EMBO reports

ADP-heptose is a newly identified pathogen-associated molecular pattern of *Shigella flexneri*

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Transaction Report:

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

1st Editorial Decision 17 September 2018

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below. As I had outlined in my earlier mail, I asked the referees if your study could be published with minor revisions and if the referees feel that it complements the earlier study from the Shao laboratory and if it can stand on its own.

As you will see from the comments below, the referees acknowledge that your study provides further confirmation that ADP-heptose represents the PAMP instead of HBP. However, the referees also point out that the conclusiveness of the study relies to some extent on the earlier publication by the Shao lab and that a set of further experiments would be required to make it fully conclusive and concise and stand on its own. Moreover, both referees consider the scope of the study rather limited and referee 1 suggests publication in a more specialized journal in the summary evaluation sheet returned with the report. Given the moderate support from the referees and given that a number of further experiments would be required to make the study a strong candidate for publication in EMBO reports, we had to conclude that we cannot offer to publish it in EMBO reports.

I am very sorry to disappoint you on this occasion and I hope you will view the possibility of a transfer favourably. If this is the case, please use the link below to transfer the manuscript directly.

REFEREE REPORTS
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Referee #1:

Garcia-Weber and coworkers aim to shed light into bHBP as a PAMP using as read-out TIFA oligomerization and production of IL8 (which is kept inside cells by treatment with monensin). Of note, I have reviewed this work under EMBO scoop protection policy due to the recent work of Shao lab published in Nature. In this work, authors provided experimental evidence demonstrating that ADP-Hep, but not HBP, is the PAMP sensed by ALK1-TIFA protein. The identification of

ADP-HEp as PAMP was based on an unbiased screen interrogating a transposon mutant library of Y. pseudotuberculosis.

The work of Garcia-Weber largely confirms the evidence that ADP-Hep is the PAMP sensed by ALKP1-TIFA. In this case, authors have followed a genetic approach testing Shigella mutants, and also infecting cells with the different mutants and complementing strains. This latter experiment is a nice addition to the state-of-the-art after Shao's team work and provides a more biological context to the recognition of this PAMP. This reviewer also prefers to infect/challenge HeLA cells (as done by Garcia-Weber) than 293T cells (as done by Shao'steam). However, the results testing Shigella hldE mutant (and even a Salmonella hldE mutant) were already reported in the work published in PlosPathogens 2017 by this lab. Therefore, this EMBO Report submission mostly demonstrates that indeed HBP might not be the PAMP sensed. In fact, one of my main concerns is related to the discrepancy on the interpretation of the HBP results between both studies. Shao's team provides experimental evidence demonstrating that HBP is NOT a PAMP, whereas Garcia-Weber et al still seems to imply that there is room for HBP to behave as a bona-fide PAMP. Authors should clarify this aspect, and I believe important they do discuss Shao's team findings in greater detail about the role of HBP as PAMP directly sense by cells.

An experiment that will bring together everything in Gaecia-Weber's work will be to assess inflammatory responses in cells deficient on ALPK1 (perhaps by siRNA). Other experiment that perhaps should be included in this manuscript is to shed light into the notion whether the type III secretion system might be the vehicle to deliver (or not) ADP-HEP. This suggestion at the end of the discussing is confusing considering that Shigella (and Salmonella) hlde mutants can be found inside cells and are responsible for the activation of TIFA (refer to PlosPathogens 2017).
This is reviewer is not experience on the chemistry to synthesize HBP, however I believe important to show, at least, the NMR data and not only indicate the values. Also, there is no information on the quality of the preparation and the final yield.

Referee #2:

This is an elegant analysis of TIFA-NFkB activation by Shigella, identifying ADP-heptose, rather than βHBP, as a ligand for TIFA oligomerisation. The authors have shown that synthesised βHBP, whilst inducing IL8 and GFP-TIFA oligomerisation, does so with delayed kinetics compared to Shigella lysate, suggesting that βHBP is not the direct inducing agent of Alpk1 (which the authors previously identified as the receptor for HBP). Use of bacterial genetic mutants and mutant complementation identifies the critical step in the biosynthetic pathway, revealing a requirement for the production of ADP-heptose. This is first tested using bacterial lysates to stimulate cells in the presence of digitonin. The authors then go on to test the LPS biosynthesis pathway mutants during Shigella infection. The data is clearly presented, robust and well controlled. It is my opinion that the work supports that from the Shao laboratory, which identified an essential role for hldE from Yersinia in the biosynthesis of ADP-heptose as an NFkB-inducing agent.
In terms of progression, this manuscript provides only minor progress from the Shao work, in that it independently identifies ADP-heptose (not HBP) as the PAMP inducing TIFA and IL-8 during Shigella infection. However, the data presented whilst a little limited in scope are broadly conclusive and robust with appropriate controls. The work could be published with minor revisions.

Shortcomings:
- Whilst the genetic analysis is thorough, this work does not directly show that ADP-heptose is the inducing agent of the TIFA-NFkB pathway. The data shown does stand on its own but without the published Shao paper, this work would have benefited from experiments to test that synthetic ADP-heptose is the direct inducing agent.
- Only IL-8 is analysed as a downstream affect for TIFA activation - I would like to see at least one additional cytokine to support this.
- 4B - it would be more appropriate if the authors included WT Shigella in their studies, rather than referring to historical data, especially as they then comment on the milder impact on bacterial infectivity observed in the ΔβhldE +pHldA+pHldC expressing bacteria compared to the hldE mutant.
- In terms of the statistics, I believe that a students t-test is inappropriate when multiple comparisons

are made - an ANOVA would be the better test.
- In relation to figure 3, the hldD and waaC mutant lysates induce TIFA oligomerisation and IL8 activity similar to WT, based on this, it is not clear why the authors believe that there is an accumulation of the PAMP in these mutants and why the activity is referred to as "massive" in both figure 3 and then later in figure 4 (where there is not WT strain for comparison due to differences in infectivity).
- Can the authors comment on whether IL-8 and TIFA activation is detected in non-infected cells within infected conditions? This isn't clear to me.

Additional Correspondence 17 September 2018

As you probably expect, we are disappointed by the rejection of the paper. We are fully convinced that our work is a stand-alone story that is very complementary to the work published by F. Shao and that a second report on the role of ADP-heptose as a PAMP will still have an important scientific echo in the field considering that we investigate a different pathogen. Our previous story showing that ALPK1 controls TIFA/TRAF6-dependent innate immunity against HBP (Milivojevic et al. Plos Pathogens, 2017) did not prevent the publication of Thomas Meyer on Helicobacter pylori in _Cell reports_ (Zimmermann et al. 2017). Both publications are cited together and reinforce the novelty of the findings. Furthermore, our story and F. Shao's are complementary because they both come from different angles. F. Shao found ADP-heptose from a bacterial screen whereas we identified it from the comparison with synthetic HBP.

In addition, I would like to highlight the fact that the second reviewer mentioned that the study can be published with minor revisions. I agree that the first reviewer is less convinced by the novelty of our work. However, he mentioned that our work is ″a nice addition to the state-of-the-art after Shao's team work and provides a more biological context to the recognition of this PAMP″. We can clarify our manuscript regarding the role of HBP and experimentally address the additional points that he raised. Finally, we are fully convinced that the work requested by both reviewers can be addressed in a 4-week period.

In the light of these clarifications, I was wondering whether you might reconsider your decision and accept our manuscript for revision.

Additional Correspondence 28 September 2018

Thank you for your letter asking us to reconsider our decision and invite revision of your manuscript for EMBO reports. I have meanwhile carefully read it and I also re-read the referee reports and discussed it further with the editorial team and you have also provided further feedback.

- I notice that referee 1 asked to assess inflammatory responses in ALPK1-deficient cells. The discrepancy between your findings on those in the manuscript from the Shao lab on the role of HBP as PAMP can be discussed in the text.
- Referee 2 indicated that the treatment with synthetic ADP-heptose would be required to unambiguously show that it is the inducing agent. I think, similar experiments have been done in the related paper from the Shao lab and as such, the effect has been demonstrated. Yet, I agree with Referee 2 that this experiment would make the current paper stronger.
- Additional cytokines apart form IL-8 should be tested and WT Shigella be included.
- The other comments are rather minor and some can be addressed in the text.

You have meanwhile provided further feedback and indicated that you can address all concerns listed above within 4 weeks. In particular you will provide further experiments showing that

wildtype S. flexneri infection (30 minutes) and ADP-heptose stimulation fail to induce TIFA oligomerization in ALPK1-deficient cells. Furthermore, you indicated that you will repeat the infection experiments with more cytokines and that you will include WT Shigella.

I agree with the outlined experiments and invite you to revise your manuscript for EMBO reports. Please address all referee concerns in the manuscript and also provide a detailed point-by-point response.

You have submitted your manuscript as Scientific Report. Please note that we can only accommodate up to five figures for this format. In case the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article (with a separate Results and Discussion section). Please also note our limit of 25,000 plus/minus 20,000 characters for Scientific Reports (Main text, Materials and Methods as well as Figure legends do not count).

Supplementary/additional data - General information:
The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification: you have correctly specified the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Note that you can also insert a paragraph "Data information" at the end of each figure legend that specifies the number of independent experiments and the statistical information for all panels in the figure. E.g. "Data information: (B-D) Data correspond to the mean plus/minus SD of 3 independent experiments. For comparison between mock and treated conditions statistical significance ... etc ".
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends have to contain a basic description of n, P and the test applied.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).
- a synopsis image and summary text. All EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large. For the larger image the height is variable. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

1st Revision - authors' response 17 October 2018

EMBOR-2018-46943V1 (Garcia-Weber et al.)

Point-by-point response

Referee #1:

Garcia-Weber and coworkers aim to shed light into bHBP as a PAMP using as read-out TIFA oligomerization and production of IL8 (which is kept inside cells by treatment with monensin). Of note, I have reviewed this work under EMBO scoop protection policy due to the recent work of Shao lab published in Nature. In this work, authors provided experimental evidence demonstrating that ADP-Hep, but not HBP, is the PAMP sensed by ALK1-TIFA protein. The identification of ADP-HEp as PAMP was based on an unbiased screen interrogating a transposon mutant library of Y. pseudotuberculosis.

The work of Garcia-Weber largely confirms the evidence that ADP-Hep is the PAMP sensed by ALKP1-TIFA. In this case, authors have followed a genetic approach testing Shigella mutants, and also infecting cells with the different mutants and complementing strains. This latter experiment is a nice addition to the state-of-the-art after Shao's team work and provides a more biological context to the recognition of this PAMP. This reviewer also prefers to infect/challenge HeLA cells (as done by Garcia-Weber) than 293T cells (as done by Shao'steam). However, the results testing Shigella hldE mutant (and even a Salmonella hldE mutant) were already reported in the work published in PlosPathogens 2017 by this lab. Therefore, this EMBO Report submission mostly demonstrates that indeed HBP might not be the PAMP sensed. In fact, one of my main concerns is related to the discrepancy on the interpretation of the HBP results between both studies. Shao's team provides experimental evidence demonstrating that HBP is NOT a PAMP, whereas Garcia-Weber et al still seems to imply that there is room for HBP to behave as a bona-fide PAMP. Authors should clarify this aspect, and I believe important they do discuss Shao's team findings in greater detail about the role of HBP as PAMP directly sense by cells.

As suggested by **Reviewer #1**, a paragraph discussing Shao's data and whether bHBP can be considered as a PAMP is included in the revised manuscript (page 14) as follows:

"In conclusion, our data rule out a major contribution of bHBP in *S. flexneri* infection and identify ADP-heptose as a new potent bacterial PAMP that can be detected down to a concentration of 10-10 M. The delay in inflammatory signaling observed in response to bHBP suggested that this bacterial metabolite is not directly recognized by host defense mechanisms. At the time of finalizing this manuscript, Zhou *et al*. confirmed this hypothesis by showing that bHBP must be converted into ADP-heptose 7-phosphate by host adenylyltransferase enzymes of the NMNAT family to induce inflammatory signaling [24]. Although bHBP is a bacteria-derived factor triggering an immune response, its classification as a genuine PAMP is challenged by the fact that it is not directly sensed via the interaction with a cognate pathogen recognition receptor."

In the revised manuscript, we strengthened the other important findings of our study, namely the identification of ADP-heptose as a PAMP and its role in *S. flexneri* infection. We now provide data showing that synthetic ADP-heptose induces rapid oligomerization and cytokine production, and show that sensing occurs down to 10-10 M (**Figure 3E and 3F, revised manuscript**). These results, documenting the sensitivity of ADP-heptose detection in cells, constitute an important addition to Shao's publication. Of note, Zhou *et al*. only monitored NF-kB luciferase activity in cells electroporated with 1, 10 and 100 mM ADP-heptose. Furthermore, we show that, as for *S. flexneri* infection, ADP-heptose-induced TIFA oligomerization and cytokine production is ALPK1-dependent (see paragraph below, **Figure 5, revised manuscript**).

An experiment that will bring together everything in Gaecia-Weber's work will be to assess inflammatory responses in cells deficient on ALPK1 (perhaps by siRNA).
As suggested by **Referee #1**, inflammatory responses of ALPK1-deficient cells were included in the revised manuscript. We now show that the oligomerization of TIFA induced in response to synthetic ADP-heptose or *S. flexneri* infection is abolished in cells transfected with an ALPK1-targeting siRNA. As expected, oligomerization is restored upon expression of a siRNA-resistant ALPK1 cDNA construct, demonstrating that the effect is truly ALPK1-dependent (**Figure 5A, revised manuscript**). In line with these results, we also added data showing that the production of cytokines (IL-8, IL-6 and IL-4) induced in response to ADP-heptose or *S. flexneri* infection is strongly impaired in ALPK1-depleted cells (**Figure 5B, revised manuscript**). Overall, these results show that, as with *S. flexneri*, ADP-heptose-induced inflammatory responses are ALPK1-dependent. They also identify the role of ALPK1 in the mechanism of ADP-heptose sensing and strengthen our data showing that ADP-heptose is a key PAMP involved in *S. flexneri* infection.

Other experiment that perhaps should be included in this manuscript is to shed light into the notion whether the type III secretion system might be the vehicle to deliver (or not) ADP-HEP.
We agree with **Referee #1** that characterizing the delivery mechanism of ADP-heptose during *S. flexneri* infection is an important question. However, we believe that addressing this question goes beyond the frame of this manuscript. Because of the role of type III secretion (T3S) in the entry mechanism of *S. flexneri*, this question is difficult to address in a conclusive manner. This is illustrated by results obtained with the T3S-deficient mutant D*mxiD* [Figures for referees not shown.].

Infection with the D*mxiD* mutant fails to trigger oligomerization of TIFA at 30 minutes (panel A), showing that functional T3S is required to trigger ADP-heptose-induced signaling. However, since this mutant does not invade epithelial cells (panel B), the absence of inflammatory response cannot be attributed to the sole defect of ADP-heptose translocation through the T3S apparatus. A delivery mechanism based on the release of ADP-heptose from damaged bacteria upon rupture of the internalization vacuole would also be impaired upon infection with the D*mxiD* mutant, and therefore an inhibition of inflammatory signaling would also be observed in this case. As the result shown above does not allow us to conclude on the mechanism of ADP-heptose delivery during infection, it was not included in the revised manuscript.

…This suggestion at the end of the discussing is confusing considering that Shigella (and Salmonella) hlde mutants can be found inside cells and are responsible for the activation of TIFA (refer to PlosPathogens 2017).
We are sorry if this point was not clear enough. We show in Milivojevic et al. (Figure 5I) and in Garcia-Weber et al. (**Figure 4B-D, revised manuscript**) that, although the D*hldE* mutant is very invasive, it is unable to trigger early oligomerization of TIFA. This result shows that functional T3S is not sufficient to trigger inflammatory signaling and that bacterial synthesis of ADP-heptose is

required as well. The revised text was modified (page 15) as follows:
…"The mechanism of ADP-heptose sensing will have to be thoroughly characterized. We show in our study that it is ALPK1-dependent. This result is consistent with the study by Zhou et al. who elegantly reported that the kinase ALPK1 is indeed the cytosolic immune receptor for both ADP-L-β-D-heptose and ADP-D-β-D-heptose [24]. More work is required to identify the respective contribution of these two metabolites during *S. flexneri* infection, understand how they are delivered in the cytoplasm of host cells and characterize the spatio-temporal dynamics of ALPK1 activation. In particular, it will be interesting to investigate whether they are injected in infected cells by the type III secretion apparatus of bacteria, and whether they diffuse via gap junctions to trigger TIFA oligomerization and cytokine production in uninfected bystander cells as previously described [3]. Alternative and non-exclusive mechanisms implicating the release of ADP-heptose from damaged bacteria upon rupture of the internalization vacuole or the contribution of a cellular transporter will also have to be investigated."…

This is reviewer is not experience on the chemistry to synthesize HBP, however I believe important to show, at least, the NMR data and not only indicate the values. Also, there is no information on the quality of the preparation and the final yield.
As suggested, we have now included the 1H-NMR spectrum of bHBP in its sodium salt form as **Figure EV1 of the revised manuscript**. It is similar to the spectrum shown in Vincent et al. (**reference 14, revised manuscript**) who synthesized bHBP via a comparable synthetic route. The final isolated yield of crude bHBP as well as the retention time measured by RP-HPLC, were added to the **Materials and Methods section of the revised manuscript (page 25).**
We also included as **Figure EV2 of the revised manuscript** the 1H-NMR (600 MHz, D2O) and 31P-NMR (243 MHz) spectra of synthetic ADP-L-glycero-b-D-manno-heptose. Yield of crude ADP-heptose is provided in the Materials and Methods section of the revised manuscript (page 26). Analytical data of final product was in full accordance with previously results as published in Zamyatina et al. (**reference 26, revised manuscript**).

Referee #2:

This is an elegant analysis of TIFA-NFkB activation by Shigella, identifying ADP-heptose, rather than bHBP, as a ligand for TIFA oligomerisation. The authors have shown that synthesised bHBP, whilst inducing IL8 and GFP-TIFA oligomerisation, does so with delayed kinetics compared to Shigella lysate, suggesting that bHBP is not the direct inducing agent of Alpk1 (which the authors previously identified as the receptor for HBP). Use of bacterial genetic mutants and mutant complementation identifies the critical step in the biosynthetic pathway, revealing a requirement for the production of ADP-heptose. This is first tested using bacterial lysates to stimulate cells in the presence of digitonin. The authors then go on to test the LPS biosynthesis pathway mutants during Shigella infection. The data is clearly presented, robust and well controlled. It is my opinion that the work supports that from the Shao laboratory, which identified an essential role for hldE from Yersinia in the biosynthesis of ADP-heptose as an NFkB-inducing agent.

In terms of progression, this manuscript provides only minor progress from the Shao work, in that it independently identifies ADP-heptose (not HBP) as the PAMP inducing TIFA and IL-8 during Shigella infection. However, the data presented whilst a little limited in scope are broadly conclusive and robust with appropriate controls. The work could be published with minor revisions. Shortcomings: - Whilst the genetic analysis is thorough, this work does not directly show that ADP-heptose is the inducing agent of the TIFA-NFkB pathway. The data shown does stand on its own but without the published Shao paper, this work would have benefited from experiments to test that synthetic ADP-heptose is the direct inducing agent.
As suggested by **Referee #2**, we now provide new data showing that ADP-heptose can directly induce inflammatory signaling. First, we show that, unlike bHBP, chemically synthesized ADP-heptose induces TIFA-GFP oligomerization at 30 minutes in a dose-dependent manner (**Figure 3E, revised manuscript**). Oligomerization is still significant at 10-10 M, showing that ADP-heptose sensing occurs down to this concentration limit. This result is an important addition to Shao's publication. Of note, Zhou et al. only monitored NF-kB luciferase activity in cells electroporated in the presence of 1, 10 and 100 mM ADP-heptose. We also show that ADP-heptose induces IL-8 expression in the same range of concentrations (**Figure 3F, revised manuscript**).
Finally, we show that, in agreement with previous data obtained upon *S. flexneri* infection

(Milivojevic et al. 2017), ADP-heptose-induced TIFA oligomerization (**Figure 5A, revised manuscript**) and cytokine production (**Figure 5B, revised manuscript**) are ALPK1-dependent. Altogether, these additional results strongly support the identification of ADP-heptose as a new bacterial PAMP and its role upon *S. flexneri* infection. They also confirm the critical role of ALPK1 in ADP-heptose sensing.

- Only IL-8 is analysed as a downstream affect for TIFA activation - I would like to see at least one additional cytokine to support this.
In the initial manuscript, we focused on the analysis of IL-8 because this chemokine is known to be the critical factor for the recruitment of PMNs during *S. flexneri* infection. However, we agree with **Reviewer #2** that it was important to analyze more cytokines. A cytokine multiplex assay showed that, in addition to IL-8, the secretion of IL-6 and IL-4 was significantly enhanced after ADP-heptose sensing or *S. flexneri* infection. Furthermore, we showed that this induction is dependent on ALPK1 (**Figure 5B, revised manuscript**). By showing that ADP-heptose induces the secretion of multiple cytokines, these results indicate that the detection of this bacterial PAMP has a broad impact during infection.

- 4B - it would be more appropriate if the authors included WT Shigella in their studies, rather than referring to historical data, especially as they then comment on the milder impact on bacterial infectivity observed in the Db*hldE +pHldA+pHldC expressing bacteria compared to the hldE mutant.*
As recommended by **Reviewer #2**, infection rates of wt and D*hldE* + pHldE *S. flexneri* were added to Figure 4B (**Figure 4B, revised manuscript**). Since data confirmed that these two strains were much less invasive than the other mutants, they were excluded for quantification of inflammatory signaling and IL-8 production (**Figure 4 C-E, revised manuscript**).

- In terms of the statistics, I believe that a students t-test is inappropriate when multiple comparisons are made - an ANOVA would be the better test.
As recommended by **Reviewer #2,** all data were reanalyzed with one-way (**Figure 2 B-E, Figure 3 B-F, Figure 4 B-E, revised manuscript**) or two-way (**Figure 2F, Figure 5, revised manuscript**) ANOVA followed by Tukey's multiple comparisons test.

- In relation to figure 3, the hldD and waaC mutant lysates induce TIFA oligomerisation and IL8 activity similar to WT, based on this, it is not clear why the authors believe that there is an accumulation of the PAMP in these mutants and why the activity is referred to as "massive" in both figure 3 and then later in figure 4 (where there is not WT strain for comparison due to differences in infectivity).
Regarding **Figure 3**, we agree with **Reviewer #2** that the comparison between wt and D*hldD* or D*waaC* lysates does not allow us to conclude on an accumulation of ADP-heptose. In the revised manuscript, the notion of ″accumulation″ was removed.
Regarding **Figure 4**, we meant that infection with D*hldD* or D*waaC* mutants led to massive signaling compared to infection with the other tested mutants (D*hldE*, simple and double complemented mutants). To avoid confusion, the adjective "massive" was removed from the **revised manuscript**.

- Can the authors comment on whether IL-8 and TIFA activation is detected in non-infected cells within infected conditions? This isn't clear to me.
As shown in Milivojevic *et al*., TIFA oligomerization can be observed in both infected and uninfected bystander cells. However, this is only visible at low MOI when some cells are left uninfected. To make this point more visible, we added bacteria to **Figure 4A of the revised manuscript**. An example of a bystander cell with TIFA oligomers is shown upon wt infection. The concept of bystander cell activation is now presented in the introduction (page 4) as follows:
…"In line with a previous study reporting that uninfected bystander epithelial cells constitute the main source of IL-8 during *S. flexneri* infection [6], TIFA and TRAF6 oligomerization as well as NF-kB activation were found in both infected and uninfected bystander cells [3]."…
The following sentence was also added to describe Figure 4A (page 11).
…"As previously reported [3], infection with wt bacteria induced rapid oligomerization of TIFA-GFP in both infected cells and uninfected bystander cells."

Regarding IL-8, we now provide IL-8 images of *S. flexneri* infection confirming that most IL-8 is

produced by uninfected bystander cells. This is shown in **Figure EV3 of the revised manuscript**.

As previously reported (Kasper et al. *Immunity*, 2010), we propose that the mechanism of bystander cell activation results from a mechanism of gap junction-mediated cell-cell communication and hypothesize that ADP-heptose may diffuse via gap junctions. This point is discussed at the end of the Results/Discussion section (page 15) with the following sentence:
…"In particular, it will be interesting to investigate whether they are injected in infected cells by the type III secretion apparatus of bacteria, and whether they diffuse via gap junctions to trigger TIFA oligomerization and cytokine production in uninfected bystander cells as previously described [3]"…

2nd Editorial Decision 24 October 2018

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed.

REFEREE REPORTS

Referee #1:

I commend the authors for meeting the most important issues raised in my first assessment of this work. The present study nicely complements the current state-of-the-art.

Referee #2:

The authors have adequately addressed the reviewers concerns.

My one remaining comment would be that western blots for the depletion of Alpk1 should be included in the manuscript as the amount of depletion observed by the authors in these experiments is important information for the reproducibility of the data by others.

2nd Revision - authors' response 26 October 2018

I thank you very much for your interest in our work. We're delighted that both reviewers considered that the manuscript has been improved and that it can now be published.

Regarding ALPK1 western blots, there is unfortunately no ALPK1 antibody allowing the detection of endogenous ALPK1. Therefore, the experiment suggested by Reviewer #2 to monitor the efficiency of siRNA-mediated depletion cannot be performed.

We know by experience that knockdown efficiency in HeLa cells is really high. For most proteins tested, we reach 80-90% depletion at 72 hours. An efficient knockdown of ALPK1 is consistent with the very strong impact of its depletion on TIFA oligomerization and cytokine production as observed in Figure 5A and 5B of the revised manuscript or in Milivojevic et al. (Plos Pathogens, 2017). We agree with Reviewer #2 that documenting knockdown efficiency would have been informative. However, considering that siRNA-mediated depletion in HeLa cells is very straightforward and that we provide the transfection protocol and the reference of the siRNA used in our study, we are convinced that knockdown conditions will be easy to reproduce in other labs. Furthermore, we would like to highlight the rescue experiment (Figure 5A) that unambiguously confirms the specificity of ALPK1 depletion.

In 2016, when we started working on ALPK1, we assessed the efficiency of ALPK1 knockdown in an indirect manner. We checked by microscopy that our ALPK1-targeting siRNA was able to induce the depletion of a fluorescently tagged-ALPK1 cDNA construct. We observed a very strong reduction in fluorescence intensity, suggesting that depletion was indeed effectively occurring. We

could repeat such experiment but this is only an indirect that would not fully address the point raised by Reviewer #2.

The ALPK1 siRNA used in Garcia-Weber et al. is identical to the one used in Milivojevic et al. It has been validated in HeLa cells by qPCR by the manufacturer Ambion and all the validation data are available on the Ambion-ThermoFisher website under the name of the sequence that we provide in the manuscript. For your information:
https://www.thermofisher.com/order/genomedatabase/browse/sirna/keyword/s37074
To emphasize on the validation of the siRNA and the rescue experiment in the manuscript, I suggest to modify the Materials and Methods section as follows:
…"Reverse transfection of siRNAs was carried out using RNAiMAX according to the manufacturer's instructions (Invitrogen ThermoFisher). HeLa cells, seeded in 96-well plates (8,000 cells/well) were reverse transfected with 20 nM siRNA and used 72 h after transfection. As a control, cells were transfected with a non-targeting sequence (4390843) from Ambion (ThermoFisher). As previously described [3], for ALPK1-depletion, cells were transfected with a validated ALPK1 siRNA (s37074) from Ambion (ThermoFisher). Validation data are available on the manufacturer's website. To demonstrate knockdown specificity, an ALPK1 rescue experiment was performed as previously described [3]. Briefly, 48 h after siRNA transfection, cells were transfected with a siRNA-resistant ALPK1 cDNA construct (pCMV-ALPK1) or an empty vector (pCMV) using Fugene 6 (Roche)"….

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: Arrieumerlou
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-46943V2-Q

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	At least 3 biological replicates were performed for each piece of data. For each biological replicate, each data point was performed in triplicate.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Images were taken in an unbiased way with an automated fluorescence microscope (ImageXpress Micro, Molecular Devices, Sunnyvale, USA) and quantifications were made automatically by running image analysis scripts with MetaXpress software. See Materials and Methods section for further details.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. Statistical analysis was described in all Figure legends. One-way (for 1 variable) and two-way (for more than 2 variables) ANOVA followed by Tukey post-test was performed to assess differences between groups.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Sample size was two small to perform normality tests. We assume normality from larger comparable datasets with D'Agistino and Pearson omnibus normality rest (Graph pad).
Is there an estimate of variation within each group of data?	Standard deviation or Standard Error of the mean are showed and taken into account in the ANOVA test.
Is the variance similar between the groups that are being statistically compared?	Yes it is, refer to the standard deviation or standard error of the mean in the figures.

C- Reagents

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/

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).	See materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	See materials and methods section. All cells were cultured for less than a month. They were routinely tested for mycoplasma (Test). No contamination was found.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. 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	NA
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).	Data sets are available on demand.
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).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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