Sumoylation controls host anti-bacterial response to the gut invasive pathogen Shigella flexneri

Sabrina Fritah, Nouara Lhocine, Filip Golebiowski, Joelle Mounier, Alexandra Andrieux, Gregory Jouvion, Ronald T. Hay, Philippe Sansonetti and Anne Dejean

Corresponding authors: Anne Dejean and Philippe Sansonetti, Institut Pasteur

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>19 December 2013</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>16 January 2014</td>
</tr>
<tr>
<td>Correspondence</td>
<td>22 January 2014</td>
</tr>
<tr>
<td>Correspondence</td>
<td>27 January 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>19 May 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>13 June 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>04 July 2014</td>
</tr>
<tr>
<td>Accepted</td>
<td>08 July 2014</td>
</tr>
</tbody>
</table>

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.)

Editor: Nonia Pariente

1st Editorial Decision 16 January 2014

Thank you for your patience while your study was under peer-review over the Christmas holidays. We have now received the three enclosed reports on it. As you will see, although all the referees find the topic of interest and appropriate for EMBO reports if suitably revised, they all raise some issues that need to be experimentally addressed before publication here can be considered.

As the reports are below, I will not detail them here. However, all referees point to the need of increasing the cohesiveness of the different results reported to make a stronger story by, for example, validating that some of the transcription factors identified by your proteomics study in HeLa cells is affected during Shigella infection, ideally in mice. In addition, the evidence of the role of SUMOylation in the inflammatory response needs to be strengthened, as indicated by referees 1 and 3, and the contribution of SUMOylation to cell cohesion (through modification of cytoskeletal components) explored, as referee 2 indicates.
In addition, it would be important to clarify the role of SUMO2 versus SUMO1, to bolster the claims made in the study, as referee 1 suggests, although we would not necessarily make this a precondition for acceptance. However, toning down this aspect of your study would deter from its conceptual novelty and we would thus encourage you to address this point if possible. The experiment raised by referee 3 in his/her point 1 would not be required for publication.

Please note that it is our policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Do not hesitate to get in touch with me if I can be of any help during the revision of your study. I look forward to receiving its revised version.

REFEREE REPORTS:

Referee #1:

The authors have investigated the functions of sumoylation in affecting the defense of mammalian cells and mice to infection by Shigella flexneri. They demonstrated that overexpression of SUMO-2 impairs Shigella invasion of HeLa cells in vitro. Using a mouse model haploinsufficient for Ubc9, they also demonstrated that sumoylation affects invasion of intestinal epithelial cells and mucosal inflammation in vivo. Quantitative proteomic studies revealed that Shigella infection of HeLa cells affects sumoylation of a number of transcription factors, including factors with roles in inflammation. Consistent with these findings, analysis of gene expression in intestines of Ubc9 haploinsufficient mice revealed that the pro-inflammatory transcriptional response was affected compared to wild type mice.

Shigella infection is an intestinal disease, causing diarrhea, cramping and vomiting. When occurring in malnourished children, Shigella infection can be life threatening. Understanding the host defense mechanisms affecting Shigella invasion of the intestinal epithelia and, and the underlying consequences of infection, is therefore important and has the potential to lead to new approaches to prevent and treat this disease. The studies reported by Fritah et al., demonstrating a role for sumoylation in affecting Shigella invasion and downstream inflammatory responses, are therefore significant.

Overall the manuscript is well written and the data are all of high quality. However, there are a number of issues and concerns that could be addressed to improve the manuscript. In particular, evidence that sumoylation plays a functionally important role in affecting transcription and inflammatory response pathways needs to be strengthened.

1) Results in Figure 4 are interpreted to suggest that sumoylation regulates the pro-inflammatory transcriptional response to Shigella infection in vivo. However, it is also possible that the observed differences in gene expression in Ubc9+/+ and Ubc9+-/ mice represent a secondary (and indirect) consequence of differences in sumoylation, with the primary effect of sumoylation being at the level of epithelial invasion. As shown in Figure 2, levels of epithelial invasion are much higher in Ubc9+-/ mice. Can wild type and heterozygous Ubc9 mice be infected with different levels of Shigella so that equivalent levels of invasion are observed? If this is possible, comparing levels of gene expression between wild type and heterozygous mice under conditions were infection levels are equivalent would provide a clearer view of direct effects of sumoylation on transcriptional control in this experimental system.

2) The suggestion that sumoylation plays a role in regulating gene expression in response to Shigella infection in vivo stems in part from the proteomic studies performed in infected HeLa cells. More direct evidence (IP westerns) that sumoylation of the identified transcription factors are affected by Shigella infection would strengthen the study. Evidence that sumoylation of any one of the factors identified in HeLa cells is differentially affected following infection of wild type and heterozygous Ubc9 intestinal epithelia in mice would also significantly increase the strength and validity of the proteomics study.

© European Molecular Biology Organization
Referee #2:

The authors explore the relationship between Shigella flexneri infection (in mice and HeLa cells) and the SUMOylation pathway (HeLa cells overexpressing Tap-tagged SUMO, HeLa cells in which the SUMO E1 enzyme subunit SAE2 is downregulated by siRNA, Ubc9+/− mice originally described in Nacerddine et al 2005). Key findings are:

1. HeLa cells that overexpress TAP-tag SUMO2 but not TAP-tag SUMO1 are less susceptible to Shigella infection, cells with reduced sumoylation capacity (siRNA SAE2) are more susceptible.
2. The intestine of 3 day old newborn Ubc9+/− mice is significantly more invaded by Shigella than wt mice.
3. The intestine of 3 day old newborn Ubc9+/− mice is significantly more permeable than that of wt mice, even when uninfected.
4. Inflammatory response is highly upregulated in infected Ubc9+/− mice (IFN-y, Cxcl-1, IL-6 were confirmed by qPCR)
5. a SUMO proteome analysis (SILAC/mass spec) of infected versus non-infected TAP-tag SUMO2 HeLa cells leads to the identification of a number of proteins whose sumoylation status seems to be altered upon infection, including several candidate transcription factors known to be sumoylated and known to contribute to inflammatory response such as c-Fos and PPARγ.

From these findings the authors conclude that SUMO2 conjugation impairs Shigella invasion of epithelial cells in vitro, that sumoylation regulates intestinal permeability, and that sumoylation is required to restrict epithelial invasion and control mucosal inflammation.

Overall this is an interesting study, well written and technically sound. However, the most exciting finding - 3 day old newborn Ubc9+/− mice have a barrier defect - is not appropriately discussed and should also be better addressed experimentally. This finding may well explain all other findings in mice (higher infection, strong transcriptional response to the infection - upregulation of cytokines) and be related to findings in the HeLa cells.

The Dejean lab reported previously that global SUMO1 and SUMO2 sumoylation is unaffected in adult Ubc9+/− mice and that stress induced sumoylation in the corresponding MEFs was only very slightly altered (Nacerddine et al 2005). They also found that inducible knockout of Ubc9 significantly altered cellular organisation and cytoskeletal organisation in the intestine. Their data indicated "that sumoylation appears as a main regulator of the baso-apical polarity and mechanical stability of the enterocytes." (Demarque et al 2011).

In light of these findings, the most obvious hypothesis is that sumoylation of one or several cytoskeletal or cell adhesion components needs sumoylation and is exceptionally sensitive to Ubc9 levels. Reduced sumoylation of this factor weakens the barrier to an extent that favours infection. The HeLa cell experiments (Figure 1) show that the initial infection efficiency is influenced by levels of sumoylation. Could this be caused by an effect that is related to the mouse findings (a function of SUMO in cellular organisation and cell-cell contacts)?

There are certainly a number of cytoskeleton candidates in the diverse SUMO proteome lists from the Hay lab that could be tested for susceptibility to altered Ubc9/SUMO2 levels by IP/immunoblotting and discussed in the context of the current study.

Downstream changes in the transcriptional program are of course interesting, but in my mind much less surprising considering current knowledge on transcription factor sumoylation.

At least some of the candidates shown in the transcription network in Figure 3 should be verified: IP / immunoblotting to show changed sumoylation in response to infection. After all, pulldown / mass-spec is an initial screen, not proof.

The authors findings suggest that SUMO2 is more relevant than SUMO1 - however this is based on overexpressing Tap-TAG SUMO. If the authors want to make a strong point out of this, a few additional straight-forward experiments are needed. First, SUMO conjugates should be shown in untransfected versus transfected cells (using SUMO antibodies) to allow conclusion about fold-increase of SUMO conjugates upon overexpression. Second, the effect of SUMO1 and SUMO2 reduction by siRNA depletion for infection should be tested.

Minor point:
Supplemental Figure legend 4 is full of spelling mistakes.
Referee #3:

In summary, the authors evaluate the role of SUMO in the regulation of cellular invasion and host inflammatory signaling in response to Shigella flexneri infection. They generally conclude that SUMO plays several roles in restricting Shigella virulence. They also report potentially new Sumoylated substrates and those that may be differentially regulated by bacterial infection. What I like about this study is that it is short, yet provides valuable information in an easily attainable format. The authors do a very good job of not over interpreting their data and not drawing conclusions that are unwarranted. The majority of experiments are properly controlled (with a small number of exceptions outlined below). In addition, the study has potential to be of significant value to the Shigella and sumoylation communities. What I am more concerned about in the manuscript is that the direct relationships between the cell-based studies, the biochemistry, and the in vivo experiments are unclear. Rather than digging into the core mechanism of one or two of the findings, the authors present a survey of, what appears to be, potentially unlinked biological phenomenon. Experiments that link these observations would be very helpful in this regard. Thus, while there are many positive aspects to this work, I have enough concerns to warrant a second evaluation of the manuscript prior to publication.

Point-1: The authors knock down (RNAi) the sumo E1. It would be valuable to also knock down Ubc9 in the cell based invasion studies, which would then link the story in a more comprehensive manner. The authors could also examine the inflammatory response genes in vitro using this approach. Perhaps most importantly, knocking down the E2 would demonstrate that the cell-based findings are robust and that Shigella is sensitive to inhibition of multiple points if the sumoylation pathway.

Point-2: A major concern is that the authors do not confirm the regulation of their sumoylated hits using a second approach (e.g. quantitative western blots). At least a handful (5 to 10) of the identified transcription factors (or other differentially Sumoylated proteins) need to be confirmed to validate the SILAC experiment. In addition, it would be best to look at endogenous Sumo in these studies.

Point-3: The authors evaluate transcriptional regulation in Ubc +/- mice. I found the analysis of these data difficult to interpret in regard to the comparison between transcriptional response and the up/down regulation of sumoylated T.Fs. Can the authors draw any conclusions about the link between sumoylation of TFs and the transcriptional response output during Shigella infection? For example, are there differences in levels or activity of sumoylated TFs that explain the increase in INF-g, Cxcl-1, Il-6 observed in UBC9 +/- mice? Likewise, can the authors demonstrate a link between the regulation of these TFs by Sumo and the regulation of cytokine expression during Shigella infection in vitro? MKL-1 may be a good test candidate. Again, this gets back to the general point that the current study is somewhat disjointed, and any molecular links between the experiments would significantly enhance the study.

Minor comments:

- On page 5, the authors indicate that the Ube9 heterozygote has no phenotype (Nacerdinne et al.), but do not explain the rationale for using a het versus a null. I assume this mutation is lethal. If so, it would be good to add this information here.

- On page 7, the authors perform SILAC experiments. They present the technique and the controls together, but do not include the results from Shigella infection in the section headlining that the Shigella experiments (top of page 7). Either retitle this section or, more preferably, add the results of the Shigella experiment under this headline.
Thank you for your interest in our manuscript entitled 'Sumoylation controls host anti-bacterial response to the gut invasive pathogen /Shigella flexneri/'. We do appreciate the reviewers' comments and your own perspective on our work.

We would like to argue, in general, that this manuscript is already rich in original data regarding the role of SUMO in host protection against infection. So far, there is not a single publication that has analysed the function of this system to such degree of detail, including a true attempt at deciphering the process /in vivo/. We are well aware that by doing so, we are exposing ourselves more than if we had focused only on an /in vitro/ model of infection, and how SUMO regulates /Shigella/ invasion in a cell line. This raises comments from reviewers that make, in a way, the core of their concern: what is the relation between /in vitro/ observations/which, by essence focus on the mechanics of bacterial internalisation into cells, and /in vivo/ observation that tend to divert the focus towards epithelial rupture, tissue invasion and inflammation?

We would like to first address this point and then address issues that are more « SUMO-specific ». The bottom line here is the uncertainty about whether the control of epithelial inflammation by SUMO - as we clearly demonstrate using Ubc9+/- newborn mice in comparison to wt mice - is linked to a decreased capacity of bacterial invasion of individual cells, or to a control of the inflammatory response (reviewers 1 and 2, point 1). Considering the intrinsic link between epithelial invasion and inflammation in the pathogenesis of shigellosis, we would like to stress that setting this as an alternative between two polarized options makes no sense. Invasion causes inflammation which in turn enhances invasion by disruption of the epithelial barrier, etc... This is an intrinsic vicious circle. /Shigella/ itself has « adopted » an antiinflammatory strategy by expressing elaborate anti-inflammatory effectors (i.e. IpgD, OspG, OspF, IpaH), demonstrating that regarding co-evolution, it had to solve the issue of cell entry AND survival in the face of inflammation. Thus, to us, the /in vitro/ demonstration that /Shigella/ causes alterations in the sumoylation status of inflammation-related transcription factors makes total sense and needs to be considered in the /in vivo/ context. Coming back to trying to experimentally sort cell invasion and inflammation: this is currently beyond reach because /Shigella/ is a human specific pathogen and even a relevant model like the newborn mouse has its limitations. For instance, the suggestion of performing a dose-response kind of experiment (reviewer 1, point 1), i.e. trying to adjust the concentration of inoculated bacteria to the difference of epithelial « susceptibility » between Ubc9-/+ and wt mice is impossible. Below a certain threshold, there is no triggering of the response, and beyond the concentration in current use, especially in highly susceptible mice like Ubc9-, pups die very quickly, making these experiments an impossible endeavor.

We would therefore like to argue that disentangling this « Gordian knot » is, for the moment, impossible (cutting not being the solution...). As an element antagonizing /Shigella/ entry (proven /in vitro/ and /in vivo/: see evaluation of invasion efficiency), strengthening the epithelial barrier (proven /in vivo/), and affecting transcription factors involved in inflammatory responses, thus decreasing the inflammatory response to an infectious aggression (not
addressed /in vitro/ in parallel to SILAC identification, done /in vivo/ with discussion about the « vicious circle » discussed above), we consider that for the time being, we have gone as far as possible within the limits of the experimental models to provide a coherent /in vitro/-/in vivo/ view of the regulatory function of SUMO in the course of /Shigella/ infection.

Regarding the more SUMO-related issues, a common concern of all three reviewers (reviewer 1, point 1- reviewer 2, point 2- reviewer 3, point 2) is the need for validation of the SILAC experiment by IP/blotting to show alteration of the sumoylation status of some of the candidate transcription factors upon /Shigella/ infection. We will address this point and focus on PPARg and c-Fos, two key mediators of inflammation which we found hypsumoylated in the SILAC assay upon infection. We will perform this experiment by comparing levels of sumoylated PPARg and c-FOS in infected /versus/ non-infected conditions in transfected HeLa cells. We will also attempt the same type of experiments in untransfected cells, though the probability of seeing the sumoylated forms at endogenous levels is extremely low. Indeed, transcription factors are modified at very low levels and their sumoylated forms are hardly, if at all detectable at the endogenous levels in cells. To our knowledge, sumoylation of endogenous PPARg and c-FOS by IP/blotting has never been reported in steady state conditions.

In summary, we feel that elucidating the role of sumoylation in epithelial barrier strength including the identification of the putative cytoskeletal or cell adhesion SUMO substrates involved (reviewer 2, point 1) and deciphering the role of sumoylation in inflammation (reviewer 3, point 3) constitute two whole studies in their own right that would go far beyond the scope of the present study. As for the second point, role of SUMO in inflammation, we provide important mechanistic insight in showing that /Shigella/ causes alteration of the sumoylation status of key transcriptional regulators of inflammation towards a pro-inflammatory response, which is in keeping with what we observe /in vivo/. After adding the experiments aimed at validating the SILAC approach, we feel our work fullfills the journal's criteria in providing «concise studies with key novel observations of physiological importance », but with « less emphasis on detailed mechanistic understanding ».

If you share our view, which we hope, we would be happy to start with the experiments and send you a revised manuscript within two months.

Looking forward to your reply.

Correspondence - editor 27 Jan 2014

Many thanks for your letter detailing your revision plans. I was out of the office all of last week and could therefore not look at your file in detail until today.

Your approach to validate the SILAC experiments seem excellent, and I do understand the technical difficulty in trying to detect the normally low levels of endogenous SUMO conjugates.

I also appreciate the difficulty and limitations of working with a human pathogen such as Shigella in newborn mice, and understand that the experiment suggested by referee 1 in his/her point 1 cannot...
be addressed. Your point regarding the inter-dependency of invasion, inflammation and disruption of epithelial integrity during the course of shigellosis is well taken.

I wonder, however, whether it would be possible to identify cytoskeletal proteins (previously reported to be SUMOylated) that are differentially SUMOylated in infected vs non-infected cells. Could this be done at the same time as you validate the SILAC experiment? This would allow you to discuss the results in the context of your previous findings on the role of SUMOylation in the organization of the intestinal epithelium.

Lastly, would it be possible to relate TF SUMOylation in cells with an effect in cytokine expression? I am unsure if there are suitable cell lines for such an experiment, but if so, this would go some way toward closing the circle between the observations reported.

I would like to stress that we remain very interested in the study, but feel its revision should try to address the points raised above, especially the validation of the SILAC and identification of cytoskeletal proteins involved in the phenotypes observed.

I look forward to hearing from you.

Correspondence - authors
27 January 2014

Thank you very much for your reply.

We will definitely address the first point (validation of the SILAC experiment on selected substrates in cotransfection conditions).

As for the second point ‘identify cytoskeletal proteins (previously reported to be SUMOylated) that are differentially SUMOylated in infected vs non-infected cells’, we will look in the literature for such proteins and if any, we will do the experiment. However we did not find any cytoskeletal or cell adhesion proteins affected in our SILAC experiment, so the requested experiment will remain really artificial and will be difficult to include in the discussion section.

We should be able to send you the revised version within a few weeks.

Thank you again for your interest in our work.
Referee #1:

The authors have investigated the functions of sumoylation in affecting the defense of mammalian cells and mice to infection by Shigella flexneri. They demonstrated that overexpression of SUMO-2 impairs Shigella invasion of HeLa cells in vitro. Using a mouse model haploinsufficient for Ubc9, they also demonstrated that sumoylation affects invasion of intestinal epithelial cells and mucosal inflammation in vivo. Quantitative proteomic studies revealed that Shigella infection of HeLa cells affects sumoylation of a number of transcription factors, including factors with roles in inflammation. Consistent with these findings, analysis of gene expression in intestines of Ubc9 haploinsufficient mice revealed that the pro-inflammatory transcriptional response was affected compared to wild type mice.

Shigella infection is an intestinal disease, causing diarrhea, cramping and vomiting. When occurring in malnourished children, Shigella infection can be life threatening. Understanding the host defense mechanisms affecting Shigella invasion of the intestinal epithelia and, and the underlying consequences of infection, is therefore important and has the potential to lead to new approaches to prevent and treat this disease. The studies reported by Fritah et al., demonstrating a role for sumoylation in affecting Shigella invasion and downstream inflammatory responses, are therefore significant.

Overall the manuscript is well written and the data are all of high quality. However, there are a number of issues and concerns that could be addressed to improve the manuscript. In particular, evidence that sumoylation plays a functionally important role in affecting transcription and inflammatory response pathways needs to be strengthened.

Point 1) Results in Figure 4 are interpreted to suggest that sumoylation regulates the pro-inflammatory transcriptional response to Shigella infection in vivo. However, it is also possible that the observed differences in gene expression in Ubc9+/+ and Ubc9+/- mice represent a secondary (and indirect) consequence of differences in sumoylation, with the primary effect of sumoylation being at the level of epithelial invasion. As shown in Figure 2, levels of epithelial invasion are much higher in Ubc9+/- mice. Can wild type and heterozygous Ubc9 mice be infected with different levels of Shigella so that equivalent levels of invasion are observed? If this is possible, comparing levels of gene expression between wild type and heterozygous mice under conditions were infection levels are equivalent would provide a clearer view of direct effects of sumoylation on transcriptional control in this experimental system.

The bottom line here is the uncertainty about whether the control of epithelial inflammation by SUMO - as we clearly demonstrate using Ubc9+/- newborn mice in comparison to wt mice - is linked to a decreased capacity of bacterial invasion of individual cells, or to a control of the inflammatory response. Considering the intrinsic link between epithelial invasion and inflammation in the pathogenesis of shigellosis, we would like to stress that setting this as an alternative between two polarized options makes no sense. Invasion causes inflammation which in turn enhances invasion by disruption of the epithelial barrier, etc... This is an intrinsic vicious circle. Shigella itself has « adopted » an anti-inflammatory strategy by expressing elaborate anti-inflammatory effectors (i.e. IpgD, OspG, OspF, IpaH), demonstrating that regarding co-evolution, it had to solve the issue of cell entry AND survival in the face of inflammation. Thus, to us, the in vitro demonstration that Shigella causes alterations in the sumoylation status of inflammation-related transcription factors makes total sense and needs to be considered in the in vivo context. Coming back to trying to experimentally sort cell invasion and inflammation: this is currently beyond reach because Shigella is a human specific pathogen and even a relevant model like the newborn mouse has its limitations. For instance, the reviewer's suggestion of performing a dose-response kind of experiment, i.e. trying to adjust the concentration of inoculated bacteria to the difference of epithelial « susceptibility » between Ubc9-/+ and wt mice is impossible. Below a certain threshold, there is no triggering of the response, and beyond the concentration in current use, especially in highly susceptible mice like Ubc9-/-, pups die very quickly, making these experiments an impossible endeavor.

We would therefore like to argue that disentangling this « Gordian knot » is, for the moment, impossible (cutting not being the solution...). As an element antagonizing Shigella entry (proven in vitro and in vivo : see evaluation of invasion efficiency), strengthening the epithelial barrier (proven in vivo), and affecting transcription factors involved in inflammatory responses, thus decreasing the inflammatory response to an infectious agression (not addressed in vitro in parallel to SILAC identification, done in vivo with discussion about the « vicious circle » discussed above), we consider that for the time being, we have gone as far as possible within the limits of the experimental models to provide a coherent in vitro-in vivo view of the regulatory function of SUMO in the course of Shigella infection.
Point 2) The suggestion that sumoylation plays a role in regulating gene expression in response to Shigella infection in vivo stems in part from the proteomic studies performed in infected HeLa cells. More direct evidence (IP westerns) that sumoylation of the identified transcription factors are affected by Shigella infection would strengthen the study. Evidence that sumoylation of any one of the factors identified in HeLa cells is differentially affected following infection of wild type and heterozygous Ubc9 intestinal epithelia in mice would also significantly increase the strength and validity of the proteomics study.

As requested by the reviewer, we performed some validation of the SILAC experiment. Given the difficulty to detect low levels of endogenous SUMO conjugates, we analyzed by quantitative immunoblotting the sumoylation status of two of the transcriptional regulators associated with digestive functions present in the short list of the proteomic analysis (Fig. 3E), c-FOS and SATB1 (special AT-rich sequence-binding protein-1). We could confirm, for both ectopically expressed substrates, the decrease in SUMO-2-modified forms induced by Shigella infection in HeLa cells. We have added these data in the new Fig. S3 and in the Result section (page 9, lines 8 to 12).
Referee #2:

The authors explore the relationship between Shigella flexneri infection (in mice and HeLa cells) and the SUMOylation pathway (HeLa cells overexpressing Tap-tagged SUMO, HeLa cells in which the SUMO E1 enzyme subunit SAE2 is downregulated by siRNA, Ubc9+/- mice originally described in Nacerddine et al. 2005). Key findings are:
1. HeLa cells that overexpress TAP-tag SUMO2 but not TAP-tag SUMO1 are less susceptible to Shigella infection, cells with reduced sumoylation capacity (siRNA SAE2) are more susceptible.
2. The intestine of 3 day old newborn Ubc9+/- mice is significantly more invaded by Shigella than wt mice.
3. The intestine of 3 day old newborn Ubc9+/- mice is significantly more permeable than that of wt mice, even when uninfected.
4. Inflammatory response is highly upregulated in infected Ubc9+/- mice (IFN-γ, Cxcl-1, IL-6 were confirmed by qPCR).
5. A SUMO proteome analysis (SILAC/mass spec) of infected versus non-infected TAP-tag SUMO2 HeLa cells leads to the identification of a number of proteins whose sumoylation status seems to be altered upon infection, including several candidate transcription factors known to be sumoylated and known to contribute to inflammatory response such as c-Fos and PPARγ.

From these findings the authors conclude that SUMO2 conjugation impairs Shigella invasion of epithelial cells in vitro, that sumoylation regulates intestinal permeability, and that sumoylation is required to restrict epithelial invasion and control mucosal inflammation.

Overall this is an interesting study, well written and technically sound.

Point 1) However, the most exciting finding - 3 day old newborn Ubc9+/- mice have a barrier defect - is not appropriately discussed and should also be better addressed experimentally. This finding may well explain all other findings in mice (higher infection, strong transcriptional response to the infection - upregulation of cytokines) and be related to findings in the HeLa cells.

The Dejean lab reported previously that global SUMO1 and SUMO2 sumoylation is unaffected in adult Ubc9+/- mice and that stress induced sumoylation in the corresponding MEFs was only very slightly altered (Nacerddine et al. 2005). They also found that inducible knockout of Ubc9 significantly altered cellular organisation and cytoskeletal organisation in the intestine. Their data indicated "that sumoylation appears as a main regulator of the baso-apical polarity and mechanical stability of the enterocytes." (Demarque et al. 2011).

In light of these findings, the most obvious hypothesis is that sumoylation of one or several cytoskeletal or cell adhesion components needs sumoylation and is exceptionally sensitive to Ubc9 levels. Reduced sumoylation of this factor weakens the barrier to an extent that favours infection.

The HeLa cell experiments (Figure 1) show that the initial infection efficiency is influenced by levels of sumoylation. Could this be caused by an effect that is related to the mouse findings (a function of SUMO in cellular organisation and cell-cell contacts)?

There are certainly a number of cytoskeleton candidates in the diverse SUMO proteome lists from the Hay lab that could be tested for susceptibility to altered Ubc9/SUMO2 levels by IP/immunoblotting and discussed in the context of the current study.

The bottom line here is the uncertainty about whether the control of epithelial inflammation by SUMO - as we clearly demonstrate using Ubc9+/- newborn mice in comparison to wt mice - is linked to a decreased capacity of bacterial invasion of individual cells, or to a control of the inflammatory response. Considering the intrinsic link between epithelial invasion and inflammation in the pathogenesis of shigellosis, we would like to stress that setting this as an alternative between two polarized options makes no sense. Invasion causes inflammation which in turn enhances invasion by disruption of the epithelial barrier, etc… This is an intrinsic vicious circle. Shigella itself has « adopted » an anti-inflammatory strategy by expressing elaborate anti-inflammatory effectors (i.e. IpgD, OspG, OspF, IpaH), demonstrating that regarding co-evolution, it had to solve the issue of cell entry AND survival in the face of inflammation. Thus, to us, the in vitro demonstration that Shigella causes alterations in the sumoylation status of inflammation-related transcription factors makes total sense and needs to be considered in the in vivo context. We would therefore like to argue that disentangling this « Gordian knot » is, for the moment, impossible (cutting not being the solution…). As an element antagonizing Shigella entry (proven in vitro and in vivo : see evaluation of invasion efficiency), strengthening the epithelial barrier (proven in vivo), and affecting transcription factors involved in inflammatory responses, thus decreasing the inflammatory response to an infectious aggression (not addressed in vitro in parallel to SILAC identification, done in vivo with discussion about the « vicious circle » discussed above), we consider that for the time being, we have gone as far as
possible within the limits of the experimental models to provide a coherent in vitro-in vivo view of the regulatory function of SUMO in the course of Shigella infection.

Coming back to trying to elucidate the role of sumoylation in epithelial barrier strength, including the identification of the putative cytoskeletal or cell adhesion SUMO substrates involved, the SILAC approach failed to reveal any cytoskeletal or cell adhesion protein differentially sumoylated upon Shigella infection. Clarifying this issue will thus constitute a whole study in its own right that would go far beyond the scope of the present study and will definitely constitute the subject of our future efforts. This point is now mentioned in the Discussion section (page 11, bottom).

However, as requested by the reviewer, we adopted a candidate approach focusing on the GAP junction channel protein connexin 43 (Cx43). Indeed previous work from our lab had shown that connexin activity and notably Cx43 is important for Shigella invasion (Tran Van Nhieu et al., Nat Cell Biol, 2003; Clair et al., Exp Cell Res, 2008). In addition, Cx43 has been reported to be SUMO-modified with sumoylation increasing the ability of Cx43 to form gap junctions (Kjenseth et al., JBC, 2012). We intuitively thought that SUMO-2-mediated increased formation of gap junctions should account for enhanced invasion of epithelial cells by Shigella.

HeLa cells were transfected with the indicated plasmids and infected with the invasive strain of Shigella (M90T) for 0h and 1h (see figure). Whole-cell lysates were subjected to immunoprecipitation followed by Western blot analysis with antibodies as indicated. Below is the quantification of SUMO-2-Cx43 relative to Cx43 and normalized to 1 at time zero. (n=3; error bars, s.d. *P<0.05). Based upon the observation that sumoylation of Cx43 improves the formation of gap junctions, thus possibly improving the invasion level by Shigella, we were surprised to observe that Shigella actually induced a decrease of the SUMO-2-modified forms of Cx43 while invading cells. This observation indeed makes it unlikely that this process accounts for the enhanced level of Shigella invasion observed in Ubc9+/− mice.

Point 2) Downstream changes in the transcriptional program are of course interesting, but in my mind much less surprising considering current knowledge on transcription factor sumoylation. At least some of the candidates shown in the transcription network in Figure 3 should be verified: IP / immunoblotting to show changed sumoylation in response to infection. After all, pulldown / mass-spec is an initial screen, not proof.

As requested by the reviewer, we performed some validation of the SILAC experiment. Given the difficulty to detect low levels of endogenous SUMO conjugates, we analyzed by quantitative immunoblotting the sumoylation status of two of the transcriptional regulators associated with digestive functions present in the short list of the proteomic analysis (Fig. 3E), c-FOS and SATB1 (special AT-rich sequence-binding protein-1). We could confirm, for both ectopically expressed substrates, the decrease in SUMO-2-modified forms induced by Shigella infection in HeLa cells. We have added these data in the new Fig. S3 and in the Result section (page 9, lines 8 to 12).
Point 3) The authors findings suggest that SUMO2 is more relevant than SUMO1 - however this is based on overexpressing Tap-TAG SUMO. If the authors want to make a strong point out of this, a few additional straight-forward experiments are needed.

First, SUMO conjugates should be shown in untransfected versus transfected cells (using SUMO antibodies) to allow conclusion about fold-increase of SUMO conjugates upon overexpression.

These data were/are present in the submitted version of the manuscript (see Fig. 1A).

Second, the effect of SUMO1 and SUMO2 reduction by siRNA depletion for infection should be tested.

As agreed to with the editor, we did not address this point.

Minor point:
Supplemental Figure legend 4 is full of spelling mistakes.

This has been corrected.
Referee #3:

In summary, the authors evaluate the role of SUMO in the regulation of cellular invasion and host inflammatory signaling in response to Shigella flexneri infection. They generally conclude that SUMO plays several roles in restricting Shigella virulence. They also report potentially new Sumoylated substrates and those that may be differentially regulated by bacterial infection. What I like about this study is that it is short, yet provides valuable information in an easily attainable format. The authors do a very good job of not over interpreting their data and not drawing conclusions that are unwarranted. The majority of experiments are properly controlled (with a small number of exceptions outlined below). In addition, the study has potential to be of significant value to the Shigella and sumoylation communities. What I am more concerned about in the manuscript is that the direct relationships between the cell-based studies, the biochemistry, and the in vivo experiments are unclear. Rather than digging into the core mechanism of one or two of the findings, the authors present a survey of, what appears to be, potentially unlinked biological phenomenon. Experiments that link these observations would be very helpful in this regard. Thus, while there are many positive aspects to this work, I have enough concerns to warrant a second evaluation of the manuscript prior to publication.

Point-1: The authors knock down (RNAi) the sumo E1. It would be valuable to also knock down Ubc9 in the cell based invasion studies, which would then link the story in a more comprehensive manner. The authors could also examine the inflammatory response genes in vitro using this approach. Perhaps most importantly, knocking down the E2 would demonstrate that the cell-based findings are robust and that Shigella is sensitive to inhibition of multiple points if the sumoylation pathway.

As agreed to with the editor, we did not address this point.

Point-2: A major concern is that the authors do not confirm the regulation of their sumoylated hits using a second approach (e.g. quantitative western blots). At least a handful (5 to 10) of the identified transcription factors (or other differentially Sumoylated proteins) need to be confirmed to validate the SILAC experiment. In addition, it would be best to look at endogenous Sumo in these studies.

As requested by the reviewer, we performed some validation of the SILAC experiment. Given the difficulty to detect low levels of endogenous SUMO conjugates, we analyzed by quantitative immunoblotting the sumoylation status of two of the transcriptional regulators associated with digestive functions present in the short list of the proteomic analysis (Fig. 3E), c-FOS and SATB1 (special AT-rich sequence-binding protein-1). We could confirm, for both ectopically expressed substrates, the decrease in SUMO-2-modified forms induced by Shigella infection in HeLa cells. We have added these data in the new Fig. S3 and in the Result section (page 9, lines 8 to 12).

Point-3: The authors evaluate transcriptional regulation in Ubc +/- mice. I found the analysis of these data difficult to interpret in regard to the comparison between transcriptional response and the up/down regulation of sumoylated TFs. Can the authors draw any conclusions about the link between sumoylation of TFs and the transcriptional response output during Shigella infection? For example, are there differences in levels or activity of sumoylated TFs that explain the increase in INF-g, Cxcl-1, Il-6 observed in UBC9 +/- mice? Likewise, can the authors demonstrate a link between the regulation of these TFs by Sumo and the regulation of cytokine expression during Shigella infection in vitro? MKL-1 may be a good test candidate. Again, this gets back to the general point that the current study is somewhat disjointed, and any molecular links between the experiments would significantly enhance the study.

As for this point, role of SUMO in inflammation, we provide here important mechanistic insight in showing that Shigella causes alteration of the sumoylation status of key transcriptional regulators of inflammation towards a pro-inflammatory response, which is in keeping with what we observe in vivo. We believe a more detailed analysis of the link between changes in the sumoylation status of the identified TFs and the pro-inflammatory
transcriptional response triggered by *Shigella* infection constitutes a whole study in its own right that would go far beyond the scope of the present study. This will definitely constitute the subject of our future efforts.

**Minor comments:**

-On page 5, the authors indicate that the Ubc9 heterozygote has no phenotype (Nacerdinne et al.), but do not explain the rationale for using a het versus a null. I assume this mutation is lethal. If so, it would be good to add this information here.

We have added this information in the revised manuscript (page 5, bottom)

-On page 7, the authors perform SILAC experiments. They present the technique and the controls together, but do not include the results from Shigella infection in the section headlining that the Shigella experiments (top of page 7). Either retitle this section or, more preferably, add the results of the Shigella experiment under this headline.

As requested by the reviewer, we have retitled this section (page 7)
Thank you for your patience while we have your revised manuscript has been under peer-review. Referee 2 was unfortunately unavailable to assess this revised version, and referee 1 was asked to evaluate your answers to referee 2's initial concerns. As you will see from the reports below, both referee 1 and 3 are now positive about the publication of your study 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 issues/corrections have been addressed, and the study has been adapted to EMBO reports format, as follows.

- In going through the figures in preparation for acceptance, our routine screening has identified some bands in the tubulin blots of figure 1A that are rather similar. To avoid unpleasant problems that might arise after publication, it would be good to provide the original blots for this figure, to be published as source data.

In general, we now encourage the publication of original source data - particularly for electrophoretic gels and blots, but also for graphs - with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the other figures (we will publish the blots related to figure 1A, as mentioned above) and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version.

- It is a precondition for publication in EMBO reports that authors agree to make all data that cannot be published in the journal itself freely available, where possible in an appropriate public database. In the case of mass spectrometry datasets, they should be deposited in a machine-readable format (e.g. mzML if possible) in one of the major public database, for example Pride (http://www.ebi.ac.uk/pride/) or PeptideAtlas (http://www.peptideatlas.org) and authors should follow the MIAPE recommendations (http://www.psidev.info/index.php?q=node/91).

This should be specified in the main text in the first instance where the data are mentioned, with the relevant accession code (which can also be included in the Methods section under the appropriate subheading).

- There seems to be some information missing in the legend to figures 1 and 4 regarding how many times the experiments were independently performed ("n") and the number of technical replicates performed in each experiment. Please ensure that this information is available in all relevant main and supplementary figure legends.

- At over 39,500 characters, your manuscript greatly exceeds the length limits of our journal (which are normally 28,500 characters). We could increase this to 30,000-31,000, but you will need to shorten the manuscript text. Shortening may be made easier by combining the Results and Discussion into a single section, which we require, and which will help eliminate the redundancy that is inevitable when discussing the same experiments twice. However, it will probably also be necessary to be more succinct in the introduction and results section. Please note that the Material and Methods section cannot be shortened any further and, indeed, that information regarding the statistical analyses performed needs to be included in the main manuscript.

- As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the edited abstract (I have not modified the title) below my signature and let me know if you do NOT agree with any of the changes.

- Every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text - I have added my proposal for this text below- as well as 2-3 one sentence bullet points that summarise the paper. These should be complementary to the abstract - i.e. not repeat the same text. This is a good place to include, as appropriate, key acronyms and
quantitative and organism (yeast, mammalian cells, etc) information. We would thus need you to supply a 550 pixels wide by 400 pixels high graphic outlining the main message of the study, and the bullet points to accompany the standfirst.

Do let me know if you would like to modify the standfirst blurb:

"This study shows that in response to Shigella invasion, SUMOylation of host transcription factors leads to an impairment of Shigella epithelial cell invasion, mucosal inflammation and intestinal permeability.

2-3 bullet points"

- Lastly, please ensure that you submit high-resolution files of each main and supplementary figure, as well as a Word document of the manuscript text.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

******************************************************************************

Edited abstract

Shigella flexneri, the etiological agent of bacillary dysentery, invades the human colonic epithelium and causes its massive inflammatory destruction. Little is known about the post-translational modifications implicated in regulating the host defence pathway against Shigella. Here we show that SUMO-2 impairs Shigella invasion of epithelial cells in vitro. Using mice haploinsufficient for the SUMO E2 enzyme, we found that sumoylation regulates intestinal permeability and is required to restrict epithelial invasion and control mucosal inflammation. Quantitative proteomics reveals that Shigella infection alters the sumoylation status of a restricted set of transcriptional regulators involved in intestinal functions and inflammation. Consistent with this, sumoylation restricts the pro-inflammatory transcriptional response of Shigella-infected guts. Altogether, our results show that the SUMO pathway is an essential component of host innate protection, as it reduces the efficiency of two key steps of shigellosis: invasion and inflammatory destruction of the intestinal epithelium.

******************************************************************************

REFEREE REPORTS:

Referee #1:

The authors have addressed many of the major questions and issues raised during the primary review. Notably, they have now validated a number of SUMO substrates identified in the proteomic studies. However, there appears to be some confusion about the question raised by this reviewer and reviewer two over the importance of sumoylation in regulating epithelial invasion versus downstream functions in regulating inflammation. Rather than addressing this question directly, the authors argue that "....setting this as an alternative between two polarized options makes no sense". The question being raised does not have to do with alternatives. Given the finding that epithelial invasion by Shigella is much is much higher in Ubc9+/− mice, the sensible question that was being asked was whether sumoylation is playing a direct role in cell invasion. The authors do go on to address this question in part, in the response to reviewer 2, by evaluating effects of infection on connexin 43. They do also present a mostly fair and balanced summary of the findings in the discussion. Thus, overall the responses to all three reviewer's are largely positive and strengthen the
findings.

Referee #3:

As requested, the authors have performed validation of ectopically expressed Sumoylated targets and present this data in the Supplementary Material. The authors are unable to conduct experiments that would clarify the link between Sumoylation and transcription-mediated inflammation, citing technical unfeasibility. However, given that the work presented is technically sound, the findings are of significant value to the scientific community, and because the limitations of the work are clearly stated, I have no further issues concerning publication.

2nd Revision - authors’ response 04 July 2014

I thank you for your patience while we implemented the corrections.

Regarding the issue in Figure 1A, when doing immunoblotting for SUMO profile analyses, the tubulin blot was done as a loading control for the samples. Following your comments and added now as source data, you will find that protein loading was also assessed by three different ponceau stainings in addition to tubulin immunoblot. We apologize for this confusion and to avoid any further one, we modified the Figure 1A accordingly and present the ponceau staining of the membranes corresponding to each SUMO profile, in addition to the tubulin blot.

In agreement with the EMBO reports policy, the proteomic data have now been deposited in the Pride database with the dataset identifier PXD001100. In the result section, the following sentence has been added: “The mass spectrometry data are available via the PRIDE repository (dataset identifier PXD001100).” This information is also included in the material and methods section as well as the reference of the Pride database.

As for the pdf files corresponding to the originals of each figures, we would appreciate not to have to do that, indeed the present paper results from a long-standing study and the first author, who performed the majority of the experiments described in the present paper has left for several years now to do a second post-doc abroad and I feel it would be an unreasonable imposition to now go back to the archives to retrieve the originals. However I can certify that I checked all of the originals at the time and the figures perfectly reflect the originals.

The information describing statistical analyses has now been moved from supplementary information to the main text as well as indication of the number of biological and technical replicates. If possible we would like to modify the standfirst blurb as follows: “This study shows that the SUMO pathway is essential in the host response to Shigella invasion by regulating the interplay between intestinal permeability and transcriptional immune response.” The following sentences could highlight this work:

- This is the first report of a role of SUMO in infection by Gram-negative bacteria
- SUMO regulates *Shigella* epithelial invasion both *in vitro* and *in vivo*
- *Shigella* infection decreases sumoylation of transcriptional regulators

We are happy that the reviewers are positive for the publication of this work and we thank you for your consideration and interest in publishing it.
I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.