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Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β

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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 12 May 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, all three referees consider the study as interesting and important in principle, but raise a number of major concerns regarding the conclusiveness of part of the data. I will not repeat all individual points here, but one concern is that, in part of the data, rather small differences/effects are observed. Related to this, the referees think that more rigorous data quantification and statistical analysis, in particular of the immunofluorescence data, will be needed. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, in which the referees' concerns need to be addressed in an adequate manner and to their satisfaction. Please do not hesitate to contact me at any time in case you would like to discuss any aspect of the revision further.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing

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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 your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors have addressed an important issue, however, a number of issues will have to be resolved in order to publish this work. The concerns follow.

Major issues
Fig 4C is misleading! The merge is incorrect because they reduced the red channel in the merge.
- Fig 5C is misleading! The inset is incorrect, because signals are completely blown up compared to the original signal. How can I still believe their measurements after these changes?
- They only looked at GORASP2 (GRASP55). It is very strange that they did not comment on the existence of another GRASP. They should mention it and investigate the role of GRASP65.
- They should check in all knockdowns that the expression levels of IL-1beta and IL-18 are not affected.
- Fig 3A, is IL-1beta in or on a vesicle? -> protease protection.
- Fig 1A, Atg5 knockouts can still secrete a lot of IL-1beta. How is this possible?
- Does NEM block secretion? Does it accumulate IL-1beta vesicles?
- Does nigericin cause cell death? Is actin or LDH released?

Minor issues
- Show a better blot in Fig 3A.
- Fig 5C. The authors state that "GRASP is juxtaposed to LC3", but since both proteins are scattered throughout the cell this does not mean anything.

- Fig 5C, is GRASP still localized at the Golgi?
- How was Fig 6F quantified?

Referee #2 (Remarks to the Author):

The authors proposed a direct link between autophagy and secretory pathway, using secretion of IL1B and IL18 as a model of unconventional type of secretion, independent on ER targeting signal peptide. Using murine bone marrow macrophages, they have shown that nigericin (inflammasome agonist) and Alum perform better in combination with starvation and mTOR inhibition, as more IL1B was secreted upon LPS stimulation. Using Atg5 Fl/Fl LysM Cre BMMs they demonstrated that increase in Il1B secretion is Atg5 dependent, and further on the involvement of LC3, Rab8 and Exocyst in secretion of IL1B was proposed by immunofluorescence co-localization of IL1B with Rab8, LC3 and Sec6 respectively. The authors show that inhibition of cathepsin B and GRASP silencing negatively regulate secretion, as well as silencing of ASC, NLRP3 and Rab8. In the end, they named the whole process "autosecretion", and indeed they show that it is not specific only for IL1B and IL18, but that HMGB1 secretion (as non-caspase-1 substrate) is also increased upon same treatments.

This reviewer finds the idea of secretory pathway-autophagy crosstalk interesting, yet work in this paper is preliminary, many experiments are not adequately performed and conclusions are not very strong.
Major comments:

1. The authors mention several times "basal autophagy" as negative regulator of IL1B secretion (e.g.Fig S2A), but they never provide corresponding LC3 blot which would give information how basal it is, and is there basal autophagy or it is not significant. If authors show more secreted IL1B from Atg5 KO BMMs upon nigericin treatment(FigS2A), this does not mean that basal autophagy plays role, ruther specifically induced autophagy upon inflammasome activation which can not be driven in starved conditions where starvation induced autophagy takes place-as they show in Fig1A. Starvation pushes up IL1B secretion in WT BMMs as well as Atg5KO- what connects these two things authors did not seek to answer. This should be clarified in more details.

2. The authors provided very weak data on Exocyst involvement in process they named "autosecretion". Showing one picture of colocalization with Sec6, not quantified properly, they made a too early conclusion. If exocyst plays role in "autosecretion" more subunits of complex should be considered, in silencing experiments as well as in IF.
Could they rescue the secretion by expressing Rab8 exogenously? What happens if Rab8 in GDP vs. GTP locked state is expressed? How do they explain secretion pathway of IL1B in terms of published story on Exo84 and RalB regulating ULK1 and Beclin/Vps34 complex (Bodemann et al.)?

3. The authors show opposite data than Harris et all.(2011), and they do not give clear explanation. They wanted to propose a direct link, but there is no evidence in their data for any direct link, neither towards autophagy machinery, neither towards secretory pathway.

Additional comments:
1. Authors do not show error bars in many figures: (Fig. 1. B and C; Fig 2.B and Fig B and G.), suggesting that experiment was not done in appropriate number of repetitions-only once.

2. Authors do not indicate anywhere the number of cells they analyzed in all immunofluorescence co-localization experiments - suggesting the Pearson correlation coefficient was calculated from only one cell pointed in the picture, and if so, the analysis is completely inappropriate. The description: s.e.m n{greater than or equal to}3 is not clear, neither logic for such a type of experiment (IF quantification). Did they calculate Pearson coefficient for 3 cells in 3 experiments, or what?

3. Authors do not show any WB of caspase-1 activity, which I find extremely important if one wants to demonstrate inflammasome activity. Specially because authors show that HMGB1 can also be secreted via "autosecretion".
Also, experiments on IL1B would be much stronger if followed by corresponding blot showing Pro-IL1B and IL1B.
Atg5 WB was not provided anywhere, though Atg5 Fl/Fl Cre+/- BMMs were used in several experiments. Cathepsin B blot shoud be also shown.

4. In Fig.1A I would like to see t-test P value for IL1B; Atg5 Fl/Fl Cre-;full media; nigericin and Atg5 Fl/Fl Cre+; starvation; nigericin. Just by looking at the graph, difference is quite high-pointing that Atg5 is not essential for starvation induced increase in secretion of IL1B. This seems obvious.

5. The purpose of experiment in Fig.3A is not clear, final conclusion is confusing. If they wanted to support link to autophagy by looking at the co-fractionation of pro-IL1B with p62, I find much more important that they have focused themselves more on Atg5. Authors could also provide IF pictures of IL1B and Atg5, followed with proper quantification.

6. Authors show that difference in IL1B secretion in p62 wt and p62 KO BMMs is non-significant, yet still this does not disprove possible degradation of IL1B via autophagy.
Authors should either exclude Fig. 3 or explain better the purpose. Also, IL1B input signal is very weak, p62 band appears in broader fractions upon nigericin treatment. Does this suggest that nigericin treatment induces more aggregates? Is p62 colocalized on those vesicles containing LC3

and IL1B? If so, what is the role of p62 in such a type of autophagy which targets specifically pro-IL1B?

7. Authors tried to point the role of GRASP in secretion of IL1B. Authors silenced GRASP and noticed effect on autophagy flux and IL1B secretion. Is this specific and direct effect?

Referee #3 (Remarks to the Author):

Manuscript: Autophagy-based autosecretory pathway for extracellular delivery of IL-1ß
Dupont et al

At first glance this paper appears well controlled and thorough. However, it is apparent that a lot of the differences they report are small to the point of questioning whether they are biologically relevant. Their immunofluorescence studies are quite nice but lack quantitation so you are again wondering whether the observations have biological relevance.

Major comments:
* The data listed below are of questionable biological relevance due the small relative differences:
- Pharmacological induction of autophagy by mTOR inhibition with p242 increased secretion of IL-1b (Figure 1C; 40 vs 55 pg/mL and 35 vs 65 pg/mL - no error bars or statistics)
- Data in Figure 2A (approx. 1000 vs 550 pg/mL) and 2B (275 vs 250 pg/mL; no error bars or statistics)
- Data in Figure 4B (approx. 350 vs 225 pg/mL)
- Data in Figure 6B (approx. 50 vs 33 pg/mL; no error bars)
- Data in Figure 6G (approx. 52 vs 28 pg/mL; no error bars)
The authors MUST conduct statistical analyses and perhaps analyze more samples to produce more robust data!
* Figure 3B appears to be normalized to something (depicts % IL-1ß (pg/mL) but the figure does not specify what this normalization is, is it normalized to controls for both wt and p62-/- or to levels observed in nigericin stimulated wt cells as it appears to be. The data in Figure 1E also appears to be normalized. This is concerning because the authors have not detailed how this normalization was done (it appears that all samples have been normalized to levels observed in nigericin-treated scrambled controls) and also implies that they are trying to hide the REAL differences (conceivably a difference of 10 pg/mL could yield a 50% difference if the levels are very low). The authors MUST provide the raw values in pg/mL.
* For ALL studies, as the authors postulate that autophagy induction regulations SECRETION of IL-1ß/IL-18, it is critically important to measure, A. IL-1ß/IL18 transcription B. the intracellular levels of IL-1ß/IL-18. For example, for the data in Figure 2E, it needs to be shown that transcription/mRNA levels are comparable and intracellular levels of both pro-IL1- and mature IL-1ß are increased (or perhaps comparable if the proteins are degraded by alternative mechanisms) in nigericin/starvation CA-074 Me treated cells vs nigericin/starvation control treated cells? Intracellular IL-1ß/IL18 levels could be measured either by western blot analysis of cell lysates or by FACS (and for western blot analysis it would be nice to compare the levels in the lysate to those in the corresponding supernatants).
* It would be nice if the authors had performed their studies with at least two independent inflammasome-activating stimuli. For some reason and despite confirming in the first figure that starvation potientiates IL-1ß release following exposure to Alum, they use nigericin as the sole inflammasome stimulus for all subsequent experiments. Please repeat a subset of critical experiments (Figures 2, 3 and 6) using at a minimum, one additional independent inflammasome-activating stimulus.
* Given the extensive use of this technique by the authors, a more thorough explanation of the relevance of the 'line tracing analysis of fluorescence signal intensity' is required. For example, what change in intensity is classified as positive? Also, it needs to be described in the methods how these graphs were derived, how many independent sections were analyzed etc. It would also be nice if these images were larger (you can not discern a thing in Figure 4A) and some estimation of how often different proteins co-localize needs to be provided (derived from analysis of multiple images/fluorescent peaks, e.g. % LC3+ puncta that are also IL-1ß+) - does this degree of localization change +/- autophagy induction and +/- inflammasome activation?
* Biochemical binding studies would vastly improve and support the immunofluorescence

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colocalization studies. For example, can the authors immunoprecipitate LC3 and see pro-IL-1ß associated or can the authors immunoprecipitate Rab8a and see IL-1ß associated etc?
* Conversely, p62/IL1- immunofluorescence colocalization studies +/- nigericin (or other inflammasome activators) are warranted to support the data in Figure 3 - it is somewhat surprising that the authors did not do this given the reliance on this technique for other analyses. Can the authors demonstrate differential localization of pro-IL-1ß vs mature IL-1ß with p62? (to support the contention that autophagasomes provide a milleu that catalyzes inflammasome activation/pro-IL-1ß processing).
* The conclusion that, "GRASP...in resting cells was mostly localized aligned (? needs some editing) within the perinuclear Golgi (Figure 5A)..." is not supported by any staining data - the authors need to provide colocalization data to make this conclusion.
* Why haven't the authors determined what happens to GRASP when cells are exposed to nigericin + starvation? This is a glaring omission given that their main point is that it is only in the context of inflammasome activation and autophagy induction (not basal autophagy) that IL-1ß secretion is augmented - is GRASP activity increased during starvation conditions? (that would presumably be responsible for the secretion of IL-1ß). Similarly, the data presented in Figure 6 relates to the effect of GRASP knock down on basal autophagy - it would have been appropriate to perform these analyses with cells that exposed to nigericin + starvation.
* An alternative interpretation of the data in Figure 6F (to the one provided by the authors that knock-down of GRASP impacts on autophagasome INITIATION but not MATURATION) is that there is actually decreased RATES of maturation in GRASP knock-down cells (how many cells were analyzed?) as you have a less autophagasomes to start with. If the rate of maturation is the same that you may expect less GFP-RFP+ puncta in the GRASP knock-down in comparison to the scrambled control. Having said that, the numbers are so low for GFP-RFP+ that it would be hard to see any difference, so generally this data is pretty weak.
* The GRASP/LC3 colocalization data is quite poor - the idea that you can analyze, 'partial pixel overlap' and arbitrarily define it as greater than 10% and then calculate significance (Figure 5E; I actually can not find reference to this in the text) is pushing it a little. This data should be removed.
* The HMGB1 data is quite out of place and actually in my opinion impacts negatively on their primary conclusion that it is the interplay between inflammasome activation (which presumably functions to increase the intracellular pool of mature IL-1ß) and autophagy induction that regulates IL-1ß secretion. The fact that the authors state that HMGB1 is, "...protein not connected to proteolytic processing in the inflammasome..." begs the question of why they co-treated the cells with nigericin and starvation - do you still see an increase with starvation alone, is nigericin affecting inflammasome-independent processes?
* For all siRNA experiments, were the results confirmed using more than one independent siRNA? If so, this data needs to be presented in supplementary form, if not, it needs to be done.

Minor comments:
-The authors need to specify how IL-1ß/IL-18 levels were measured, there is no description in the methods whether this was by ELISA and I assume they are measuring levels in the tissue culture supernatants - this needs to be clarified for all experiments.
-What is, "G; means and range" referring to in the legend to figure 3?
-Last paragraph p6 - this should reference Figure 4A not 3A.
-The authors need to show evidence of efficient deletion of Atg5 in the cre+ animals.
-It needs to be clarified in the figure legends for all IL-1ß immunofluorescence whether the staining is referring to pro-IL-1ß or mature IL-1ß.

1st Revision - authors' response 23 August 2011

Below (a-c) is the overall summary of the revisions, followed by our detailed point-for-point response to reviewers' criticisms:
(a) As requested by the editor, additional statistical analyses were carried out throughout. We now explain that both the old and the new Pearson's colocalization coefficient graphs have been derived from 3 independent experiments (5 fields/experiment for a total number of fields 15), which is now clarified in the legends. The error bars in graphs are standard deviations (mislabeled in the original legends –our apologies - as standard errors of the mean; now corrected in figure legends), which should additionally attest to the robustness of the data.

(b) Main experimental additions in response to reviewers' requests are as follows: (i) Autophagyenhanced IL-1ß secretion is now affirmed by immunoblots of the secreted-into-the-medium mature IL-1ß and capase-1; (ii) IL-18 secretion has been firmed up statistically; (iii) in addition to nigericin, silica has been used throughout as an independent inflammasome inducer; (iv) the use of exogenously expressed Rab8a is now added to the previously shown siRNA knockdowns; (v) Pearson's colocalization coefficients (3 independent experiments, 5 fields per experiment -now specified in figure legends) are included throughout; (vi) both GRASP55 and GRASP65 have been examined; (vii) HMGB1 has been studied in greater detail (now in a separate figure); (viii) expression controls for proIL-1ß under various treatment conditions have been included; (ix) extensive LDH controls for release into the medium due to cell death or membrane damage are now included ruling out this as a potentially contributing source to autophagy-based enhancement of IL-1ß secretion; and (x) localization of GRASP55 (the mammalian GRASPs that is responsible for the majority of effects) relative to the Golgi and LC3 has been examined in greater detail. The results of these extensive and probing additional experiments are consistent with and further extend our original conclusions.
(c) The above changes resulted in: (i) a total of 25 new figure panels containing new data (Fig. 1B,E,H; Fig. 2C,G; Fig. 3C,F; new Fig. 4; new Fig. 6; Fig. S1A,E,F; Fig. S2A-D; Fig. S3D; Fig. S4C,D,G; Fig. S5C; new Fig. S6); (ii) deletion of old Fig. 3 as recommended by one of the reviewers; and (iii) revision, replacements, or relocation to the supplementary data of a number of datasets in compliance with reviewers' criticisms, including Figs. 1C,F; Fig. 2B; Fig. 6A; and Fig. S1B.

The following is our point-for point response to editor's and reviewers' comments.

Editor
One concern is that, in part of the data, rather small differences/effects are observed. Related to this, the referees think that more rigorous data quantification and statistical analysis, in particular of the immunofluorescence data, will be needed.

(i) We have added further repeats to experiments where statistical analysis was not performed (Figs. 1C,F; 2B; 6A;S5B) and determined additional Pearson's coefficients for immunofluorescence colocalization analysis (Figs. 3C; 4D; S4C,G). (ii) All Pearson's coefficients for immunofluorescence colocalization have been calculated from 3 independent experiments, examining for each experiment 5 fields with one or more cells (Figs. 1L; 3C; 4D; S3C; S4C,G). (iii) Regarding the robustness of the data, error bars shown in all graphs represent standard deviations (with the exception of Fig. S2A-D) not standard errors. We apologize for the mistake on our part of not indicating that these were standard deviations, and have corrected the legends throughout.

Referee #1:
The authors have addressed an important issue, however, a number of issues will have to be resolved in order to publish this work. The concerns follow.
Major issues
1. Fig 4C is misleading! The merge is incorrect because they reduced the red channel in the merge.

We thank the reviewer for the observation. The panel in question, containing LC3-Sec6-IL-1ß colocalization has been replaced with new images and moved to the supplement (now Suppl. Fig. S4E) along with other Sec6 data (p. 7, 2nd paragraph). Furthermore, we now provide, in addition to the previously shown line tracing fluorescence coincidence analysis (Suppl. Fig. S4F,I), a full Pearson's colocalization coefficient statistical analysis derived from 3 independent experiments with 5 fields per experiment (Suppl. Fig. S4G,J).

2. Fig 5C is misleading! The inset is incorrect, because signals are completely blown up compared to the original signal. How can I still believe their measurements after these changes?

We now provide completely new images and data (Fig. 4B-D), showing immunofluorescence analyses (images, line graphs and Pearson's colocalization coefficients) GRASP55 (GORASP2) in response to these (and addressing subsequent) comments by this reviewer.
We have removed the offending figure (although the levels were increased equally for all channels to show detail, we agree with the reviewer and have removed the main panels and insets in

question), and stayed away from providing insets (which were solely intended to facilitate visualization of smaller profiles).

3. They only looked at GORASP2 (GRASP55). It is very strange that they did not comment on the existence of another GRASP. They should mention it and investigate the role of GRASP65.

We agree and thank the reviewer for this key comment. We have addressed both GRASP55 (GORASP2) and GRASP65 (GORASP1) as follows: (i) Fig. 4 shows GRASP55 knockdown effects on IL-1ß secretion and GRASP55 (GORASP2) localization relative to LC3 in control cells vs. nigericinstimulated cells. (ii) A partial knockdown of GRASP65 with the GRASP55 knockdown effects on LC3-II formation is shown in Suppl. Fig. S5C. These experiments are described on p. 7 and 8.

4. They should check in all knockdowns that the expression levels of IL-1beta and IL-18 are not affected.
We now provide data indicating no changes in pro-IL-1ß expression with all relevant knockdowns (Fig. S1, new panels E & F; p. 5, 2nd paragraph).

5. Fig 3A, is IL-1beta in or on a vesicle?
As per reviewer #2 (point 6) request/recommendation these data/figure have been removed.

6. Fig 1A, Atg5 knockouts can still secrete a lot of IL-1beta. How is this possible?
The following explanation has been inserted in the text (p. 7): "We interpret the incomplete reduction in IL-1ß secretion in the absence of Atg5 as a net result of two opposing effects -one described here as a product of positive contribution of induced autophagy on extracellular delivery of IL-1ß and the other being the recently reported negative regulation of IL-1ß secretion by autophagy (Harris et al, 2011; Nakahira et al, 2010; Zhou et al, 2011)."

7. Does NEM block secretion? Does it accumulate IL-1beta vesicles?
We interpreted the reviewer's comment as a reference to N-ethyl maleimide, a reagent that has been used to study SNARE-dependent fusion in vitro. Since our studies were done in vivo (using intact cells), we did not carry out the NEM experiments, given membrane permeability issues and peloitropic effects of this alkylating agent also used to inhibit deubiquitination enzymes.

8. Does nigericin cause cell death? Is actin or LDH released?
Nigericin as well as caspase-1 activation (an event downstream of nigericin action) can cause cell death. So, a possibility that the IL-1ß in the supernatant was the result of cell death and nonspecific release into the culture supernatant was a reasonable issue to consider, and this point is well taken by the reviewer. We now show extensive kinetic analyses of IL- relative to LDH release from cells upon nigericin and silica treatment. We find that IL-1ß secretion precedes LDH release, demonstrating that it is not a result of cell death or membrane permeability issues. This is now shown in Fig. S2A-D, and described in the text (p. 5, 2nd paragraph).

Minor issues
Show a better blot in Fig 3A.
This dataset, the corresponding figure, and the text, have been deleted as per reviewer #2's request. Fig 5C.

The authors state that "GRASP is juxtaposed to LC3", but since both proteins are scattered throughout the cell this does not mean anything.
The images and measurements in new Fig. 4 and description in the text (p. 8, top paragraph) should illustrate these points better.

Fig 5C, is GRASP still localized at the Golgi?
GRASP55 localization relative to the Golgi marker GM130 is shown in new Fig. S6. The majority of GRASP55 remains with the Golgi, but peripheral profiles emerge (Fig. 4B).

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How was Fig 6F quantified?
We apologize for not describing this. Published methods for tandem RFP-GFP-LC3 analysis (differentiating early autophagosomes that are GFP+RFP+ and autolysosomes that are GFP-RFP+ due to acidification sensitivity of GFP and relative insensitivity of RFP) were used and the references are now included (p. 8, 2nd paragraph).

Referee #2:
The authors proposed a direct link between autophagy and secretory pathway, using secretion of IL1B and IL18 as a model of unconventional type of secretion, independent on ER targeting signal peptide.
Using murine bone marrow macrophages, they have shown that nigericin (inflammasome agonist) and Alum perform better in combination with starvation and mTOR inhibition, as more IL1B was secreted upon LPS stimulation. Using Atg5 Fl/Fl LysM Cre BMMs they demonstrated that increase in Il1B secretion is Atg5 dependent, and further on the involvement of LC3, Rab8 and Exocyst in secretion of IL1B was proposed by immunofluorescence co-localization of IL1B with Rab8, LC3 and Sec6 respectively. The authors show that inhibition of cathepsin B and GRASP silencing negatively regulate secretion, as well as silencing of ASC, NLRP3 and Rab8. In the end, they named the whole process "autosecretion", and indeed they show that it is not specific only for IL1B and IL18, but that HMGB1 secretion (a snon-caspase-1 substrate) is also increased upon same treatments.
This reviewer finds the idea of secretory pathway-autophagy crosstalk interesting, yet work in this paper is preliminary, many experiments are not adequately performed and conclusions are not very strong.

Major comments:
1. (a)The authors mention several times "basal autophagy" as negative regulator of IL1B secretion (e.g.Fig S2A), but they never provide corresponding LC3 blot which would give information how basal it is, and is there basal autophagy or it is not significant.
This is now documented in Fig. S1A. If one compares LC3-II conversion in Atg5Fl/Fl Cre- with Atg5Fl/Fl Cre+, there is basal LC3-II in Cre- but not in Cre+ cells.

(b) *If authors show more secreted IL1B from Atg5 KO BMMs upon nigericin treatment (FigS2A), this does not mean that basal autophagy plays role, rather specifically induced autophagy upon inflammasome activation which can not be driven in starved conditions where starvation induced autophagy takes place-as they show in Fig1A. Starvation pushes up IL1B secretion in WT BMMs as well as Atg5KO- what connects these two things authors did not seek to answer. This should be clarified in more details.*

Basal autophagy is necessary, as shown by several groups (Harris et al, 2011; Nakahira et al, 2010; Zhou et al, 2011), to prevent (not to induce, but to suppress) spontaneous inflammasome induction. What we show here is that, unlike the basal autophagy (which counteracts inflammasome activation), induced autophagy (e.g. by starvation) acts to promote (and not suppress) inflammasome-dependent IL-1ß secretion. We now include, as requested by the reviewer, the following explanation in the text (pp. 6-7): "We interpret the incomplete reduction in IL-1ß secretion in the absence of Atg5 as a net result of two opposing effects -one described here as a product of positive contribution of induced autophagy on extracellular delivery of IL-1ß and the other being the recently reported negative regulation of IL-1ß secretion by autophagy (Harris et al, 2011; Nakahira et al, 2010; Zhou et al, 2011).

2. The authors provided very weak data on Exocyst involvement in process they named "autosecretion". Showing one picture of colocalization with Sec6, not quantified properly, they made a too early conclusion. If exocyst plays role in "autosecretion" more subunits of complex should be considered, in silencing experiments as well as in IF. Could they rescue the secretion by expressing Rab8 exogenously? What happens if Rab8 in GDP vs. GTP locked state is
How do they explain secretion pathway of IL1B in terms of published story on Exo84 and RalB regulating ULK1 and Beclin/Vps34 complex (Bodemann et al.)?

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The following has been done to address these important questions raised by the reviewer: (i) We show new data (Fig. 3F) with overexpression of exogenous Rab8, as requested by the reviewer. Expression of Rab8aS22N (dominant negative form) inhibited IL-1ß secretion (new panel, Fig. 3F), in keeping with the siRNA data (Fig. 3D), as stated in the text (p.7). (ii) Our repeated attempts to knockdown Sec6 have not resulted in a successful downregulation in macrophages. We also attempted to knockdown Exoc1 (Sec3), Exoc8 (Exo84), Exoc4 (Sec8) with different protocols (single transfection 48 h; single transfection 72 h; and double transfection 48 h). We interpret this as a potentially vital role of the exocyst in macrophages. Accordingly, we have moved Sec6 localization data to the supplement (Suppl. Fig. 4). We underscore however that the Sec6 and IL-1ß colocalization is not sporadic or coincidental, as we have carried out further statistical analysis (3 independent experiments) and the Pearson's colocalization coefficient turned out to be high (near 0.6). This is shown in Suppl. Fig. S4G and discussed on p. 7)

3. The authors show opposite data than Harris et all.(2011), and they do not give clear explanation. They wanted to propose a direct link, but there is no evidence in their data for any direct link, neither towards autophagy machinery, neither towards secretory pathway.

We thank the reviewer for the opportunity to clarify these issues:
(i) Our data are not the opposite of Harris et al., (Harris et al., 2011)
who have, like others (Harris et al, 2011; Nakahira et al, 2010; Zhou et al, 2011), shown that basal autophagy suppresses IL- 1 secretion. Our data on basal autophagy are concordant with these findings, as our data also show that in full medium (basal autophagy conditions), Atg5Fl/Fl Cre+ cells (autophagy incompetent) produce more IL-1ß (Fig. S2E). This is "the first half" - the negative/suppressing (and indirect -as shown by Nakahira et al., 2010 and Zhou et al., 2011) role of basal autophagy in inflammasome activation and IL-1ß secretion. Thus, our work does not contradict
nor oppose the data by Harris, but affirms these findings instead.
(ii) Our work however exceeds these findings (with which we agree) to reveal another role ("the second half") of autophagy -when it is induced -to promote IL-1ß secretion. It is upon the induction of autophagy, as shown in Fig. 1A, that our data extend past the reports of the negative role of basal autophagy reported by others (Harris et al, 2011; Nakahira et al, 2010; Zhou et al, 2011). The points we are making here are covered by the explanation we included in the answer to the comment 1b above.
(iii) We do provide a direct link between the factors (GRASP) shown to govern unconventional secretion and autophagic apparatus (Fig. 4, new data). We furthermore show that GRASP55 controls autophagy (Fig. 5A-D). Finally, we provide new data showing the specificity of action of mammalian GRASP55 (GORASP2) in its effects on LC3 (new data in Fig. S5C) and on autosecretion (new data in Fig.5A). These new data are covered in the text on p. 8, middle section.

Additional comments:
1. Authors do not show error bars in many figures: (Fig. 1. B and C; Fig 2.B and Fig B and G.), suggesting that experiment was not done in appropriate number of repetitions-only once.

We have now added additional repeats and provide statistics for the experiments in question (now in Fig. 1C,D,E,F,H; Fig. 2B,C; Fig. 6A; Fig. S5B).

2. Authors do not indicate anywhere the number of cells they analyzed in all immunofluorescence colocalization experiments - suggesting the Pearson correlation coefficient was calculated from only one cell pointed in the picture, and if so, the analysis is completely inappropriate. The description: s.e.m n{greater than or equal to}3 is not clear, neither logic for such a type of experiment (IF quantification). Did they calculate Pearson coefficient for 3 cells in 3 experiments, or what?

Both the old and new Pearson's colocalization coefficient graphs have been derived from 3 independent experiments (≥5 fields/experiment for a total number of fields ≥15). This is now clarified in all relevant legends and in Materials and Methods (p. 13).

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3. (a) Authors do not show any WB of caspase-1 activity, which I find extremely important if one wants to demonstrate inflammasome activity. Specially because authors show that HMGB1 can also be secreted via "autosecretion". Also, experiments on IL1B would be much stronger if followed by corresponding blot
showing Pro-IL1B and IL1B.

As requested, blots of cellular pro-IL-1β and secreted mature IL-1β are now shown in new Fig. 1B and results described on p. 5, first Results section.
(b) Atg5 WB was not provided anywhere, though Atg5 Fl/Fl Cre+/- BMMs were used in several experiments.
As requested, Atg5 immunoblot for Atg5Fl/Fl Cre+ and Cre- BMMs is now shown in new Suppl. Fig.S1A, p. 5, top paragraph.
(c) Cathepsin B blot shoud be also shown.
As requested, cathepsin B blot is now shown (both cellular and secreted) in new Fig. 2G, and results discussed on p. 7, top paragraph.

4. In Fig.1A I would like to see t-test P value for IL1B; Atg5 Fl/Fl Cre-;full media; nigericin and Atg5 Fl/Fl Cre+; starvation; nigericin. Just by looking at the graph, difference is quite high-pointing that Atg5 is not essential for starvation induced increase in secretion of IL1B. This seems obvious.

As requested, the P values are specified not only in Fig. 1A but throughout. Please see our detailed explanation in response to the comment #3, and in the text, end of p5 and top of p. 6.

5. The purpose of experiment in Fig.3A is not clear, final conclusion is confusing. If they wanted to support link to autophagy by looking at the co-fractionation of pro-IL1B with p62, I find much more important that they have focused themselves more on Atg5. Authors could also provide IF pictures of IL1B and Atg5, followed with proper quantification.
We agree with the reviewer and have deleted the p62 co-fractionation data. See further explanations under point 6.

6. Authors show that difference in IL1B secretion in p62 wt and p62 KO BMMs is non-significant, yet still this does not disprove possible degradation of IL1B via autophagy. Authors should either exclude Fig. 3 or explain better the purpose. Also, IL1B input signal is very weak, p62 band appears in broader fractions upon nigericin treatment Does this suggest that nigericin treatment induces more aggregates? Is p62 colocalized on those vesicles containing LC3 and IL1B? If so, what is the role of p62 in such a type of autophagy which targets specifically pro-IL1B?

We have now excluded these data, as proposed by the reviewer. We understand that p62 analysis can be easily confused with degradation, and much more work will be needed in future experiments to clearly establish its role in secretion.

7. Authors tried to point the role of GRASP in secretion of IL1B. Authors silenced GRASP and noticed effect on autophagy flux and IL1B secretion. Is this specific and direct effect?

We provide new data narrowing down specificity of the action of mammalian GRASP55 (GORASP2) in autosecretion (new data in Fig.4A). The same specificity has been observed with autophagic induction (Figs 5A,B and S5C), indicating a molecular link between GRASP55 and autophagy. The new and GRASP55-sepcific data are discussed in the text on p.7 and p. 8.

Referee #3:

Manuscript: Autophagy-based autosecretory pathway for extracellular delivery of IL-1β Dupont et al At first glance this paper appears well controlled and thorough. However, it is apparent that a lot of the differences they report are small to the point of questioning whether they are biologically relevant. Their immunofluorescence studies are quite nice but lack quantitation so you are again wondering whether the observations have biological relevance.

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We very much appreciate Reviewer's 3 incisive comments and thank him/her for a very thorough examination of our data and challenging but excellent recommendations for improvement. Below are our point-for-point responses; in this paragraph we present our overall response to the reviewer's general comments: (a) Additional statistical significance analyses were carried out throughout. It is now clarified that both the old and the new Pearson's colocalization coefficient graphs have been derived from 3 independent experiments (≥5 fields/experiment for a total number of fields ≥15), which is now stated in the legends. (b) The error bars in graphs are actually standard deviations (mislabeled – our apologies - as standard errors of the mean; this is now corrected in figure legends), which should attest to a better robustness of the data. (c) We now add immunoblot data (e.g. Fig. 1B) as an independent
ethodology that further support our findings and conclusions. (d) In addition to IL-1β, our additional data extend autophagy-dependent unconventional secretion to HMGB1. We submit to the reviewer that since IL-1β and HMGB1 secretion pathways have remained largely a mystery, our findings, which uncover the previously unappreciated fact that autophagy (and Atg5) control these pathways, are of high biological relevance.

Major comments:
* *The data listed below are of questionable biological relevance due the small relative differences:*
- Pharmacological induction of autophagy by mTOR inhibition with p242 increased secretion of IL-1b
(Figure 1C; 40 vs 55 pg/mL and 35 vs 65 pg/mL - no error bars or statistics)
- Data in Figure 2A (approx. 1000 vs 550 pg/mL) and 2B (275 vs 250 pg/mL; no error bars or statistics)
- Data in Figure 4B (approx. 350 vs 225 pg/mL)
- Data in Figure 6B (approx. 50 vs 33 pg/mL; no error bars)
- Data in Figure 6G (approx. 52 vs 28 pg/mL; no error bars)

We apologize for absence of statistics in some experiments (this has been remedied by additional repeats) and a mistake in error bar definitions - they are standard deviations not standard errors (now corrected in figure legends). Thus the data and differences are more robust than what they might have appeared. The pp242 data have been moved to the supplement; we note that since different concentrations were used these should be taken as repeats).

* *Figure 3B appears to be normalized to something (depicts % IL-1ß(pg/mL) but the figure does not specify what this normalization is, is it normalized to controls for both wt and p62-/- or to levels observed in nigericin stimulated wt cells as it appears to be.*

These data have been deleted, as requested by reviewer #2.

The data in Figure 1E also appears to be normalized. This is concerning because the authors have not detailed how this normalization was done (it appears that all samples have been normalized to levels observed in nigericin-treated scrambled controls) and also implies that they are trying to hide the REAL differences (conceivably a difference of 10 pg/mL could yield a 50% difference if the levels are very low).
Raw data (pg/ml of IL-1β) are now shown instead of normalization, as requested.
* *For ALL studies, as the authors postulate that autophagy induction regulations SECRETION of IL- 1β/IL-18, it is critically important to measure, A. IL-1β/IL18 transcription B. the intracellular levels of IL-1β/IL-18. For example, for the data in Figure 2E, it needs to be shown that transcription/mRNA levels are comparable and intracellular levels of both pro-IL1-ßand mature IL-1ßare increased (or perhaps comparable if the proteins are degraded by alternative mechanisms) in nigericin/starvation CA-074 Me treated cells vs nigericin/starvation control treated cells?*
Intracellular IL-1β/IL18 levels could be measured either by western blot analysis of cell lysates or by FACS (and for western blot analysis it would be nice to compare the levels in the lysate to those in the corresponding supernatants).

As requested by the reviewer pro-IL1β mRNA levels have been measured and no differences in expression have been observed (Figs. S1E, S3D, S4D; p. 5, middle paragraph, p. 7, top and middle paragraphs). In addition, pro-IL-1β protein levels did not change (Fig. S1F; p. 5, middle paragraph).

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** It would be nice if the authors had performed their studies with at least two independent inflammasomeactivating stimuli. For some reason and despite confirming in the first figure that starvation potientiates IL-1ßrelease following exposure to Alum, they use nigericin as the sole inflammasome stimulus for all subsequent experiments. Please repeat a subset of critical experiments (Figures 2, 3 and 6) using at a minimum, one additional independent inflammasome-activating stimulus.*

As requested, we have carried out the key experiments with another inflammasome agonist – silica (Fig. 1E,H; Fig 2C; Suppl. Fig. S2C,D).

** Given the extensive use of this technique by the authors, a more thorough explanation of the relevance of the 'line tracing analysis of fluorescence signal intensity' is required. For example, what change in intensity is classified as positive? Also, it needs to be described in the methods how these graphs were derived, how many independent sections were analyzed etc. It would also be nice if these images were larger (you can not discern a thing in Figure 4A) and some estimation of how often different proteins colocalize needs to be provided (derived from analysis of multiple images/fluorescent peaks, e.g. % LC3+ puncta that are also IL-1β+) - does this degree of localization change +/- autophagy induction and +/- inflammasome activation?*

All key colocalization experiments have now Pearson's colocalization coefficient analysis (3 independent experiments, 5 fields per experiment) in addition to tracings. The tracings (a standard feature built into the standard Zeiss LSM software) are used in a conventional way, and are nothing more than a standard cell biological way of presenting imaging data. Coupled with Pearson's coefficients that have now been derived for all key image analyses, we believe that these concerns have been appropriately and professionally addressed.

** Biochemical binding studies would vastly improve and support the immunofluorescence colocalization studies. For example, can the authors immunoprecipitate LC3 and see pro-IL-1ßassociated or can the authors immunoprecipitate Rab8a and see IL-1ßassociated etc?*

These interesting experiments are beyond the scope of this study. It is a full-scale project for a graduate thesis that the second author on the present manuscript will be carrying out with conclusive results expected in 2-3 years.

** Conversely, p62/IL1-ßimmunofluorescence colocalization studies +/- nigericin (or other inflammasome activators) are warranted to support the data in Figure 3 - it is somewhat surprising that the authors did not do this given the reliance on this technique for other analyses. Can the authors demonstrate differential localization of pro-IL-1ßvs mature IL-1ßwith p62? (to support the contention that autophagasomes provide a milleu that catalyzes inflammasome activation/pro-IL-1ßprocessing).*

These valuable suggestions and experiments are beyond the scope of this study. We have removed p62 data and will carry out the necessary in depth studies (that will take over a year to complete) in future experiments.

** The conclusion that, "GRASP...in resting cells was mostly localized aligned (? needs some editing) within the perinuclear Golgi (Figure 5A)..." is not supported by any staining data - the authors need to provide colocalization data to make this conclusion.*

GRASP55 localization relative to Golgi is now shown in Fig. S6 (p. 8, top paragraph).

** Why haven't the authors determined what happens to GRASP when cells are exposed to nigericin + starvation? This is a glaring omission given that their main point is that it is only in the context of inflammasome activation and autophagy induction (not basal autophagy) that IL-1ßsecretion is augmented - is GRASP activity increased during starvation conditions? (that would presumably be responsible for the secretion of IL-1β). Similarly, the data presented in Figure 6 relates to the effect of GRASP knock down on basal autophagy - it would have been appropriate to perform these analyses with cells that exposed to nigericin + starvation.*

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Additional experiments with GRASP effects (now attributed specifically to GRASP55) have been carried out. These data are shown in new Fig. 4 (p. 8).

** An alternative interpretation of the data in Figure 6F (to the one provided by the authors that knockdown of GRASP impacts on autophagasome INITIATION but not MATURATION) is that there is actually decreased RATES of maturation in GRASP knock-down cells (how many cells were analyzed?) as you have a less autophagasomes to start with. If the rate of maturation is the same that you may expect less GFP-RFP+ puncta in the GRASP knock-down in comparison to the scrambled control. Having said that, the numbers are so low for GFP-RFP+ that it would be hard to see any difference, so generally this data is pretty weak.*

We appreciate the comment by the reviewer. However, the tandem GFP-RFP data are commonly and routinely presented as in our study. We point out that there is no difference in GFP-RFP+ levels and that GFP+RFP+ and Total correspond in full. The statistical analysis is described in the legend and shows appropriate significance. The issues brought up by the reviewer would bring into question about 90% of the current literature published on autophagy. While we appreciate the constructive intentions and concerns, these some of the best assays and measures we presently have in the field of autophagy and represent the current gold standard.

** The GRASP/LC3 colocalization data is quite poor - the idea that you can analyze, 'partial pixel overlap' and arbitrarily define it as greater than 10% and then calculate significance (Figure 5E; I actually can not find reference to this in the text) is pushing it a little. This data should be removed.*

In keeping with the reviewer's comment, we have removed the data in question. In further compliance with the reviewer's criticism, new analyses and standard Pearson's colocalization coefficients are provided (new Fig. 4B-D).

** The HMGB1 data is quite out of place and actually in my opinion impacts negatively on their primary conclusion that it is the interplay between inflammasome activation (which presumably functions to increase the intracellular pool of mature IL-1 ß) and autophagy induction that regulates IL- 1ßsecretion. The fact that the authors state that HMGB1 is, "...protein not connected to proteolytic processing in the inflammasome..." begs the question of why they co-treated the cells with nigericin and starvation - do you still see an increase with starvation alone, is nigericin affecting inflammasome-independent processes?*

New studies have shown that HMGB1, while not proteolytically processed by the inflammasome components, does depend on inflammasome for secretion (Keller et al, 2008; Lamkanfi, 2011; Lamkanfi et al, 2010; Willingham et al, 2009). As surprising as it may appear at first, these links have been made by others, so our data on HMGB1 are not out of place. They do show that autosecretion is a broader process than the typical proteolytic substrates such as IL-1β. We believe that additional data (Fig. 6A and B) enrich the study and that the reviewer will recognize the benefits of this aspect of the present work.

** For all siRNA experiments, were the results confirmed using more than one independent siRNA? If so, this data needs to be presented in supplementary form, if not, it needs to be done.*

The multiple siRNAs used are described in Material and Methods. In response to the reviewer comment, we have carried out additional analyses of potentially relevant off target effects for positive siRNA knockdowns and such effects have now been ruled out (Fig. S1F; p.5).

Minor comments:
The authors need to specify how IL-1ß/IL-18 levels were measured, there is no description in the methods whether this was by ELISA and I assume they are measuring levels in the tissue culture supernatants - this needs to be clarified for all experiments.

In compliance with the reviewer's request this is now addressed in greater detail in Material and Methods. For example: "For all conditions, cell-free supernatants were assayed by Western blots

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after TCA precipitation; for mouse IL-1β p17 (R&D), caspase-1 p10 (Santa Cruz Biotechnology), HMGB1 (Abcam) and Cathepsin B (R&D) or by ELISA for mouse IL-1β (R&D), IL-18 (MBL) and HMGB1 (IBL)."

What is, "G; means and range" referring to in the legend to figure 3?
This figure has been deleted.

Last paragraph p6 - this should reference Figure 4A not 3A.
Thank you. Corrected.

The authors need to show evidence of efficient deletion of Atg5 in the cre+ animals.
This is now shown in Fig. S1A.

It needs to be clarified in the figure legends for all IL-1ßimmunofluorescence whether the staining is referring to pro-IL-1ßor mature IL-1β.
The antibody from Abcam does not discriminate between the pro-IL1β and mature IL-1β, and the experiments were not designed to distinguish the pro-form form the mature form.

References:

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Keller M, Ruegg A, Werner S, Beer HD (2008) Active caspase-°©-1 is a regulator of unconventional protein secretion. Cell 132: 818-°©-831 Lamkanfi M (2011) Emerging inflammasome effector mechanisms. Nature reviews Immunology 11: 213-°©-220

Lamkanfi M, Sarkar A, Vande Walle L, Vitari AC, Amer AO, Wewers MD, Tracey KJ, Kanneganti TD, Dixit VM (2010) Inflammasome-°©-dependent release of the alarmin HMGB1 in endotoxemia. J Immunol 185: 4385-°©-4392

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Willingham SB, Allen IC, Bergstralh DT, Brickey WJ, Huang MT, Taxman DJ, Duncan JA, Ting JP (2009) NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-°©-1 activation, necrosis, and HMGB1 release via inflammasome-°©- dependent and -°©-independent pathways. J Immunol 183: 2008-°©-2015

Zhou R, Yazdi AS, Menu P, Tschopp J (2011) A role for mitochondria in NLRP3 inflammasome activation. Nature 469: 221-°©-225

2nd Editorial Decision 20 September 2011

Thank you for sending us your revised manuscript. After some delay due to problems with the availability of our referees during the past summer holiday season, all three referees have seen the manuscript again, and all three referees are now supportive of publication of the study here. Still, referee 1 puts forward a number of points that still need to be addressed in his/her view before we can finally accept the manuscript. I would therefore like to ask you to deal with these issues in an amended manuscript. Also, I would like to ask you to mention the recent Cell paper on CFTR secretion (http://www.sciencedirect.com/science/article/pii/S0092867411008191) in your discussion section.

In addition there are a number of editorial issues that need further attention:

- An author contributions section needs to be included into the main body of the manuscript text after the acknowledgements section.

- Please add scale bars, together with an explanation for figures 5, S3A, S4E; and an explanation for the scale bar in figure S4.

- In cases where cropping and pasting of lanes has been performed, we require a proper indication (like a white space or a black line between) together with an explanation in the figure legend as a standard procedure. Could you please confirm that all lanes come from the same gel in figure S5C and modify the figure and the legend accordingly? According to our policies, we also need to see the original scans for this panel.

- We have now started encouraging the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

I am sorry to have to be so insistent at this late stage, but we will still do our best to make it for a 2011 publication date.

Thank you very much in advance for your cooperation.

Yours sincerelty

Editor
The EMBO Journal

―――

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The revised version clarifies some of my issues but a large number of key points remain unaddressed. This could be an important paper for the field but the onus is on the authors if they want to publish this work in a highly respectable journal. I have listed my comments as new issues and previously

New issues
- Figure 3A: the blue channel does not match the merged image.

- their new term "autosecretion" (=self-secretion) does not sound appropriate for this process.

- How physiological is secretion via autophagosomes? Why does a cell need to be starved before treatment with nigericin/silica/amyloid fibrils/etc to release IL-1beta? They have Atg5 KO mice, so can they measure whether their IL-1beta serum level is indeed lower in these mice?

Previous major issues
previously: Fig 4C is misleading! The merge is incorrect because they reduced the red channel in the merge.
Now: They replaced the figure, which is now of a much better quality.

previously: Fig 5C is misleading! The inset is incorrect, because signals are completely blown up compared to the original signal.
Now: They replaced the figure with a better one, although this image does not show so much since they removed the insets because of the earlier criticism. I think that insets/zooms are informative, as long as no manipulations are done that are not stated in the legend.

previously: They only looked at GORASP2 (GRASP55). It is very strange that they did not comment on the existence of another GRASP. They should mention it and investigate the role of GRASP65.
Now: They say that GRASP65 could not be knocked down efficiently, which is shown in Fig S5C. Fig S5A and B are unclear. In the text and the figure itself it is mentioned that GRASP55 is knocked down, however, in the figure legend it is said that GRASP65 is knocked down. This is confusing. How can starvation + nigericin (Fig4A) lead to less secretion of IL-1beta (~70pg/ml) than full medium + nigericin (~375 pg/ml, Fig S5A)?

previously: They should check in all knockdowns that the expression levels of IL-1beta and IL-18 are not affected.
Now: They measured it and there are no problematic effects.

previously: Fig 3A, is IL-1beta in or on a vesicle? -> protease protection.
Now: The entire fractionation figure is gone. It is strange that such an informative experiment has disappeared. At least it would be informative to know whether IL-1beta is in an autophagosome or on it.

previously: Fig 1A, Atg5 knockouts can still secrete a lot of IL-1beta. How is this possible?
Now: The authors state the following.
"We interpret the incomplete reduction in IL-1ß secretion in the absence of Atg5 as a net result of two opposing effects - one described here as a product of positive contribution of induced autophagy on extracellular delivery of IL-1ß and the other being the recently reported negative regulation of IL-1ß secretion by autophagy"

My concern. This does not resolve the issue This experiment still suggests that Atg5 is not critically important for IL-1beta secretion. How is IL-1beta secreted in Atg5 KO cells?

Previously: Does nigericin cause cell death? Is actin or LDH released?
Now: They measured it, however the cell death is substantial (~8-~45% for the nigericin). How much % of the IL-1beta of the total is really secreted? What is the % of the IL-1beta in the supernatant minus the % cell death?

TPreviously: Does NEM block secretion? Does it accumulate IL-1beta vesicles?
Now: No such experiment has been done.
"We interpreted the reviewer's comment as a reference to N-ethyl maleimide, a reagent that has been used to study SNARE-dependent fusion in vitro. Since our studies were done in vivo (using intact cells), we did not carry out the NEM experiments, given membrane permeability issues and peloitropic
effects of this alkylating agent also used to inhibit deubiquitination enzymes."

My concern. NEM experiments in intact cells can be done and they have been done before (for instance Zemskov et al, 2011, PLoS ONE).

Previous minor issues
Previously: they should show a better blot in Fig 3A
Now: the entire figure is removed without replacement.

Previously: They say that "GRASP is juxtaposed to LC3", but since both proteins are scattered throughout the cell this does not mean so much. For me it is also unclear at how many cells we are looking in this figure.
Now: Now Fig 4B. The image is a bit improved, however, it is still not very convincing. For instance, where is the rightmost arrow pointing at?

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Previously: Fig 5C, is GRASP still localized at the Golgi?
Now: a GRASP localization has been done in control cells, but not in nigericin treated cells, which was essentially the question. (Figure 5C showed nigericin treated cells in the previous manuscript).

previously: How was Fig 6F quantified?
Now: Now it is shown.

Referee #2 (Remarks to the Author):

The authors have done a very thorough work in successfully responding to all raised issues. There are no further comments.

Referee #3 (Remarks to the Author):

All concerns adequately addressed.

2nd Revision - authors' response 20 September 2011

Thank you for the letter and re-review of our manuscript. We have now addressed the remaining points raised by reviewer 1, complied with editorial requests, and addressed editorial queries. The revised manuscript has been submitted for your consideration.

In summary, 5 figures have modifications and text contains changes as follows: (i) Fig 4B, an arrow in question now points to a different colocalization profile in the same cell (in response to reviewer 1); Figs. 5C and S3A and S4A,E,H have size bars added or more clearly marked (editorial request); Suppl. Fig. S6 now contains image analysis of GRASP55 vs Golgi in cells treated with nigericin instead of untreated (reviewer 1). (ii) Textual changes encompass inclusion of the Cell paper on CFTR in the discussion (editor), and an explanation in Fig. S4A legends for differences in the amounts of IL-1ß in Figs S4A vs S5A - simply different cell numbers in two separate experiments (reviewer #1).

The following is our pint-for-point response to editor's and reviewers' comments:

Editor:

1. I would like to ask you to mention the recent Cell paper on CFTR secretion (http://www.sciencedirect.com/science/article/pii/S0092867411008191) in your discussion section.

This is now, as requested, referenced and discussed (p. 11): "A recent study that appeared while this work was in revision suggests that autosecretion or an akin unconventional secretion process equally dependent on GRASP and autophagic machinery may facilitate plasma membrane delivery of mutant CFTR, potentially expanding the range of autosecretory substrates to integral membrane proteins (Gee et al., 2011)".

2. In addition there are a number of editorial issues that need further attention:

- An author contributions section needs to be included into the main body of the manuscript text after the acknowledgements section.

Done, as requested (p. 13).

- Please add scale bars, together with an explanation for figures 5, S3A, S4E; and an explanation for the scale bar in figure S4.

Scale bars (5 μm) have been added or revised as requested for figures 5, S3A, S4E, S4.

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- Could you please confirm that all lanes come from the same gel in figure S5C and modify the figure and the legend accordingly? According to our policies, we also need to see the original scans for this panel.

All lanes are from the same gel. All lanes are from the same gel (one intervening lane was cut out due to redundancy). This is now stated in Fig. S5C legend. The original scan is attached as a separate file. The explanation for the appearance that the images came form different gels is provided in the footnote .
The following is the explanation for a missing lane: “For the Fig. S5C, we deleted one lane (the GRASP2 lane) in the Western blot. In the raw data (attached) you can see that there are 4 loading lanes, Scr, GRASP1, GRASP2 and GRASP1+2. We deleted in the main Fig. S5C the GRASP2 loading line, because these GRASP2 data are redundant with the main figure Fig. 5A GRASP2/LC3 western blot. The figure S5C is not coming from 2 different gels, i.e. it is all from the same gel except that the GRASP2 lane was deleted for scientific presentation purposes to avoid gratuitous extra lanes. This is apparent from the supplementary source data file.”

- We have now started encouraging the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

We are wiling and will provide source files as per above. We will prepare the files as soon as we hear about the final decision.

Referee #1 :

The revised version clarifies some of my issues but a large number of key points remain unaddressed. This could be an important paper for the field but the onus is on the authors if they want to publish this work in a highly respectable journal. I have listed my comments as new issues and previously

New issues
- Figure 3A: the blue channel does not match the merged image.

We are not sure we understand completely. The merge image is a simple composite/overlay of the 3 images of individual channels. No changes have been made.

- their new term "autosecretion" (=self-secretion) does not sound appropriate for this process.

Autosecretion was in the original submission. At that time the reviewer did not object. We do believe that autosecretion is a good term as it refers to autophagy’s role in secretion (hence "autosecretion"). The term has been retained.

- How physiological is secretion via autophagosomes? Why does a cell need to be starved before treatment with nigericin/silica/amyloid fibrils/etc to release IL-1beta? They have Atg5 KO mice, so can they measure whether their IL-1beta serum level is indeed lower in these mice?

The cell does not need to be starved before the treatment but at the same time. This is now clarified in Materials and Methods (p. 12): "Starvation and other treatments (except for macrophage priming with LPS done in advance) were carried out concurrently (i.e. initiated at the same time)." We thank the reviewer for the opportunity to clarify.

The in vivo studies were not requested/suggested in the first round of review. We are not fully sure how such studies would be conducted but may consider them as a topic for a potential follow-up study by us or others.

Previous major issues
- previously: Fig 4C is misleading! The merge is incorrect because they reduced the red channel in the merge.
Now: They replaced the figure, which is now of a much better quality.

We thank the reviewer for acknowledging that thin has been improved.

- previously: Fig 5C is misleading! The inset is incorrect, because signals are completely blown up compared to the original signal.
Now: They replaced the figure with a better one, although this image does not show so much since they removed the insets because of the earlier criticism. I think that insets/zooms are informative, as long as no manipulations are done that are not stated in the legend.

We agreed with the reviewer in the previous round. We removed the inset. We think that the way the figure appears now is sufficient to draw conclusions, although we do agree with the new, albeit somewhat circuitous comment, that an inset for smaller profiles would help. However, we are reluctant to add any new insets based on the previous reviewer's comment specifically for this figure.

- previously: They only looked at GORASP2 (GRASP55). It is very strange that they did not comment on the existence of another GRASP. They should mention it and investigate the role of GRASP65.
Now: They say that GRASP65 could not be knocked down efficiently, which is shown in Fig S5C. Fig S5A and B are unclear. In the text and the figure itself it is mentioned that GRASP55 is knocked down, however, in the figure legend it is said that GRASP65 is knocked down. This is confusing.

We thank the reviewer for noticing this. It is our mistake. We now clarify this in Fig. S5 legend.

- How can starvation + nigericin (Fig4A) lead to less secretion of IL-1beta (~70pg/ml) than full medium + nigericin (~375 pg/ml, Fig S5A)?

Please note that the data were not normalized and that in different experiments the absolute numbers of cells ended up being different. This is now clarified by stating in Fig. S5A legend that: "the number of cells in this experiment (2x105 cells) was 2.8 times higher than in Fig. 4A (0.7x105) ".

- previously: They should check in all knockdowns that the expression levels of IL-1beta and IL-18 are not affected.
Now: They measured it and there are no problematic effects.

We thank the reviewer for acknowledging that this has been resolved.

- previously: Fig 3A, is IL-1beta in or on a vesicle? -> protease protection.
Now: The entire fractionation figure is gone. It is strange that such an informative experiment has disappeared. At least it would be informative to know whether IL-1beta is in an autophagosome or on it.

This was removed based on another reviewer's explicit request. We do appreciate the reviewer's point, but we also agree with the other reviewer's view that this specific aspect needs much more work before it is ready. These are ongoing studies.

- previously: Fig 1A, Atg5 knockouts can still secrete a lot of IL-1beta. How is this possible?
Now: The authors state the following.
"We interpret the incomplete reduction in IL-1ßsecretion in the absence of Atg5 as a net result of two opposing effects - one described here as a product of positive contribution of induced autophagy on extracellular delivery of IL-1ßand the other being the recently reported negative regulation of

IL-1ßsecretion by autophagy"

My concern. This does not resolve the issue This experiment still suggests that Atg5 is not critically important for IL-1beta secretion. How is IL-1beta secreted in Atg5 KO cells?

It appears that the reviewer believes that Atg5 is absolutely critical for any form of autophagy, that Atg5Fl/Fl ¨LysM-Cre excision is 100%, and is not completely satisfied with our explanation. In response to that, we now add an additional sentence to acknowledge that "… we cannot exclude the possibility of slight leakiness of the Atg5 fl/fl LyzM-Cre system or the existence of additional pathways." (p. 10)".

- Previously: Does nigericin cause cell death? Is actin or LDH released?
Now: They measured it, however the cell death is substantial (~8-~45% for the nigericin). How much % of the IL-1beta of the total is really secreted? What is the % of the IL-1beta in the supernatant minus the % cell death?

We would be reluctant to devise a new derivative measure - to ratio IL-1ß secretion vs LDH release - what we believe may be the reviewer's intent/suggestion. The data shown in un-derivatized form demonstrate that IL-1ß secretion precedes LDH release. The reader can compare the raw IL-1ß levels over time with LDH data over time, and not be under the impression that we deriviatized data into a convenient result. No change has been made.

- Previously: Does NEM block secretion? Does it accumulate IL-1beta vesicles?
Now: No such experiment has been done.
"We interpreted the reviewer's comment as a reference to N-ethyl maleimide, a reagent that has been used to study SNARE-dependent fusion in vitro. Since our studies were done in vivo (using intact cells), we did not carry out the NEM experiments, given membrane permeability issues and peloitropic effects of this alkylating agent also used to inhibit deubiquitination enzymes."
My concern. NEM experiments in intact cells can be done and they have been done before (for instance Zemskov et al, 2011, PLoS ONE).

Even if NEM can be added to cells as the reviewer stated it will affect more than SNARE function as we already explained (deubiquination is affected). No change.

- Previous minor issues
Previously: they should show a better blot in Fig 3A
Now: the entire figure is removed without replacement.

See the above explanation regarding the removal of p62 data (following another reviewer's recommendation).

- Previously: They say that "GRASP is juxtaposed to LC3", but since both proteins are scattered throughout the cell this does not mean so much. For me it is also unclear at how many cells we are looking in this figure.
Now: Now Fig 4B. The image is a bit improved, however, it is still not very convincing. For instance, where is the rightmost arrow pointing at?

We now show an arrow pointing to a different profile showing overlap in Fig. 4B (cell on the right, bottom row).

- Previously: Fig 5C, is GRASP still localized at the Golgi?
Now: a GRASP localization has been done in control cells, but not in nigericin treated cells, which was essentially the question. (Figure 5C showed nigericin treated cells in the previous manuscript).

As per reviewer's request we have now substituted GRASP55 colocalization to the Golgi in untreated cells with images from nigericin treated cells (Fig. S6).

previously: How was Fig 6F quantified?
Now: Now it is shown.

We thank the reviewer for acknowledging that this has been resolved.

Referee #2:

The authors have done a very thorough work in successfully responding to all raised issues. There are no further comments.

We thank the reviewer on the positive assessment.

Referee #3:

All concerns adequately addressed.

We thank the reviewer for the endorsement of our revisions.

Thank you for your consideration.

3rd Editorial Decision 30 September 2011

Thank you for sending us your re-revised manuscript. In the meantime, I have now had a chance to consult once more with referee 1. As you will see below, he/she is disappointed with the changes you have made. He/she feels strongly that the term 'autosecretion' should not be used and that 'unconventional secretion' is more appropriate as this is how the process has been first introduced into the literature based on findings in model organisms. I have to say that I agree with the referee, also in the light of the nomenclature used in the recent Cell paper on CFTR. I therefore need to insist on following this referee's request. Furthermore, the referee still has issues with the conclusiveness of part of the data and puts forward suggestions for experiments. Partly based on this caveat, but also more generally, the referee thinks that your claims need to be toned down, in particular in the discussion section. I should add that the referee is supportive of publication of the study here overall, but really feels that the concerns need to be taken into account in the final version. I therefore urge you to accommodate the caveats he/she points out and to soften the claims in a final version of the manuscript text.

I am looking forward to your final version.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The single gene for GRASP was shown to be required for the secretion of a signal sequence lacking protein Acba in Dictyostelium in 2007. It was suggested that this might involve a membrane bound autophagsome like intermediate because the secretion of AcbA was observed only upon starvation of cells. In 2010, Malhotra and Subramani's groups, independently, revealed the involvement of a number of genes products in the secretion of AcbA ortholog called Acb1 in yeast. These include: GRASP, a number of Atg genes, proteins required for MVB formation, the first clear identification of two T-SNAREs (Tlg2 and Sso1), NSF, and a few other components. A potential pathway of Acb1

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secretion was outlined in these papers and the authors,suggested the involvement of a secretory autophagosome for the secretion of Acb1.
The authors state "We thus define one of the first biogenesis functions of autophagy in mammalian cells, and show that at least one type of unconventional secretion utilizes autophagic machinery in mammalian cells. We termed this type of unconventional secretion autosecretion, and the corresponding pathway the autosecretory pathway."

This paper is potentially important only because it confirms that unconventional secretion in mammalian cell might use the same components as in the yeast, flies and the slime mold. There is, however, no new mechanistic insight. I also fail to understand the new terminology, autosecretion, for an old and well-established process. The literal meaning of autosecretion is self-secretion. This is simply wrong, as IL-1fl secretion probably involves 100 proteins. They state somewhere that because this is mediated by autophagomes it is better to call the process autosecretion. There is no data anywhere in the paper that IL-1fl is in an autophagsome. This is unfair to the others who have been working on unconventional secretion for much longer than the authors. Sitia and colleagues made the first observation on the secretion of IL-1fl by a membrane bound compartment, and they even suggested that it was likely to be some sort of an autophagosome like structure. This was in a paper published in 1992 in EMBO J. Please read that paper and cite it.
My concerns with the data follow.

Figure 1: localization of LC3 and IL-1fl: What is this LC3 containing compartment? It cannot be an autophagosome? Do these structures accumulate when the terminal fusion events are inhibited? Only then can these authors claim the LC3 containing compartments as intermediates in the IL-1fl secretory pathway. The authors were asked to carry out a simple test with NEM treatment but they have argued about non-specificity and membrane permeability problems. This is an excuse. They should have done the experiment and based on the results described the possible problems with the interpretation. Since the v and the t-SNAREs involved in this process are not known, this simple test could have supported their observation.

Rab8a. Why is IL-1fl in the nucleus? Are the Rab8a positive elements the endosomes? They are far too big to be autophagsomes.

GRASP55. No one who has worked with this protein is going to like the localization of GRASP reported in this paper. It is all over the place. The authors are using commercial antibodies and it is likely the reagent is far from optimal for these experiments. Did they affinity purify the antibody? Does it only bind and immunoprecipitate one band in normal and starved cells? The appearance of this antibody on a few punctate elements upon starvation also warrants caution. Would it not be better to transfect a tagged GRASP for these studies, since this is a key issue?

Figure 5C. The authors state that GRASP knockdown reduces the number of LC3 containing autophagosomes. Why do they call these punctate elements autophagosomes? They could just as well be endosomes or a structure on the ER.

Problems with the discussion
The authors start with "The data presented in this work outline the principles of the autosecretory pathway in mammalian cells." The authors have shown three known proteins of the unconventional secretory pathway to perhaps also work in the potential secretion of IL-1fl in mammalian cells. This work does not outline the principle of the autosecretory pathway. There data does not even show that Il-1fl is contained in an autophagome; that it is contained in a membrane-bounded compartment (membrane fractionation is necessary for that); where it is loaded into a compartment for secretion, and where does this process begin? They have also not shown what new components might be involved in this process. Finally, they have not shown that the compartment containing the IL-1fl is en route to secretion. So why start the discussion with such overblown statements?

The authors write "The autosecretion pathway in mammalian cells includes GRASP, one of the peripheral Golgi proteins involved in lateral organization of Golgi ribbons. Although the role of GRASP in alternative secretory pathway has been studied , its exact mechanism of action has not been elucidated (Kinseth et al, 2007; Nickel & Rabouille, 2009). We observed here a potentially telling connection betweeen GRASP and autophagy, by showing that GRASP affects autophagy induction, which places GRASP upstream of autophagy execution, including the conjugation

systems involved in LC3 lipidation." This is an insult to others who showed much more clearly the involvement of GRASP in the secretion of signal sequence lacking protein in Dictyostelium, cerevisiae, P.Pastoris, Drosophila and more recently in the mouse cells. See above my concerns for the claim of the authors on the involvement of GRASP. There data does not add anything about the stage of the action of GRASP.

The paper is important for the field because it indicates that the mammalian cells might use the same set of components as observed in many other cell types. However, the paper does not reveal any new mechanism about the secretion of signal sequence lacking proteins and it is unnecessary to claim more than what is being shown. Importantly, they do not show anywhere that this process does not involved the role of the conventional secretory pathway. For that they should thank the original findings of Roberto Sitia and colleagues (EMBO J, 1992). Please check that paper of Sitia and colleagues and at least cite the reference. The authors should refrain from coining another term for this secretory pathway, it is misleading, and what is wrong with the original description of Sitia and colleagues. Coming up with a new-misleading term- is not likely to improve the impact of the paper.

In sum, if the editors and the authors want my support for the publication of the paper, the authors will have to tone down their claims and certainly not call this process auto secretion.

3rd Revision - authors' response 03 October 2011

Thank you for your editorial comments and re-review of our revised manuscript by reviewer #1. We have now complied with your editorial recommendation and reviewer's demand to replace the term autosecretion with "unconventional secretion" and toned down the discussion in compliance with reviewer's request and your instructions.
We have complied in full and removed the term "autosecretion"1. We do feel that in addition to complying with the request we owe an explanation. We intended to use the term "autosecretion" to specify the type of unconventional secretion that our study addressed, since the term unconventional secretion is broader and most likely is not a single uniform pathway but refers to several potentially different mechanisms according to the published reviews and opinions in this filed. However, since the reviewer #1 insists on the use of the term unconventional secretion, we have now complied in full with this request. We certainly did not mean to be unfair to others working on this or related unconventional secretory pathways and regret if our intent to be more specific was misconstrued.
We have toned down the claims in the discussion section and elsewhere. As an example, the opening sentence now reads: "The data presented in this work outline several elements of the autophagy-based unconventional secretory pathway in mammalian cells" (replacing the original sentence: "The data presented in this work outline the principles of the autosecretory pathway in mammalian cells"). Further, we have toned down other statements whenever possible -for example, replacing "key" with "relevant" as in the following sentence: "A relevant aspect of the process described here is that induction of autophagy is required to observe the manifestations of this type of unconventional secretion." Additional examples are delineated below.

The following is the list of changes to implement the replacement of the term "autosecretion":

Title, change: "Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β"
Abstract, change: "This process, which is a type of unconventional secretion, expands the functional manifestations of autophagy beyond autodigestive and quality control roles in mammals. It enables a subset of cytosolic proteins, devoid of signal peptide sequences and unable to access the conventional pathway through the ER, to enter an autophagy-based secretory pathway facilitating their exit from the cytoplasm."

Introduction, deleted the concluding sentence ("We termed this type of unconventional secretion autosecretion, and the corresponding pathway the autosecretory pathway" has been removed).

Results, text change, p.8: "A similar downregulation of IL-18 secretion (instead of "autosecretion") was observed with GRASP55 knockdown (Suppl. Fig. S5B)".

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Results, section title change, p.8: “Autophagy-based unconventional secretion is not limited to proteolytically processed inflammasome substrates”

Results, text change, p. 8: “We next wondered whether the unconventional secretion (instead of “autosecretion”) process described above…”

Results, text change, p.9: “These experiments show that autophagy-based unconventional secretion affects release of HMGB1 in a manner similar to IL-1β. Our findings broaden the spectrum of autophagy-based unconventional secretion (instead of “autosecretion”) substrates, and establish this type of unconventional secretion as a more general process in extracellular delivery of cytosolic proteins.”

Discussion, opening sentence: “The data presented in this work outline several elements (instead of “the principles”) of the autophagy-based unconventional secretory pathway (instead of “autosecretion”) in mammalian cells.”

Discussion, p.9 “…and we propose that autophagy-based unconventional secretion may be a key coupler between the metabolism and inflammation”

Discussion, p.10: “…in part the genetic associations between autophagy risk loci and disease states may stem from altered autophagy-based unconventional secretion of inflammatory cytokines.”

Discussion, p. 10: “The autophagy-based unconventional secretion pathway (instead of “autosecretion”) in mammalian cells includes GRASP…”

Discussion, p. 10: “The finding that Rab8a plays a functional role in autophagy-based unconventional
secretion (instead of “autosecretion”)…”

Discussion, p. 11: “Broadening the scope of autophagy-based alternative secretion pathway (instead of
“autosecretion”) is the observation that it facilitates exit from cells of the alarmin HMGB1.”

Discussion, p. 11: “We propose (instead of “postulate”) here that autophagy-based alternative secretion
pathway (instead of “autosecretion”) may be used for extracellular delivery of a spectrum of cytosolic
proteins…”

Discussion, p. 11: “A recent study that appeared while this work was in revision suggests that an unconventional secretion process, also dependent on GRASP and autophagic machinery, may facilitate
plasma membrane delivery of mutant CFTR, potentially expanding the range of substrates to integral
membrane proteins…(all references to “autosecretion” removed form this sentence).

Discussion, end: “Given the capacity for either bulk transport or selectivity when coupled with autophagic adaptors, it is likely that autophagy-based unconventional secretion (instead of “autosecreton”) serves a potentially broad spectrum of yet to be uncovered physiological functions”.

Fig. 6 caption. HMGB1 is an autophagy-based alternative secretion (instead of “autosecretion”) substrate.

The following is our point-for-point response to reviewer’s comments:

This paper is potentially important only because it confirms that unconventional secretion in mammalian cell might use the same components as in the yeast, flies and the slime mold. There is, however, no new mechanistic insight.

We appreciate reviewer’s point emphasizing the prior fundamental findings in yeast, flies and the

slime mold, and have modified and toned down the manuscript accordingly. For the record, we respectfully disagree with reviewer's statements that our study has no mechanistic insights and that its only importance is in confirming the role of autophagy in unconventional secretion in mammalian cells. Among other points, finding that GRASP55 participates in autophagy induction cannot be labeled "confirmatory" as this has not been previously shown. IL-1ß secretion and secretion of HMGB1 were far form being understood. Rab8a and exocyst roles have not been studied in this context. Inflammasome linkage with autophagy-based unconventional secretion is a new mechanistic insight of potentially high significance for mammalian systems and human disease.

2. I also fail to understand the new terminology, autosecretion, for an old and well-established process. The literal meaning of autosecretion is self- secretion. This is simply wrong, as IL-1ß secretion probably involves 100 proteins. They state somewhere that because this is mediated by autophagomes it is better to call the process autosecretion. There is no data anywhere in the paper that IL-1ß; is in an autophagsome. This is unfair to the others who have been working on unconventional secretion for much longer than the authors. Sitia and colleagues made the first observation on the secretion of IL-1ß by a membrane bound compartment, and they even suggested that it was likely to be some sort of an autophagosome like structure. This was in a paper published in 1992 in EMBO J. Please read that paper and cite it.

Upon reviewer's insistence, we have changed the term "autosecretion" to "unconventional secretion". We intended to use the term "autosecretion" to specify the type of unconventional secretion that our study addressed, since unconventional secretion may not be a single, uniform pathway at least according to the published reviews and opinions in this filed. This is reflected in the descriptive reference to this specifc process in the text as autophagybased unconventional secretion. We certainly did not mean to be unfair to others working on this or related pathways and apologize if our intent to be more specific was misconstrued. We have also, as requested, cited the 1992 (a 1990 reference to be exact) study by Sitia et al., in two places to appropriately honor this seminal work.

The work by Sitia and colleagues, (Rubartelli, A., F. Cozzolino, et al. (1990). "A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence." The EMBO journal 9(5): 1503-1510") is now cited on p. 10 and p. 11. On p. 10 we state: " In keeping with this model of a muster station for inflammasome components, activation and subsequent extracellular delivery, is the translocation of pro-IL-1β to membranous organelles upon stimulation with the inflammasome agonist nigericin, as previously observed by R. Sitia and colleagues (Rubatelli et al., 1990) who have established early on that this process does not follow the conventional secretory pathway." On p. 11, we state: "To be eligible for export outside of the cell without invoking a pore mechanism, cytosolic proteins first need to be brought somehow into the lumen of vesicular carriers, as previously shown by others (Rubartelli et al, 1990)."

3. Figure 1: localization of LC3 and IL-1ß: What is this LC3 containing compartment? It cannot be an autophagosome? Do these structures accumulate when the terminal fusion events are inhibited? Only then can these authors claim the LC3 containing compartments as intermediates in the IL-1ß secretory pathway. The authors were asked to carry out a simple test with NEM treatment but they have argued about non- specificity and membrane permeability problems. This is an excuse. They should have done the experiment and based on the results described the possible problems with the interpretation. Since the v and the t- SNAREs involved in this process are not known, this simple test could have supported their observation.

Our decision, delineated in the point-for-point response accompanying our first revision (when we provided 25 new experimental panels), not to carry out NEM experiments was based on the lack of specificity with NEM effects. We have explained this and the reviewer acknowledges.

We fail to understand how this simple but (as both the authors and the reviewer agree) highly nonspecific assay would improve our study without an in-depth follow up to discern the effects on SNAREs, ubiquitination and potentially other processes that are likely to be affected by NSF.

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4. Rab8a. Why is IL-1ß in the nucleus?

To accommodate this reviewer's criticism, the following explanation and amendment has been provided in the supplement to explain the nuclear staining with IL-1ß (Suppl. Figs. S3 and S4): "Some of the nuclear staining with IL-1ß in BMMs (e.g. Suppl. Figs. S3 and S4) may be nonspecific, but we cannot rule out functional roles. IL-1ß family members such as IL-1ß and IL-1ß are known to be distributed between the cytoplasm and the nucleus, reflecting their dual functionality or to counter excessive inflammation (Luheshi et al, 2009a). Of further note is that IL-1ß has been observed in the nucleus of microglia, a major macrophage cell type of the CNS (Luheshi et al, 2009b)."

5. Are the Rab8a positive elements the endosomes? They are far too big to be autophagsomes.

We are unsure about the intent of this comment. Not all Rab8a profiles in a cell are autophagosomes or autophagosome-related profiles, and this has not been stated or implicated in the text. We are unsure what precise size limits the reviewer refers to regarding autophagic organelles in mammalian cells.

6. GRASP55. No one who has worked with this protein is going to like the localization of GRASP reported in this paper. It is all over the place. The authors are using commercial antibodies and it is likely the reagent is far from optimal for these experiments. Did they affinity purify the antibody? Does it only bind and immunoprecipitate one band in normal and starved cells? The appearance of this antibody on a few punctate elements upon starvation also warrants caution. Would it not be better to transfect a tagged GRASP for these studies, since this is a key issue?

We have now modified Fig. S6 by reinstating the already presented to the reviewer in one of the previous versions immunofluorescence colocalization alaysis with GRASP55 in resting cells where there is a tight colocalization between GRASP55 and GM130. This was previously shown but removed in subsequent rounds of review since the reviewer asked for nigericin treated cells instead. Suppl. Fig. S6 now contains two sets of panels, both of them representing experiments expressly performed on request by this reviewer and both previously viewed by this reviewer. Panel A shows resting cells (there is a tight localization between GRASP55 and the Golgi). Panel B shows nigericin treated cells where a fraction of GRASP55 disperses. The text has been modified and now reads: "GRASP55 in resting cells is mostly localized aligned within the perinuclear Golgi (Fig. 4B; Suppl. Fig. S6A). However, a fraction of it dispersed upon treatment of cells with the inflammasome agonist nigericin (Fig. S6B) and was found juxtaposed and partially overlapping with LC3 profiles (Fig. 4B,C)." GRASP55 in resting macrophages (not stimulated with inflammasome agonist nigericin) displays typical Golgi localization, so there should be no grounds for suspicions concerning the antibody. We remind the reviewer that he/she has seen this before but asked for nigericin instead. Again, this has been reinstated in Suppl. Fig. SA6, to accommodate the reviewer's concern. In response to a new suggestion 4 concerning expressing a tagged construct, we have carried out experiments with endogenous protein precisely to avoid the likely criticism that a protein over-expressed from a transgene may not reflect endogenous protein localization.

This has not been previously brought up by the reviewer and is now being introduced in the third round of re-review. We believe that the resting localization of GRASP55 in unstimulated macrophages (Suppl. Fig. S6) and additional experiments with siRNA knockdowns whereby this antibody recognizes specifically GRASP55 should adequately address the reviewer's concerns.

7. Figure 5C. The authors state that GRASP knockdown reduces the number of LC3 containing autophagosomes. Why do they call these punctate elements autophagosomes? They could just as well be endosomes or a structure on the ER.

We have used two gold standard measures of autophagy induction - LC3 lipidation and the tandem RFP-GFP-LC3 probe. The text has been modified to explain this as follows: "In addition to being required for IL-1ß secretion, GRASP55 showed functional effects on LC3 and autophagy, tested by employing two core assays (Mizushima et al, 2010): LC3-II lipidation and the RFP-GFP-LC3

tandem probe."

If our usage of these well established autophagy assays were to be deemed wrong, as per reviewer's comment, about 95% of the current literature on autophagy would have to be called invalid.

Problems with the discussion

8. The authors start with "The data presented in this work outline the principles of the autosecretory pathway in mammalian cells." The authors have shown three known proteins of the unconventional secretory pathway to perhaps also work in the potential secretion of IL-1ß in mammalian cells. This work does not outline the principle of the autosecretory pathway. There data does not even show that IL-1ß is contained in an autophagome; that it is contained in a membrane- bounded compartment (membrane fractionation is necessary for that); where it is loaded into a compartment for secretion, and where does this process begin? They have also not shown what new components might be involved in this process. Finally, they have not shown that the compartment containing the IL- 1ß is en route to secretion. So why start the discussion with such overblown statements?

As per reviewer's comments and requests, we have changed the discussion and the starting paragraph now reads: "The data presented in this work outline several elements of the autophagybased unconventional secretory pathway in mammalian cells. This type of unconventional secretion is shown here to support the extracellular delivery of inflammasome substrates, in particular IL-1ß and IL-18 and may potentially have a broader number of clients. A relevant aspect of the process described here is that induction of autophagy is required to observe the manifestations of this type of unconventional secretion. Since basal autophagy suppresses spurious induction of inflammasome (Nakahira et al, 2010; Zhou et al, 2011), autophagy provides both avoidance of unscheduled inflammasome activation and a platform for extracellular delivery of inflammasome substrates. Since a number of hormones, cytokines, pathogen associated molecular patterns, and danger associated molecular patterns (Deretic, 2011; Tang et al, 2010) are known to induce or inhibit autophagy, a link between autophagy and secretion of major immunomodulatory cytokines such as IL-1ß could significantly influence the extent and duration of inflammation. Connections between metabolic syndrome, high fat diet and inflammasome activity are now beginning to be appreciated (Vandanmagsar et al, 2011; Wen et al, 2011), and we propose that autophagy-based unconventional secretion may be a key coupler between the metabolism and inflammation. Since a number of genetic links have been found between autophagy and idiopathic inflammatory diseases or infectious diseases with significant inflammatory components (Levine et al, 2011) it is possible that at least in part the genetic associations between autophagy risk loci and disease states may stem from altered autophagy-based unconventional secretion of inflammatory cytokines.

9. The authors write "The autosecretion pathway in mammalian cells includes GRASP, one of the peripheral Golgi proteins involved in lateral organization of Golgi ribbons. Although the role of GRASP in alternative secretory pathway has been studied , its exact mechanism of action has not been elucidated (Kinseth et al, 2007; Nickel & Rabouille, 2009). We observed here a potentially telling connection betweeen GRASP and autophagy, by showing that GRASP affects autophagy induction, which places GRASP upstream of autophagy execution, including the conjugation systems involved in LC3 lipidation." This is an insult to others who showed much more clearly the involvement of GRASP in the secretion of signal sequence lacking protein in Dictyostelium, cerevisiae, P.Pastoris, Drosophila and more recently in the mouse cells. See above my concerns for the claim of the authors on the involvement of GRASP. There data does not add anything about the stage of the action of GRASP.

Others have undeniably shown in model organisms that GRASP controls unconventional secretion, as acknowledged throughout our manuscript. Our statements here indicate that GRASP also controls autophagy initiation, which has not been addressed before. It is unfortunate that the reviewer views this as an insult (instead of a set of scientific findings) since this was certainly not our intention. We hope that the extensive textual modifications introduced in earnest throughout the manuscript alleviate this reviewer's concern.

10. The paper is important for the field because it indicates that the mammalian cells might use the same set of components as observed in many other cell types. However, the paper

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does not reveal any new mechanism about the secretion of signal sequence lacking proteins and it is unnecessary to claim more than what is being shown.

Please see the above paragraph regarding the new mechanisms.

11. Importantly, they do not show anywhere that this process does not involved the role of the conventional secretory pathway. For that they should thank the original findings of Roberto Sitia and colleagues (EMBO J, 1992). Please check that paper of Sitia and colleagues and at least cite the reference. The authors should refrain from coining another term for this secretory pathway, it is misleading, and what is wrong with the original description of Sitia and colleagues. Coming up with a new- misleading term- is not likely to improve the impact of the paper.

In compliance with reviewer's requests we have deleted any reference to the offending term (autosecretion), as detailed in the above paragraphs. We refer to and cite the findings by Sitia et al. (p. 10, end of second paragraph; we believe that the reviewer refers to the 1990 paper not 1992

Rubartelli, A., F. Cozzolino, et al. (1990). "A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence." The EMBO journal 9(5): 1503-1510.

12. In sum, if the editors and the authors want my support for the publication of the paper, the authors will have to tone down their claims and certainly not call this process auto secretion.

We have made adjustments throughout the manuscript as per reviewer's request. Thank you for your consideration.

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