Inhibition of the PtdIns(5) kinase PIKfyve disrupts intracellular replication of Salmonella


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1st Editorial Decision 09 July 2009

Thank you for submitting your research manuscript to The EMBO Journal editorial office. I did now receive reports from three referees that I had asked to comment on your work. As you will see, they all appreciate the potentially interesting finding that PIKfyve might be essential for macropinosome/lysosome fusion and contribute to Salmonella replication. However, their comments also reveal significant shortcomings in the actual experimental support for these claims. More specifically, all three request to abrogate PIKfyve function by alternative means (siRNA and tested dominant-negatives) followed by careful quantification of the resulting effects that should also include SFV's/SIF's characterization by EM. Ref#2 demands a better connection between the cell biological effects of PIKfyve with its role in Salmonella replication, an aspect that will have to be resolved to present a more coherent and functionally relevant case. All in all, we do realize that your paper contains very interesting initial observations. However, the reports also reveal the very preliminary state of analysis that would need significant further experimentation with a currently uncertain outcome to eventually reach the rather high demands and standards set by our referee's.

Appreciating not only the current problems, but also the potential of your study, and certainly conditioned on the fact that you were willing and able to provide the necessary and convincing experimental support for your claims, we would be willing to assess suitability of an appropriately revised version in the future. Given the amount of critiques I also would like to mention that we are able to extend the deadline for revisions upon request by the authors to allow sufficient time for experiments. IN this respect I really urge you to take the comments of our referee's into serious consideration as we will have no hesitation to reject the study if their valuable points have not been appropriately addressed.

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Finally, I have to remind you that it is EMBO Journal policy to allow a single round of revisions only, which means that the final decision on acceptance or rejection will depend entirely on the content of the final version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Kerr et al. report that PIKfyve is essential for intracellular replication of Salmonella typhimurium. Using a fluorescence-based content mixing assay the authors also find that PIKfyve is required for fusion of macropinosomes with lysosomes. These conclusions are based on the use of a pharmacological inhibitor and overexpression of the catalytically inactive K1831M mutant. Overall this manuscript sheds light on the functions of PIKfyve, but in its present form it is not sufficiently convincing to merit publication in the EMBO Journal.

Specific points:

1. A major limitation of the present study is the methods used to inhibit the activity of PIKfyve. Even though YM201636 is useful as a selective inhibitor, the specificity of this inhibitor has only been tested on a limited cohort of kinases so far, and the working concentration (800 uM) is rather high. Overexpression of the K1831M mutant is prone to indirect effects due to sequestration of any proteins that preferentially bind to this form of PIKfyve. The authors should use RNAi technology to verify the importance of PIKfyve on Salmonella replication and macropinosome-lysosome fusion.

2. The characterization of SFVs and SIFs in the absence of PIKfyve function is rather superficial. These structures should be examined by electron microscopy.

3. The title of Figure 2 is an overinterpretation of the data. All it shows is that 2xFYVE and PIKfyve are recruited to macropinosomes with similar kinetics. Moreover, in the absence of any statistical treatment it is difficult to judge how representative the data are.

4. The manuscript needs to be checked for typos.

Referee #2 (Remarks to the Author):

In this work, Kerr et al report a study of the role of PIKfyve in the intracellular replication of Salmonella and on fusion between macropinosomes and LE/Lys. Using a pharmacological inhibitor of PIKfyve (YM201636), the authors performed experiments in A431 cells infected with S. Typhimurium that led them to propose that the activity of PIKfyve interferes with the intracellular replication of Salmonella. Kerr et al then analysed the role of PIKfyve during macropinocytosis and in macropinosome maturation, by using YM201636 and a dominant-negative catalytically inactive PIKfyve. They conclude that PIKfyve regulates macropinosome-LE/Lys fusion by mechanism that is distinct from that involving the small GTPase Rab7. Next, the authors analysed the role of PIKfyve in a) the formation of the characteristic LAMP1 enriched tubules (SIFs) that appear in epithelial-like cells infected with Salmonella and b) in the activity of the SPI-2 T3SS. Interfering with PIKfyve activity seemed to dramatically affect the appearance of SIFs and of effector translocation by the SPI-2 T3SS. Finally, Kerr et al perform experiments to study the effect of inhibiting PIKfyve in the intracellular replication of salmonella in macrophages. Here, the authors propose that interference with PIKfyve activity disturbs not only the ability of Salmonella to replicate within macrophages but also the bactericidal activity of phagocytes.
The interaction between intracellular microbial pathogens and host phosphoinositides and its regulators is an important topic of broad biological significance. The analysis of the role of PIKfyve in macropinocytosis is well executed, interesting, and sheds some light in this particular process. However, this paper of has two major general problems. One is that it is very unclear whether the observations made regarding PIKfyve and macropinocytosis correlate to a possible role of PIKfyve during intracellular replication of Salmonella. In the end, the two experimental models appear disjointed and the paper is not a coherent story. The other major issue is that the PIKfyve-Salmonella analyses have too many experimental flaws. Overall, there is no strong evidence that inhibition of PIKfyve disrupts intracellular replication of Salmonella. Even if PIKfyve might play a role during intracellular replication of Salmonella, this study does not clarify at which stage this is happening and what is the mechanism. Furthermore, there is a significant imprecision in the writing that disturbs the reading. A possible role of PIKfyve with intracellular Salmonella is however suggested (by what I would define as the preliminary experiments shown) and this is meaningful and interesting, but this issue needs to be analysed separately and much more carefully.

Specific comments:

1. There are several problems with the experiments from which the authors conclude that "Inhibition of PIKfyve activity interferes with the replication and survival of intracellular Salmonella":
   a) No quantitative data is presented. Quantitative and reproducible data from two different methods (microscopy and CFUs) for the same time point should be presented.
   b) 24 hrs of infection appears too long to me. Intracellular replication of Salmonella is normally analysed between 8 and 16 hrs post-infection (p.i.). Such long infection time can lead to artefacts due to bacterial overgrowth and cell lysis.
   c) It would be important to analyse more than one time-point. The authors do this, but use microscopy at 24 hr p.i. and CFUs at 12 hr p.i. The 1.47-fold reduction by CFUs was observed as a result of how many experiments? Is it statistically significant? Such small difference is clearly within the experimental error of this type of experiments.
   d) It would be important to analyse the effect of the YM201636 PIKfyve inhibitor when added to cells at different times. For example, is there an effect on intracellular replication of Salmonella when the YM201636 inhibitor is added to cells between 2-4 hrs p.i.?
   e) What is the effect on cell viability caused by the 26 hr incubation with YM201636? The authors analysed a possible effect of the inhibitor on bacterial growth in broth (which is an important control but that could easily be on supplementary data or even data not shown) but is there any effect on cells infected or not infected with Salmonella?
   f) It would be important that the role of PIKfyve on the intracellular replication of Salmonella be analysed after inhibiting PIKfyve by different means (not only pharmacological but also siRNA and dominant-negative).
   g) I also have an issue with the cell line used. Why A431 cells? This cell line is not physiologically relevant to Salmonella pathogenesis and to my knowledge there is no historical reason for using it (as for example HeLa cells, also not physiologically relevant but a common cell line used to study the intracellular replication of Salmonella throughout the years). This can lead to discrepancies in observations that will only bring confusion into the field. Furthermore, S. Typhimurium SL1344 forms filaments within this cell line. This is actually a consequence of a defect in bacterial cell division (Henry et al, Mol Microbiol, 2005, 56:252-267). Therefore, it seems rather inappropriate that such strain/cell line combination is used to study intracellular replication of Salmonella. It could be helpful to use a bacterial strain (such as ATCC 14208) that does not form filaments or a cell line in which SL1344 also does not form filaments.

2. There are also many problems with the experiments in which the authors conclude that "inhibition of PIKfyve activity interferes with SCV development". I will not list all, many are similar to those mentioned above. It seems to me that the authors are analysing a too late time point. What is the effect of PIKfyve inhibition on SCV maturation between 0-2 hrs p.i.? By that time, SCVs have already interacted with LAMP1-positive compartments in a Rab7-dependent manner (see Meresse et al, 1999, EMBO J). Assuming that PIKfyve is important for SCV maturation my guess is that the protein is acting much earlier than what was analysed by the authors. For example, it is striking that translocation of PipB was not observed in cells transfected with PIKfyve(K1831M)-GFP [By the way, what happens if cells are transfected with PIKfyve(S318A)-GFP?]. The most likely explanation is that upon PIKfyve inhibition the SCV does not acidify and therefore the SPI-2 T3SS is never assembled or activated. On the other hand, if indeed the SPI-2 T3SS is not active in
PIKfyve-inhibited cells I find it very strange that the effect on intracellular replication of Salmonella observed by CFU analysis is so mild (1.5 fold). The authors should analyse directly if upon PIKfyve inhibition the SCV acidifies and if the SPI-2 T3SS is expressed. Also, in the images shown in Figure 4A how do we know that the cells are infected? When analysing the cells for the presence of SIFs were the bacteria immuno-labelled to identify infected cells? As is not possible that the filamentous appearance of SL1344 interferes with the scoring for LAMP1-rich tubules?

3. The macrophage experiments also have its problems. Most of all they are very confusing. First, the data does not support that PIKfyve inhibition prevents intracellular replication of Salmonella. The "explanation" that in YM201636-treated cells the macrophage bactericidal activity is affected is rather weak. Then why in Figure 5A do we appear to see more bugs in DMSO-treated than in YM201636-treated (but again, what is the effect of the drug on macrophage viability?). Why some experiments were done with RAW264.7 cells and the others with BMDMs? Also, the authors claim that SCVs in BMDMs treated with YM201636 are LAMP1-negative. Which percentage? Is this also observed (and in which percentages) in other cell lines used in this study (RAW264.7 and A431)? What about in HeLa cells? This raises the very important question of knowing whether inhibition of PIKfyve affects integrity of the SCV membrane. The "LAMP1-negative SCVs" can actually be cytosolic bacteria, which is also an alternative explanation of why SPI-2 T3SS effector translocation was not observed in PIKfyve-inhibited cells.

4. Even if "Salmonella exploit macropinocytosis to gain entry into" non-phagocytic cells, how do we know that a possible role of PIKfyve in the intracellular replication of Salmonella is related to the function of PIKfyve in macropinosome-LE/Lys fusion? There is no data in this manuscript directly supporting that idea.

5. It would be interesting to analyse if PIKfyve-GFP, PIKfyve[K1831M]-GFP, and PIKfyve(S318A)-GFP are recruited to the SCV and, if so, characterise the dynamics and kinetics of PIKfyve recruitment.

6. I find it strange that such a low concentration (5 ug/ml) of gentamicin has been used in Salmonella infections - has this been reported before? To my knowledge, the usual protocol is first to kill extracellular bacteria with 50-100 ug/ml gentamicin for about one hour and then reduce the antibiotic concentration to 10-20 ug/ml for the remaining infection time (see papers from the labs of Brumell, Hensel, Holden, Meresse, or Steele-Mortimer).

7. "By doing so the adherent SCV is expanded to form extensive LAMP1-positive SIFs in which the bacterium then replicate" - what does this mean? Adherent SCV? Salmonella replicate within a tubulated SCV but not within the tubules.

8. What is a "highly efficient" intracellular pathogen? How is the "efficiency" of an intracellular pathogen measured?

9. In the introduction, the two consecutive sentences starting with "Orally ingested..." need to be re-written. The uptake of Salmonella by CD-18 expressing phagocytes is independent of the SPI-1 T3SS.

10. Intracellular Salmonella delivers effectors into host cells via the SPI-2 T3SS and not via SPI-2 (page 3, line 15).

11. In the discussion, the entire paragraph starting with "To accommodate the expanding..." contains wrong interpretations of the data and is a very poorly written description of the features of intracellular replication of Salmonella. Most of all, the data presented in the manuscript does not allow one to conclude, or even to suggest, that the possible effect of PIKfyve inhibition on intracellular replication of Salmonella is related to the observed role of PIKfyve on fusion between macropinosomes and LE/Lys. Furthermore, and again, intracellular Salmonella does not replicate within SIFs. Also, are there effectors that rely upon SIFs for their functions? Which experiments/papers show this?

12. The authors should write Salmonella enterica serovar Typhimurium (Salmonella enterica italicised) and abbreviate to S. Typhimurium (S italicised) or to S. typhimurium (all italicised).
13. Page 6, line 8, Salmonella should be italicised (and in several other places in the text).

14. Please write Salmonella-containing vacuole and not Salmonella Containing Vacuole.

15. Please write SCV maturation and not SCV development.

Referee #3 (Remarks to the Author):

This manuscript presents a series of studies designed to determine whether the PtdIns(5) kinase, PIKfyve, is required for the intracellular replication of Salmonella. The authors show that while a PIKfyve inhibitor does not inhibit Salmonella growth in culture, it does inhibit intracellular replication of the bacteria. The authors find that wildtype PIKfyve and PI3P transiently associate with macropinosomes and present evidence that the catalytic activity of PIKfyve, is responsible for disappearance of PtdIns3P from macropinosomes. The authors also provide data that PIKfyve is required for fusion of micropinosomes with late endosomes, and further, that PIKfyve is required for formation of Salmonella induced filaments.

This is a novel, and potentially very important study. Little is known about the cellular functions of PIKfyve or PtdIns(3,5)P2, especially in infection. However, the lack of specificity of YM201636 [1], the PIKfyve inhibitor, along with the absence of direct data showing that the PIKfyve(K1831M) is catalytically inactive, raise questions about whether the reagents utilized were the most effective reagents available, for specific inhibition of PIKfyve catalytic activity. Moreover, the fact that active PIKfyve is part of a large multimeric complex brings up concerns about the relevance of studies that overexpress PIKfyve alone.

1. In light of the above, it is critical that the authors use a non-related approach, for example RNAi silencing, to show that when PIKfyve is knocked-down (shown by western blot) they obtain the same results provided by YM201636.

2. There are no published reports that the PIKfyve(K1831M) mutant is catalytically inactive. This data should be provided. The authors should either test this mutation in vitro, or show that overexpression of this mutant affects cellular levels of PtdIns(3,5)P2. It is not sufficient to assume that the K1831M mutant behaves similarly to the K1831E mutant. Furthermore, the expression of the K1831E mutant in COS cells had very little effect on the levels of PtdIns(3,5)P2 [2].

3. The results presented in Figure 2 are difficult to interpret.
   A. The time scales of each experiment should be the same. As presented, it appears that wild-type PIKfyve GFP comes on and off the macropinosome prior to GFP-2xFYVE. This does not fit with the statement in the manuscript that : PIKfyve-GFP is however recruited with a similar kinetic profile to that of the PI(3)P-specific probe GFP-2xFYVEHrs"
   B. The authors do not explain why the presumed catalytically inactive PIKfyve(K1831M) mutant behaves differently than GFP-2xFYVE. They each contain PI3P binding domains, and lack PtdIns 5-kinase activity. The authors should test the localization of these three GFP tagged proteins in cells were PIKfyve has been knocked down. Also RNAi knockdown would bypass the problem of having the mutant PIKfyve also serve as a reporter for PtdIns3P
   C. Each of the graphs represent the behavior of a single macropinosome. The authors should either show the averages plus error bars of a statistically significant number of macropinosomes, or show individual tracings of multiple macropinosomes.

4. The authors state that homotypic fusion of macropinosomes is not affected by the PIKfyve(K1831M) mutant. This should be shown, and also tested in knock-down experiments.

5. The authors state that the PIKfyve(S318A)-GFP mutant did not prevent fusion of macropinosomes with the late endosome. It is not clear what this means. The effect of this mutation PIKfyve activity is not clear. Plus in discussing this mutant (top of page 10) the authors mention "the lipid phosphatase activity of PIKfyve". Is this a typo?
6. In Figure 3, the authors should show a scale of the heat map. Also, because Dextran-647 is shown as blue, it would make the heat map less confusing to show no fluorescence as black rather than blue, or to pseudo-color the Dextran-647 a color that is not blue. Also, in Figure 3C, the authors need to show the results of GFP-Rab7(Q67L) alone.

7. The authors report that treatment with YM201636 combined with transfection with GFP-Rab7(Q67L) does not rescue the YM201636 phenotype. They interpret this to mean that PIKfyve and Rab7 affect macropinosome-late endosomal/lysosomal fusion via independent pathways. However, these results do not distinguish between parallel pathways or a single ordered pathway.

8. A further problem with the experiments shown in Figure 3, are potential effects of the PIKfyve(K1831M) mutant on loading Dextran-647 into late endosomes/lysosomes.

9. In Figure 4, it is not apparent which are the SIF structures. The authors should show an enlarged view of a region that contains these structures. Also the level of expression of each GFP construct is very different. This is particularly important for comparison of PIKfyve-GFP with the PIKfyve(K1831M) mutant.

10. The data regarding PipB is also unclear. In cells with PIKfyve-(K1831M) there is no visible HA-PipB2. The loss of PipB2 protein is consistent with a lack of SCV maturation. However, it is also consistent with these cells having not been infected, or PIKfyve-K1831M blocking the expression or stability of this protein. Control experiments that rule out these possibilities should be presented.

11. Comparison of the behavior of PIKfyve(K1831M) in Figure 2C with Figure 4A does not make sense. The simplest explanation of Figure 2C is that the K1831M mutant is dominant negative and inhibits endogenous PIKfyve. This would explain why GFP-FYVE comes off the membrane, while the PIKfyve(K1831M)-GFP mutant does not. However, if the PIKfyve(K1831M)-GFP mutant is dominant negative, then there should be a large number of LAMP1 positive vacuoles in Figure 4A. Again, RNAi knock-down of PIKfyve should provide more straightforward answers.

12. The authors should show the data that Salmonella replication was reduced 1.47-fold in YM201636 treated cells.

Minor points.
On page 10 and 11, the authors refer to Figure 3B, but meant 3C.

Similarly, on page 10 they referred to Supplementary Figure 2 instead of Supplementary Figure 1.


requested, we have applied RNAi technology to deplete the PIKfyve protein. Unfortunately, two antibodies reported to detect PIKfyve [(1) & Abnova (H00200576-A01)] failed to detect the endogenous enzyme in our cell type of interest. We therefore selected siRNAs based on the ability to induce large endosomal vacuoles as observed by others (2) when they depleted PIKfyve using siRNA. Analysis of RNAi-treated cells that displayed the large endosomal vacuoles resulted in a decrease in the intracellular replication of Salmonella consistent with the other strategies used to modulate PIKfyve’s activity (see Figure 1). In addition, we extended this approach to also deplete VAC14 by siRNA. VAC14 is part of the regulatory complex that activates PIKfyve. RNAi depletion of Vac14 gave the same phenotype as PIKfyve. The application of these 4 independent strategies to ablate PIKfyve activity provides conclusive evidence that PIKfyve activity is essential for intracellular replication of Salmonella.

Referee #1 (Remarks to the Author):

Kerr et al. report that PIKfyve is essential for intracellular replication of Salmonella typhimurium. Using a fluorescence-based content mixing assay the authors also find that PIKfyve is required for fusion of macropinosomes with lysosomes. These conclusions are based on the use of a pharmacological inhibitor and overexpression of the catalytically inactive K1831M mutant. Overall this manuscript sheds light on the functions of PIKfyve, but in its present form it is not sufficiently convincing to merit publication in the EMBO Journal.

Specific points:

1. A major limitation of the present study is the methods used to inhibit the activity of PIKfyve. Even though YM201636 is useful as a selective inhibitor, the specificity of this inhibitor has only been tested on a limited cohort of kinases so far, and the working concentration (800 uM) is rather high. Overexpression of the K1831M mutant is prone to indirect effects due to sequestration of any proteins that preferentially bind to this form of PIKfyve. The authors should use RNAi technology to verify the importance of PIKfyve on Salmonella replication and macropinosome-lysosome fusion.

   >>> This was a common point from multiple referees (see above for more detailed response). I am uncertain if it is an error on part of this referee or formatting but we used 800 nanoM not 800 microM of YM201636. Others routinely use this concentration in the published literature.

2. The characterization of SFVs and SIFs in the absence of PIKfyve function is rather superficial. These structures should be examined by electron microscopy.

   >>> The referee is correct. Therefore we have added EM analysis of Salmonella infected cells (Figure 6 & 8) in control and YM201636 treated cells.

3. The title of Figure 2 is an over interpretation of the data. All it shows it that 2xFYVE and PIKfyve are recruited to macropinosomes with similar kinetics. Moreover, in the absence of any statistical treatment it is difficult to judge how representative the data are.

   >>> We have significantly modified the data presented in this figure. As requested we have included multiple examples and standardized the acquisition conditions. In addition, we have examined the recruitment of the 2xFYVE in cells treated with YM201636. When PIKfyve activity is inhibited, the 2xFYVE domain accumulates and is retained on the macropinosome. This is consistent with an
increase in the levels of PI(3)P due to a absence of conversion to PI(3,5)P2. Therefore, PIKfyve activity is required to observe the transient accumulation of PI(3)P.

4. The manuscript needs to be checked for typos.

>> We have modified the manuscript to reflect this comment.

Referee #2 (Remarks to the Author):

In this work, Kerr et al report a study of the role of PIKfyve in the intracellular replication of Salmonella and on fusion between macropinosomes and LE/Lys. Using a pharmacological inhibitor of PIKfyve (YM201636), the authors performed experiments in A431 cells infected with S. Typhimurium that led them to propose that the activity of PIKfyve interferes with the intracellular replication of Salmonella. Kerr et al then analysed the role of PIKfyve during macropinocytosis and in macropinosome maturation, by using YM201636 and a dominant-negative catalytically inactive PIKfyve. They conclude that PIKfyve regulates macropinosome-LE/Lys fusion by mechanism that is distinct from that involving the small GTPase Rab7. Next, the authors analysed the role of PIKfyve in a) the formation of the characteristic LAMP1 enriched tubules (SIFs) that appear in epithelial-like cells infected with Salmonella and b) in the activity of the SPI-2 T3SS. Interfering with PIKfyve activity seemed to dramatically affect the appearance of SIFs and of effector translocation by the SPI-2 T3SS. Finally, Kerr et al perform experiments to study the effect of inhibiting PIKfyve in the intracellular replication of salmonella in macrophages. Here, the authors propose that interference with PIKfyve activity disturbs not only the ability of Salmonella to replicate within macrophages but also the bactericidal activity of phagocytes.

The interaction between intracellular microbial pathogens and host phosphoinositides and its regulators is an important topic of broad biological significance. The analysis of the role of PIKfyve in macropinocytosis is well executed, interesting, and sheds some light in this particular process. However, this paper of has two major general problems. One is that it is very unclear whether the observations made regarding PIKfyve and macropinocytosis correlate to a possible role of PIKfyve during intracellular replication of Salmonella. In the end, the two experimental models appear disjointed and the paper is not a coherent story. The other major issue is that the PIKfyve-Salmonella analyses have too many experimental flaws. Overall, there is no strong evidence that inhibition of PIKfyve disrupts intercellular replication of Salmonella. Even if PIKfyve might play a role during intracellular replication of Salmonella, this study does not clarify at which stage this is happening and what is the mechanism. Furthermore, there is a significant imprecision in the writing that disturbs the reading. A possible role of PIKfyve with intracellular Salmonella is however suggested (by what I would define as the preliminary experiments shown) and this is meaningful and interesting, but this issue needs to be analysed separately and much more carefully.

Specific comments:

1. There are several problems with the experiments from which the authors conclude that "Inhibition of PIKfyve activity interferes with the replication and survival of intracellular Salmonella".

a) No quantitative data is presented. Quantitative and reproducible data from two different methods (microscopy and CFUs) for the same time point should be presented.
b) 24 hrs of infection appears too long to me. Intracellular replication of Salmonella is normally analysed between 8 and 16 hrs post-infection (p.i.). Such long infection time can lead to artefacts due to bacterial overgrowth and cell lysis.

c) It would be important to analyse more than one time-point. The authors do this, but use microscopy at 24 hr p.i. and CFUs at 12 hr p.i. The 1.47-fold reduction by CFUs was observed as a result of how many experiments? Is it statistically significant? Such small difference is clearly within the experimental error of this type of experiments.

>> In the first submission we nominated to present a single time point for comparison of the Salmonella replication under different conditions. In response to the reviewers requests we have included our quantitative data for multiple time points over a 24 h period post-infection for the two different methods used (microscopy and CFUs). This has been generated from multiple independent experiments. The extension of this analysis provides greater detail however no changes in the conclusions were made.

d) It would be important to analyse the effect of the YM201636 PIKfyve inhibitor when added to cells at different times. For example, is there an effect on intracellular replication of Salmonella when the YM201636 inhibitor is added to cells between 2-4 hrs p.i.?

>> We do not understand the motivation for this experiment. I do not see what conclusions can be gained from inhibiting this process post-infection. Clearly a mixture of SCVs at different points of maturation will be present when the inhibition commences and interpretation of any outcome will be complex.

e) What is the effect on cell viability caused by the 26 hr incubation with YM201636? The authors analysed a possible effect of the inhibitor on bacterial growth in broth (which is an important control but that could easily be on supplementary data or even data not shown) but is there any effect on cells infected or nor infected with Salmonella?

>> Examination of the viability of the cells using a standard tunnel assay indicated no increase in the level of cell death when cells were treated with YM201636. This data is shown a supplementary figure 1.

f) It would be important that the role of PIKfyve on the intracellular replication of Salmonella be analysed after inhibiting PIKfyve by different means (not only pharmacological but also siRNA and dominant-negative).

>> This was a common point from multiple referees (see above for more detailed response).

g) I also have an issue with the cell line used. Why A431 cells? This cell line is not physiologically relevant to Salmonella pathogenesis and to my knowledge there is no historical reason for using it (as for example HeLa cells, also not physiologically relevant but a common cell line used to study the intracellular replication of Salmonella throughout the years). This can lead to discrepancies in observations that will only bring confusion into the field. Furthermore, S. Typhimurium SL1344 forms filaments within this cell line. This is actually a consequence of a defect in bacterial cell division (Henry et al, Mol Microbiol, 2005, 56:252-267). Therefore, it seems rather inappropriate that such strain/cell line combination is used to study intracellular replication of Salmonella. It could be helpful to use a bacterial strain (such as ATCC 14208) that does not form filaments or a cell line in which SL1344 also does not form filaments.
A431 are an accepted model cell line for macropinocytosis while HeLa are not. In fact HeLa cells display very little constitutive macropinocytosis. Therefore to enable comparison of macropinosome maturation with or without Salmonella infection we required the use of a cell line other than HeLa cells. While less common, A431 cells have been used to investigate Salmonella infection previously (for example see (3,4)). The subtle variations between sub-strains of Salmonella will require careful comparative investigation. We have a strong preference to limit this study to the SL1344 strain as it represents a widely used strain within the field.

2. There are also many problems with the experiments in which the authors conclude that "inhibition of PIKfyve activity interferes with SCV development". I will not list all, as many are similar to those mentioned above. It seems to me that the authors are analysing a too late time point. What is the effect of PIKfyve inhibition on SCV maturation between 0-2 hrs p.i.? By that time, SCVs have already interacted with LAMP1-positive compartments in a Rab7-dependent manner (see Meresse et al, 1999, EMBO J). Assuming that PIKfyve is important for SCV maturation my guess is that the protein is acting much earlier than what was analysed by the authors.

We have added extensive data to now examine the impact of PIKfyve inhibition at multiple time points during the 24 hr period post infection (see new Figures 2, 3, 5, 6 and 8). In particular in Figure 5 we now quantify the level of Lamp1 recruitment to SCV at 1 h p.i.

For example, it is striking that translocation of PipB was not observed in cells transfected with PIKfyve(K1831M)-GFP [By the way, what happens if cells are transfected with PIKfyve(S318A)-GFP?]. The most likely explanation is that upon PIKfyve inhibition the SCV does not acidify and therefore the SPI-2 T3SS is never assembled or activated. On the other hand, if indeed the SPI-2 T3SS is not active in PIKfyve-inhibited cells I find it very strange that the effect on intracellular replication of Salmonella observed by CFU analysis is so mild (1.5 fold). The authors should analyse directly if upon PIKfyve inhibition the SCV acidifies and if the SPI-2 T3SS is expressed. Also, in the images shown in Figure 4A how do we know that the cells are infected? When analysing the cells for the presence of SIFs were the bacteria immuno-labelled to identify infected cells? As is not possible that the filamentous appearance of SL1344 interferes with the scoring for LAMP1-rich tubules?

Firstly, we have replaced the IF data (Figure 5 and 6) with that using YM201636 to enable the requested dual co-labelling of Salmonella and Lamp 1 or PipB to be presented. Secondly, we have extended our analysis to examine the Salmonella protein SseA a non-secreted chaperone protein required for SPI-2 T3SS assembly. This protein is expressed when the SPI-2 loci is induced. As for PipB inhibition of PIKfyve activity prevented the expression of this protein in infected cells. This is consistent with a failure to induce the expression of SPI-2 T3SS system. We have also quantified the total levels of these reporter proteins using western immunoblotting.

We are aware of multiple published approaches applied to analyze the pH of macropinosome derived SCV (i.e. in epithelial cells). Steele-Mortimer’s group developed an elegant “ratiometric method based on the pH sensitivity of fluorescein [fluorescein isothiocyanate (FITC)] fluorescence” (5). This assay relies on the delivery of such fluid phase markers from the late endosomes and lysosomes to the SCV. Our observations that inhibition of PIKfyve blocks content mixing of fluid phase markers like dextrans for both macropinosomes and SCVs in epithelial cells prevents application of this approach. Monitoring the direct spectral properties of the FITC-labeled Salmonella is prone to photobleaching (6) and is only suitable for short time frames post-infection. We have monitored the co-localisation of the acidotropic dye LysoTracker with SCV and observed limited co-localization. This observation is consistent with other published observations (7) but conflicts with other reports. Therefore it appears that the application of lysotracker to monitor pH depends on the experimental conditions used (ie the host cell or bacterial strains).
3. The macrophage experiments also have its problems. Most of all they are very confusing. First, the data does not support that PIKfyve inhibition prevents intracellular replication of Salmonella. The "explanation" that in YM201636-treated cells the macrophage bactericidal activity is affected is rather weak. Then why in Figure 5A we do appear to see more bugs in DMSO-treated cells than in YM201636-treated (but again, what is the effect of the drug on macrophage viability?).

>> As originally documented, the observations within the BMDM's with respect to intracellular replication were less clear than in epithelial cells. To further clarify this data we have extended the analysis to examine multiple time points within 24hr post infection (figure 6). Within BMDM's monitoring the level of RFP-Salmonella indicates that more Salmonella replication occurs in control cells when compared to YM201636 treated cells. This is consistent with that observed in A431 cells. The variation occurs when you measure viable bacteria recovered from the cell lysates. Within control BMDMs the number of viable Salmonella recovered at the later time-points decreased relative to the early time points. This is consistent with the expected active antibacterial pathways present in macrophages. In contrast, in YM201636-treated BMDMs, the number of viable Salmonella recovered increased over the time-course. The most likely explanation for these observations is that inhibition of PIKfyve prevents intracellular replication of Salmonella but also disrupts the ability of macrophages to carry out an effective anti-bacterial response. A similar moderate level of replication under these conditions was also observed for the A431 cells.

Why some experiments were done with RAW264.7 cells and the others with BMDMs? Also, the authors claim that SCVs in BMDMs treated with YM201636 are LAMP1-negative. Which percentage? Is this also observed (and in which percentages) in other cell lines used in this study (RAW264.7 and A431)? What about in HeLa cells?

>> We have replaced all the data using RAW264.7 cells and only present work using the BMDMs. Furthermore we have quantified the proportion of cells with Lamp1-positive SIFs (see Figure 5).

This raises the very important question of knowing whether inhibition of PIKfyve affects integrity of the SCV membrane. The "LAMP1-negative SCVs" can actually be cytosolic bacteria, which is also an alternative explanation of why SPI-2 T3SS effector translocation was not observed in PIKfyve-inhibited cells.

>> We failed to detect any evidence of increased cytoplasmic Salmonella in the YM201636 treated cells when analysed by EM (new Figure 5C) or by IF microscopy. We do not consider the escape of Salmonella into the cytoplasm to be the cause of the observed inhibition of Salmonella replication.

4. Even if "Salmonella exploit macropinocytosis to gain entry into" non-phagocytic cells, how do we know that a possible role of PIKfyve in the intracellular replication of Salmonella is related to the function of PIKfyve in macropinosome-LE/Lys fusion? There is no data in this manuscript directly supporting that idea.

>> We have performed a modified content mixing assay to monitor the delivery of LE/Lys content (dextran) to a SCV (see Figure 5A). Inhibition of PIKfyve blocked the delivery of dextran to the SCV. This observation correlates with that observed during macropinocytosis in non-infected cells.

5. It would be interesting to analyse if PIKfyve-GFP, PIKfyve[K1831M]-GFP, and PIKfyve(S318A)-GFP are recruited to the SCV and, if so, characterise the dynamics and kinetics of PIKfyve recruitment.
> While we would consider it beyond the focus of this manuscript it is an interesting suggestion and one we had already evaluated. To date, our attempts to perform these experiments have failed. This reflects the unsuitability of the PIKfyve-GFP fusions for the required long-term (hours) spatial and temporal live cell imaging.

6. I find it strange that such a low concentration (5 ug/ml) of gentamicin has been used in Salmonella infections - has this been reported before? To my knowledge, the usual protocol is first to kill extracellular bacteria with 50-100 ug/ml gentamicin for about one hour and then reduce the antibiotic concentration to 10-20 ug/ml for the remaining infection time (see papers from the labs of Brumell, Hensel, Holden, Meresse, or Steele-Mortimer).

>> The referee is correct, we have utilized the usual protocol documented and we have therefore modified the manuscript accordingly.

7. "By doing so the adherent SCV is expanded to form extensive LAMP1-positive SIFs in which the bacterium then replicate" - what does this mean? Adherent SCV? Salmonella replicate within a tubulated SCV but not within the tubules.

8. What is a "highly efficient" intracellular pathogen? How is the "efficiency" of an intracellular pathogen measured?

9. In the introduction, the two consecutive sentences starting with "Orally ingested..." need to be re-written. The uptake of Salmonella by CD-18 expressing phagocytes is independent of the SPI-1 T3SS.

10. Intracellular Salmonella delivers effectors into host cells via the SPI-2 T3SS and not via SPI-2 (page 3, line 15).

11. In the discussion, the entire paragraph starting with "To accommodate the expanding..." contains wrong interpretations of the data and is a very poorly written description of the features of intracellular replication of Salmonella. Most of all, the data presented in the manuscript does not allow one to conclude, or even to suggest, that the possible effect of PIKfyve inhibition on intracellular replication of Salmonella is related to the observed role of PIKfyve in fusion between macropinosomes and LE/Lys. Furthermore, and again, intracellular Salmonella does not replicate within SIFs. Also, are there effectors that rely upon SIFs for their functions? Which experiments/papers show this?

12. The authors should write Salmonella enterica serovar Typhimurium (Salmonella enterica italicised) and abbreviate to S. Typhimurium (S italicised) or to S. typhimurium (all italicised).

13. Page 6, line 8, Salmonella should be italicised (and in several other places in the text).

14. Please write Salmonella-containing vacuole and not Salmonella Containing Vacuole.

15. Please write SCV maturation and not SCV development.

>> Points 7-15. We have considered each of these points and modified the manuscript in response. These comments will help clarify the manuscript and we thank the referee for identifying them.
Referee #3 (Remarks to the Author):

This manuscript presents a series of studies designed to determine whether the PtdIns(5) kinase, PIKfyve, is required for the intracellular replication of Salmonella. The authors show that while a PIKfyve inhibitor does not inhibit Salmonella growth in culture, it does inhibit intracellular replication of the bacteria. The authors find that wildtype PIKfyve and PI3P transiently associate with macropinosomes and present evidence that the catalytic activity of PIKfyve, is responsible for disappearance of PtdIns3P from macropinosomes. The authors also provide data that PIKfyve is required for fusion of micropinosomes with late endosomes, and further, that PIKfyve is required for formation of Salmonella induced filaments.

This is a novel, and potentially very important study. Little is known about the cellular functions of PIKfyve or PtdIns(3,5)P2, especially in infection. However, the lack of specificity of YM201636 [1], the PIKfyve inhibitor, along with the absence of direct data showing that the PIKfyve(K1831M) is catalytically inactive, raise questions about whether the reagents utilized were the most effective reagents available, for specific inhibition of PIKfyve catalytic activity. Moreover, the fact that active PIKfyve is part of a large multimeric complex brings up concerns about the relevance of studies that overexpress PIKfyve alone.

>> It should be noted that the specificity of the YM201636 inhibitor remains controversial. The subsequent report for Shisheva’s group does evoke consideration when using the YM201636 drug. However, their conclusions are based on indirect assay’s and not direct inhibition of the recombinant 110beta catalytic subunit of class IA PI 3-kinase. Likewise the proposed off-target action of the drug is not confirmed by RNAi depletion. The original specificity observed by Jefferies et al for YM201636 was independently reproduced using a highly related compound (MF4 which lacks an amino group on the pyridine ring) in a recent report (8).

1. In light of the above, it is critical that the authors use a non-related approach, for example RNAi silencing, to show that when PIKfyve is knocked-down (shown by western blot) they obtain the same results provided by YM201636.

>> This was a common point from multiple referees. We have performed the requested siRNA experiments targeting both PIKfyve and Vac14 a subunit from the regulatory complex that activates PIKfyve (see above for more detailed response). Unfortunately, two antibodies reported to detect PIKfyve ((1) Abnova (H00200576-A01)) failed to detect the endogenous enzyme in our cell type of interest. We therefore selected siRNA’s based on the ability to induce large endosomal vacuoles as observed by others (2) when they depleted PIKfyve using siRNA.

2. There are no published reports that the PIKfyve(K1831M) mutant is catalytically inactive. This data should be provided. The authors should either test this mutation in vitro, or show that overexpression of this mutant affects cellular levels of PtdIns(3,5)P2. It is not sufficient to assume that the K1831M mutant behaves similarly to the K1831E mutant. Furthermore, the expression of the K1831E mutant in COS cells had very little effect on the levels of PtdIns(3,5)P2 [2].

>> The characterization of the kinase dead mutant PIKfyve(K1831M) is published (1). It is well established that mutation of this critical Lys residue generates a kinase dead mutant. Like other reported mutants, namely PIKfyve(K1831E), its expression disrupts the endomembrane integrity to induce large endosomal vacuoles. Given this consistent induced phenotype we do not consider it essential to examine enzyme activity of recombinant protein nor to attempt to detect a difference total PI(3,5)P2 levels in our transient expression assays.
3. The results presented in Figure 2 are difficult to interpret.

A. The time scales of each experiment should be the same. As presented, it appears that wild-type PIKfyve GFP comes on and off the macropinosome prior to GFP-2xFYVE. This does not fit with the statement in the manuscript that PIKfyve-GFP is however recruited with a similar kinetic profile to that of the PI(3)P-specific probe GFP-2xFYVEHrs

>> The referee is correct and we have therefore repeated these experiments using identical capture conditions.

B. The authors do not explain why the presumed catalytically inactive PIKfyve(K1831M) mutant behaves differently than GFP-2xFYVE. They each contain PI3P binding domains, and lack PtdIns 5-kinase activity. The authors should test the localization of these three GFP tagged proteins in cells were PIKfyve has been knocked down. Also RNAi knockdown would bypass the problem of having the mutant PIKfyve also serve as a reporter for PtdIns3P

>> We would propose that the PIKfyve(K1831M) mutant membrane recruitment is different to that of GFP-2xFYVE because it acts as a dominant-interfering mutant. To test this more directly we have examined the recruitment of GFP-2xFYVE when PIKfyve is inhibited using YM201636. When PIKfyve activity is inhibited the 2xFYVE domain accumulates over time on the macropinosome which is consistent with an increase in the levels of PI(3)P due to a absence of conversion to PI(3,5)P2.

C. Each of the graphs represent the behavior of a single macropinosome. The authors should either show the averages plus error bars of a statistically significant number of macropinosomes, or show individual tracings of multiple macropinosomes.

>> We now present multiple individual tracings of multiple macropinosomes in the new Figure 3

4. The authors state that homotypic fusion of macropinosomes is not affected by the. This should be shown, and also tested in knock-down experiments.

>> We have added a supplementary figure showing the homotypic fusion in the cells expressing the PIKfyve(K1831M) mutant. Similar results were observed when PIKfyve was inhibited using YM201636.

5. The authors state that the PIKfyve(S318A)-GFP mutant did not prevent fusion of macropinosomes with the late endosome. It is not clear what this means. The effect of this mutation PIKfyve activity is not clear. Plus in discussing this mutant (top of page 10) the authors mention "the lipid phosphatase activity of PIKfyve". Is this a typo?

>> The referee is correct and text modified to remove this error.

6. In Figure 3, the authors should show a scale of the heat map. Also, because Dextran-647 is shown as blue, it would make the heat map less confusing to show no fluorescence as black rather than blue, or to pseudo-color the Dextran-647 a color that is not blue. Also, in Figure 3C, the authors need to show the results of GFP-Rab7(Q67L) alone.
Based on this comment we surveyed numerous colleagues and majority preferred the style we used. However if the journal has a preferred style we will gladly accommodate that.

7. The authors report that treatment with YM201636 combined with transfection with GFP-Rab7(G67L) does not rescue the YM201636 phenotype. They interpret this to mean that PIKfyve and Rab7 affect macropinosome-late endosome/lysosome fusion via independent pathways. However, these results do not distinguish between parallel pathways or a single ordered pathway.

We agree with this point and have modified the manuscript in response to this comment.

8. A further problem with the experiments shown in Figure 3, are potential effects of the PIKfyve(K1831M) mutant on loading Dextran-647 into late endosomes/lysosomes.

The dextran loading was determined by measuring the total fluorescence signal within both samples. No significant difference was observed.

9. In Figure 4, it is not apparent which are the SIF structures. The authors should show an enlarged view of a region that contains these structures. Also the level of expression of each GFP construct is very different. This is particularly important for comparison of PIKfyve-GFP with the PIKfyve(K1831M) mutant.

We have replaced this analysis of SIF formation with data from cells treated with YM201636 to inhibit PIKfyve. The quantification of lamp1-positive SIFs in Salmonella infected cells is now presented.

10. The data regarding PipB is also unclear. In cells with PIKfyve-(K1831M) there is no visible HA-PipB2. The loss of PipB2 protein is consistent with a lack of SCV maturation. However, it is also consistent with these cells having not been infected, or PIKfyve-K1831M blocking the expression or stability of this protein. Control experiments that rule out these possibilities should be presented.

We have replaced this analysis of Salmonella effector secretion of HA-PipB2 with data from cells treated YM201636 to inhibit PIKfyve. The new data presented clearly shows infection based on DAPI labeling and immunoblotting of the Salmonella DnaK protein. Furthermore this analysis was extended to include quantification of PipB2 by immunoblotting and also to directly analyze a non-secreted Salmonella protein SseA which is a chaperone required for assembly of the SPI2 Type III secretion system.

11. Comparison of the behavior of PIKfyve(K1831M) in Figure 2C with Figure 4A does not make sense. The simplest explanation of Figure 2C is that the K1831M mutant is dominant negative and inhibits endogenous PIKfyve. This would explain why GFP-FYVE comes off the membrane, while the PIKfyve(K1831M)-GFP mutant does not. However, if the PIKfyve(K1831M)-GFP mutant is dominant negative, then there should be a large number of LAMP1 positive vacuoles in Figure 4A. Again, RNAi knock-down of PIKfyve should provide more straightforward answers.

We do not agree with this comment the original data clearly showed a large number of dilated vacuoles only in the cells expressing PIKfyve(K1831M)-GFP mutant. Regardless we have replaced this analysis as described above (referee 3, point 9)
12. The authors should show the data that Salmonella replication was reduced 1.47-fold in YM201636 treated cells.

>> We have extended this analysis to now include multiple time points within 24 hrs post infection. This data is now shown.

Minor points.

On page 10 and 11, the authors refer to Figure 3B, but meant 3C.

Similarly, on page 10 they referred to Supplementary Figure 2 instead of Supplementary Figure 1.

>> We have modified the manuscript to reflect these comments.


Your revised manuscript has now been re-reviewed by one of the original referees. Although overall still encouraging publication, this referee still expresses technical concerns that will have to be singled out before we can eventually consider publication. Specifically, appropriate controls for the knockdown will have to be incorporated into the paper. This is of major importance, as the presumably DN-constructs and chemical inhibitors might not be as established and specific as presented. A more careful interpretation of these complementary tools would therefore also be indicated. With the still expressed interest in the study, we would be happy to assess a modified, ultimate version of your paper that would also provide the opportunity to incorporate the indicated live imaging data.

Yours sincerely,

Editor
EMBO Journal

REFEREE REPORT

Referee #3 (Remarks to the Author):

The revised manuscript of Kerr et al is significantly improved. However, there remains a critical outstanding issue about the extent to which PIKfyve and PI(3,5)P2 levels are depleted, and whether there is a possibility of off targets providing the observed phenotypes.

The addition of complementary siRNA studies has the potential to ameliorate these concerns. Thus, it is essential that the authors document the knockdown efficiency achieved in their studies. This should be done by immunoblotting with endogenous anti-PIKfyve and anti-Vac14 antibodies to determine the degree of depletion of these proteins. Detection of endogenous PIKfyve has been published by several groups including a paper published by one of the authors of the present manuscript [1]. Other examples of detection of PIKfyve [2-9] and Vac14 [3-10] are listed below.

The reason why documentation of the knockdown is crucial, is that there are problems with the other two techniques used by the authors, that are likely not correctable within the time frame of this manuscript.

The problem with the PIKfyve K1831M mutation, is that there is no published data about its effects on PI3,5P2 levels. It is unfortunate that the published PIKfyve K1831E mutant was not provided to the authors. However, even for the published PIKfyve K1831E mutant, the effect on PI3,5P2 levels has not been thoroughly documented. Thus in the current manuscript the authors should indicate that while the PIKfyve K1831M mutant has a dominant effect on the phenotypes measured, they were unable to measure the extent of depletion of PI3,5P2 levels.

The problem with the studies that used the YM201636 PIKfyve inhibitor, is that YM201636 also inhibits Akt [11]. Moreover, in studies of a related compound, MF4, it appears that Akt might also be inhibited [12]. Note that in Figure 5E of de Lartigue et al., the levels of phosphorylated Akt were much lower in most of the MF4 time points when compared with the control.

Thank you for the opportunity to resubmit our manuscript. We have carried out the final control experiments and modified the manuscript according to the requests from reviewer 3. We hope that this satisfies these technical concerns and the manuscript is now suitable for publication. We have highlighted the modified text in red. In addition, to the new supplementary figure documenting the siRNA efficiency we have added the data examining the pH of the SCV by monitoring the acidotropic probe lysostracker.

Response to individual points raised by reviewer 3.

Point 1/

Thus, it is essential that the authors document the knockdown efficiency achieved in their studies. This should be done by immunoblotting with endogenous anti-PIKfyve and anti-Vac14 antibodies to determine the degree of depletion of these proteins.

We have added a supplementary figure to the manuscript that documents the efficiency of the siRNA for both PIKfyve and Vac14. This was performed using both RT-PCR and western immunoblotting. We purchased additional commercial PIKfyve antibodies and evaluated all available antiserum for its ability to detect the recombinant GFP-human PIKfyve by immunoblotting. Only one of the antiserum tested was positive and this antibody was used to detect endogenous PIKfyve in the new supplementary figure. Unfortunately this antibody did not detect PIKfyve in fixed cell monolayers and is therefore not suitable for immunofluorescence applications. Additional earlier work not presented confirmed that the PIKfyve siRNA used is able to reduce the expression of the GFP recombinant protein based on immunoblotting for GFP.

Point 2/
Thus in the current manuscript the authors should indicate that while the PIKfyve K1831M mutant has a dominant effect on the phenotypes measured, they were unable to measure the extent of depletion of PI3,5P2 levels.

We have added this point to the first paragraph of the discussion.

Point 3/
The problem with the studies that used the YM201636 PIKfyve inhibitor, is that YM201636 also inhibits Akt [11].

While I would argue that this statement is not accurate (see below). We have added a sentence to the results to indicate that the originally reported specificity of the YM201636 for PIKfyve has more recently come under question.

Shisheva and co-workers state “Thus, the dual inhibitory effect of YM201636 on both PIKfyve and insulin-activated class IA PI 3-kinase makes unclear the relative contribution of the PIKfyve inhibition to the reduced Akt phosphorylation and GLUT4 translocation in response to insulin.” (1) This manuscript concludes that YM201636 may also inhibit the insulin-activated class IA PI 3-kinase that in turn will result in altered Akt phosphorylation. Therefore YM201636 does not directly inhibit Akt.