THE EMBO JOURNAL

Manuscript EMBO-2016-96272

The Shigella type III effector IpgD recodes Ca^{2+} signals during invasion of epithelial cells

ChunHui Sun, Benjamin Wacquier, Daniel I. Aguilar, Nathalie Carayol, Kevin Denis, Sylviane Boucherie, Cesar Valencia-Gallardo, Ceren Simsek, Christophe Erneux, Alexandre Lehman, Jost Enninga, Laurence Arbibe, Philippe Sansonetti, Geneviève Dupont, Laurent Combettes & Guy Tran Van Nhieu

Corresponding author: Guy Tran Van Nhieu, Collège de France

Review timeline:

Submission date:	07 December 2016
Editorial Decision:	24 January 2017
Revision received:	09 April 2017
Editorial Decision:	24 May 2017
Additional Correspondence	25 May 2017
Additional Correspondence	31 May 2017
Revision received:	03 June 2017
Accepted:	13 June 2017

Editor: Ieva Gailite

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 24 January 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below. I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers, but particularly focusing on the further analysis of IpgD role in phosphoinositide metabolism and the potential competition with PLC, as well as the validation of results in an epithelial cell system. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns raised at this stage.

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

We generally allow three months as standard revision time. Please contact us in advance if you need an extension of the revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

This study examined the role of the PI(4,5)P2 Shigella phosphatase ipgD in affecting the Ca2+ signal induced by Shigella when invading cells. In previous work the authors showed that infection starts with forming an actin enriched domain rich in PI(4,5)P2 to evoke Ca2+ signals that are required for Shigella invasion. In the present work the authors conclude that in early infection ipgD partially hydrolysis PI(4,5)P2 to reduce and delay the Ca2+ signaling that may function to allow invasion before cell death.

The evidence in support of the main conclusion are quite weak and other alternatives were hot excluded. A major flaw in the results is the physiological relevance.

Presentation of the data is sloppy. For example, in no way in the manuscript it is indicated what the ipgD mutant is actually supposed to do. This reviewer is assuming it lacks PI(4,5)P2 hydrolytic activity but I am not sure. The authors refer the reader to their previous work. I had to go to all their previous papers and yet never found this mutant. In previous work they only mention ipgB and ipgC. The authors them mention the use of the ipgD-C438S mutant, again with no clear indication what this mutant supposed to do. It is also difficult to follow the Figures and some are not even labeled correctly or at all. An example, what NI stands for in Figure 1e, what the symbols stand for in Figure 6a and so on.

All the experiments were performed with HeLa cells. In fact Shigella invade enterocytes in the intestine. Enterocytes are very different from HeLa cells. To start with enterocytes are polarized and Shigella enters the cells through the luminal membrane that has a very different lipid composition than HeLa cells plasma membrane. In enterocytes the IP3 receptors are clustered at the apical pole, an arrangement not seen in HeLa cells. In enterocytes the Ca2+ signal starts at the apical pole and propagates to the basal pole or remains confined to the apical pole. No such signal occur in HeLa cells. Thus, it is not clear at all how relevant the reported findings to the physiological setting where Shigella invade enterocytes. At the very minimum these studies should be performed with enterocytes cell line grown on permeable support and better if they are done with intestinal organoids.

The authors show that IP3R1 is recruited to the invasion sites and this is affected by the ipgD mutant. A change in IP3 receptors arrangement and clustering can account to the effect of ipgD on Ca2+ signaling. There is no way to know from the data presented if the main effect of ipgD was altering IP3 receptors localization of PI(4,5)P2 hydrolysis.

It is not clear at all why the IP3 levels are higher in cells invaded by ipgD than wild-type shigella. If the mutant simply lost PI(4,5)P2 hydrolysis the IP3 levels should be as in untreated cells. This data is not presented. If the level is lower than in untreated HeLa cells (the NI condition?) than it is not clear why it should be so.

The authors attribute the effect of ipgD to partial hydrolysis of PI(4,5)P2. This is not examined to any extent. Measuring pH-GFP is not sufficient. Does pH-GFP clusters in response to invasion, in what time course, is it sensitive to PI(4,5)P2 depletion? Does partial PI(4,5)P2 depletion reverses the effect of ipgD, etc..

Figure 2: Most of these results were already published in the NC paper.

Figure 3: FRAP data, any of these time constant statistically different. In general, the statistical aspect of the results is not documented very well and not always given. This should be provided for all experiments.

Figure 4: The model does not achieve what models supposed to be. Having a set of equations that reproduce the observed signals does not mean much. A model has a value only if it provides fresh testable predictions. The model does not achieve this and has no value as presented.

Figure 5: For these results to have any meaning it should be compared to the Ca2+ signals observed in uninfected control cells.

Referee #2:

The study by Sun et al. analyzes the role of the Shigella effector IpgD, a PtdIns(4,5)P2 4-phosphatase, for calcium signaling during uptake of the bacterial pathogen by epithelial cells. Specifically, the authors show that at Shigella invasion sites IpgD (i) reduces the second messenger inositol (1,4,5) triphosphate (InsP3) by hydrolyzing the precursor PtdIns(4,5)P2, (ii) elicits long-lasting local calcium signals with atypical dynamics and amplitude, and (iii) restricts actin dynamics and prevents calcium-dependent cell detachment.
The manuscript is well written, and the story unfolds in a straight-forward manner. The study recapitulates and builds on findings and methods of a previous study by the same group (Tran Van Nhieu et al., 2013, Nat Commun 4:1567). The novelty of the current study is grounded in the identification of a major Shigella player in the calcium-dependent processes, the type III-secreted effector IpgD. This result seems not too unexpected, given the well-known substrate of IpgD, PtdIns(4,5)P2, i.e. the precursor of InsP3.

Specific points
1) The phenotypes of the S. flexneri ipgD mutant strain described in Fig. 1 should be complemented by using wild-type IpgD and, as a control, the catalytically inactive mutant enzyme IpgD_C438S.

2) Fig. 7AB: Provide statistics. Is the data significant? Also, the data shown in Fig. 7C should be quantified.

3) Fig. 3E: The FRAP measurements indicate a difference in actin dynamics at invasion sites between foci around ipgD mutant bacteria and control areas. What (which effector(s)) accounts for this difference?

4) Labeling (figure legends):
- Fig. 1D: provide P-value for *** (not **).
- Fig. 3BC: provide P-value for *** and * (not **).
- Fig. 6A: the keys for the symbols used are missing.
- Fig. 7B: ...at the indicated incubation time for cells...
- Fig. 7C: the number of independent experiments should be indicated.
- Fig. 7D: the figure shows only the P-value * (not **, ***).
- Fig. 5A/Fig. 7B: add headings to the figures to better guide the reader. Fig. 5A: the red and blue curves could be amended with a title (WT, ipgD mutant) in addition to or instead of the color code. Fig. 7B: "Talin" and "Capn4" could be added.

Referee #3:

The authors examine the dynamics of calcium during infection of cells by Shigella flexneri. They focus on the role of a bacterial type 3 secreted effector protein, IpgD, in regulating this process. They conclude that the phosphatase activity of IpgD controls calcium through its ability to control cellular levels of IP3. The experiments are well designed and executed, and the paper is well written (with a few typos). I think their finding that IpgD confines calcium signaling to the region of the infection foci is very important for our understanding of how pathogens can exploit/modify calcium during invasion and will be a paradigm for the field. I have only a few minor suggestions for the authors consideration.

Major Comments:

-The first paragraph of the results is a bit confusing as several issues are blended together (maybe a model would help orient the reader to start if the authors really want to keep all the data in the paper?). The first issue relates to localized PIP2 hydrolysis by IpgD. This is somewhat known/expected, but doesn't help so much with understanding where the paper is going from the start. PIP2 is not really the subject of the paper and after Figure 1A does not get mentioned. I recommend removing the PIP2 data and starting directly with measurements of IPs, which is the heart of the story.

-If the authors believe that IpgD is competing with PLC for PIP2 substrate, it is worth showing that with their assays. Does PLC inhibition impair IP3 production? Another possibility is that IpgD affects PLC recruitment to invasion foci, not just access to substrate (since there is a lot of PIP2 at invasion foci, with both WT and ipgD mutant). There are also other pathways that can generate IP3 - I mention this not to suggest experiments, but for the authors to consider in their model if PLC inhibitors have no effect. Either way, their story is exciting because IpgD alters IP3 and global calcium signaling.

Minor Comments:

-RATPs need to be defined

-Figure 1B, a legend would help to understand the graph better (and typically WT=black bars, mutant=open bars)

-Figure 1E, x-axis label is confusing... maybe use "Column elution time (min)"

-Figure 1F, needs legend

-several typos and grammatical errors were noted

1st Revision - authors' response 09 April 2017

EMBOJ-2016-96272R

Point-by-point answers

Referee #1
We were disappointed that referee 1 was not as positive as the other referees. We have tried to better explain the rationale of the work in this revised version, as detailed in response to referee 2. To address the relevance issue raised by the referee and following exchanges with the editors, we performed additional experiments with polarized Caco-2/TC7

- ..."A major flaw in the results is the physiological relevance."
In preliminary experiments, we tried growing the cells on BioCoat filters. Polarized TC7 cells grown on these filters turned out to be refractory to loading, or to load very erratically, with the Ca^{2+} fluorescent probe. We were not able to find in the literature article describing Ca^{2+} imaging on these cells using this set up. Because of this, and also because of the high cost of these filters, we therefore used TC7 cells plated on glass for a 7 days period, in a set up enabling cell polarization that was previously used by us and other teams. To illustrate cell polarization, we show the apical ZO-1 staining in 7 days-old TC7 monolayers in Fig S2.

Using this polarized cell system, we were able to show recruitment of InsP3 receptor type III at Shigella invasion sites, and to confirm the role of IpgD in the down-regulation of this recruitment. These results are now presented in Figs 1E and 1F of the revised mansucript.

While loading of the Ca^{2+} indicator was not sufficient to allow us to perform high speed imaging to analyze local Ca2+ responses, we were able to analyze global Ca^{2+} responses and show that as for HeLa cells, IpgD inhibited the elicitation of Ca^{2+} responses during prolonged infection kinetics. These results are presented in Figs 5D and 5E.

We also analyzed the effects of bacterial infection on focal adhesions in this cell system. As for HeLa cells, we also observed that IpgD delayed the disassembly of focal adhesion during infection, an effect that was not observed with catalytically inactive IpgD C438S. These results are presented in Fig S4.

We would like to point out that because of the poor invasion efficiency of *Shigella* in these polarized cells, we were not able to perform all the experiments that were performed with HeLa cells. However, we hope that the referee will agree with us that the experiments shown with this more relevant cell system support our main findings.

- **" ...in no way in the manuscript it is indicated what the *ipgD* mutant is actually supposed to do... I had to go to all their previous papers and yet never found this mutant...This reviewer is assuming it lacks PI(4,5)P2 hydrolytic activity but I am not sure."**

The *ipgD* mutant corresponds to a mutant in which the *ipgD* allele has been disrupted and has been used in the various references (Konradt et al., 2011; Mellouk et al., 2014; Niebuhr et al., 2002; Weiner et al., 2016) that we cited in the introduction. It was also used in Fig.S12 of our previous Nature Communications 2013 paper, that sets the ground of the present study. Also, we explicitly introduced IpgD as a phosphatidyl (4, 5)bisphosphate ($PI(4, 5)P_2$)-4-phosphatase with related references in p. 3, and to the "catalytically inactive IpgD C438S and GFP-IpgD C438S mentionned p.4 and p. 7. We do not feel that the text is ambiguous concerning the description of this mutant.

- **The authors show that IP3R1 is recruited to the invasion sites and this is affected by the ipgD mutant. A change in IP3 receptors arrangement and clustering can account to the effect of ipgD on Ca2+ signaling. There is no way to know from the data presented if the main effect of ipgD was altering IP3 receptors localization of PI(4,5)P2 hydrolysis.**

  We believe that IP3R1recruitment is linked to $InsP_3$ production in a diffusion-capture process at *Shigella* invasion sites, as discussed in our previous report (Tran Van Nhieu et al., Nat. Comm. 2013). Since we show in the present submitted work that InsP3 synthesis is regulated by IpgD-mediated $PI(4,5)P_2$ hydrolysis, we feel that the IP3R1 recruitment results are in good agreement with the role of IpgD in $InsP_3$-mediated Ca^{2+} responses.

- **It is not clear at all why the IP3 levels are higher in cells invaded by ipgD than wild-type shigella. If the mutant simply lost PI(4,5)P2 hydrolysis the IP3 levels should be as in untreated cells. This data is not presented. If the level is lower than in untreated HeLa cells (the NI condition?) than it is not clear why it should be so.**

  As shown previously, *Shigella* invasion leads to PLC activation (Tran Van Nhieu et al., Nat. Comm 2013). In the present submission, we show that IpgD triggers a decrease in InsP3 levels, as evidenced by the higher levels of InsP3 in cells infected with the *ipgD* mutant relative to wild-type *Shigella*. We believe that the decrease in $InsP_3$ levels is linked to the reduced availibility of this PLC substrate at invasion sites. We hope that this is now clearer in this revised version.

- **The authors attribute the effect of ipgD to partial hydrolysis of PI(4,5)P2. This is not examined to any extent. Measuring pH-GFP is not sufficient. Does pH-GFP clusters in response to invasion, in what time course, is it sensitive to PI(4,5)P2 depletion? Does partial PI(4,5)P2 depletion reverses the effect of ipgD, etc..**

  The effects of IpgD on $PI(4,5)P_2$ levels have been well documented in several published reports by the Bernard Payrastre's team. We agree with referee 3 that the $PI(4,5)P_2$ and GFP-PH results may distract from the message on $InsP_3$-mediated signals and have removed these data from the main Figures.

- **Figure 2: Most of these results were already published in the NC paper.**

  With all respect due, we do not agree. No characterization of the Ca2+ responses induced by the *ipgD* mutant was performed in the Nature Communication 2013 article. In the present manuscript we highlight the key role of IpgD in recoding Ca^{2+} signals induced during bacterial infection. We hope that the rationale for this present work is better introduced in this revised version (please, see response to referee 2).

- **Figure 3: FRAP data, any of these time constant statistically different. In general, the statistical aspect of the results is not documented very well and not always given. This should be provided for all experiments.**

  Statistical analysis indicates that the differences between time constants are significant. This is now indicated on the revised Fig 3. Also, we are now showing statistical analysis for all experiments when relevant, as suggested by the referee, in the legends to Figures.

- **The model does not achieve what models supposed to be. Having a set of equations that reproduce the observed signals does not mean much. A model has a value only if it provides fresh testable predictions. The model does not achieve this and has no value as presented.**

  With all respect due, we disagree with the referee. It was not obvious at all that modifying the model that was previously proposed (Tran Van Nhieu et al., Nature Comm. 2013) according to the observed differences between WT and *ipgD* mutant (and only these ones) would reproduce what is observed with the mutant. In this sense, the set of equations was not built to reproduce the observed signals, as suggested by the Referee. Moreover, Fig. 4D (Ca^{2+} responses induced by WT Shigella or by the *ipgD* mutant over prolonged infection kinetics) are a prediction of the model, confirmed experimentally in Fig. 5A. So the model clearly provided fresh testable predictions.

- **Figure 5: For these results to have any meaning it should be compared to the Ca2+ signals observed in uninfected control cells.**

  For all experiments, we ensure that the observed Ca^{2+} responses are not spontaneous responses prior to data acquisition. These controls are routine control practice for experimentators in the Ca^{2+} imaging field. We do not feel that showing "flat" traces indicative of the absence of Ca^{2+} responses cells in the absence of stimuli present interest.

Referee #2

We thank the referee for his insightful suggestions. We would like to comment /clarify on the role of IpgD as a Ca^{2+} modulator. While PI(4,5)P2 hydrolysis is expected to prevent InsP3 synthesis, it was not clear if PI(4,5)P2 depletion was sufficiently severe to be the case. This is particularly true in the case of bacterial infection, for which the overall cellular levels of PIP(4,5)P2 levels are certainly not expected to be limiting compared to the levels of injected IpgD, at least at the initial stages of invasion. An important notion that emerges from our studies, is that the local elicitation of Ca^{2+} responses is controlled by IpgD at the site of bacterial invasion. By controlling the PIP2 levels at its point source, IpgD down-regulates InsP3 production to prevent global Ca^{2+} release and to restrain Ca^{2+} signals at invasion sites. The modeling performed in this work was key to provide a mechanistic basis for these unexpected observations. The model also stresses the role of IpgD in the slow dynamics and varying amplitude of the atypical global Ca^{2+} responses induced by bacteria prior to their inhibition at later stages of infection. We think that these findings were not sufficiently stressed in the original submission and have tried to clarify these points in this revised version by inserting the following sentences:

- " $PI(4, 5)P_2$ is the main substrate used by phospholipases C (PLCs) to produce $InsP_3$, and it is expected that a general depletion of $PI(4,5)P_2$ will impact on $InsP_3$ levels. However, as opposed to agonists acting diffusely and synchronously on cell surface receptors triggering $InsP_3$-mediated Ca^{2+} release, *Shigella* only interacts with a discrete area of the cell plasma membrane and invasion events are not synchronized. As a consequence, the overall levels of cell substrates targeted by injected type III effectors are not expected to be limiting, in particular at the onset of the invasion process. It is therefore unclear whether the $PI(4,5)P_2$ phosphatase activity of IpgD could affect Ca^{2+} signaling during bacterial invasion. Any detected effects could provide insights into the local aspect of bacterial stimulation and the transition from local to global Ca2+ responses. These reasons prompted us to study the effects of IpgD on Ca^{2+} signaling during bacterial invasion."

Specific points

1) The phenotypes of the S. flexneri ipgD mutant strain described in Fig. 1 should be complemented by using wild-type IpgD and, as a control, the catalytically inactive mutant enzyme IpgD_C438S.

As suggested by referee 3, the data relevant to $PI(4,5)P_2$ recruitment at invasion sites using the GFP-PH $_{PLC\text{-}\delta}$ probe were removed from the main Figures. For reasons that we explained during our previous exchange with the editor linked to the collaborative set up implicated, we were not able to perform additional experiments for InsP3-H^3 measurements. As suggested by this referee, we performed quantification of InsP3R1 recruitment at invasion sites of the ipgD mutant strains complemented with the IpgD C438S catalytically inactive form or wt IpgD. The results now shown in Fig EV2, indicate the role of IpgD catalytic activity in the decreasing IP3R1 recruitment at invasion sites.

2) Fig. 7AB: Provide statistics. Is the data significant? Also, the data shown in Fig. 7C should be quantified.

Statistics are now provided for Fig 7B. Instead of showing the scan of the representative gels in Fig 7A, the quantification shown in Fig 7B now corresponds to the average of at least 4 independent experiments. The data were statistical different for values between the 90 mn-150 min incubation period in a Wilcoxon ($p < 0.05$) and Ancova ($p < 0.01$) test for the talin and calpain blots, respectively.

Fig 7D now represents the average of three determinations corresponding to the representative gel shown in Fig 7C.

3) Fig. 3E: The FRAP measurements indicate a difference in actin dynamics at invasion sites between foci around ipgD mutant bacteria and control areas. What (which effector(s)) accounts for this difference?

We believe that differences in actin dynamics at invasion sites are due to IpgD itself. As previously observed by Niebuhr et al., 2000 and as quantified in Fig 3C, the *ipgD* mutant strain induces foci that are less dense in polymerized actin. At the time this defect was attributed to the disconnection of cortical actin from the plasma membrane by IpgD through its PI(4,5)P2 hydrolytic activity. More recent works by Viaud et al, 2014 suggest that through PI5P synthesis, IpgD could stimulate the Rac GEF Tiam-1 and actin polymerization. We also reported that InsP3- through local Ca2+ signals could also contribute to actin polymerization.

However, while less restrictive in diffusion than foci induced by WT *Shigella*, this difference was not sufficient to explain the global versus local Ca2+ responses observed in the two strains and that the difference in InsP3 synthesis was also key.

We have tried to clarify this point by inserting the following sentences:

- p. 6, l. 12:

" We also reported that $InsP_3$-mediated signalling contributes to actin polymerization at *Shigella* invasion sites (Tran Van Nhieu et al., 2013), suggesting that through the regulation of $InsP_3$ levels and confinement of Ca^{2+} signals reported in this study, IpgD may also affect actin dynamics. Since actin polymerization may in turn restrict diffusion, IpgD could therefore control Ca2+ signals by dampening $InsP_3$ synthesis at its point source, as well as its diffusion at invasion sites."

-p. 7, l. 5:

Thus, foci induced by the *ipgD* mutant show and a restriction of diffusion, which, while less pronounced than that observed for wild-type *Shigella*, is likely to restrict diffusion of $InsP_3$ and impact on bacterial-induced Ca^{2+} signals.

4) Labeling (figure legends):
- Fig. 1D: provide P-value for * (not **).**
ok
- Fig. 3BC: provide P-value for * and * (not **).**
ok
- Fig. 6A: the keys for the symbols used are missing.
keys now mentioned.

- Fig. 7B: ...at the indicated incubation time for cells...
ok
- Fig. 7C: the number of independent experiments should be indicated.
This is now mentioned in 7D. " The average integrated intensity of the talin band was determined from at least three independent experiments for the indicated bacterial strain."
- Fig. 7D: the figure shows only the P-value * (not **, *).**
ok
- Fig. 5A/Fig. 7B: add headings to the figures to better guide the reader. Fig. 5A: the red and blue curves could be amended with a title (WT, ipgD mutant) in addition to or instead of the color code. Fig. 7B: "Talin" and "Capn4" could be added.
We apologize for all the mistakes and thank the referee for his careful reading. All changes were made as suggested.

Referee #3
We thank the referee for his positive and constructive comments.

Major comments:

- "... PIP2 is not really the subject of the paper and after Figure 1A does not get mentioned. I recommend removing the PIP2 data and starting directly with measurements of IPs, which is the heart of the story."
We agree that the PIP2 data are not at the heart of this story and followed the referee's suggestion, by removing them from the main Figures to better focus in InsP3.
We have re-rewritten the corresponding part to clarify this point in the first paragraph as follows:
" During *Shigella* invasion, the atypical duration of bacterial-induced local Ca^{2+} responses were shown to depend on the confinement of $InsP_3$ and enrichment of $InsP_3$ receptors (IP3Rs) at bacterial entry sites (Tran Van Nhieu et al., 2013). We reasoned that through its action on $PI(4, 5)P_2$, IpgD could regulate $InsP_3$-mediated signaling.

We first analyzed the effects of IpgD on the recruitment of the $InsP_3$ receptors at invasion foci induced by an *ipgD* mutant isogenic to wild-type *Shigella* (Niebuhr et al., 2002). As shown in Figs 1A and 1B and as previously observed in HeLa cells, the $InsP_3$ receptor type 1 (IP3R1) was enriched by 1.5-fold as early as five minutes following bacterial challenge for both wild-type and *ipgD* mutant strains. While this enrichment factor only moderately increased for wild-type *Shigella* at later stages of foci formation, however, invasion foci induced by the *ipgD* mutant showed a drastic increase in IP3R1 enrichment, reaching 2.2 fold after 30 min (Fig 1B). Consistent with a role for IpgD-mediated hydrolysis of $PI(4, 5)P_2$ in regulating

IP3R1 recruitment, invasion foci induced by an *ipgD* mutant complemented with catalytically inactive IpgD C438S also showed a similar increase of IP3R1 enrichment compared to the *ipgD* strain complemented with wild-type IpgD (Figs EV1A and EV1B)." ....

-If the authors believe that IpgD is competing with PLC for PIP2 substrate, it is worth showing that with their assays. Does PLC inhibition impair IP3 production?
We thank the referee for his insightful comment. We performed additional experiments and show in this revised manuscript that PLC inhibition by U73122 abolishes global and local responses induced by WT and ipgD mutant. These results are now shown in Fig EV5 and referred to in the text p. 5, l. 24:
-" Consistent with InsP3-mediated Ca^{2+} release, cell treatment with the PLC inhibitor U73122 virtually abolished local and global responses induced by wild-type *Shigella* and the *ipgD* mutant (Fig EV5)."

Minor comments:

-RATPs need to be defined
we have inserted p. 3, l.15: "... local Ca^{2+} responses with durations exceeding several seconds termed RATPs for Responses Associated with Trespassing Pathogens"

-Figure 1B, a legend would help to understand the graph better
to the Figure, now Fig EV2 in the Expanded View p. 2, as follows:
" The enrichment fold of $PI(4,5)P_2$ at invasion foci ± SEM was determined from the levels of the GFP-$PH_{PLC\delta}$ probe at the indicated time points (Materials and Methods). WT *Shigella*: black squares bars; *ipgD* mutant: empty diamonds."

-Figure 1E, x-axis label is confusing... maybe use "Column elution time (min)"
Former Fig 1E, now Fig 1C has been modified as suggested.

-Figure 1F, needs legend
Legend to former Figure 1F, now Fig 1D, have been modified.

-several typos and grammatical errors were noted
The ms was re-read and many typos / syntax were fixed.

2nd Editorial Decision 24 May 2017

Thank you for submitting a revised version of your manuscript. The manuscript has now been seen by all original referees. While referees #2 and #3 find that their concerns have been sufficiently addressed, referee #1 requests further clarification of the effects of InsP3 receptor clustering vs change in InsP3 synthesis. I agree with referee #1 that experimental investigation of this question should be added to the manuscript before it can be accepted for publication here. Therefore, I would like to invite you to submit a revised manuscript, addressing this final remaining concern.

Please feel free to contact me if have any further questions regarding the revision. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I am looking forward to seeing the final revised version.

REFEREE REPORTS

Referee #1:

I appreciate the effort of the authors to address my major concerns. Although it would have been better to address the physiological significance using polarized cells grown on filter support, the results provided with the TC7 cells are adequate providing the infection was restricted to the luminal membrane. This should be clarified by the authors.

However, I find that several points have not been adequately addressed:

The response to the point of whether the effects of invasion on Ca2+ signaling is due to changes in IP3/PI(4,5)P2 levels or rearrangement of IP3 receptors is not satisfactory. It is still not clear to me how the authors can distinguish between the two. This is not a secondary issue but relate to the main interpretation of the results.

I also fail to see what the modeling add to the manuscript. Again, there is no obvious prediction made by the model that leads to a fundamental observation or an experiment. The model largely reproduces the results and takes large amount of journal space.

Referee #2:

The authors did a thorough job revising the manuscript and addressed my concerns in a satisfactory manner. In particular, the role of IpgD modifying local calcium concentrations (at bacterial entry sites) rather than global calcium levels is now more convincingly outlined.

Referee #3:

The authors have addressed my comments, and I am in support of publication. I think this is an important paper for the field.

Additional Correspondence - authors 25 May 2017

Thank your for your mail. We are particularly pleased that referees 2 and 3 found the paper convincing and that it would make an important contribution in the field. We think that experiments aiming to address the request of referee 1 at distinguishing between the PIP2 hydrolysis - IP3 levels and IP3R clustering / recruitment would be very difficult to design and to interpret beyond what is already shown.

First, we do not claim that the effects of IpgD during Shigella invasion on Ca2+ signals are linked to PIP2 hydrolysis / IP3 depletion or IP3R clustering. We actually believe that they are interdependent and both required, as discussed in the manuscript. We think that experiments aiming at

distinguishing between the PIP2 hydrolysis - IP3 levels and IP3R clustering / recruitment would be very difficult to design and to interpret beyond what is already shown.

Indeed, we previously showed that enrichment of IP3R at Shigella invasion sites was key to explain the local responses with atypically long durations that are observed at Shigella invasion sites (Tran Van Nhieu et al. Nature Commmunications 2013). Thus, IP3R clustering does affect Ca2+ responses during Shigella invasion, a notion that we further extend in this submission. That IP3-dependent Ca2+ stimulation affects IP3R clustering has been shown in various models and is generally accepted in the field, including by major experts (Tateishi, et al., and Mikoshiba, J. Biol. Chem, 2005; Rahman et al., and Taylor, Nature 2009). This point was also alluded to in our previous publication in the Shigella model. How this occurs is a long lasting debate that we do not pretend to address in this submission. From works in the Taylor's lab, it was speculated that IP3R oligomerization could be triggered by IP3 binding. As previously discussed in the Nature Commmunications 2013 paper, we certainly agree with this view, since it explains the clustering and enrichment of IP3R at Shigella invasion sites by a simple diffusion-capture process. In brief, we believe that during Shigella invasion, as in any model implicating IP3-dependent Ca2+ responses, IP3 levels and IP3R clustering / enrichment are interdependent and will impact on Ca2+ responses.

Regarding the model input, we believe that this section strengthens our argumentation. Reviewer #1 claims that there is no modeling prediction, which is not the case as Fig. 4D is a theoretical prediction, which is then validated by the experiment shown in Fig. 5A.

Because of their interdependency, we believe that the testing of the respective roles of IP3 levels and IP3 receptor clustering on Ca2+ signals would be very difficult if not impossible to unambiguously tackle experimentally.

Additional Correspondence - editor 31 May 2017

I have now received comments from one of the original referees, and they are in agreement that experimental extrication of the signaling inputs would be too effort-intensive to include in the scope of this revision. However, upon rereading of your manuscript I did notice that this complexity of signaling does not become obvious to a non-expert. I would therefore like to ask you to include a discussion on the interplay between InsP3 synthesis and InsP3 receptor clustering in Ca2+ response in the manuscript. Since the discussion part is very brief at the moment, and this point appears to cause some confusion, I think it would be very beneficial for our readers to have a more detailed discussion available.

2nd Revision - authors' response 03 June 2017

The authors made the requested changes and submitted the final version of their manuscript.

3rd Editorial Decision 13 June 2017

Thanks very much for making the final modifications in the revised version of the manuscript. I'm sorry for the delay in communicating the decision; I was away at a conference last week and have now finally had a chance to take a look at the revised manuscript. I am now happy to inform you that your manuscript has been accepted for publication in the EMBO Journal. Congratulations!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Guy TRAN VAN NHIEU
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-96272R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For all data sets involving statistics, we used as a general rule a minimal size of 30 objects in three independent experiments. For experiments with high variances such as those concerning cytotoxicity assays presented in Fig 6B or the scoring of focal adhesions in Fig 6D, up to several thousands cells / adhesions were scored. For the Western-blot analysis shown in Fig 7 involving a single determination per time point, statistitcs were performed on values obtained from 3 to 5 independent experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Non applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Non applicable
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Non applicable
For animal studies, include a statement about randomization even if no randomization was used.	Non applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Blinding of the sample was performed for the analysis of focal adhesions (scoring, size estimation in Figs 6 and EV5.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Non applicable
5. For every figure, are statistical tests justified as appropriate?	Yes. The tests used to analyze data from the different experimental procedures are mentionned below. Wilcoxon test: Fig 1B, Fig 1F, Fig EV1B, Fig S1B, Fig 2B, Fig 5D, Fig EV2B, Fig EV3C-E,Fig EV4B, Fig 5C, Fig 3B, Fig 6E, Fig S4, Fig EV5B. Unpaired t-test: Fig 3C, Fig 6B, Fig EV5C, Fig 7E, Fig 6D,Fig S4C. Dunn sum rank test: Fig 3E. ANCOVA test:Fig 7B
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Shapiro-Wilk normality testing was performed
Is there an estimate of variation within each group of data?	yes
Is the variance similar between the groups that are being statistically compared?	F-tests have been performed to determine equal or unequal variance in t-tests.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The antibodies used are described in the Materials and Methods - Bacterial strains, cell line and reagents section. "The rabbit polyclonal antibody against the type1 InsP3 receptor was from ABR Affinity Bioreagents. The monoclonal antibody against the type 3 InsP3 receptor was from Thermofisher. The anti-Shigella LPS FlexV polyclonal antibody was described previously (Mounier et al., 2009). The mouse monoclonal anti-capn4 antibody and anti-talin clone 8D4 antibodies were from Biovision and Sigma corporation, respectively. The phalloidin-A488, phalloidin-A633, anti-rabbit IgG-Alexa547 were from Thermo Fisher Scientific. "
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Non applicable

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Non applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Non applicable
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Non applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Non applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Non applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Non applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Non applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Non applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Non applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Non applicable

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Non applicable
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We have included with our submisson a 5-Figures Expanded View as well as an appendix containing additional 7 Figures. We will be happy to deposit additional data sets in other repositories upon request if our article is accepted.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Non applicable
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Non applicable
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	The text code for the stochastic simulations of Shigella-induced Ca2+ responses is shown in the Appendix section.

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Non applicable