nature portfolio

Peer Review File

Shigella flexneri evades septin-mediated cell-autonomous immunity via protein ADP-riboxanation

Corresponding Author: Professor Xiaoyun Liu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this work, Tang et al. employed multiple strategies—including western blotting, mass spectrometry, and microscopy—to demonstrate that OspC effectors enable S. flexneri to evade septin cage entrapment. Mechanistically, they show that OspC catalyzes ADP-riboxanation of SEPT9 at Arg561, a critical residue essential for stabilizing septin hetero-oligomers.
The writing style and scope align well with the aims of Nature Communications. Therefore, the journal should consider this manuscript for publication. I have some minor to moderate comments to help further improve the manuscript.
1. Data Availability: The authors should provide a link to access the raw mass spectrometry (MS) data,for example, ProteomeXchange.
2. Modified Site Assignment: For the ADP-riboxanation sites identified via MS/MS, did the authors manually inspect the spectra and assign fragment ions for all potential modified peptides? Please clearly describe the procedure for modified site assignment in the Methods section.
3. The rational of using both spectral counting and MaxLFQ (maxquant) for label-free quantification needs to be discussed. Usually, intensity-based label-free quantification will be more accurate than the spectral counting method.
4. For Table 1, how is the modification rate calculated? Some brief information here will be helpful. Because the modification will impact the ionization efficiency of the peptides strongly, it will be hard to calculate the modification rates just based on the peptide intensity information.
5. Residue Specificity: ADP-ribosylation can also occur on glutamic acid residues. For the peptide sequence shown in Figure 2C, is it possible that the modification occurs on glutamic acid rather than arginine? Please clarify.
6. Experimental Details: a) For each experiment—such as immunoprecipitation (IP) or MS analysis—the authors should specify the number of cells used.
b) The flow rate used in the nanoLC-MS analysis should be provided.
c) The authors should specify the number of biological and technical replicates used for the MS quantification analysis.
d) For label-free data analysis, please specify the download date and the number of protein entries in the FASTA file used.

Reviewer #2

(Remarks to the Author)
The study by Tang et al. explores how the bacterial pathogen Shigella flexneri evades host defense mechanisms by targeting septins, a family of proteins that form cage-like structures to entrap cytosolic bacteria. The key findings are as follows: S. flexneri uses its Type Three Secretion System (T3SS) effector proteins from the OspC family to modify septins via ADP-riboxanation, a form of post-translational modification closely related to ADP-ribosylation. SEPT9 is identified as a primary target among septin proteins. OspC effectors target SEPT9 and modify it at Arginine 561 (Arg561), disrupting the NC-interface between adjacent SEPT9 subunits. This disruption leads to disassembly of septin octamers, thereby inhibiting septin cage formation.
Additionally, both OspC and another effector protein, OspG, act in concert to inhibit septin cage assembly. Mutant strains lacking these effectors show increased septin cage entrapment of bacteria, indicating a cooperative role in evading host defense. This study reveals that S. flexneri has evolved two distinct mechanisms to counteract septin-mediated cell-autonomous immunity.
This study offers novel insights into the mechanisms by which S. flexneri evades host cell defenses through targeted modifications of septins. Overall, the experimental rigor of the study is commendable. This robustness is not surprising given

that two labs with a successful track record in studying host-pathogen interactions—specifically, shigella cage entrapment by septins and effector-based modification of cellular targets—collaborated on this research.
The experiments presented are well-designed and executed to a high standard. The experimental setup largely builds on the authors' previous work on eIF3 and caspase modifications.
The relevance of the study lies in the fact that it identifies a direct modification of septins by a bacterial toxin or effector ("an overdue finding"). OspCs are phylogenetically and functionally conserved across multiple bacterial pathogens, which further underscores the significance of this finding.
However, the authors face an inherent challenge: they are investigating a highly complex system involving multiple effectors, some of which are partially redundant or dispensable for the biological outcome—Shigella infection. Furthermore, they report promiscuous substrate activity of their effector, adding another layer of complexity: OspCs modify eIF3, caspase-11 and now SEPT9. A previously identified additional effector OspG targets septins indirectly by ubiquitination via CAND1 phosphorylation .
It is quite common for two or more effectors from the same bacterial species to target the same host protein or pathway (functional redundancy or effector convergence). Likewise, it is also possible for individual effectors to target multiple substrates. However, these substrates are often functionally and/or structurally related.
Thus, further investigation into the biological significance of the septin modification by the authors would be beneficial.

Major points:
#1
Line 143: "SEPT9 was post-translationally modified in a manner dependent on both the enzymatic activity and ARD domain of OspC3 (Fig. S2A and S2B)."
This interpretation might be misleading and requires further clarification. It suggests that both, the enzymatic N-terminal domain and the ARD (C-terminal domain) are required for the modification, which is not fully supported by the data. In Figures S2A and S2B (Flag-IP, ADPr), a band is still visible for the N-terminal domain (enzymatic domain) alone—albeit strongly reduced compared to the full-length protein. Taken together with the results in Figure S2F, this indicates that the post-translational modification of SEPT9 depends on its catalytic N-terminal domain. However, the modification is strongly enhanced by septin-binding mediated by the C-terminal ARD.
Please clarify.

#2 Fig. The results from Fig. 2D+E clearly indicate that OspCs exhibit a high level of promiscuity concerning modified residues and substrate specificity. This is a significant problem of the study. Therefore, all findings must be interpreted with great caution. In the majority of their experiments, the authors overexpress the effector OspC along with the target or substrate protein under investigation using vectors with strong promoters ensuring high expression levels. However, high concentrations of OspC can lead to reduced specificity. It is important to note that physiologically, the number of effector molecules delivered by a T3SS is very limited. High septin concentrations may mask the modification of other preferred (potentially more physiological) substrates due to the excessive supply.
Experiments employing bacterial infection and endogenous proteins are therefore eligible.
In Fig. 2H+I+J, the authors perform cell infections (still with Flag-SEPT9 overexpression).
#2.1 In Fig. 2H, the ADP-ribosylation of SEPT9 without infection is an interesting finding. Why does this ADP-ribosylation almost disappear in Fig. 2I and 2J? Please clarify.
#2.2 Flag-tagged SEPT9 always runs between 72 and 100 kDa, but in Fig. 2H, it appears closer to 100 kDa. Please clarify.
#2.3 As mentioned above, infection experiments with endogenous protein levels would significantly enhance the significance of the observations.
-The authors should make efforts to infect cells without overexpression of septins, perform immunoprecipitation (IP) of endogenous septins, and probe for the studied modification.
-A classical yet informative assay could be employed to determine the extent of endogenous SEPT9 modification during infection. Specifically, lysates from infected cells—either following immunoprecipitation of SEPT9 or directly from whole-cell extracts—could be subjected to an in vitro post ADP-ribosylation reaction using recombinant OspC and labeled NAD^+ (e.g., biotinylated or radiolabeled). If SEPT9 is already efficiently ADP-ribosylated during infection with wild-type Shigella, minimal to no additional labeling would be detected. In contrast, infection with a catalytic-dead Shigella or KO mutant strain would leave SEPT9 unmodified, resulting in a strong signal upon in vitro labeling.
This approach could provide insights into the efficiency of SEPT9 modification in vivo and could help estimate substrate preferences of OspC under near-physiological conditions. Moreover, when using total lysates, other OspC substrates will also be labeled, enabling comparison of modification levels and preferences between SEPT9 and alternative targets. While the presence of multiple ADP-ribosylation sites on OspC substrates might complicate signal interpretation, this assay remains valuable for evaluating the physiological relevance of OspC substrate specificity and activity during infection.

#3 – Fig. 4D and Fig. S8D (Fluorescence Imaging Quality):
The fluorescence images presented in Fig. 4D (and S8D) are currently not informative to the reader. In particular, bacteria are difficult to detect in the DAPI channel due to the weak staining color and overlap with host nuclei. Given the authors' imaging capabilities, it is recommended to acquire 4-channel fluorescence images. Labeling bacteria with a distinct fluorophore (e.g., GFP or a green dye), SEPT9 in red, and Flag in far-red (as done in Fig. 4E) would significantly enhance clarity and interpretability. This approach would allow clear spatial distinction.

#4 -Fig. 4D and Fig. S8C (Individual Line Scans)
The individual line scans shown in Fig. 4D and Fig. S8C exhibit relatively noisy profiles and offer limited informational value to the reader. Since single line scans are inherently qualitative and not suited for statistical evaluation, they do not robustly support conclusions about fluorescence intensity differences between strains or conditions.

A more informative and quantitative approach would involve using intracellular bacteria as a mask to extract fluorescence intensities from the corresponding regions in other channels (e.g., SEPT9 and Flag). This would allow for the rapid quantification of signal intensities across hundreds of bacteria, enabling meaningful statistical comparisons. Implementing such an analysis would significantly enhance the quantitative rigor and clarity of the presented data.

#5 – Fig. 4E (Image Selection and Magnification):
The magnified region in Fig. 4E is not optimally chosen. The selected area (EH/AA) includes a large oversaturated region that provides limited information about individual bacteria. Other fields of view within the same dataset appear to better illustrate the phenotype, similar to vector control conditions, where single, clearly caged bacteria are visible. A more representative and well-balanced zoomed area would substantially improve the figure's impact.

#6 – Fig. 5B (Quantitative Validation of Phenotype):
Fig. 5B effectively demonstrates the individual and combined roles of OspC and OspG in bacterial caging. However, to provide a more complete picture of their contribution to Shigella pathogenesis, a complementary CFU assay using the same bacterial strains would be highly beneficial. A side-by-side comparison of intracellular survival or replication across the different effectors would clarify the impact of both effectors on overall infection.

#7 – Disruption of the Septin Octamer
The authors propose that OspC effector proteins destabilize SEPT9-containing septin octamers via ADP-ribosylation, leading to a shift toward tetramers. This hypothesis is supported by co-expression experiments in E. coli (Fig. 4B) and also overexpression experiments in 293T cells (Fig. 3E) look promising.
However, there is a possibility to test the proposed model in the context of Shigella infection. The Mavrakis lab has developed a toolbox (available via Addgene) that enables the investigation of specific septin–septin interactions (Martins et al. 2023). Using the splitGFP-SEPT9 construct, the GFP signal is expected to be lost during infection with wild-type Shigella due to OspC activity, whereas the signal should be maintained in infections with a catalytic-dead OspC or KO mutant, where no modification occurs.

Minor point:
Line 127: "catalytic mutant". I prefer the terms 'catalytically dead/inactive' or 'enzymatically inactive', as they more clearly indicate the loss of enzymatic function. That said, this remains a matter of personal preference.

Summary:
The authors present a highly interesting and timely study with an overall solid methodological framework. This reviewer considers the findings to be a long-awaited contribution to both the host–pathogen interaction and septin biology fields — specifically, the demonstration of direct septin modification by a bacterial effector or toxin. With the addition of some further experimental evidence supporting the specificity and function of this modification in a more physiological context, the manuscript will be suitable for publication.
Recommendation: Major Revision

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
Bacteria can be trapped in cage-like structures containing septin, key components of the cytoskeleton. These septin cages represents an important aspect of cell-autonomous immunity. In this manuscript the authors examine how a facultative intracellular bacterial pathogen, Shigella flexneri, can evade septin cages during infection. Building on prior work, they show that type 3 secreted proteins of the OspC family can suppress septin cages through a chemical modification (ADP-riboxanation) of septin 9 at a critical amino acid required for cage assembly. Their findings provide a new mechanism for evasion of septin cages by a bacterial pathogen. Furthermore, their findings show how OspC acts cooperatively with OspG to evade septin cages, thus underlining the importance of this cell autonomous immune defense. The paper is well written and the data and interpretations quite clear and convincing. I am in support of publication and have only minor comments for the authors consideration.

Minor comments:
-line 26 and line45, use either type 3 secretion or type III secretion consistently throughout
-line 257 "Since ADP-riboxanation of arginine basically converts it to a negatively charged residue…". This is not really accurate and could be worded better.
-line 306, "a double-knockout mutant (dospCdospG). Notably, septin cage formation was increased to an even higher level (31%). This is an exciting finding . The authors should comment on whether the more prevalent septin cages appear the same (morphologically, position within the cell, etc), or perhaps distinct, from cages observed in WT infected cells or single mutant infected cells.
-is bacterial intracellular growth, or cell-to-cell spread, altered in the double-knockout mutant (dospCdospG)?

-Figure 5A, D. It is very difficult to see bacteria within septin cages. The use of insets with higher magnification would likely help.
-Figure S7B. Where is the 10RK mutant?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors did an excellent job in addressing our concerns and we do not have any comments.

Reviewer #2

(Remarks to the Author)
The authors have made sufficient efforts to address the reviewer's main point of criticism -namely, to demonstrate the modification of endogenous SEPT9 in the context of infection at endogenous protein levels. The quality of several immunofluorescence images has also been significantly improved. Additional infection experiments further support the physiological relevance of the effector. I therefore support the publication of the presented work.
Since SEPT9 has been identified as a new target of a bacterial effector, the presented study represents an important contribution to host–pathogen interaction and septin research.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The authors have addressed my comments.

We thank the editor and reviewers for their constructive feedback and positive comments. We are grateful for their overall interest in our study. As suggested by reviewers, we carried out further investigations and made substantial improvements to significantly strengthen our manuscript message. We updated all figure panel labels to lowercase to comply with journal publication guidelines. Detailed point-to-point responses are provided below and all edits to the manuscript are highlighted in yellow for ease of reference.

REVIEWER COMMENTS

Reviewer #1:

In this work, Tang et al. employed multiple strategies—including western blotting, mass spectrometry, and microscopy—to demonstrate that OspC effectors enable S. flexneri to evade septin cage entrapment. Mechanistically, they show that OspC catalyzes ADP-riboxanation of SEPT9 at Arg561, a critical residue essential for stabilizing septin hetero-oligomers.
The writing style and scope align well with the aims of Nature Communications. Therefore, the journal should consider this manuscript for publication. I have some minor to moderate comments to help further improve the manuscript.

Response: We thank the reviewer for their positive feedback and support for publication.

1. Data Availability: The authors should provide a link to access the raw mass spectrometry (MS) data, for example, ProteomeXchange.

Response: We deposited raw MS data to ProteomeXchange and included a “Data Availability” section, which provides details of our proteomic datasets. The processed data (mostly in Excel format) have also been included as supplemental materials (see Supplemental Data 1-2).

2. Modified Site Assignment: For the ADP-riboxanation sites identified via MS/MS, did the authors manually inspect the spectra and assign fragment ions for all potential modified peptides? Please clearly describe the procedure for modified site assignment in the Methods section.

Response: We manually inspected spectra for all modified peptides. To ensure clarity and transparency, we provided a detailed description of the procedure in the Methods section (see lines 604-619).

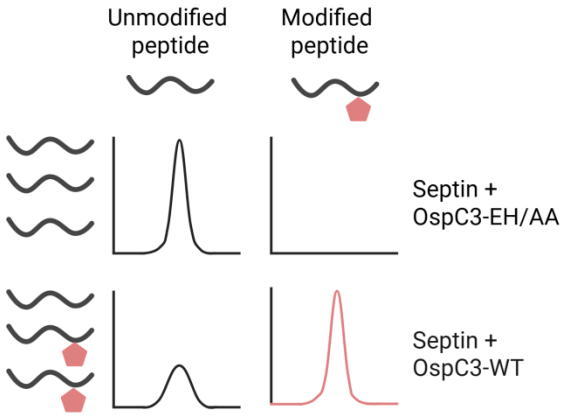
3. The rational of using both spectral counting and MaxLFQ (maxquant) for label-free quantification needs to be discussed. Usually, intensity-based label-free quantification will be more accurate than the spectral counting method.

Response: We agree that intensity-based quantification is generally more accurate than spectral counting. While the intensity-based approach is superior for detecting subtle differences, spectral counting is efficient for identifying the most robust proteomic changes. Therefore, for the initial proteomic screen to identify potentially modified targets (Fig. 1A), we used spectral counting for rapid assessment to tease out the most significant substrates. When these hits undergo further testing, the likelihood of not dropping out is usually higher.

In contrast, when we evaluated impact of ADP-riboxanation on interaction strength between different septin subunits, intensity-based quantification was used because differences were relatively modest (by approximately 50%, see Fig. 3E). As suggested, we clarified our rationale in the revised manuscript (see lines 650-652 and 664-665).

4. For Table 1, how is the modification rate calculated? Some brief information here will be helpful. Because the modification will impact the ionization efficiency of the peptides strongly, it will be hard to calculate the modification rates just based on the peptide intensity information.

Response: We agree that modifications often change ionization efficiency of peptides. Therefore, intensity readouts of modified and unmodified peptides cannot be compared directly (i.e., they are somewhat analogous to different currencies). To circumvent this problem, modification rate was calculated as percentage decrease of peptide intensities for unmodified peptides only. We have two experimental conditions (i.e., septins co-expressed with either wild-type OspC or catalytically inactive OspC, see the figure below) and compare differences of unmodified signals (denoted by black chromatographic peaks) between these two cases. For clarity we included description of this calculation process in the Methods section (lines 616-619).



5. Residue Specificity: ADP-ribosylation can also occur on glutamic acid residues. For the peptide sequence shown in Figure 2C, is it possible that the modification occurs on glutamic acid rather than arginine? Please clarify.

Response: We agree this is interesting. It is true that canonical ADP-ribosylation can target a broader range of residues, including glutamic acid. However, ADP-riboxanation is distinct from canonical ADP-ribosylation in terms of its modification chemistry. As demonstrated in our previous study (Li et al., *Nature* 2021), this modification occurs specifically on arginine residues. This specificity is derived from additional deamination of arginine's guanidinium group, thus yielding an oxazolidine ring structure in modified substrates. Glutamic acids, in principle, are not able to undergo such a unique chemical reaction. Additionally, mutational studies with substitution of modified Arg561 to either Ala or Lys further support the proposed modification site (new Fig. 2i). We clarified this with text updates in our revised manuscript (lines 611-614).

6. Experimental Details: a) For each experiment—such as immunoprecipitation (IP) or MS analysis—the authors should specify the number of cells used.
b) The flow rate used in the nanoLC-MS analysis should be provided.
c) The authors should specify the number of biological and technical replicates used for the MS quantification analysis.
d) For label-free data analysis, please specify the download date and the number of protein entries in the FASTA file used.

Response: These experimental details have been included in the revised manuscript.
a) 1-2 × 10^7 293T cells were used for IP (line 445) and MS analysis (line 577), respectively.
b) The flow rate of nanoLC-MS was set at 300 nL/min (line 594).
c) Two and four biological replicates were used for ADP-ribosylome (Fig. 1c and line 825) and interactome (Fig. 4a and line 881) analyses, respectively.
d) The FASTA file was downloaded in April 2021, containing 20,388 entries with the identifier UP000005640 (line 654).

Reviewer #2 (Remarks to the Author):

The study by Tang et al. explores how the bacterial pathogen Shigella flexneri evades host defense mechanisms by targeting septins, a family of proteins that form cage-like structures to entrap cytosolic bacteria. The key findings are as follows: S. flexneri uses its Type Three Secretion System (T3SS) effector proteins from the OspC family to modify septins via ADP-riboxanation, a form of post-translational modification closely related to ADP-ribosylation. SEPT9 is identified as a primary target among septin proteins. OspC effectors target SEPT9 and modify it at Arginine 561 (Arg561), disrupting the NC-interface between adjacent SEPT9 subunits. This disruption leads to disassembly of septin octamers, thereby inhibiting septin cage formation. Additionally, both OspC and another effector protein, OspG, act in concert to inhibit septin cage assembly. Mutant strains lacking these effectors show increased septin cage entrapment of bacteria, indicating a cooperative role in evading host defense. This study reveals that S. flexneri has evolved two distinct mechanisms to counteract septin-mediated cell-autonomous immunity.

This study offers novel insights into the mechanisms by which S. flexneri evades host cell

defenses through targeted modifications of septins. Overall, the experimental rigor of the study is commendable. This robustness is not surprising given that two labs with a successful track record in studying host-pathogen interactions—specifically, shigella cage entrapment by septins and effector-based modification of cellular targets—collaborated on this research. The experiments presented are well-designed and executed to a high standard.

The experimental setup largely builds on the authors' previous work on eIF3 and caspase modifications. The relevance of the study lies in the fact that it identifies a direct modification of septins by a bacterial toxin or effector (“an overdue finding”). OspCs are phylogenetically and functionally conserved across multiple bacterial pathogens, which further underscores the significance of this finding.

Response: We thank the reviewer for their highly positive comments on our work, in terms of both scientific significance and quality. It is encouraging to read that experts in the field consider this work (i.e., direct septin modification by bacterial proteins) as an “overdue” finding.

However, the authors face an inherent challenge: they are investigating a highly complex system involving multiple effectors, some of which are partially redundant or dispensable for the biological outcome—Shigella infection. Furthermore, they report promiscuous substrate activity of their effector, adding another layer of complexity: OspCs modify eIF3, caspase-11 and now SEPT9. A previously identified additional effector OspG targets septins indirectly by ubiquitination via CAND1 phosphorylation.

It is quite common for two or more effectors from the same bacterial species to target the same host protein or pathway (functional redundancy or effector convergence). Likewise, it is also possible for individual effectors to target multiple substrates. However, these substrates are often functionally and/or structurally related. Thus, further investigation into the biological significance of the septin modification by the authors would be beneficial.

Response: We acknowledge the complexity of bacterial infection involving multiple effectors, such as functional redundancy and substrate promiscuity. In our study we clearly show that OspCs and OspG work together to counteract septin cage entrapment via two distinct modifications, thereby facilitating bacterial cell-to-cell spread and replication (see results of our newly conducted experiments in Fig. 5e-i).

Major points:
#1 Line 143: “SEPT9 was post-translationally modified in a manner dependent on both the enzymatic activity and ARD domain of OspC3 (Fig. S2A and S2B).”
This interpretation might be misleading and requires further clarification. It suggests that both, the enzymatic N-terminal domain and the ARD (C-terminal domain) are required for the modification, which is not fully supported by the data. In Figures S2A and S2B (Flag-IP, ADPr), a band is still visible for the N-terminal domain (enzymatic domain) alone—albeit strongly reduced compared to the full-length protein. Taken together with the results in Figure S2F,

this indicates that the post-translational modification of SEPT9 depends on its catalytic N-terminal domain. However, the modification is strongly enhanced by septin-binding mediated by the C-terminal ARD.
Please clarify.

Response: We agree with the reviewer's interpretation of our data. Indeed the modification depends on the catalytic domain and can be strongly enhanced by ARD domain-mediated interaction. We revised the original claim in the text accordingly (see lines 143-145 and 149-151).

#2 Fig. The results from Fig. 2D+E clearly indicate that OspCs exhibit a high level of promiscuity concerning modified residues and substrate specificity. This is a significant problem of the study. Therefore, all findings must be interpreted with great caution. In the majority of their experiments, the authors overexpress the effector OspC along with the target or substrate protein under investigation using vectors with strong promoters ensuring high expression levels. However, high concentrations of OspC can lead to reduced specificity. It is important to note that physiologically, the number of effector molecules delivered by a T3SS is very limited. High septin concentrations may mask the modification of other preferred (potentially more physiological) substrates due to the excessive supply. Experiments employing bacterial infection and endogenous proteins are therefore eligible. In Fig. 2H+I+J, the authors perform cell infections (still with Flag-SEPT9 overexpression).

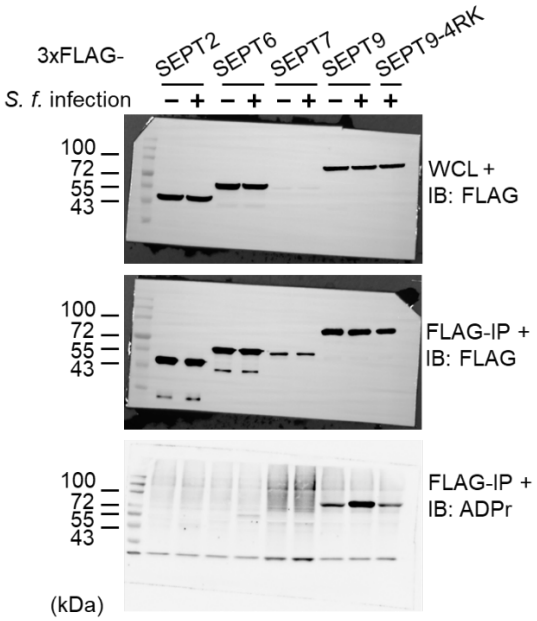
Response: We agree that overexpression of either effectors or substrates can lead to modification results that are sometimes unlikely to occur in physiologically relevant conditions (e.g., bacterial infection). As suggested, we repeated infection-based experiments and examine modification status of endogenous septins (at least for SEPT9). Below, we address each of the reviewer's specific comments.

#2.1 In Fig. 2H, the ADP-ribosylation of SEPT9 without infection is an interesting finding. Why does this ADP-ribosylation almost disappear in Fig. 2I and 2J? Please clarify.

Response: We acknowledge the observed discrepancy in levels of endogenously ADP-ribosylated SEPT9 between Fig. 2H and Fig. 2I/J. To clarify this, we repeated experiments under identical conditions and ensure reproducibility with multiple replicates (new Fig. 2i). Previous differences may result from repeated use of α-ADPr antibody, leading to reduced sensitivity for detecting weak endogenous modifications of SEPT9.

#2.2 Flag-tagged SEPT9 always runs between 72 and 100 kDa, but in Fig. 2H, it appears closer to 100 kDa. Please clarify.

Response: From the original uncropped blot the gel ran unevenly, thereby making SEPT9 appear higher than expected (see Rebuttal Figure 1).



Rebuttal Figure 1. Uncropped original images corresponding to Fig. 2H.

#2.3 As mentioned above, infection experiments with endogenous protein levels would significantly enhance the significance of the observations.
-The authors should make efforts to infect cells without overexpression of septins, perform immunoprecipitation (IP) of endogenous septins, and probe for the studied modification.

Response: We immunoprecipitated endogenous SEPT9 and probed with α-ADPr antibody (new Fig. 2j). Immunoblotting data revealed modification of endogenous SEPT9 during *Shigella* infection in an OspC-dependent manner, and this modification was confirmed by mass spectrometry analysis (new Fig. S6e).

-A classical yet informative assay could be employed to determine the extent of endogenous SEPT9 modification during infection. Specifically, lysates from infected cells—either following immunoprecipitation of SEPT9 or directly from whole-cell extracts—could be subjected to an in vitro post ADP-ribosylation reaction using recombinant OspC and labeled NAD^+ (e.g., biotinylated or radiolabeled). If SEPT9 is already efficiently ADP-ribosylated during infection with wild-type Shigella, minimal to no additional labeling would be detected. In contrast, infection with a catalytic-dead Shigella or KO mutant strain would leave SEPT9 unmodified, resulting in a strong signal upon in vitro labeling.
This approach could provide insights into the efficiency of SEPT9 modification in vivo and could help estimate substrate preferences of OspC under near-physiological conditions. Moreover, when using total lysates, other OspC substrates will also be labeled, enabling comparison of modification levels and preferences between SEPT9 and alternative targets.
While the presence of multiple ADP-ribosylation sites on OspC substrates might complicate signal interpretation, this assay remains valuable for evaluating the physiological relevance of OspC substrate specificity and activity during infection.

Response: The proposed in vitro ADP-ribosylation reaction with infected cell lysates, in principle, can be informative in determining extent of modifications. The concept of analyzing the proportion of unmodified septins during infection as an indirect readout of modification rate is similar to the approach we described in response to reviewer #1 (see point #4). However, the success of such assays relies on a sizeable fraction of substrates (i.e., septins) being modified prior to in vitro reactions. In reality, we learned from years of experience that rates of effector-catalyzed modifications during infection are generally very low (e.g., <5% of total protein pools). Such low rates are largely due to the very limited number of effector molecules delivered by the T3SS, a point raised by the reviewer (see point #2). Considering this, we would need to distinguish 95% unmodified from 100% unmodified by in vitro NAD^+ labeling and immunoblotting assays, which is far beyond quantification accuracy of Western blotting.

Another complication may arise from the fact that some modifications (i.e., SEPT9 at R561) may require presence of septin complexes or higher-order assemblies, which are likely disrupted during cell lysis. In support of this, R561 modification was not detected in prokaryotic co-expression systems or in vitro ADP-ribosylation reactions (see Fig. S6A and S6B).

As an alternative to in vitro labeling experiments, we directly measured the percentage of unmodified septins under infection conditions by mass spectrometry (see response to point #4 from reviewer #1 for methodological details, and refer to newly added Fig. 2j, Fig. S6e, and Table 2 for corresponding data). In general, quantification accuracy of mass spectrometry is much higher than that of immunoblotting assays. Mass spectrometry analysis revealed that modification rates were approximately 4% in cells infected by wild type *Shigella* and 12% with the complementation strain (Δ*ospC*+pOspC1/C3).

#3 – Fig. 4D and Fig. S8D (Fluorescence Imaging Quality):
The fluorescence images presented in Fig. 4D (and S8D) are currently not informative to the reader. In particular, bacteria are difficult to detect in the DAPI channel due to the weak staining color and overlap with host nuclei. Given the authors' imaging capabilities, it is recommended to acquire 4-channel fluorescence images. Labeling bacteria with a distinct fluorophore (e.g., GFP or a green dye), SEPT9 in red, and Flag in far-red (as done in Fig. 4E) would significantly enhance clarity and interpretability. This approach would allow clear spatial distinction.

Response: We repeated experiments using GFP-expressing bacteria (new Fig. 4d and S8f), which indeed substantially improved clarity and interpretability of our data.

#4 -Fig. 4D and Fig. S8C (Individual Line Scans)
The individual line scans shown in Fig. 4D and Fig. S8C exhibit relatively noisy profiles and offer limited informational value to the reader. Since single line scans are inherently qualitative and not suited for statistical evaluation, they do not robustly support conclusions

about fluorescence intensity differences between strains or conditions.

A more informative and quantitative approach would involve using intracellular bacteria as a mask to extract fluorescence intensities from the corresponding regions in other channels (e.g., SEPT9 and Flag). This would allow for the rapid quantification of signal intensities across hundreds of bacteria, enabling meaningful statistical comparisons. Implementing such an analysis would significantly enhance the quantitative rigor and clarity of the presented data.

Response: We appreciate the suggestion. By using bacteria as a mask, extracted fluorescence intensities were further normalized to the total mean fluorescence intensity of the respective individual cells. Statistical analysis revealed that both R561 and the NC-interface are required for FLAG-SEPT9 recruitment to *Shigella* (new Fig. 4d–e and S8c–d). In comparison, fluorescence intensities of total *Shigella*-associated SEPT9 (including both transfected and endogenous proteins) were not significantly different among the same set of transfected samples (new Fig. 4f and S8e). We updated text in the Methods (line 569-573) and Results section (line 288-298) accordingly.

Due to overwhelming signal of host nuclei in the DAPI channel (as mentioned in point #3), we replaced DAPI with GFP-labeled bacteria to improve visualization (new Fig. 4d and S8c).

#5 – Fig. 4E (Image Selection and Magnification):
The magnified region in Fig. 4E is not optimally chosen. The selected area (EH/AA) includes a large oversaturated region that provides limited information about individual bacteria. Other fields of view within the same dataset appear to better illustrate the phenotype, similar to vector control conditions, where single, clearly caged bacteria are visible. A more representative and well-balanced zoomed area would substantially improve the figure's impact.

Response: We replaced this magnified region with a more representative and well-balanced zoomed area to support our conclusions (new Fig. 4g).

#6 – Fig. 5B (Quantitative Validation of Phenotype):
Fig. 5B effectively demonstrates the individual and combined roles of OspC and OspG in bacterial caging. However, to provide a more complete picture of their contribution to Shigella pathogenesis, a complementary CFU assay using the same bacterial strains would be highly beneficial. A side-by-side comparison of intracellular survival or replication across the different effectors would clarify the impact of both effectors on overall infection.

Response: We conducted CFU assays as suggested. Though single deletion mutants did not show any growth defects intracellularly, we did observe reduced replication of the double mutant (see lines 331-334 and new Fig. 5g–i). Given that septin cages restrict intracellular mobility and cell-to-cell spread of cytosolic bacteria, we also carried out a plaque formation assay (see comments of reviewer #4). These new results revealed collaborative contribution of these two effectors to *Shigella* pathogenesis (new Fig. 5e–i, lines 327-338).

#7 – Disruption of the Septin Octamer
The authors propose that OspC effector proteins destabilize SEPT9-containing septin octamers via ADP-ribosylation, leading to a shift toward tetramers. This hypothesis is supported by co-expression experiments in E. coli (Fig. 4B) and also overexpression experiments in 293T cells (Fig. 3E) look promising.
However, there is a possibility to test the proposed model in the context of Shigella infection. The Mavrakis lab has developed a toolbox (available via Addgene) that enables the investigation of specific septin–septin interactions (Martins et al. 2023). Using the splitGFP-SEPT9 construct, the GFP signal is expected to be lost during infection with wild-type Shigella due to OspC activity, whereas the signal should be maintained in infections with a catalytic-dead OspC or KO mutant, where no modification occurs.

Response: We agree that direct visualization of SEPT9–SEPT9 interactions during infection with the splitGFP-SEPT9 construct would further validate our hypothesis within a physiologically relevant context. Nonetheless, we need to consider that minimal amounts of effectors are delivered by T3SSs during infection (see point #2). Together with modification rate of septins as low as <5% during *Shigella* infection, it is very likely that the suggested assay will not be sensitive enough to detect impact of OspC on septin-septin interactions.

Minor point:
Line 127: “catalytic mutant”. I prefer the terms 'catalytically dead/inactive' or 'enzymatically inactive', as they more clearly indicate the loss of enzymatic function. That said, this remains a matter of personal preference.

Response: We revised the terms to 'catalytically dead/inactive' throughout the manuscript.

Summary:
The authors present a highly interesting and timely study with an overall solid methodological framework. This reviewer considers the findings to be a long-awaited contribution to both the host–pathogen interaction and septin biology fields — specifically, the demonstration of direct septin modification by a bacterial effector or toxin. With the addition of some further experimental evidence supporting the specificity and function of this modification in a more physiological context, the manuscript will be suitable for publication.
Recommendation: Major Revision

Response: We thank the reviewer for their positive evaluation and suggestions to strengthen our manuscript message.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This

is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thank you, we are grateful for ECRs to evaluate our work.

Reviewer #4 (Remarks to the Author):

Bacteria can be trapped in cage-like structures containing septin, key components of the cytoskeleton. These septin cages represents an important aspect of cell-autonomous immunity. In this manuscript the authors examine how a facultative intracellular bacterial pathogen, Shigella flexneri, can evade septin cages during infection. Building on prior work, they show that type 3 secreted proteins of the OspC family can suppress septin cages through a chemical modification (ADP-riboxanation) of septin 9 at a critical amino acid required for cage assembly. Their findings provide a new mechanism for evasion of septin cages by a bacterial pathogen. Furthermore, their findings show how OspC acts cooperatively with OspG to evade septin cages, thus underlining the importance of this cell autonomous immune defense. The paper is well written and the data and interpretations quite clear and convincing. I am in support of publication and have only minor comments for the authors consideration.

Response: We thank the reviewer for their enthusiasm.

Minor comments:
-line 26 and line45, use either type 3 secretion or type III secretion consistently throughout

Response: We revised text to type III secretion and ensure consistency throughout the manuscript.

-line 257 “Since ADP-riboxanation of arginine basically converts it to a negatively charged residue…”. This is not really accurate and could be worded better.

Response: We modified text for improved clarity (lines 267-269).

-line 306, “a double-knockout mutant (dospCdospG). Notably, septin cage formation was increased to an even higher level (31%). This is an exciting finding. The authors should comment on whether the more prevalent septin cages appear the same (morphologically, position within the cell, etc), or perhaps distinct, from cages observed in WT infected cells or single mutant infected cells.

Response: We agree that a bacterial strain triggering >30% septin cage entrapment can help to transform the community. We did not observe significant morphological or positional differences in septin cages under experimental conditions we tested. To address this in depth future studies will be required by employing super-resolution and/or high-content microscopy.

-is bacterial intracellular growth, or cell-to-cell spread, altered in the double-knockout mutant (dospCdospG)?

Response: We performed both plaque formation and CFU assays. Overall, the double mutant exhibited stronger phenotypes/defects in these new assays (new Fig. 5e–i, lines 325-338).

-Figure 5A, D. It is very difficult to see bacteria within septin cages. The use of insets with higher magnification would likely help.

Response: We included insets with higher magnification (new Fig. 5a, 5c).

-Figure S7B. Where is the 10RK mutant?

Response: We incorporated the 10RK panel (new Fig. S7b).

Response: We appreciate very much the comments from all the reviewers and thank you for your support as well as appreciation of our work!

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors did an excellent job in addressing our concerns and we do not have any comments.

Reviewer #2 (Remarks to the Author):

The authors have made sufficient efforts to address the reviewer's main point of criticism -namely, to demonstrate the modification of endogenous SEPT9 in the context of infection at endogenous protein levels. The quality of several immunofluorescence images has also been significantly improved. Additional infection experiments further support the physiological relevance of the effector. I therefore support the publication of the presented work.
Since SEPT9 has been identified as a new target of a bacterial effector, the presented study represents an important contribution to host–pathogen interaction and septin research.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

The authors have addressed my comments.