty present in their revised manuscript.

Referee #3:

The authors did provide more data and have answered my several questions. However, as the points 2 and 4 in major concerns, I still cannot understand why autophagy membrane sequestrates ASC specks away from peripheral nucleus where the autophagy cargo is degraded in lysosomes. Please check the level of ASC in Triton-X100 insoluble fraction using subcellular fractionation assay to confirm the sequestration of ASC speck.

It is difficult to reconcile the various levels of detected LC3-II across the experiments since the levels differ mostly at baseline

(mock-treated) (FIG.3B; EV3B/3F). In fact, there seem to be a very high baseline level of autophagy as indicated by LC3-II, which is hard to reconcile for cells growing normally in media with ample glucose and serum.

Adriana R. Mantegazza, Ph.D.
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July 22nd, 2022

Karin Dumstrei, Ph.D.
Senior Editor
The EMBO Journal

Dear Dr. Dumstrei,

We are very thankful for the opportunity to address the reviewers' comments and submit a final revised version of our manuscript EMBOJ-2022-11116, entitled "SLC15A4 favors inflammasome function via mTORC1 signaling and autophagy restraint in dendritic cells" by Cynthia López-Haber, et al., to be considered for publication in *The EMBO Journal*.

Please find below our responses to the reviewers' comments. We have highlighted in blue the new incorporations in the manuscript text, and added the requested information to the Figure legends. We hope that you find the revised manuscript suitable for publication in The EMBO Journal.

Thank you very much again for the opportunity to submit our work!

Yours sincerely,

Adriana R. Mantegazza

Referee #2:

Here the authors fairly addressed all my questions either experimentally or by discussing it. I support and congratulate the authors for their experimental and scientific novelty present in their revised manuscript.

We thank the reviewer for his positive comment!

Referee #3:

1. The authors did provide more data and have answered my several questions. However, as the

points 2 and 4 in major concerns, I still cannot understand why autophagy membrane sequestrates ASC specks away from peripheral nucleus where the autophagy cargo is degraded in lysosomes. Please check the level of ASC in Triton-X100 insoluble fraction using subcellular fractionation assay to confirm the sequestration of ASC speck.

In our previous publication (*PLos Pathogens 2017*; "Increased autophagic sequestration in AP-3 deficient cells...") we showed that by treating bone marrow-derived DCs (BMDCs) with digitonin at conditions that only permeabilize the plasma membrane, and then attempting to detect ASC-GFP with an anti-GFP antibody, we observed decreased detection of ASC-GFP in AP-3 knock-out cells, compared to WT cells, by flow cytometry, which suggests that the speck is at least partly sequestered (internal membranes not permeabilized by digitonin prevent ASC detection). Also, Shi et al. (*Nature Immunol 2012*) showed that ASC is ubiquitylated and targeted for autophagosomal degradation.
We now extend our observations to describe that mTORC1 signaling is required for ASC positioning away from the nucleus, and mTORC1 signaling is modulated by SLC15A4 (which is a cargo of AP-3). We believe that due to the insoluble nature of the oligomerized ASC speck fibrils, the Triton-X100 insoluble fraction would be confounded by the insoluble nature of the speck. Considering this, we have modified the text on section *SLC15A4 promotes inflammasome perinuclear positioning away from autophagic membranes* to incorporate a reference to the differential permeabilization finding, and to indicate that our data suggest that ASC specks are at least partly sequestered by autophagic membranes.

2. It is difficult to reconcile the various levels of detected LC3-II across the experiments since the levels differ mostly at baseline (mock-treated) (FIG.3B; EV3B/3F). In fact, there seems to be a very high baseline level of autophagy as indicated by LC3-II, which is hard to reconcile for cells growing normally in media with ample glucose and serum.

The levels of LC3 vary among cell types (in the cases that the reviewer indicates, these were tissue-resident DCs, BMDCs or BM-derived macrophages), and LC3-II may also be present on compartments that are not autophagosomes (as described in Klionsky et al, *Autophagy 2021*). It is also worth mentioning that the immunoblots were developed using horseradish peroxidase-conjugated antibodies and chemiluminescence, and using two different detection equipment (as indicated in Materials and Methods). For these reasons, we normalized LC3-II/LC3-I induction over time and/or upon stimulation with respect to time 0, and represented data as fold induction. We now incorporated the normalization strategy in Materials and Methods.

We hope the reviewer will find these additions satisfying.

2nd Revision - Editorial Decision **1st Aug 2022**

Dear Adriana,

Thank you for submitting your revised manuscript. I have looked at everything and all looks good.

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

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

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

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

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods.

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods.
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods.
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods.
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods.

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods.

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments.

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods and Figure legends.
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and Methods.
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods.
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods.

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Materials and Methods.
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Materials and Methods.

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods.
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section.
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	