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# Proteasomal degradation within endocytic organelles mediates antigen cross-presentation

Debrup Sengupta, Morven Graham, Xinran Liu and Peter Cresswell.

**Review timeline:**

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29th March 2018

Thank you for submitting your manuscript on a role for phagosomal proteasome in MHC-I antigen processing.

The manuscript has now been reviewed by three expert referees whose comments are provided below. As you can see, while all referees consider the findings novel and interesting, they also raise some critical points that need to be addressed before they can support publication here.

In particular, the referees are concerned that: i) the presence of active proteasome in phagosomes and its role in cross-presented peptides processing are not sufficiently supported by the experimental data; and ii) the mechanism through which the proteasome enters the phagosome needs to be elucidated. In addition, referee #1 and #2 find that the effects of overexpressed Rab mutants on vesicle trafficking and phagosome maturation have to be investigated.

Addressing these issues through decisive additional data as suggested by the referees would be essential to warrant publication in The EMBO Journal. Given the overall interest of your study, I would thus like to invite you to revise the manuscript in response to the referee reports. Please note that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

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REFEREE REPORTS

Referee #1:

Sengupta and Cresswell propose that active proteasome within phagosomes can mediate antigen degradation for cross-presentation by bone-marrow derived dendritic cells. This is an intriguing

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hypothesis which, if true, could address various unexplained findings regarding the cell biology of cross-presentation. However unfortunately the evidence presented fails to convince this reviewer that the conclusions drawn by the authors are correct, and significantly more experimentation would be required to provide such evidence.

The paper starts by showing that HEK293 transfectants and bone marrow-derived dendritic cells expressing constitutively active mutants of Rab5a or Rab22a or dominant negative mutants of Rab7 display enhanced cross-presentation of phagocytosed antigens. While this is clearly demonstrated, the authors do not undertake an effort to characterize the effect of these mutants on vesicle trafficking in the cells. The different papers cited in support of the claim that these mutants delay/inhibit phagosome maturation all used a macrophage cell line, in one of them in conjunction with mycobacterial infection. Thus some evidence on the effects of these mutants especially on phagosome maturation (EEA-1/Lamp acquisition, antigen degradation..) in BM-DCs would be welcome. Moreover, the paper cited in support of using the Rab5 mutant reports enhanced phagocytosis of latex beads. It is fairly standard in the type of study presented here to control for equal efficiency of phagocytosis, which appears even more important given this data.

The key data of the paper are obtained using TAP-deficient cells. Fig. 3 shows that cross-presentation by TAP-deficient BM-DCs can be reconstituted partly by transfecting human beta2m, and almost fully by expressing in addition one of the mutants presumably delaying phagosome maturation. The authors conclude that reduced phagosomal maturation and the availability of a stable pool of cell surface Kb molecules may be the key... to cross-presentation. Surprisingly here the authors fail to cite a previous demonstration of efficient and proteasome-dependent cross-presentation of phagocytosed antigen by TAP-deficient BM-DCs (Merzougui et al, Embo Rep 2012). The findings in that paper parallel those in the paper under review, in that normalization of class I on the cell surface alone (using a more efficient approach than transfecting human beta2m) restores cross-presentation. It is important to cite this published evidence which will at the same time corroborate the observation of TAP-independent, proteasome-dependent cross-presentation. It would also be appropriate to cite work from the Eisenlohr group showing TAP-independent, proteasome dependent presentation by class II molecules (Miller et al., Nat Med 2015), an observation that may well involve compartments where both class I and class II molecules can be loaded.

Given the key hypothesis of a role of vesicular proteasome in endosomal cross-presentation, it seems of critical interest to test proteasome inhibition in this experimental setting, however surprisingly this is not done. The authors should test the effect of epoxomicin in cross-presentation by TAP ko BM-DCs expressing human beta2m and/or Rab mutants.

Fig. 4 then shows microscopy data meant to demonstrate that active proteasome is recruited to phagosomes. However I feel that only electron microscopy studies could provide strong evidence for the presence of proteasome in phagosomes. The experiment meant to show active proteasome also fails to convince. Phagosomes purified by sucrose gradients can be strongly contaminated with other subcellular compartments and cytosolic components so that such preparations usually are examined by immunoblot for contaminating organelle markers. Moreover here also electron microscopy could help to convince readers. Using active site probes would also be a useful complementation. It is also unclear why proteasome complexes would remain associate with latex beads in the presence of detergents. Finally the choice of Lamp for co-localisation with Lmp2 surprises given that expression of Rab mutants expected to reduce phagosome fusion with Lamp-positive vesicles is a key element of the experimental strategy.

Fig. 5 shows that proteasome inhibition reduces cell surface class I expression by TAP ko BM-DCs expressing or not human beta 2m, and that this is prevented by adding synthetic SIINFEKL. However it is not possible to establish a causal relationship between peptide production by endosomal proteasome and cell surface class I based on this evidence. This experiment shows that the proteasome is involved in some step stabilizing class I on the cell surface or promoting class I export to it, but not necessarily that it produces peptide ligands in endosomes.

In another experiment the authors interpret the fact that human beta2m transfection of TAP ko BM-DCs does not restore endogenous presentation by class I as evidence against the existence of an alternative transporter. Presently no evidence for transport of class I ligands by an alternative

transporter into phagosomes is known. However, as the authors will be aware, the best candidate transporter for such a role (TAP-L) is expressed exclusively in late endosomes/lysosomes so that reconstitution of endogenous class I presentation by it is not expected, and the failure of its reconstitution by human beta2m is not an argument against its existence.

Finally did the authors consider that (likely inactive) proteasome complexes may appear in autophagosomes that may fuse with bead-containing vesicles?

Referee #2:

In a manuscript entitled "Proteasomal degradation within endocytic organelles can mediate antigen cross-presentation", Sengupta and Cresswell provide evidence that active proteasomes are present within the phagosomal lumen and generate peptides contributing to cross-presentation. This is quite an interesting, and provocative, argument. The manuscript is well-written and all the experiments performed are well-done. The main point missing, I feel, is to provide more direct evidence that the proteasome is present as a "large" structure in the lumen, and address the kinetic and mechanic of its recruitment.

They first show in Fig.1 that constitutively active forms of the small GTPases Rab5, and Rab22 significantly enhanced cross-presentation. In the introduction of these experiments, they indicate regarding these mutant GTPases: "Expecting that Rab mutants that restrict phagosomal maturation would further inhibit acidification and proteolysis and enhance cross-presentation...". They should provide evidence that these GTPases effectively restrict phagosome maturation in their conditions (perhaps by monitorig the acquisition of late phagosome markers such as Rab7 and/or LAMP1).

In Fig. 2, they show that a proteasome inhibitor inhibits antigen presentation (which is to be expected even if proteasomes were exclusively in the cytoplasm), through the TAP-independent pathway. To characterize their system, they could perhaps also test the effect of bafilomycin, which inhibits vacuolar processing. It might also be interesting to look if the cross-presentation of Ova is also affected when higher concentrations of ovalbumin on beads are used. Indeed, it was shown that in such condition Ova presentation is TAP-independent suggesting that the whole processing occurs in the lumen (Bertholet et al., 2006). Accordingly, Ova presentation at higher doses should still be proteasome-dependent if these structures are in the lumen.

In Fig. 3, they show that Ova cross-presentation is TAP-independent through well-defined experiments.

In Fig. 4, they use immunofluorescence to show that some of the immunoproteasome subunit LMP2 is observed within the lumen of phagosomes. The demonstration that the proteasome is indeed in the lumen is critical to this manuscript. Accordingly, additional evidence should be provided. For example, they could look at other markers of the proteasome by IF. What would, however, be a more convincing piece of evidence would be to isolate phagosomes and perform blue-native gels in non-denaturing conditions to look for the whole structure, rather than isolated subunits. They could distinguish whether the proteasome complex is loss by protease treatment to "shave" proteins and structures outside the organelle. One could assume that the proteasome is recruited during maturation of phagosomes. It would thus be important to look at phagosomes isolated at different time points to see when the structure is recruited (again, by using more than one marker). This recruitment is also likely to involve autophagy capturing the structures from the cytoplasm and delivering them to phagosomes. Experiments with either 3-methyladenine or with ATG5 KD would provide interesting clues.

Fig. 5 is fine.

Referee #3:

This manuscript starts by examining 3 Rab proteins (Rab5a, 7a, & 22a), which were already known to be involved in vacuole maturation, for their effects on antigen presentation of ingested protein particles. The authors found that mutant versions of these Rab proteins, which should prevent phagosomes from maturing and thereby restrain their degradative capacity, enhanced presentation of internalized extracellular antigens on class I MHC molecules (cross presentation). Earlier publications demonstrated that cross presentation is promoted in a less hydrolytic environment and the present findings using the Rabs to interfere with maturation give the expected result of increased antigen presentation. The authors then characterize the requirements of this form of cross presentation and find a TAP-independent and proteasome-dependent component. These findings are fine but are not without precedence or earth shaking.

The most interesting and surprising finding is that active proteasomes are found inside of phagocytic vesicles and I think that this is a key discovery, if adequately characterized, that could elevate the paper to be appropriate for EMBO. However, there are four related issues that need to be resolved before the impact of these findings can be assessed. If the further experiments were to demonstrate the validity and importance of the discovery, then this report would be appropriate and of interest for EMBO.
The first issue is whether proteasomes are found in phagosomes under normal physiological conditions or only under conditions where modified Rab proteins are overexpressed. This issue would be of importance in any case, but is of heightened concern because the phagosomes in the Rab-overexpressing cells look highly abnormal; they are greatly enlarged and contain multiple beads (something that is not normally seen). Other vesicles are similarly abnormally enlarged. Also, there is very little proteasome enzymatic activity in phagosomes from normal cells (Fig 4G). It is conceivable that the modified and overexpressed Rab proteins are creating abnormal conditions wherein proteasomes end up in phagosomes (which could occur, e.g., from a cellular adaptation to the abnormal conditions or unnatural fusion events). Therefore, it is critical to assess whether intraphagosomal proteasomes are found in normal cells. The authors state that they tried to assess this in normal cells but were unable to resolve this because of the limits of their optical microscopy. However, this issue should be able to be resolved using higher resolution methods (e.g. electron microscopy). If normal phagosomes contain proteasomes and this is not a rare event, then this would be the beginning of an important discovery, although more would be needed as described in the next points. The second issue is that the paper would be greatly strengthened if the mechanism by which proteasomes enter phagosomes was elucidated. Since these are large particles without a surrounding membrane, they are almost certainly not being delivered intact into vesicles by a transporter or direct fusion event and this is what makes the finding surprising and very interesting. One could imagine these particles could be delivered by autophagy, assembled inside the vesicles or end up in this location via some novel mechanism. It would be important to resolve the underlying mechanism. The third issue is whether the intraphagosomal proteasomes are really involved in protein degradation in phagosomes (not just in the hydrolysis of small peptide substrates by detergent liberated/activated proteasomes, which is the level of analysis in the current manuscript). Is there ubitquitin and ubiquitination machinery inside of phagosomes and can such machinery conjugate ubiquitin to internalized substrates (which is needed for many substrates to be degraded by proteasomes) in phagosomes? Is there actually proteasome-dependent degradation of internalized proteins in phagosomes (e.g. this could be looked at by evaluating whether ovalbumin is being hydrolyzed off of internalized beads in a proteasome-dependent manner or other methods). The fourth issue is that the connection between the presence of intra-phagosomal proteasomes and antigen presentation is circumstantial. It appears in Fig. 4 that only a fraction of phagosomes contains proteasomes (and the percent of these vesicles should be quantified). If the authors' model is correct, then only the proteasome-containing phagosomes should contain the cross presented peptide-MHC class I complexes in TAP-negative cells; this is potentially testable. Such experiments or potentially other ones might be able to make a stronger connection between the intravesicular proteasomes and antigen presentation.

There are a number of specific technical points that should also be addressed as follows:

1. There are only a few images of phagosomes containing proteasomes. In the two optical sections in Fig 4A (Rab5ACA and Rab22ACA), intraphagosomal proteasomes appear to be present in the interior of a phagocytized bead and this is in optical sections that are not being taken at the bottom or top of a bead. The same may be seen in Fig4B and Fig4F as well, but since these images are of a whole cell or 3D rendering it is unclear if the proteasomes are outside of the bead and superimposed

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optically. Having proteasomes in the center of beads is hard to understand because these particles are solid and this appearance makes one worry about an artifact. How often is this seen and how do the authors explain this finding?

2. As noted above, the percent of phagosomes (and the percent of cells with phagosomes) that contain proteasomes should be quantified and shown. Is this a rare or common event?

3. In Figure 2 the potential for toxicity from proteasome inhibitors contributing to inhibitions should be better evaluated. The control that was shown was adding extracellular synthetic peptide; this primarily measures surface MHC I molecules. A better control is to evaluate the presentation of intracellular epitope that does not require proteasome cleavage (e.g. from a minigene).

4. The background (no antigen) is not shown in any of the antigen presentation experiments and should be shown or put in the legend. (minor point)

5. In Figure 1, why are the dominant negative constructs only shown in 293T cells but not in the dendritic cells? (minor point)

6. In Fig 2, the proteasome inhibition of 293T cells is partial (and this may also be true in DCs but this is hard to tell without knowing the background in these assays). Is greater inhibition seen in 293T cells at higher but non-toxic doses of epoxomicin? Is there a proteasome independent component of antigen presentation? (minor point).

7. In Fig 3A&B, it is unclear how the calculation of percent is done for the Rab groups. Is the comparator a control transfected cell without the TAP inhibitor, a Rab overexpressing cell without the TAP inhibitor, or a control cell with the TAP inhibitor. This should be clarified. (minor point)

8. In Fig 4G the authors should provide (at least in the legend) data on the levels of substrate hydrolysis without proteasome inhibitor since the substrate is not proteasome-specific (i.e. not just give the fold-increase). (minor point)

9. In Fig 5A, the results of the peptide titration are odd in that there are higher levels of H-2Kb with 2uM peptide compared to 8uM peptide? The authors should check that there is not a labeling error. (minor point)

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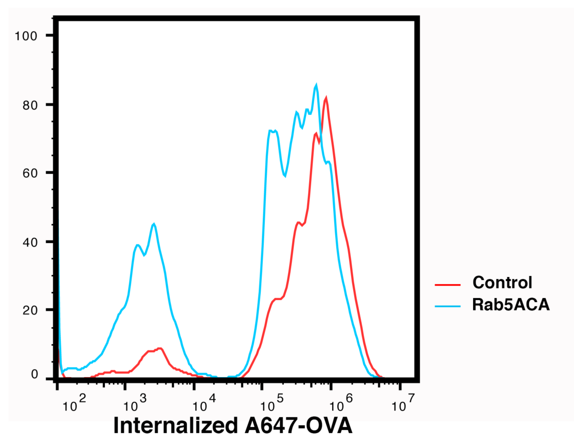
**Response to the referee's comments**
Referee #1:

Sengupta and Cresswell propose that active proteasome within phagosomes can mediate antigen degradation for cross-presentation by bone-marrow derived dendritic cells. This is an intriguing hypothesis which, if true, could address various unexplained findings regarding the cell biology of cross-presentation. However unfortunately the evidence presented fails to convince this reviewer that the conclusions drawn by the authors are correct, and significantly more experimentation would be required to provide such evidence.

The paper starts by showing that HEK293 transfectants and bone marrow-derived dendritic cells expressing constitutively active mutants of Rab5a or Rab22a or dominant negative mutants of Rab7 display enhanced cross-presentation of phagocytosed antigens. While this is clearly demonstrated, the authors do not undertake an effort to characterize the effect of these mutants on vesicle trafficking in the cells. The different papers cited in support of the claim that these mutants delay/inhibit phagosome maturation all used a macrophage cell line, in one of them in conjunction with mycobacterial infection. Thus some evidence on the effects of these mutants especially on phagosome maturation (EEA-1/Lamp acquisition, antigen degradation..) in BM-DCs would be welcome. Moreover, the paper cited in support of using the Rab5 mutant reports enhanced phagocytosis of latex beads. It is fairly standard in the type of study presented here to control for equal efficiency of phagocytosis, which appears even more important given this data.

**We thank the reviewer for pointing out these problems. To address the issue of whether the Rab mutants impact phagosomal maturation we analyzed the kinetics of phagosomal degradation of OVA by BMDC expressing the relevant Rab mutants, now presented in Fig EV1. The assays show that the rate of OVA degradation is slower in BMDCs expressing Rab5ACA, Rab22ACA or Rab7ADN compared to control. This is consistent with published studies in macrophage cell lines. We also tested the efficiency of phagocytosis of Alexa647-OVA coated latex beads by control or Rab5ACA-expressing BMDC under the conditions used for the cross-presentation assays. We actually observed a reduction in uptake in BMDCs expressing Rab5CA (see the figure below). This suggests that the increased cross-presentation induced by Rab5CA may actually be an under representation of the cross-presentation potential of the cells.**



The key data of the paper are obtained using TAP-deficient cells. Fig. 3 shows that cross-presentation by TAP-deficient BM-DCs can be reconstituted partly by transfecting human beta2m, and almost fully by expressing in addition one of the mutants presumably delaying phagosome maturation. The authors conclude that reduced phagosomal maturation and the availability of a stable pool of cell surface Kb molecules may be the key... to cross-presentation. Surprisingly here the authors fail to cite a previous demonstration of efficient and proteasome-dependent cross-presentation of phagocytosed antigen by TAP-deficient BM-DCs (Merzougui et al, Embo Rep 2012). The findings in that paper parallel those in the paper under review, in that normalization of class I on the cell surface alone (using a more efficient approach than transfecting human beta2m) restores cross-presentation. It is important to cite this published evidence which will at the same time corroborate the observation of TAP-independent, proteasome-dependent cross-presentation. It would also be appropriate to cite work from the Eisenlohr group showing TAP-independent, proteasome dependent presentation by class II molecules (Miller et al., Nat Med 2015), an observation that may well involve compartments where both class I and class II molecules can be loaded.

**We thank the reviewer for pointing out this oversight and the excellent suggestion to include the MHC class II work of the Eisenlohr group. We have added an extensive discussion of this topic that cites the findings of Merzougui et al. and Miller et al. on the first page of the Discussion (page 19).**

Given the key hypothesis of a role of vesicular proteasome in endosomal cross-presentation, it seems of critical interest to test proteasome inhibition in this experimental setting, however surprisingly this is not done. The authors should test the effect of epoxomicin in cross-presentation by TAP ko BM-DCs expressing human beta2m and/or Rab mutants.

**We thank the reviewer for this suggestion. We have added an experiment showing the proteasome dependence of cross-presentation mediated by $TAP1^{-/-}$ BMDCs expressing human $\beta$2m (Fig. 3E). However, since treatment of the BMDCs with epoxomicin impacts cell surface MHC class-I levels, which could affect the interpretation of the results, we performed the cross-presentation assay in the presence of an irrelevant $K^b$-binding peptide (gB) to stabilize the cell surface MHC-I (Fig EV2). We observed a dose-dependent reduction in antigen cross-presentation by $TAP1^{-/-}$ BMDC expressing human $\beta$2m.**

Fig. 4 then shows microscopy data meant to demonstrate that active proteasome is recruited to phagosomes. However I feel that only electron microscopy studies could provide strong evidence for the presence of proteasome in phagosomes.

**We appreciate the reviewer's suggestion and performed immuno-EM analysis on resting WT BMDC not expressing any of the Rab mutants. Efforts using BMDCs following phagocytosis of latex beads were difficult to interpret because of poor delineation of the membrane surrounding the beads, but examination of the BMDCs without phagocytosis were compelling and are presented in Fig. 5A and Fig EV4. Three antibodies raised against different subunits of the proteasome ($\beta$5, $\alpha$5 and the 19S component S2) were used in conjunction with an anti-LAMP1 antibody to label endolysosomal compartments. All three proteasome subunits were detected within LAMP1-positive**

**membrane-bound compartments, indicating that proteasomes localize to the endolysosomal compartment in BMDCs even in the resting state.**

The experiment meant to show active proteasome also fails to convince.
Phagosomes purified by sucrose gradients can be strongly contaminated with other subcellular compartments and cytosolic components so that such preparations usually are examined by immunoblot for contaminating organelle markers. Moreover here also electron microscopy could help to convince readers.

**The reviewer's concern is understandable, although we would point out that the proteasome assay was performed after detergent treatment and washing the beads, which should remove contamination by other membranous vesicles. However, to alleviate the concern we added two independent approaches to examine this question. We added an alternative FACS-based proteasome degradation assay, now presented in Fig. 6B and C, and we also show that phagosomal antigen undergoes ubiquitination in experiments shown in Fig. 6 D and E.**

Using active site probes would also be a useful complementation.

**We basically agree that this could be useful. However, our concern is that an active site probe generally inhibits the proteasome by binding covalently to an active site and a number of studies have shown that inactive proteasomes can undergo lysosomal degradation following autophagy. Any observation of phagosomal proteasomes would therefore be ambiguous and subject to misinterpretation. Given that we now have three independent observations that support the idea we have chosen not to follow this approach.**

It is also unclear why proteasome complexes would remain associate with latex beads in the presence of detergents.

**We were ourselves surprised by this finding. It may involve ubiquitin-mediated interactions, suggested by the results in Fig. 6 D and E, but we have not followed this up. Nonetheless, it did allow us to test the activity of proteasomes associated with latex beads while avoiding the confounding problem of contaminating proteasome-associated membranes.**

Finally the choice of Lamp for co-localisation with Lmp2 surprises given that expression of Rab mutants expected to reduce phagosome fusion with Lamp-positive vesicles is a key element of the experimental strategy.

**We could not find a monoclonal antibody raised against early endosomal markers, (e.g. EEA-1) useable for immunofluorescence analysis of mouse BMDCs. However, the staining we observed is consistent with the LAMP1 distribution in cells expressing Rab5ACA mutants as previously described (Rosenfeld, J.L., et al. J cell Sci, 2001; Duclos, S., et. al. J cell Sci, 2000 ). In addition, it also been reported that endocytosed antigens localize to LAMP1-positive compartments in a cross-presenting DC cell line (Rodriguez A., Nat Cell Bio 1999). To the best of our knowledge there is no established causal relationship between the presence of LAMP1 and the degradative capacity of endolysosomal compartments.**

Fig. 5 shows that proteasome inhibition reduces cell surface class I expression by TAP ko BM-DCs expressing or not human beta 2m, and that this is prevented by adding synthetic SIINFEKL. However it is not possible to establish a causal relationship between peptide production by endosomal proteasome and cell surface class I based on this evidence. This experiment shows that the proteasome is involved in some step stabilizing class I on the cell surface or promoting class I export to it, but not necessarily that it produces peptide ligands in endosomes.

**The reviewer raises an important concern. It could indeed be argued that inhibition of cytosolic proteasomes is impacting a cellular process that stabilizes $K^b$ independently peptide generation or peptide delivery by TAP. However, any such process would have to be compatible with its reversibility by adding peptides exogenously. We do not know any data that argue for an additional mechanism that explains this finding. We believe that rescue of surface $K^b$ by SIINFEKL, and by the HSV-1 gB peptide data now added (Fig. EV2), strongly argues that the loss of cell surface MHC-I of $TAP1^{-/-}$ BMDC upon proteasome inhibition results from a lack of associated peptides. An alternative peptide transporter that takes cytosolically-generated peptides into the endolysosomal compartment could explain it, but no such transporter has been defined and one candidate, TAP-L, appears to have been eliminated by published data (Lawand M.,2018 Molecular Immunology, see below).**

In another experiment the authors interpret the fact that human beta2m transfection of TAP ko BM-DCs does not restore endogenous presentation by class I as evidence against the existence of an alternative transporter. Presently no evidence for transport of class I ligands by an alternative transporter into phagosomes is known. However, as the authors will be aware, the best candidate transporter for such a role (TAP-L) is expressed exclusively in late endosomes/lysosomes so that reconstitution of endogenous class I presentation by it is not expected, and the failure of its reconstitution by human beta2m is not an argument against its existence.

**These issues are now discussed, and the finding that TAP-L is not involved in antigen cross-presentation (Lawand M.,2018 Molecular Immunology) is now cited in the text (page 20).**

Finally did the authors consider that (likely inactive) proteasome complexes may appear in autophagosomes that may fuse with bead-containing vesicles?

**We did indeed consider this possibility. It was the main reason why we designed experiments to look for proteasome activity in the phagosomes, and now using three different approaches we have shown that proteasome-ubiquitin system is functional within them (Fig 6). It is indeed likely that some form of autophagy delivers active proteasome into the phagosomes/endolysosome(Cohen-Kaplan, V., et al., PNAS, 2019). However, autophagy also plays a role in multiple cellular processes making dissection of the precise mechanism in the case of cross-presentation difficult. Specifically, autophagy down-regulates cell surface MHC-I (Loi, M., et al., Cell Rep. 2016 ). We have discussed this issue in the manuscript on pages 22-23, and are working on uncoupling these processes to address the issue in future work.**

Referee #2:

In a manuscript entitled "Proteasomal degradation within endocytic organelles can mediate antigen cross-presentation", Sengupta and Cresswell provide evidence that active proteasomes are present within the phagosomal lumen and generate peptides contributing to cross-presentation. This is quite an interesting, and provocative, argument. The manuscript is well-written and all the experiments performed are well-done. The main point missing, I feel, is to provide more direct evidence that the proteasome is present as a "large" structure in the lumen, and address the kinetic and mechanic of its recruitment.

They first show in Fig.1 that constitutively active forms of the small GTPases Rab5, and Rab22 significantly enhanced cross-presentation. In the introduction of these experiments, they indicate regarding these mutant GTPases: "Expecting that Rab mutants that restrict phagosomal maturation would further inhibit acidification and proteolysis and enhance cross-presentation...". They should provide evidence that these GTPases effectively restrict phagosome maturation in their conditions (perhaps by monitorig the acquisition of late phagosome markers such as Rab7 and/or LAMP1).

**We agree with this concern and have responded to the comment in our answers to reviewer #1. It has been shown that there is an inverse correlation between phagosomal degradation and cross-presentation and our rationale for using the Rab mutants was to delay maturation and therefore the rate of phagosomal antigen degradation. Our analysis shows that the Rab mutants do reduce the phagosomal antigen degradation compared to control (Fig. EV1).**

In Fig. 2, they show that a proteasome inhibitor inhibits antigen presentation (which is to be expected even if proteasomes were exclusively in the cytoplasm), through the TAP-independent pathway. To characterize their system, they could perhaps also test the effect of bafilomycin, which inhibits vacuolar processing.

**In a previous publication from this laboratory we showed that bafilomycin inhibits antigen cross-presentation (Singh and Cresswell, 2010, Science). Our interpretation was that phagosomal acidification and partial degradation may be required to generate an appropriate fragment for translocation into the cytosol. However, the live cell imaging of Rab22ACA -expressing cells(Fig 4F; Movie EV3) shows that some OVA dissociates from the beads to gain access to vesicle positives for proteasomes. The relative roles of the two pathways in TAP-positive cells remain an area worth investigating, but lie outside the bounds of this manuscript, where we focus on the hβ2m-dependent TAP-independent pathway in BMDCs and use the Rab mutants as a tool to limit lysosomal proteolysis rather than a chemical inhibitor (Fig 2).**

It might also be interesting to look if the cross-presentation of Ova is also affected when higher concentrations of ovalbumin on beads are used. Indeed, it was shown that in such condition Ova presentation is TAP-independent suggesting that the whole processing occurs in the lumen (Bertholet et al., 2006). Accordingly, Ova presentation at higher doses should still be proteasome-dependent if these structures are in the lumen.

**We thank the reviewer for the suggestion. It could be interesting if cross-presentation by TAP1$^{-/-}$ BMDCs should become apparent without the introduction of $\beta_2$m if a higher concentration of OVA is present on the beads. We have not attempted this because the OVA concentration used to coat the beads is already quite high (10 mg/ml) and we worry that using even more could introduce sufficient peptide contamination that the results could be compromised. The data we present is sufficiently internally consistent that we do not believe this experiment is necessary.**

In Fig. 3, they show that Ova cross-presentation is TAP-independent through well-defined experiments.

**We appreciate the positive comment.**

In Fig. 4, they use immunofluorescence to show that some of the immunoproteasome subunit LMP2 is observed within the lumen of phagosomes. The demonstration that the proteasome is indeed in the lumen is critical to this manuscript. Accordingly, additional evidence should be provided. For example, they could look at other markers of the proteasome by IF.

**We also examined the localization of a different subunit of the immunoproteasome, LMP7 (Fig EV4). BMDC expressing Rab5ACA and Rab22ACA were stained with anti-LMP7 antibody and the staining pattern was similar to LMP2. In addition, as discussed above, immuno EM on resting BMDC showed endolysosomal staining with antibodies raised against three distinct proteasomal subunit, $\alpha$5, $\beta$5 and 19S2 (Fig. 5A, EV4).**

What would, however, be a more convincing piece of evidence would be to isolate phagosomes and perform blue-native gels in non-denaturing conditions to look for the whole structure, rather than isolated subunits.

**This is an excellent suggestion and we would have also preferred to isolate intact proteasomes from the phagosomal lumen. The observation that proteasomes remain associated with latex beads post-TritonX 100 extraction convinced us that proteasomal subunits are within the lumen of phagosomes. However, to remove them from the beads requires denaturation, meaning the proteasomes would not be intact. A blue native gel analysis of the Triton X 100-solubilized pool would be feasible, but then the proteasomes could be associated with cytosolic face of the phagosomal membrane, which would lead to ambiguity.**

They could distinguish whether the proteasome complex is loss by protease treatment to "shave" proteins and structures outside the organelle.

**Once again, this is an excellent suggestion and it sent us back to the literature. An earlier study showed that proteasomes associate with cytosolic side of purified phagosomes using a protease protection assay (Houda, M., 2003, Nature). The data showed that a majority of the proteasomes associated with phagosomes are degraded by the protease pronase, but a fraction was resistant to protease degradation. Notably, this fraction was much higher than that of the control cytosolic face marker, which was Rab5. This agrees with our findings and we have introduced this work into the discussion at the bottom of page 20.**

One could assume that the proteasome is recruited during maturation of phagosomes. It would thus be important to look at phagosomes isolated at different time points to see when the structure is recruited (again, by using more than one marker).

**Our new immuno EM data suggest that proteasomes gain access to the endolysosomal compartments of BMDCs at steady state, and three proteasome antibodies are used (Fig 5A EV4). This is compatible with the proposal that phagosomes recruit endolysosomal membrane and acquire proteasomes as they mature. This hypothesis is supported by live cell imaging of Rab22ACA expressing BMDC; we observe proteasomes co-localizing with OVA within first hour of phagocytosis.**

This recruitment is also likely to involve autophagy capturing the structures from the cytoplasm and delivering them to phagosomes. Experiments with either 3-methyladenine or with ATG5 KD would provide interesting clues.

**We agree that autophagy is a likely mechanism for the delivery of proteasomes into the phagosomes. However, as described above, autophagy impacts multiple cellular processes and causes the down-regulation of surface MHC-I (Loi, M., et al., Cell Rep. 2016). Uncoupling this from the effects on proteasome recruitment to the phagosome is the focus of future work.**

Fig. 5 is fine.

**We thank the reviewer for this observation.**

Referee #3:

This manuscript starts by examining 3 Rab proteins (Rab5a, 7a, & 22a), which were already known to be involved in vacuole maturation, for their effects on antigen presentation of ingested protein particles. The authors found that mutant versions of these Rab proteins, which should prevent phagosomes from maturing and thereby restrain their degradative capacity, enhanced presentation of internalized extracellular antigens on class I MHC molecules (cross presentation). Earlier publications demonstrated that cross presentation is promoted in a less hydrolytic environment and the present findings using the Rabs to interfere with maturation give the expected result of increased antigen presentation. The authors then characterize the requirements of this form of cross presentation and find a TAP-independent and proteasome-dependent component. These findings are fine but are not without precedence or earth shaking.

The most interesting and surprising finding is that active proteasomes are found inside of phagocytic vesicles and I think that this is a key discovery, if adequately characterized, that could elevate the paper to be appropriate for EMBO. However, there are four related issues that need to be resolved before the impact of these findings can be assessed. If the further experiments were to demonstrate the validity and importance of the discovery, then this report would be appropriate and of interest for EMBO.

The first issue is whether proteasomes are found in phagosomes under normal physiological conditions or only under conditions where modified Rab proteins are overexpressed. This issue would be of importance in any case, but is of heightened concern because the phagosomes in the Rab-overexpressing cells look highly abnormal; they are greatly enlarged and contain multiple beads (something that is not normally seen). Other vesicles are similarly abnormally enlarged. Also, there is very little proteasome enzymatic activity in phagosomes from normal cells (Fig 4G). It is conceivable that the modified and overexpressed Rab proteins are creating abnormal conditions wherein proteasomes end up in phagosomes (which could occur, e.g., from a cellular adaptation to the abnormal conditions or unnatural fusion events). Therefore, it is critical to assess whether intraphagosomal proteasomes are found in normal cells. The authors state that they tried to assess this in normal cells but were unable to resolve this because of the limits of their optical microscopy. However, this issue should be able to be resolved using higher resolution methods (e.g. electron microscopy). If normal phagosomes contain proteasomes and this is not a rare event, then this would be the beginning of an important discovery, although more would be needed as described in the next points.

**We thank the reviewer for these thoughts and insights, and we think that the new data we have incorporated and discussed above should satisfy the reviewer. In particular, the immuno EM analysis using LAMP1 to define endolysosomal membranes of normal BMDCs, expressing no Rab mutants, and the detection of intraluminal proteasomes using antibodies to three distinct subunits ($\alpha$5, $\beta$5 and 19 S2) is extremely convincing (Fig 5A EV4). We also observe active proteasome-dependent proteolysis of OVA in phagosomes extracted from normal BMDC (Fig. 6B,C) as well as OVA ubiquitination within them (Fig. 6D,F).**

The second issue is that the paper would be greatly strengthened if the mechanism by which proteasomes enter phagosomes was elucidated. Since these are large particles without a surrounding membrane, they are almost certainly not being delivered intact into vesicles by a transporter or direct fusion event and this is what makes the finding surprising and very interesting. One could imagine these particles could be delivered by autophagy, assembled inside the vesicles or end up in this location via some novel mechanism. It would be important to resolve the underlying mechanism.

**We agree with this comment, and as stated above we are currently focusing our efforts on this problem. We have discussed the issues involved in the updated version of the manuscript (page 22).**

The third issue is whether the intraphagosomal proteasomes are really involved in protein degradation in phagosomes (not just in the hydrolysis of small peptide substrates by detergent liberated/activated proteasomes, which is the level of analysis in the current manuscript). Is there ubitquitin and ubiquitination machinery inside of phagosomes and can such machinery conjugate ubiquitin to internalized substrates (which is needed for many substrates to be degraded by proteasomes) in phagosomes? Is there actually proteasome-dependent degradation of internalized proteins in phagosomes (e.g. this could be looked at by evaluating whether ovalbumin is being hydrolyzed off of internalized beads in a proteasome-dependent manner or other methods).

**As discussed above, we have introduced two additional assays to address this concern. We have established a FACS based proteasomal degradation assay using Alexa647-OVA-coupled beads as a substrate (Fig 6B, C), and as well as a phagosomal ubiquitination assay (Fig 6 D, E). The findings using these assays are consistent with intraphagosomal proteasomal degradation.**

The fourth issue is that the connection between the presence of intra-phagosomal proteasomes and antigen presentation is circumstantial. It appears in Fig. 4 that only a fraction of phagosomes contains proteasomes (and the percent of these vesicles should be quantified). If the authors' model is correct, then only the proteasome-containing phagosomes should contain the cross presented peptide-MHC class I complexes in TAP-negative cells; this is potentially testable. Such experiments or potentially other ones might be able to make a stronger connection between the intravesicular proteasomes and antigen presentation.

**These are very good points. The ideal approach to detecting cross-presentation derived MHC-I peptide complexes within the proteasome containing phagosomes would be to stain with the 25.D1 mAb to H2K$^{b}$-SIINFEKL complexes. However, we were unable to obtain a sufficient signal to noise ratio using this mAb. Nevertheless, we think that the combined evidence that we have provided (confocal Imaging, live cell imaging, ImmunoEM, activity assays, surface MHC-I stabilization assays in a TAP1$^{-/-}$ BMDCs in a proteasome dependent manner) strongly supports the hypothesis.**

There are a number of specific technical points that should also be addressed as follows:

1. There are only a few images of phagosomes containing proteasomes. In the two optical sections in Fig 4A (Rab5ACA and Rab22ACA), intraphagosomal proteasomes appear to be present in the interior of a phagocytized bead and this is in optical sections that are not being taken at the bottom or top of a bead. The same may be seen in Fig4B and Fig4F as well, but since these images are of a whole cell or 3D rendering it is unclear if the proteasomes are outside of the bead and superimposed optically. Having proteasomes in the center of beads is hard to understand because these particles are solid and this appearance makes one worry about an artifact. How often is this seen and how do the authors explain this finding?

**We thank the reviewer for the careful observations. The original image was an optical aberration resulting from the spherical structure of the bead and the constraints of confocal microscopy in resolving the Z-resolution. Based on our analysis, the proteasomes are actually associated with the surface of the spherical beads but the XY resolution of optical microscopy does not allow us to observe the bead and proteasomes as separate entities. In the corrected Fig 4C LMP2 staining is now visible within the phagosome that is distinct from the latex bead. Moreover, staining with a different antibody, anti-LMP7, also shows punctate proteasome staining closely associated with the beads (Fig EV4 B, C)**

2. As noted above, the percent of phagosomes (and the percent of cells with phagosomes) that contain proteasomes should be quantified and shown. Is this a rare or common event?

**The presence of proteasomes within the phagosomes/endolysosomal compartment is very common specifically in cells expressing Rab mutants. While we have not precisely quantitated the**

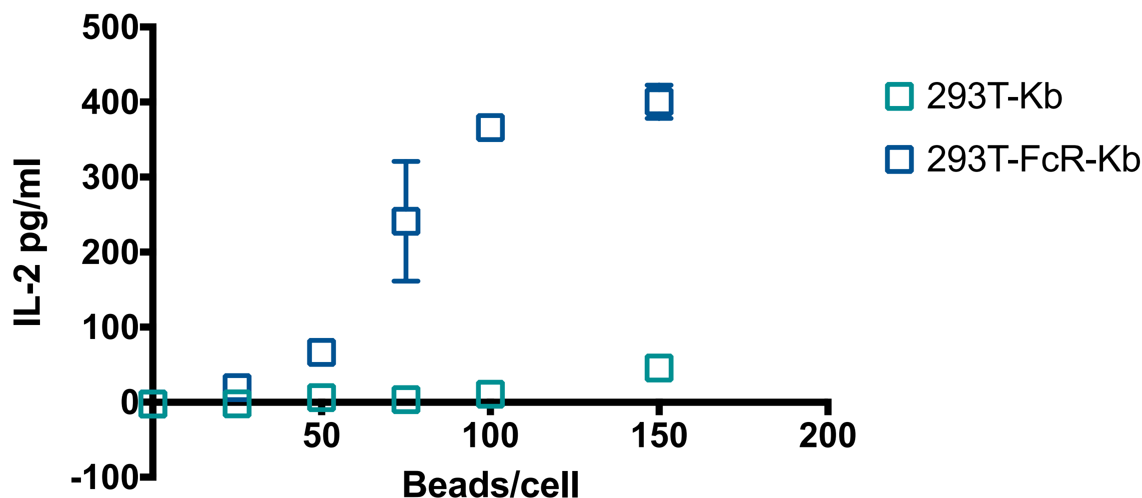
**compartments containing the proteasomes because of experimental variability our general estimate at least two thirds of the phagosomes, where we can reliably delineate the lumen, contain proteasomes.**

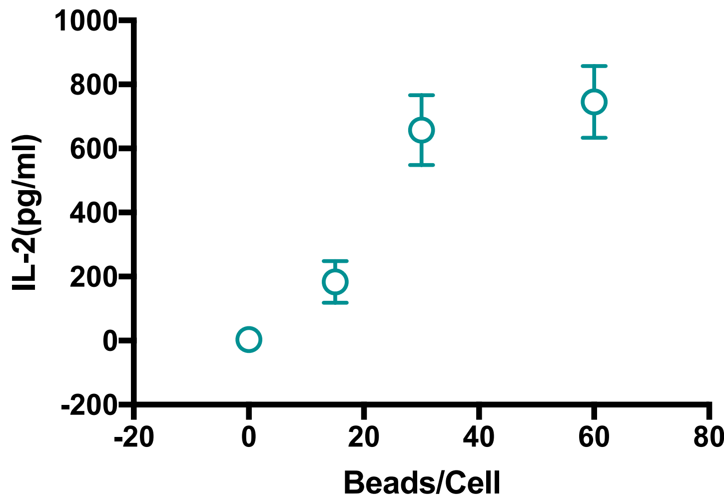
3. In Figure 2 the potential for toxicity from proteasome inhibitors contributing to inhibitions should be better evaluated. The control that was shown was adding extracellular synthetic peptide; this primarily measures surface MHC I molecules. A better control is to evaluate the presentation of intracellular epitope that does not require proteasome cleavage (e.g. from a minigene).

**We agree that this would be a very good control. However, we performed a careful characterization of toxic effects of epoxomicin on BMDCs in a recent publication from our lab (Lu, Q et al. Nat Comm, 2018). No untoward effects were found at concentrations as high as 800nM. In the experiments reported the highest dose of epoxomicin used for cell-based assays was 200nM.**

4. The background (no antigen) is not shown in any of the antigen presentation experiments and should be shown or put in the legend. (minor point)

**The figures below show examples of titrations of latex beads in 293T.FcR.K$^b$ cells and BMDCs which include the 'no antigen' control. These data are representative of the experiments in the manuscript. In addition, the 293T cell figure shows that the level of stimulation in the absence of FcR expression is virtually zero throughout the titration.**






5. In Figure 1, why are the dominant negative constructs only shown in 293T cells but not in the dendritic cells? (minor point)

**We did not use this approach for BMDCs because even with the inducible system expression of dominant negative Rab5A and Rab22A perturbed the differentiation of BMDCs. The cellular morphology was altered and the viability was reduced compared to control cells. To simplify the data and interpretation we chose to remove the dominant negative construct in 293T cells from the revised manuscript.**

6. In Fig 2, the proteasome inhibition of 293T cells is partial (and this may also be true in DCs but this is hard to tell without knowing the background in these assays). Is greater inhibition seen in 293T cells at higher but non-toxic doses of epoxomicin? Is there a proteasome independent component of antigen presentation?.

**We tested the proteasome dependence of cross-presentation by titrating the dose of epoxomicin to reduce any effects dependent on the inhibition of vital cellular processes, and it is likely that we are not inhibiting all the available pool of proteasomes. Indeed, we cannot rule out the possibility that there is a proteasome-independent component**.

7. In Fig 3A&B, it is unclear how the calculation of percent is done for the Rab groups. Is the comparator a control transfected cell without the TAP inhibitor, a Rab overexpressing cell without the TAP inhibitor, or a control cell with the TAP inhibitor. This should be clarified. (minor point)

**We apologize for the lack of clarity in explaining the analysis. We have made changes to the main text (page 12) that should improve this, and we have also included raw data from a representative experiment (Fig EV3). The comparisons are of cross-presentation by cells co-expressing the Rab mutant plus the viral inhibitor to that of cells expressing the same Rab mutant plus LacZ as a control.**

**Since the Rab mutants enhance cross-presentation to different extents, and because there is some variability in the absolute values, cross-presentation of cells co-expressing each of the Rab mutants plus LacZ is set to 100% to allow us to combine data from multiple experiments.**

8. In Fig 4G the authors should provide (at least in the legend) data on the levels of substrate hydrolysis without proteasome inhibitor since the substrate is not proteasome-specific (i.e. not just give the fold-increase). (minor point).

**Rather than disrupting the overall figure or complicating the legend we chose to include the raw data for this experiment as a supplemental Table S1. The conclusions remain unchanged.**

9. In Fig 5A, the results of the peptide titration are odd in that there are higher levels of H-2Kb with 2uM peptide compared to 8uM peptide? The authors should check that there is not a labeling error (minor point) .

**We thank the reviewer for bringing this to our attention. We actually missed it. The difference in surface $K^b$ levels with 2$\mu$M SIINFEKL versus 8$\mu$M SIINFEKL is reproducible and not because of a labeling error. We are unsure of the reason, but it may be a result of higher concentration of DMSO in the culture medium correlating with increased peptide concentration. However, the results are internally consistent at both concentrations and do not affect the take home message of the experiment.**

2nd Editorial Decision 27th November 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by the original referees, whose comments are shown below.

As you will see, while referee #1 and #2 find that their criticisms have been sufficiently addressed, referee #3 points to one unresolved issue. In particular, s/he requests you to test the specificity of vesicular proteasome staining by EM (e.g. by anti-LMP2 immuno-gold labeling of LMP2-null cells). I agree with the referees that the issue of antibody specificity is important and would be good to address. I would anticipate that you should be able to address this issue in a good way. Let me know if we need to discuss this further.

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REFEREE REPORTS.

Referee #1:

In the revised version, Sengupta and co-authors have provided some additional evidence strengthening their conclusions. Important questions remain to be answered such as how proteasome is acquired by endosomes, the demonstration of actual MHC-I loading with peptides produced by intraluminal proteasome complexes, and an evaluation of the relative contribution of this pathway in different cell types. However, the evidence presented is sufficient to make a strong point for a new pathway that likely explains previous reports of TAP-independent, proteasome-dependent cross-presentation. This very interesting but somewhat provocative paper is suitable for Embo J and will certainly inspire exciting new research and discussion on cross-presentation.

Referee #2:

The authors performed additional experiments and introduced new elements of discussion in their manuscript, making it more thorough. Although I am not entirely convinced about the significance of their findings (e.g. what is the relative contribution of proteasome inside and outside of phagosomes?), I feel that publication of the manuscript will challenge the field and promote more work to address this important issue. Accordingly, I think that the manuscript should be published in EMBO J.

Referee #3:

One of the significant concerns I had raised in my previous review of this manuscript was whether proteasomes were found in vesicles of normal cells and not just in the abnormal vacuoles that were present after manipulation of Rab protein expression. In revision, the authors have attempted to address this point by performing immuno-electron microscopy. This is a good approach and the analysis clearly shows anti-proteasome antibody labeling in vesicles. However, it is very surprising that it appears that there is almost little-to-no proteasome-labeling outside of vesicles. This is very surprising because proteasomes are very abundant in the cytosol, as is well known and also shown in their immunofluorecence analysis of their Rab-manipulated cells; In fact, one would think that proteasomes would be present in the cytosol at much higher abundance than in vesicles. This raises the specter of an artifact (antibody cross-reactions with an endosomal protein or other problems) in their immuno-gold staining of cell sections. The authors need to resolve this issue. Perhaps anti-LMP2 immuno-gold labeling of LMP2-null cells might help. If and when this issue is satisfactorily resolved I would recommend publication.

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Additional Author correspondence 28th November 2018

EMBOJ-2018-99266R - Response to reviewers.

We appreciate the generally positive sentiments regarding the manuscript, and are grateful to reviewers #1 and #2 for their consensus that the manuscript should be accepted.

Regarding reviewer #3, the major concern is that cytosolic proteasomes are not dominant in the immunoelectron microscopy images we submitted and that antibody cross-reactivity with an endosomal protein may be responsible for the endolysosomal detection staining. However, we get the same pattern with three different rabbit antisera, to b5 and 19S S2 in Fig. 5 and to a5 in Fig. S6, and the presence of such a cross-reactive antibody in each seems unlikely. The precise experiment suggested, to use BMDCs from LMP2 knockout mice, is a good one but problematic because we do not have these mice in the laboratory. However, we have data that I believe should satisfy the reviewer.

The reviewer points out that the immunoelectron microscopy images we provided do not show significant proteasome labeling in the cytosol of BMDCs, where they should be readily detectable. However, the images were chosen to emphasize the presence of proteasomes in endolysosomal compartments, which was the unexpected result that we thought needed to be convincingly established. These images were of sections double labeled with anti LAMP1 antibodies to establish that the vesicles identified were indeed endolysosomes. We chose to detect the proteasome antibodies with 15nm gold particles and LAMP1 antibodies with 5nm gold particles to better emphasize the proteasomes. Our e.m. collaborators tell us that larger particles are more easily removed during the washing stage between proteasome staining and the follow-up staining for LAMP1 and that this may play a role in the limited cytosolic proteasomal staining observed. Nevertheless, whatever the explanation we do have images of BMDCs that are singly labeled with the same anti-proteasome antibodies that clearly show cytosolic staining and two of these are attached as a TIFF. In these examples b5 is detected with 15nm gold particles and 19S S2 with 10nm gold particles. We suggest including these images as a supplemental figure and believe that this should satisfy the reviewer.

Additional Editor correspondence 6th December 2018

Thank you for sending me your point-by-point response to referee #3. I have now read it and also consulted with him/her.

The referee finds that the EM results are critical for the main message of the study. In lights of your response, s/he recommends that you provide some further revision as follow:

" 1.The inclusion of a rigorous specificity control, such using the anti-LMP2 or 5 antibodies in cells lacking these subunits (as I had previously suggested) would be ideal. This may not be easily done since the authors don't have these mutant cells (perhaps they could try 721 vs 721.174 B cells, which they have - although this might not be informative if B cells differ from dendritic cells and lack intravesicular proteasomes) and it is unknown to me if there are anti-LMP2 or 5 antibodies that will work in immunoEM. If the authors can't or won't include the above suggested control, then at least they should provide the additional things below.

2. Include a description of the basic staining specificity controls noted above (staining with an irrelevant matched antibody, second step reagent + gold without the primary antibody, etc.) and that these gave the expected negative results (data does not need to be shown).
3. I presume the authors examined many images of their immune-EM studies. Ideally, they should include quantitative data: % vesicles containing proteasomes, and some quantitative measure of staining of vesicles vs cytosol and nucleus. Minimally, they should include more images (these could be in the supplemental data) that show the spectrum of results - this will hopefully show that odd staining in single images are not generally seen. If there are "odd" results, such as the ones I have pointed out, they should be described, discussed, and appropriate caveats stated.

4. New minor point: The authors should probably cite the 2015 Baumeister Science paper (347:439) on the molecular census of proteasomes in cells as assessed by EM tomography. The cell atlas image that is displayed in this paper does not show intravesicular proteasomes. This may be a neuron vs dendritic cell difference or a limitation of a single picture. However, since it is an opposite result to the present study, it should probably be mentioned."

Please do not hesitate to contact me should you have any question about the specific points requested by the referee. I will be happy to further discuss these with you.

2nd Revision - authors' response 2nd May 2019

**Response to Reviewer #3**

**1.The inclusion of a rigorous specificity control, such using the anti-LMP2 or 5 antibodies in cells lacking these subunits (as I had previously suggested) would be ideal. This may not be easily done since the authors don't have these mutant cells (perhaps they could try 721 vs 721.174 B cells, which they have - although this might not be informative if B cells differ from dendritic cells and lack intravesicular proteasomes) and it is unknown to me if there are anti-LMP2 or 5 antibodies that will work in immunoEM. If the authors can't or won't include the above suggested control, then at least they should provide the additional things below.**

To demonstrate the specificity of the LMP2 antibody in immunoelectron microscopy we used MEF cells derived from LMP2 knockout mice as a negative control (Fig 5A). We compared the efficiency and distribution of LMP2 labeling of MEFs derived LMP2 knockout mice with MEF cells from wild type mice as well as wild type BMDC. These data are presented in Fig 5B.

**2. Include a description of the basic staining specificity controls noted above (staining with an irrelevant matched antibody, second step reagent + gold without the primary antibody, etc.) and that these gave the expected negative results (data does not need to be shown).**

The above experiment addresses this concern.

**3. I presume the authors examined many images of their immune-EM**

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**studies. Ideally, they should include quantitative data: % vesicles containing proteasomes, and some quantitative measure of staining of vesicles vs cytosol and nucleus. Minimally, they should include more images (these could be in the supplemental data) that show the spectrum of results - this will hopefully show that odd staining in single images are not generally seen. If there are "odd" results, such as the ones I have pointed out, they should be described, discussed, and appropriate caveats stated.**

We have quantified the distribution of LMP2, $\alpha$-5, $\beta$-5 and 19S S2 subunits of the proteasomes, with respect to the LAMP1 positive vacuoles (FIG 5 B, D, E). We find that 10%-15% of proteasomes localize to the LAMP1 vesicles (Fig 5E). For quantification we captured 22-23 frames of images for each cell type labeled with each of the proteasome subunit antibody, and five representative images of each are presented in appendix Fig. S3. Again, the analysis is consistent with the hypothesis that proteasomes gain access to the endolysosomal lumen.

**4. New minor point: The authors should probably cite the 2015 Baumeister Science paper (347:439) on the molecular census of proteasomes in cells as assessed by EM tomography. The cell atlas image that is displayed in this paper does not show intravesicular proteasomes. This may be a neuron vs dendritic cell difference or a limitation of a single picture. However, since it is an opposite result to the present study, it should probably be mentioned."**

We thank the reviewer for bringing this paper to our attention and we have now cited it in our discussion. The publication makes our observations even more surprising and interesting. We also agree that there might be cell-type dependent differences in proteasome localization, especially in dendritic cells, which maintain a low degradative environment in their endolysosomal compartments: this may allow proteasomes to survive and be active in such compartments in dendritic cells, a topic we are currently pursuing.

3rd Editorial Decision 15th May 2019

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Thank you for submitting a revised version of your manuscript.

As you will see, referee #3 finds that his/her remaining concerns are sufficiently addressed and recommends the manuscript for publication. However, before we can officially accept the manuscript, there are a few editorial issues concerning text and figures that I need you to address.

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REFEREE REPORTS.

Referee #3:

The revised manuscript addresses my concerns. I recommend acceptance for publication.

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Corresponding Author Name: Peter Cresswell



Journal Submitted to: EMBO journal

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## 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 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.

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)

| Question | Answer |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | N/A |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | N/A |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | N/A |
| 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. | N/A |
| For animal studies, include a statement about randomization even if no randomization was used. | N/A |
| 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. | N/A |
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| 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. | The details of the analysis are described in the figure legends. For most of experiments the results are mean of triplicates of representative experiment or mean of independent experiment. |
| Is there an estimate of variation within each group of data? | Error bars are shown. |
| Is the variance similar between the groups that are being statistically compared? | N/A |

### C- Reagents

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | Yes |
| 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 were generated in the lab or primary cells were used. |

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### 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. | Reported in the methods section. |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | Reported in the methods section. |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | Mice were not subjected to experiment. Mice were only used as the source material. |

### E- Human Subjects

| | |
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| 11. Identify the committee(s) approving the study protocol. | N/A |
| 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. | N/A |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | N/A |
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### 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'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | N/A |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | N/A |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | N/A |
| 21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | N/A |

### G- Dual use research of concern

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|---|---|
| 22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | No |