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Listeria phospholipases subvert host autophagic defenses by stalling pre-autophagosomal structures

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(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 22 May 2013

Thank you for submitting your research manuscript entitled "Listeria phospholipases subvert host autophagic defenses by stalling pre-autophagosomal structures" to our editorial office. It has now been seen by three referees whose comments to the author you will find enclosed.

While reviewer #1 is in general positive and considers your study interesting, albeit in need of further substantiation, the other two referees do not share her/his opinion. They both find that the data supporting the major novel claim of the manuscript are not conclusive, and especially referee #2 feels that the study currently lacks sufficient novelty and would therefore be better suited for a more specialized title. Based on the overall evaluation provided by the reviewers and the extensive revisions that would be required to substantiate your conclusions, I am afraid that we feel that the study is too preliminary at this stage to offer publication at The EMBO Journal.

Thank you in any case for the opportunity to consider this manuscript. I am sorry that I cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

REFEREE REPORTS

Referee #1

This interesting study by Tattoli et al, entitled 'Listeria phospholipases subvert host autophagic defenses by stalling pre-autophagosomal structures' establishes the differential role of three Listeria toxins on the induction and subsequent accumulation of pre-autophagosomal vesicles. After LLO-

mediated host membrane damage induces a transient amino acid starvation response and autophagy induction, two bacterial phospholisases seem to stall autophagosomal maturation, thus likely preventing bacterial degradation and thus promoting replication and spread. While this study provides a novel and highly interesting link of bacterial toxins / virulence factors with the autophagy pathway, I feel that this study would be more interesting to the broad readership of EMBO J if the effect of PlcA/B-mediated autophagy inhibition on bacterial degradation and replication was established more directly, as specified below.

General concerns:

While the role of PlcA/B during accumulation of pre-autophagosomal vesicles is well supported by this study, it would be highly interesting to learn more about their ability to influence overall autophagic flux. In particular, it would be highly interesting to use a lysosomal fusion assay (e.g. based on GFP-mCherry double tagged bacteria) and/or treatment with bafilomycin A1 to analyze whether there is really an increased capture and degradation of PlcA/B-negative bacteria in comparison to wt, as the authors imply.

Concerning the mechanism of action of the two phospholipases, it would be very interesting to test whether expression of an unrelated phospholipase C could rescue the PlcA/B- mutant phenotype. Conversely, is overexpression of Plc A and/or B in the absence of infection sufficient to induce the accumulation of NDP52+ vesicles?

Minor concern:

Figure 1a: This western blot would profit from a loading control, since the amount of S6K1 present in the 1h lane seems significantly lower than at other time points. Is protein abundance affected by infection, as well as phosphorylation? Maybe this point could be clarified by referring to Figure 2A already in the first results paragraph.

Referee #2

The authors investigate the response of Hela cells to infection with Listeria. They find evidence for transient amino acid starvation and reduced mTOR activity. mTOR activity was unrelated to the ability of Listeria to avoid autophagy, which the authors show is partially mediated by PlcA and PlcB.

This manuscript provides rather incremental progress over previous work. The authors published recently (Tattoli Cell Host Microbes 2012) that Shigella and Salmonella induce amino acid starvation in Hela cells; notwithstanding the more transient nature of the phenomenon in Listeria-infected cells, I don't think the data in Figs 1, 2 and 3 are particularly novel against the background of the authors' recent findings with Shigella and other bacteria.

Thereafter the authors investigate the fate of NDP52+ cytosolic granules in Listeria infected cells. These are most likely the membrane remnants of vacuoles from which Listeria has escaped based on markers found in this study on NDP52 granules and known to occur in association with membrane remnants (galectins, NDP52, ubiquitin). Targeting of membrane remnants by autophagy has been described in cells infected with Listeria and other bacteria (Dupont Cell Host Microbes 2009, Thurston Nature 2012). Escape of Listeria from autophagy is mediated by several genes (ActA Yoshikawa Nat Cell Biol 2009, InlK Dortet PLos Pathogens 2011 and PlcA/B Birmingham 2007). The role of PlcA/B in escape from autophagy was confirmed in this study. Whether the NDP52+ granules / membrane remnants represent 'stalled pre-autophagosomal structure' remains to be firmly established as the only supporting evidence is the occurrence of WIPI. Kinetic quantifications, electron microscopy and the inclusion of less ambiguous markers of autophagosome maturation (Klionsky 2012 Autophagy) will be required to firmly establish the authors' conclusion about the 'stalled' state of these structures.

technical points

Fig 1a: why would a phospho-specific antibody produce two bands while a protein-specific antibody

stains only one species? Are the antibodies specific? Is it necessary to confirm their specificity, for example by knockdown? If only one band of the phosphor-specific antibody is derived from S6K1, why would the other band also vary in intensity? A loading control may help.

Fig 1b (and 1c) The quality of the images is poor. It is hard to believe that images of that quality allow assessment whether or not mTor localizes to individual LAMP+ vesicles (as in 1c). (Independent from the image quality, using green, red and yellow rather than green, orange, and yellow would make the images
throughout the manuscript easier for the reader to interpret.)

Fig7c: Data should be presented as primary CFU of wt and mutant bacteria, rather than as ratios to enable the reader to judge primary data. If necessary the ratio can be given in an additional (supplementary?) figure.

Fig2, 3, 4, 6, 7: complementation of Listeria knockouts is missing entirely from the manuscript

Referee #3

This manuscript examines the role of bacterial PLCs during Listeria monocytogenes (Lm) escape in epithelial cells and fibroblasts. It claims that Lm use PLCs to prevent host anti-bacterial autophagy and the progression of early stage autophagosomes toward lysosomes. This is a reasonable hypothesis, given recent studies showing the involvement of diacylglycerol in pathogen-induced autophagy (Shahnazari et al., Cell Host and Microbe 2010) and showing that anti-bacterial autophagy is induced by exposed galectin 8 to cytosol after pathogens perforate vacuoles (Thurston et al., Nature 2012). The main conclusion is supported by experiments showing that, after infection, intracellular Lm deficient in PlcA and PlcB (PlcA/PlcB- Lm) become surrounded by the early autophagosome marker NDP52 to a greater extent than do wild-type Lm (Fig. 3D). The interpretation is that wild type bacteria utilize the PLCs to inhibit bacterial capture by the autophagic machinery. The conclusion stated in the title, that the PLCs stall progression of preautophagosomal structures, is supported by images showing that the autophagosomes containing wildtype Lm fail to acquire a later marker of autophagy, WIPI-2 (Fig. 5). Moreover, the protective effect provided by PLCs may act in trans, as coinfection of cells with wildtype and PlcA/PlcB- Lm conferred a slight increase in survival upon the PlcA/PlcB- Lm. Although interesting, the manuscript is inconclusive because the data supporting the major claim are weak and not quantitative. Overall, the quality of the data in the manuscript is uneven and sometimes poor.

Major Concerns:

1. None of the quantitative comparisons of labeling in cells infected with different strains of Lm normalize for differences in the efficiency of infection. Therefore, experiments such as Fig. 4D, showing the percent of cells containing NDP52 granules, do not adjust for the possibility that different percentages of cells will contain bacteria. That is, do PlcA- Lm-infected cells contain a smaller percentage of NDP52+ granules than cells infected with wildtype Lm simply because a smaller percentage of cells contain bacteria?

2. Similar concerns about variable infection efficiencies apply to the western blotting and qPCR data.

3. The claim that PlcA and PlcB inhibit autophagosome progression is not supported by quantitative data showing rates of autophagosome progression plus and minus the PLCs. Rather, suggestive images are provided. This considerably weakens the conclusions.

4. In quantifying the membrane association of Lm inside infected cells, more should be done to distinguish cytosolic vs. vacuolar Lm. Cells infected with wildtype, and PlcA/PlcB- Lm should contain two different groups: 1) Lm which escape into cytosol and 2) Lm which remain in vacuoles. Using DAPI images as representative signals of vacuole-associated Lm fails to distinguish these groups. To observe and quantify co-localization of Lm-containing vacuoles and LAMP/NDP52, the authors should exclude escaped Listeria (eg., using rhodamine-phalloidin staining to exclude cytosolic bacteria).

Minor Comments

1. Fig1A. Since the amount of S6K1 at 1h is less than that at other times, it is difficult to interpret phospho-S6K signals. This problem occurs in other blots, as well, especially Fig. S3D.

2. Figs. 1A, 1D, 2A, 3B. Exposure times should be shorter.

3. Fig. 2C. In the TKO-DAPI image, there are many bacteria-like structures. However, no bacteria-like structures are visible in WT-DAPI image. Overall, bacteria were difficult to spot in all of the DAPI images.

4. Fig4AB. The image is not clear. At 4h, most of the WT Lm should have escaped into cytosol. Is the bacterium shown in panel B still in a vacuole? If so, what is ratio of the bacteria which does not escape?

5. Figs. 5C, 5D, 5E, 6B and 6C. Quantification is necessary.

6. Fig. 6A. The infection and escape efficiencies of each strain should be reported. Also, the experiment measured only cells at 4 hours after infection. What were the PI3P levels at early timepoints?

Resubmission 22 August 2013

Point-by-point response to the reviewers' comments:

We wish to thank the reviewers for their useful comments that greatly helped us improve the quality of our study. In this revised manuscript, we provide a significant amount of new data that we believe strengthen the manuscript. The most important new data are the following:

- Using a GFP-RFP-LC3 construct as suggested by the reviewers, we analyzed the autophagic flux in cells infected by WT and PLC- *Listeria*. We observed that *Listeria* PLCs significantly inhibit autophagic flux, both at the peak of this flux (1h p.i.) as well as at later time points. These observations provide a mechanistic rationale for the observed accumulation of pre-autophagosomal structures (PAS) in *Listeria-infected* cells, which likely occurs when the autophagic flux passes under a certain threshold. The results are presented in Fig. 6A of the revised manuscript.

- We now present direct evidence that the PLCs are responsible for PAS accumulation by showing that ectopic expression of PlcA by *Listeria* PlcA/B- is sufficient to restore PAS accumulation. The results are presented in Fig. 4E of the revised manuscript.

- We provide a more detailed analysis of the composition and formation of the PLC-dependent granules. We now show that the endogenous LC3, ATG16L1 and DFCP1 accumulate in these structures, which adds to the proteins that we had already identified (Ub'ed proteins, Galectin-8, NDP52, WIPI-2). Importantly, knocking down WIPI-2 expression did not affect recruitment of the damage membrane marker Gal-8, but inhibited recruitment of endogenous LC3, thus showing that the proteins of the autophagic machinery accumulate in these granules in a stepwise and coordinated manner, in agreement with those structures being true PAS, rather than simple large aggregates containing autophagy proteins. The results are presented in the new figures S5, S6, S7, S8 and S9 of the revised manuscript.

- We repeated a number of western blots for which loading levels of S6K1 were not satisfactory. These new western blots are found in Fig. 1A, 2A, and S3D. We also added a western blot showing eIF2α phosphorylation by *Listeria* WT, which reinforces the conclusions about the transient induction of the GCN2/eIF2α/ATF3 axis by *Listeria*.

- We present improved images of mTOR staining by immunofluorescence in Figs 1B and 2C. Finally, we would like to insist on the fact that most of our results rely on the analysis of endogenous proteins, and we believe that we have analyzed one of the largest selections

of endogenous markers of membrane damage and autophagy in a bacterial autophagy study. Please find below the point-by-point response to all the comments by the referees.

Referee #1

This interesting study by Tattoli et al, entitled 'Listeria phospholipases subvert host autophagic defenses by stalling pre-autophagosomal structures' establishes the differential role of three Listeria toxins on the induction and subsequent accumulation of pre-autophagosomal vesicles. After LLO-mediated host membrane damage induces a transient amino acid starvation response and autophagy induction, two bacterial phospholisases seem to stall autophagosomal maturation, thus likely preventing bacterial degradation and thus promoting replication and spread. While this study provides a novel and highly interesting link of bacterial toxins / virulence factors with the autophagy pathway, I feel that this study would be more interesting to the broad readership of EMBO J if the effect of PlcA/B-mediated autophagy inhibition on bacterial degradation and replication was established more directly, as specified below.

General concerns:

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We thank the reviewer for proposing experiments relating to autophagic flux. In light of this comment, we have now performed the proposed experiments, and obtained interesting results that, we believe, help us understand the PLC-dependent mechanism of formation of the granules, thus explaining how *Listeria* PLCs protect against autophagy.

Indeed:
-In kinetic experiments using the GFP-RFP-LC3 construct to monitor autophagic flux, we showed that the autophagic flux was maximal at 1-2h p.i., and strongly declined at 3-4h p.i. for both *Listeria* WT and PlcA/B-, in agreement with our mTOR inhibition data. Although the kinetics were similar for both strains, we observed that the intensity of the autophagic flux was reduced in WT *Listeria* as compared to PlcA/B- *Listeria*, which likely accounts for the accumulation of preautophagosomal structures in cells infected with WT *Listeria*. The fact that the granules accumulated mainly at late time points (>2h p.i.) likely reflects the fact that the autophagic flux needs to pass under a certain threshold for this event to occur. Thus, these important new data present a rationale for the accumulation of preautophagosomal structures at late time points of *Listeria* WT infection, by showing that the autophagic flux reaches very low levels that are not observed in the case of the PlcA/B- *Listeria*. Together, these results present direct evidence that *Listeria* phospholipases inhibit autophagic flux, which represents a new and previously unanticipated role for these toxins in *Listeria* pathogenesis. Of note, while there are multiple examples of bacteria escaping from autophagy targeting, the capacity of bacterial pathogens to directly affect the autophagic flux is poorly documented. A recent example demonstrated that a *Legionella* effector blocked autophagy at the level of LC3 conjugation (Choy et al. Science 2012, 338(6110):1072-6).
-Moreover, and in line with the above results, the use of the GFP-RFP-LC3 construct allowed demonstrating that the granules were systematically yellow (thus red and green), indicative of immature autophagosomes**.**

Concerning the mechanism of action of the two phospholipases, it would be very interesting to test whether expression of an unrelated phospholipase C could rescue the PlcA/B- mutant phenotype. Conversely, is overexpression of Plc A and/or B in the absence of infection sufficient to induce the accumulation of NDP52+ vesicles?

In order to provide insights into this point, and to also respond to a concern of the Reviewer #2 about complementation, we decided to ectopically re-express PlcA into the *Listeria* PlcA/B- strain, to demonstrate that it was the expression of the phospholipases that caused the formation of the NDP52+ granules, which is a central point of our manuscript. As shown in the revised Figure 4E, expression of PlcA was sufficient to induce the formation of these granules, supporting our conclusions.

Minor concern:
Figure 1a: This western blot would profit from a loading control, since the amount of S6K1 present in the 1h lane seems significantly lower than at other time points. Is protein abundance affected by infection, as well as phosphorylation? Maybe this point could be clarified by referring to Figure 2A already in the first results paragraph.

We present new western blots in the revised manuscript for the Figures 1A, 2A and Supplementary Figure 3D, which are of better technical quality than in the previous version. We believe that the new images presented will clarify these concerns.

Referee #2

The authors investigate the response of Hela cells to infection with Listeria. They find evidence for transient amino acid starvation and reduced mTOR activity. mTOR activity was unrelated to the ability of Listeria to avoid autophagy, which the authors show is partially mediated by PlcA and PlcB. This manuscript provides rather incremental progress over previous work. The authors published recently (Tattoli Cell Host Microbes 2012) that Shigella and Salmonella induce amino acid starvation in Hela cells; notwithstanding the more transient nature of the phenomenon in Listeria-infected cells, I don't think the data in Figs 1, 2 and 3 are particularly novel against the background of the authors' recent findings with Shigella and other bacteria.

We do not agree that our manuscript only provides incremental progress over our previous study on *Shigella*/*Salmonella*. First, the effects of *Shigella* and *Salmonella* on AA starvation pathways were different, and it was thus not possible to anticipate the outcome of *Listeria* infection. Second, our previous results demonstrated that the metabolic stress response was likely driven by the insertion of the type three secretion system (TTSS) into host membranes, at least in the case of *Salmonella* (see PMID 23259056). In the case of *Listeria*, which does not have a TTSS, it needed to be seen what were the effects of the toxins on mTOR and GCN2/eIF2α/ATF3 pathways. Since *Listeria* LLO and PLC toxins both induce host membrane damage but through totally distinct mechanisms, it was interesting to observe that their impact on the modulation of mTOR and GCN2/eIF2α/ATF3 pathways was also very different, and again, we do not think that these results could have been predicted. Finally, it must be pointed out that the precise characterization of the effect of *Listeria* toxins on mTOR signaling was important for showing that PLCdependent escape of *Listeria* from autophagy was unrelated to mTOR, and for the interpretation of our new data on autophagic flux in WT versus PlcA/B- *Listeria*.

Thereafter the authors investigate the fate of NDP52+ cytosolic granules in Listeria infected cells. These are most likely the membrane remnants of vacuoles from which Listeria has escaped based on markers found in this study on NDP52 granules and known to occur in association with membrane remnants (galectins, NDP52, ubiquitin). Targeting of membrane remnants by autophagy has been described in cells infected with Listeria and other bacteria (Dupont Cell Host Microbes 2009, Thurston Nature 2012). Escape of Listeria from autophagy is mediated by several genes (ActA Yoshikawa Nat Cell Biol 2009, InlK Dortet PLos Pathogens 2011 and PlcA/B Birmingham 2007). The role of PlcA/B in escape from autophagy was confirmed in this study. Whether the NDP52+ granules / membrane remnants represent 'stalled preautophagosomal structure' remains to be firmly established as the only supporting evidence is the occurrence of WIPI. Kinetic quantifications, electron microscopy and the inclusion of less ambiguous markers of autophagosome maturation (Klionsky 2012 Autophagy) will be required to firmly establish the authors' conclusion about the 'stalled' state of these structures.

The reviewer suggests that the accumulating granules in *Listeria*-infected cells are "membrane remnants" that have been described previously, mainly in *Shigella*infected cells. Although it is very possible that such membrane remnants serve as a nucleator for the formation of these granules, we believe that it is very unlikely that the granules are only membrane remnants, and think that the granules represent pre-autophagosomal structures that are unsuccessfully trying to engulf and degrade damaged membranes. Here are the main reasons that bring us to these conclusions:
-If the NDP52+ granules were "simple" membrane remnants, those would accumulate in both PlcA/B- *Listeria* and wild type *Listeria*, since both strains express LLO, which causes rupture of the entry vacuole.
-Similarly, if the NDP52+ granules were membrane remnants, the NDP52+ granules should be affected by ActA deficiency. Indeed, ActA- *Listeria* are impaired in their cell-to-cell spread capacity and thus generate less membrane remnants. However, we found no role for ActA in the accumulation of NDP52+ granules.
-*Listeria* escapes the entry vacuole rapidly through LLO, and our mTOR/S6K1/ATF3 data support the conclusion that LLO-dependent membrane damage peaks at around 30-60' p.i. This kinetic is not compatible with the accumulation of the granules after 2h p.i.
-Although not presented in the manuscript, we performed numerous experiments with *Shigella*, which also ruptures the entry vacuole and generates membrane remnants and never observed accumulation of similar granules.
In addition, we also present new evidence that further support our interpretation that these structures indeed represent accumulating pre-autophagosomal structures, and complemented the WIPI-2 immunofluorescence with other autophagy markers:
- We used a GFP-RFP-LC3 construct and demonstrated that the granules were systematically yellow (thus red and green), indicative of immature autophagosomes.
- We detected the endogenous DFCP1 in these granules. DFCP1 is present on primordial pre-autophagosomal structures.
-In kinetic experiments using the GFP-RFP-LC3 construct to monitor autophagic flux, we showed that the autophagic flux was maximal at 1-2h p.i., and strongly declined at 3-4h p.i. for both *Listeria* WT and PlcA/B-, in agreement with our mTOR inhibition data. Although the kinetics were similar for both strains, we observed that the intensity of the autophagic flux was reduced in WT *Listeria* as compared to PlcA/B- *Listeria*, which likely accounts for the accumulation of preautophagosomal structures in cells infected with WT *Listeria*. The fact that the granules accumulated mainly at late time points (>2h p.i.) likely reflects the fact that the autophagic flux needs to pass under a certain threshold for this event to occur. Thus, these important new data provide a rationale for the accumulation of preautophagosomal structures at late time points of *Listeria* WT infection, by showing that the autophagic flux reaches very low levels that are not observed in the case of the PlcA/B- *Listeria*.
-We now demonstrate that endogenous ATG16L1 and LC3 accumulate in the granules.
-Using WIPI-2 knockdown cells, we further demonstrate that WIPI-2 is crucial for the accumulation of LC3 into NDP52+ granules. These new results are critical as they demonstrate that LC3 accumulates into these granules as a result of a dynamic autophagic input, rather than as a consequence of the aggregation of proteins, such as NDP52, which display LC3-interaction domains.
Finally, we would like to emphasize the importance of WIPI-2 as a marker of preautophagosomal structures: both the results presented in this manuscript and our previous data (see PMID 20505359) demonstrate that WIPI-2 is absent from mature autophagosomes. None of the other markers that we followed (Ubiquitin, NDP52, Galectin-8, DFCP1, ATG16L1, LC3) displayed a similar profile. In the case of bacterial autophagy, it is remarkable that as soon as the autophagosome fully encloses the bacteria, endogenous WIPI-2 staining disappears, although LC3 and ATG16L1 remain associated (as we show in the revised manuscript).

technical points
Fig 1a: why would a phospho-specific antibody produce two bands while a protein-specific antibody

stains only one species? Are the antibodies specific? Is it necessary to confirm their specificity, for example by knockdown? If only one band of the phosphor-specific antibody is derived from S6K1, why would the other band also vary in intensity? A loading control may help.

We present new western blots in the revised manuscript for the Figures 1A, 2A and Supplementary Figure 3D, which are of better technical quality than in the previous version. The S6K1 antibodies used are from Cell Signaling, and those are the ones used by most investigators studying mTOR signaling. The S6K1 antibodies (phospho and non phospho) detect mainly a doublet, corresponding to the two isoforms of S6K1: p70 and p85. The antibodies typically detect more of the p70 isoform over the p85 isoform, and the intensity of the p85 band over p70 depends on the exposure of the films: low exposed films show mainly the p70 isoform. Nevertheless, we believe that the new images presented will clarify these concerns.

Fig 1b (and 1c) The quality of the images is poor. It is hard to believe that images of that quality allow assessment whether or not mTor localizes to individual LAMP+ vesicles (as in 1c). (Independent from the image quality, using green, red and yellow rather than green, orange, and yellow would make the images throughout the manuscript easier for the reader to interpret.)

We present new images from recently performed experiments, which are of better quality. The antibody used here is the same as the one used by the group of David Sabatini who presented the original demonstration that mTOR localization to late endosomes/lysosomes was affected by AA starvation. The endogenous mTOR staining in IF with this antibody is not very bright, but has an excellent signal/noise ratio, which makes the quantification by eye on the microscope very easy.

Fig7c: Data should be presented as primary CFU of wt and mutant bacteria, rather than as ratios to enable the reader to judge primary data. If necessary the ratio can be given in an additional (supplementary?) figure.

Absolute levels of CFUs tend to vary from one experiment to another, which makes it difficult to pool several experiments together to get statistical analyses. The CFU data after co-infection experiments presented were performed four independent times and provided very robust and reproducible effects of the WT *Listeria* on the growth of the co-infected PLC- *Listeria*. The representation presented in the manuscript was chosen because it allowed showing the results of the individual experiments and performing statistical analyses.

Fig2, 3, 4, 6, 7: complementation of Listeria knockouts is missing entirely from the manuscript.

Complementation experiments are rarely performed on those widely used strains that were generated over twenty years ago by the Portnoy lab. However, in agreement with this comment, and also in response to another question by Reviewer #1 above, we decided to re-express PlcA into the *Listeria* PlcA/B- strain in order to demonstrate that the expression of the phospholipases caused the formation of the NDP52+ granules, a central point of our manuscript. As shown in the revised Figure 4E, expression of PlcA was sufficient to induce the formation of these granules, supporting our conclusions.

Referee #3

This manuscript examines the role of bacterial PLCs during Listeria monocytogenes (Lm) escape in epithelial cells and fibroblasts. It claims that Lm use PLCs to prevent host anti-bacterial autophagy and the progression of early stage autophagosomes toward lysosomes. This is a reasonable hypothesis, given recent studies showing the involvement of diacylglycerol in pathogen-induced autophagy (Shahnazari et al., Cell Host and Microbe 2010) and showing that anti-bacterial autophagy is induced by exposed galectin 8 to cytosol after pathogens perforate vacuoles (Thurston et al., Nature 2012). The main conclusion is supported by experiments showing that, after infection, intracellular Lm deficient in PlcA and PlcB (PlcA/PlcB- Lm) become surrounded by the early

autophagosome marker NDP52 to a greater extent than do wild-type Lm (Fig. 3D). The interpretation is that wild type bacteria utilize the PLCs to inhibit bacterial capture by the autophagic machinery. The conclusion stated in the title, that the PLCs stall progression of preautophagosomal structures, is supported by images showing that the autophagosomes containing wildtype Lm fail to acquire a later marker of autophagy, WIPI-2 (Fig. 5). Moreover, the protective effect provided by PLCs may act in trans, as coinfection of cells with wildtype and PlcA/PlcB- Lm conferred a slight increase in survival upon the PlcA/PlcB- Lm. Although interesting, the manuscript is inconclusive because the data supporting the major claim are weak and not quantitative. Overall, the quality of the data in the manuscript is uneven and sometimes poor.

Major Concerns:

1. None of the quantitative comparisons of labeling in cells infected with different strains of Lm normalize for differences in the efficiency of infection. Therefore, experiments such as Fig. 4D, showing the percent of cells containing NDP52 granules, do not adjust for the possibility that different percentages of cells will contain bacteria. That is, do PlcA- Lm-infected cells contain a smaller percentage of NDP52+ granules than cells infected with wildtype Lm simply because a smaller percentage of cells contain bacteria?

For all the immunofluorescence studies presented in the manuscript, we only analyzed results of experiments where the infection levels were similar. In addition, quantifications were performed in randomly selected fields from several independent experiments, thus minimizing the potential effects of heterogeneous cell-to-cell infection efficiencies. Finally, cells that were poorly infected (less than 3 bacteria observed in DAPI channel) or very heavily infected (hundreds of bacteria and defects in the host cell nuclear morphology- a sign of ongoing cell death in these *Listeria*-infected cells) were excluded from the quantifications. We clarified this point in the Methods section of the manuscript.

2. Similar concerns about variable infection efficiencies apply to the western blotting and qPCR data.

For all our WB and qPCR experiments, a Giemsa stain is systematically performed on a dedicated well, and only experiments for which good and homogeneous levels of infection are observed will be used further. Our results for both WBs and qPCR are extremely reproducible, which argues against a significant effect of infection efficiency. For instance, for analysis of S6K1 phosphorylation levels over time in cells infected with WT *Listeria*, the reviewer can notice that similar results are presented at 4 different places in the manuscript: Fig. 1A, 2A, 3B and S3D (in each experiment the WT *Listeria* is shown). As shown, very similar profiles and kinetics are observed on independent experiments performed several months apart. Regarding qPCR, in addition to the Giemsa stain, we systematically analyze both ATF3 levels and IL-8 levels, with the latter providing a good estimate of the infection efficiency.

3. The claim that PlcA and PlcB inhibit autophagosome progression is not supported by quantitative data showing rates of autophagosome progression plus and minus the PLCs. Rather, suggestive images are provided. This considerably weakens the conclusions.

With regards to the analysis of the rate of autophagosomal progression, we now present new data using cells over-expressing a GFP-RFP-LC3 construct, as proposed by Reviewer #1, which address this concern (please see response to related issue of other reviewers above). Together, these important new data present a rationale for the accumulation of pre-autophagosomal structures at late time points of *Listeria* WT infection, by showing that the autophagic flux reaches very low levels that are not observed in the case of the PlcA/B- *Listeria*.

4. In quantifying the membrane association of Lm inside infected cells, more should be done to distinguish cytosolic vs. vacuolar Lm. Cells infected with wildtype, and PlcA/PlcB- Lm should contain two different groups: 1) Lm which escape into cytosol and 2) Lm which remain in vacuoles.

Using DAPI images as representative signals of vacuole-associated Lm fails to distinguish these groups. To observe and quantify co-localization of Lm-containing vacuoles and LAMP/NDP52, the authors should exclude escaped Listeria (eg., using rhodamine-phalloidin staining to exclude cytosolic bacteria).

On the basis of our own experiments, and also the current literature on *Listeria*, we want to emphasize that we observe very little vacuole-confined *Listeria* at any time point, using LAMP2 staining. *Listeria* is extremely efficient at escaping to the host cytosol in a LLO-dependent manner. As a matter of fact, virtually all the WT *Listeria* found to be LAMP2+ were also NDP52+, suggesting that those were actually bacteria that were trapped in autophagosomes, rather than bacteria that had remained in the entry vacuole, as observed in the case of the LLO- or TKO *Listeria*. Our observations and quantifications in Fig. 2 and 3 support these conclusions.

Minor Comments

1. Fig1A. Since the amount of S6K1 at 1h is less than that at other times, it is difficult to interpret phospho- S6K signals. This problem occurs in other blots, as well, especially Fig. S3D.
As mentioned above in response to Reviewers #1 and 2, we have performed new western blots that should respond to this issue. Those new western blots are found in Figures 1A, 2A and S3D.

2. Figs. 1A, 1D, 2A, 3B. Exposure times should be shorter.
We believe that the issue about exposure times of western blots is fixed with the new improved data.

3. Fig. 2C. In the TKO-DAPI image, there are many bacteria-like structures. However, no bacteria-like structures are visible in WT-DAPI image. Overall, bacteria were difficult to spot in all of the DAPI images.
We present data with improved images for Fig. 2C in the revised version of the manuscript.

4. Fig4AB. The image is not clear. At 4h, most of the WT Lm should have escaped into cytosol. Is the bacterium shown in panel B still in a vacuole? If so, what is ratio of the bacteria which does not escape?
See our response to point #4 above.

5. Figs. 5C, 5D, 5E, 6B and 6C. Quantification is necessary.
Large-scale quantifications were not done for the experiments mentioned at the time when the IFs were performed; however, the images shown are representative of the overall effects that were observed in three or more independent experiments.

6. Fig. 6A. The infection and escape efficiencies of each strain should be reported. Also, the experiment measured only cells at 4 hours after infection. What were the PI3P levels at early timepoints?
See response to comments above about infection efficiency and cytosolic escape. The levels of PI3P at early time points were measured in some experiments but resulted in rather small effects and overall inconclusive results.

1st Editorial Decision 06 September 2013

Thank you for the re-submission of your research manuscript to The EMBO Journal. In light of the previous reports, your study has been sent to two of the original referees, whose reports have been just received.

As you will see in the attached reports, although referee #1 is as positive as s/he was in the first round of review, and referee #2 acknowledges that the manuscript has seen some improvements compared to the previous version, in his/her opinion the major concerns regarding the general interest of your manuscript already raised in the first round of review still remain. Therefore, s/he

does not recommend publication of your study in The EMBO Journal.

Given this negative evaluation and the fact that your manuscript is a re-submission of an already rejected study, I am afraid that we do not see it productive to call for yet another revised version of your manuscript at this stage and therefore we cannot offer to publish it.

I am sorry that I have to disappoint you this time. I hope, however, that the referee comments will be helpful in your continued work in this area and I thank you once more for the opportunity to consider your manuscript.

REFEREE REPORTS

Referee #1

The new data presented in the revised manuscript provide useful information that support and strengthen the findings reported in the original submission. In my opinion this study provides significant insights into the understanding of the mechanisms used by Listeria to evade the host immune system.

Referee #2

Regarding the impact of the manuscript, I stand to my original comment that Figures 1-3 provide only limited additional insight over Tattoli (Cell Host Microbe 2012), where the authors discovered that "...infection with Shigella and Salmonella triggers acute amino acid starvation (and reduced mTOR activity) due to host membrane damage." Having concluded that 'membrane damage' is the common denominator of the acute amino acid starvation / mTOR response, the observation that damage by Listeria due to LLO causes the same principal response as damage by the type three secretion systems of Gram-negative bacteria is, despite the authors reply, in my opinion not worth three figures in EMBO J.

The characterization of the NDP52 granules as stalled autophagosomal structures, however, has made good progress. Accumulation of WIPI2 and DFCP1 on NDP52 granules as well as the traffic-light RFP-GFP-LC3 assay provide convincing evidence of an aberrant autophagic structure occurring in cells infected with wt but not PLCA/B- Listeria. The proposed mechanism of autophagy stalling by PLCA/B is not without problems, given the accumulation of the PI3P-binding WIPI2 in NDP52 granules in Wortmannin-treated cells (Fig6D).

specific points

1. colors in microphotographs (1b, 2c, 3c + many others): as pointed out in my original comments, the use of orange instead of red makes it unnecessarily difficult for the reader to detect colocalization. The authors should use the conventional green / red / yellow color scheme.

Rebuttal 06 September 2013

Thanks for sending the comments regarding our publication. I have to say that i am shocked by the decision...
Reviewer #1 is extremely supportive of publication, and reviewer #2 makes a comment about the fact that he/she thinks that the first 3 figures should be more concise ("not worth three figures"). However, and this is crucial, this reviewer acknowledges "The characterization of the NDP52 granules as stalled autophagosomal structures, however, has made good progress" and "Accumulation of WIPI2 and DFCP1 on NDP52 granules as well as the traffic-light RFP-GFP-LC3 assay provide convincing evidence of an aberrant autophagic structure occurring in cells infected with wt but not PLCA/B- Listeria"...This was the main point that was raised at the first stage of revision: that we should provide better evidence that the Listeria PLCs indeed stall pre-autophagosomal structures.

I read several times the comments from both reviewers and really do not understand the reason for the decision made here. If the reviewer #2 is concerned that we put too much emphasis on Figs 1-3 because of the supposed link with our previous publication, we could reduce it to 1 or 2 figures very easily. Would it be possible to call you to discuss this issue on the phone? This work represents several years of intense work and i would like to get to the bottom of it.

2nd Editorial Decision 27 September 2013

Thank you for the submission of your revised manuscript to The EMBO Journal and please accept again my apologies for the delay in our response. As I already mentioned in a previous communication, your study was sent back to former referee #3, who has evaluated not only the manuscript, but also his/her own comments and those of the other referees. After careful consideration, s/he now believes that all major concerns have been properly addressed and your manuscript is almost ready for publication.

A minor issue, however, regarding figure 4 has been still pointed out by referee #3, as you will see below, that will need your attention before your manuscript can be finally accepted. Browsing through the manuscript myself, I have also noticed that scale bars are not included in any micrographs, neither in the main figures, nor in the supplementary data, and we require them for clarity.

I would like to mention that we now encourage 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. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels presented? The PDF files should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this initiative do not hesitate to contact me.

Thank you very much again for your patience. I am looking forward to seeing the revised, final version of your manuscript.

REFEREE REPORT

Referee #3

I have reviewed the revised manuscript, the previous three reviews and the authors' response to the earlier critiques. They have adequately addressed my concerns regarding quantification and micrograph image quality. The characterization of the preautophagic granules is sufficient to support their claims. The added time-course experiments support the claims about autophagic flux. My only concern about the quality of the new data is with Figure 4E, which should be supported by quantitative data.

I also think that the revised manuscript and authors' responses adequately address the concerns of reviewers 1 and 2. Overall, this manuscript presents a novel mechanism of microbial resistance to autophagic defense mechanisms, which should be of interest to the readership of EMBO J.

