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## **BID-mediated release of mitochondrial SMAC dampens XIAP-mediated immunity against Shigella**

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### **Transaction Report:**

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

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1st Editorial Decision

27 November 2013

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Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the three referees find your analysis interesting and insightful. They raise a number of specific concerns, in particular regarding the *in vivo* data, that I suspect that you should be able to resolve in a revision. Should you be able to address the raised concerns in full then I would like to invite you to submit a suitably revised manuscript. I should add that it is EMBO Journal policy to allow a single major round of revision only and that it is therefore important to address the raised concerns at this stage.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may

be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

#### REFEREE REPORTS

Referee #1:

This study, by Andree, et al, investigates how the XIAP-dependent innate immune response is modulated during infection by *Shigella flexneri*. The authors show that *Shigella* triggers cleavage of Bid, which may occur by calpains. This cleavage mechanism is distinct from the mechanism of Bid truncation in apoptosis, which is Caspase-8 dependent. Calpain-cleaved Bid releases SMAC, which antagonizes XIAP-dependent signaling in the absence of overt mitochondrial damage.

The role of XIAP in an in vivo inflammatory response is still not fully appreciated. The authors provide intriguing evidence that *Shigella* targets XIAP-dependent immune signaling by an alternative pathway to release SMAC, which antagonizes XIAP. The authors' model is somewhat counterintuitive in that they show that XIAP-deficient animals are more susceptible to *Shigella*, but also that *Shigella* is effectively antagonizing XIAP-mediated immune signaling. If *Shigella* was strongly antagonizing XIAP-dependent immune signaling in vivo, one would not predict a strong phenotype in XIAP-deficient animals. These data may point to more complex roles for XIAP in *Shigella* infection, which are only partially blocked by microbial interference. However, the experiments overall are well designed, well executed and the text clearly written. The points below should be addressed to better support the conclusions of the study.

1. In Fig. 2B, the authors do show that at 6h, there is less IL-8 produced in SMAC kd cells, but the effect is quite modest. This may be because there has not been sufficient accumulation of cytokine in the supernatant after the affect of *Shigella* on SMAC is observed by 6h. A 24h time point should also be included.
2. The overexpression experiments in Fig. 2C may not represent a physiological signaling response since both SMAC and the signaling components (ie., NOD1 and RIP2) were overexpressed. To better support these data, expression of NFkB-dependent cytokines should be assessed in hepatocytes from infected WT and XIAP-deficient mice (in the hepatocyte specific knockdown).
3. In Fig. 4B, the authors provide evidence that knockdown of Bid decreases release of SMAC into the cytosol. However, the accompanying immunoblot of Bid shows very poor knockdown efficiency. Moreover, in the control blot shown, the Bid kd sample appeared to have less actin signal. Thus, the data to support that Bid kd actually occurs is not convincing. This is a major concern since it is a significant point of the paper.
4. Fig. 4C did not show statistical analysis.
5. In Fig. 4E, the authors use what they term "specific calpain inhibitors". Unfortunately, both of these inhibitors are also known to inhibit the proteasome. The authors need to take care to acknowledge this ambiguity when making their conclusions. As far as I can see, these inhibitors are the only evidence to implicate calpains. To better support their conclusions, the truncated Bid protein could be analyzed by mass spectrometry to determine if the cleavage site is indeed a calpain cleavage site.

Minor comments:

1. The manuscript could benefit from additional editing.
2. Labeling of Fig. 2C should indicate that an engineered form of SMAC was used, not WT SMAC.

Referee #2:

Andree et al. report an intriguing novel anti-inflammatory mechanism in epithelial cells, which is executed during *Shigella* infection. Recent studies have indicated that invasive bacterial pathogens

such as *Shigella* have multiple evolved mechanisms to modulate host inflammatory response and block the host cell death during bacterial multiplication. In light of these bacterial strategies, the authors focus on the role of the X-linked Inhibitor of Apoptosis Protein (XIAP) with special emphasis on its activity as an essential factor in inflammatory signaling. In the present study, they indicate that *Shigella* infection of HeLa cells somehow causes the Calpain activation, which leads to the BID cleavage, and that the activated BID results in the BID-mediated release of SMAC from the mitochondria. The BID-mediated SMAC releasing into the host cytosol can prevent the XIAP activity, by which *Shigella* antagonizes the NF- $\kappa$ B-mediated inflammatory response without inducing apoptotic cell death. The authors propose how the bacterial pathogen hijacks the BID-SMAC-XIAP-Caspase-apoptosis circuit to ensure the safer replicative niche. The findings of SMAC, which is originally defined as a pro-apoptotic agent via inhibiting XIAP activation, playing important role in regulating host inflammatory response to bacterial infection of epithelial cells is also intriguing. On the other hand, the *in vivo* study using XIAP-deficient mice infected with *Shigella* via intravenous would not be ideal in this case, rather the author should consider another well-established mice model, such as mice lung model infected with *Shigella* via the nasal route. Since the route of *Shigella* infection via intravenous skips epithelial barriers, the host inflammatory and defense responses would not be reflected by *Shigella* infection of epithelial cells. I very much appreciate their findings with HeLa cells, while I am afraid of the results with mice, those might be mostly reflected by myeloid cell responses to the bacterial injection directly into the blood vessels, which seems to mimic systemic infection. The followings are my concerns and comments to improve the manuscript.

#### Major comments;

1. Fig. 2. Previous studies indicated that SMAC contributes to dampen the NOD1-RIP2-NF- $\kappa$ B activation, and XIAP mediates the NOD signaling via interaction with RIP2. After 3 h infection of cells by *Shigella*, is it possible to show the decrease in XIAP-RIP2 interaction and the ubiquitination of RIP2?
2. The NOD1-RIP2 pathway is not the sole means to activate the downstream NF- $\kappa$ B. How about MAPK contribution to the pathway during infection?
3. Fig. 2B. The levels of IL-8 in between shScr and shSMAC look marginal, though the difference is statistically significant. To make clear the SMAC suppressed XIAP-mediated NF- $\kappa$ B activation, I recommend to look at also other NF- $\kappa$ B targeting genes, such as IL-6.
4. Fig. 3A. BID undergoes Caspase-8 processing. BID is cleaved by Calpain during *Shigella* infection. In this context, Caspase-8 activation should also be included.
5. Fig. 4A shows the released SMAC from mitochondria upon *Shigella* infection, which is also accompanied by BAX accumulation. But there is no statement on the BAX involvement in the text.
6. As pointed at item 3, I would like to see in Fig. 4D with Caspase-8.
7. Fig. 4E indicates the effect of the two Calpain inhibitors, but the data look unclear to me.
8. Fig. 4F. The authors point that Calpain inhibitors suppress BID cleavage. In Fig. 4F, the Calpeptin inhibition looks more efficient than ALLN. In this connection, I would like to see whether Calpeptin also could suppress SMAC releasing into the cytosol.
9. In Fig. 4. Bergouniux et al. have recently reported the *Shigella* VirA involving in Calpain activation via degrading Calpastatin, a Calpain inhibitor (see Cell Host Microbe 2012). Do the authors demonstrate whether the *Shigella* VirA involves in the BID cleavage and SMAC production?
10. Figs 5E-G. The authors should show the basal levels of chemokines and cytokines in the XIAP-deficient and the wild type mice such as using RTPCR or ELISA.
11. Is there any difference in the levels of NF- $\kappa$ B in between the XIAP-deficient and the wild type mice? If not, perhaps due to multiple pathways to activate NF- $\kappa$ B, I am afraid of the validity of the mice model, which may not be reflected the data with epithelial cells.
12. Fig. 5F needs some explanation on the level CD45 cells being increased at 48 h post infection.
13. I encourage the authors to perform mice experiments using nasal route of lung infection with *Shigella*.

#### Minor comments;

14. Supplementary Fig. 1C legend needs to some statement on shRNA knockdown.
15. Fig. 4B needs control siRNA for siBID.

#### Referee #3:

## General comment:

How *Shigella* dampens inflammation to ensure its own survival is a topic of much interest. In this work, the authors interrogate the molecular mechanisms by which *Shigella* dampens the nod1 proinflammatory signaling pathway and show that *Shigella* disrupts the XIAP signaling pathway through a Bid-release of mitochondrial SMAC in a non-apoptotic context. Therefore, this study suggests that component of the death machinery can be diverted to exert an antagonistic function on the pro-inflammatory signaling mediated by nod1. This finding is interesting and novel since this study identifies a new signaling pathway by which *Shigella* dampens inflammation. There is also a considerable effort for a relevance *in vivo* using a model of shigella hepatitis, and with the development of hepatocyte-specific XIAP knockout mice. So far, there is no mice model that fully mimics the pathogenesis of shigellosis in the gut because of a natural resistance of rodents to oral infection with *Shigella*. Nevertheless, the hepatitis model used by the authors fully allows the investigation of their *in vitro* findings on the implication XIAP and its inhibition by BID/SMAC on shigella pathogenesis.

While the mechanism of SMAC release through Bid is clearly demonstrated, there are some weaknesses in the *in vitro* demonstration that this signaling pathway crosstalks with XIAP and dampens XIAP induced NF- $\kappa$ B/inflammation. For example, whether SMAC interacts with XIAP upon shigella infection remains to be demonstrated. The *in vivo* data suggest that XIAP deficient mice are unable to mount an immune response towards *Shigella*. However some quantifiable parameters of immune dysfunction needs to be provided to support this claim. Importantly, in the experiment using genetic ablation of Bid and SMAC, it is still unclear whether mice survival results from an efficient immune response clearing the bacterium or from the prevention of hepatocellular apoptosis that occurs in this very particular *Shigella* model of infection.

## Specific comments:

Figure 2: *Shigella* induces the release of SMAC and inhibits XIAP-mediated inflammation

-While DNA binding activity seems to be slightly more induced upon SMAC depletion (Fig 2B), the impact of SMAC depletion on cytokine production seems to be low (Fig 2A). The authors need to reinforce the demonstration that SMAC crosstalks with the inflammatory pathway by showing that *Shigella* induces the formation of a molecular complex between XIAP and SMAC (co-immunoprecipitation). Also, does Smac impairs XIAP RIP2 formation complex upon *Shigella* infection?. In other words, what is the mechanism proposed by the authors to implicate SMAC as an antagonist of XIAP signaling?

--An important statement is that SMAC release is not in relation to cell death but rather acts as an immunomodulator. Therefore, what is the impact of the stable shSMAC knockdown on cell death into *Shigella*-infected cells (LDH release)?

Figure 3: Intra-cellular *Shigella* induces the release of mitochondrial SMAC without mitochondrial damage

-The statement "intra-cellular" is unclear: does it mean that *Shigella* must enter into the cells for SMAC release? Is this the case, it has to be demonstrated by impairing *Shigella* entry.

-The analysis of SMAC release should be performed in a more relevant model like enterocytic cells (like HCT116 with a non mutated p53 status), or in hepatocytic cell line since liver infection is the *in vivo* model of infection chosen in this study.

Figure 4: Calpain-cleaved BID induces mitochondrial release of SMAC

-The statement that "nod1 is not involved in SMAC by SMAC release" is not at all demonstrated. The fact that nod1 overexpression or TRIDAP stimulation does not induce SMAC release (FigS4A) is not excluding the possibility that in the context of infection, nod1 could be a trigger, either through calpain or caspase 8 activations (since nod1 actually can induce caspase 8 activation- see the paper from da Silva Correia J, et al, . Cell Death Differ. 2006;14:830-839). This is a very important issue since the authors propose a model in which calpain signaling and nod signaling are somehow disconnected (Fig 6A). The impact of nod1 invalidation on SMAC release needs to be experimentally addressed.

Fig4C: The impact of BID invalidation of NF $\kappa$ B activation is poorly demonstrated, which might be due to an incomplete BID gene inactivation. In the MEF BID KO, the release of SMAC is totally abrogated, does NF  $\kappa$ B activity is increased in gel shift? What is the impact of BID inactivation on

cytokine expression? This is an important statement that would support the in vivo results obtained using the BID KO Shigella model of infection.

Fig 4E-F: implication of calpain activation on Bid cleavage

-Because calpain is important for shigella entry, the authors need to check whether the calpain inhibitors did not affect this initial step.

-The impact of calpain inhibitors on Bid cleavage is not convincing because of the poor quality of the blot (Fig 4E). Idem for the use of ZVAD that seems to slightly decrease the release of SMAC (Fig 4F). Caspase 8 has been shown to be induced upon shigella infection in different cell types. The experiment showing the impact of calpain inhibition on bid cleavage has to be readdressed and to include the use of specific caspase 8 inhibitor Z-IETD-FMK.

Figure 5: XIAP confers immunity against Shigella infection

Fig 5-C:

XIAP deficient mice were previously shown to be more sensitive to infection in response to bacteria such as listeria and chlamydiae. Thus, XIAP deficiency sensitizes mice to various bacterial challenge. In the experimental design shown in figure 5C, we don't know whether the difference in survival between WT and XIAP<sup>-/-</sup> relies on Shigella's invasiveness per se since there is no information provided on the sensitivity of XIAP<sup>-/-</sup> upon non invasive bacterial challenge such as BS176 or the M90T delta IpaB strain. Because XIAP confer sensitivity to Shigella is a major finding of this paper, the control experiment with the use of non invasive strain should be done, with the expectation that XIAP deficiency did not impact mice survival upon non invasive bacterial challenge, as compared to the WT mice.

Fig 5D : XIAP deficient mice are unable to mount an immune response towards Shigella

We have no information on the immunological status of the infected mice. The parameters have been well described in the Martino paper and include apoptosis in both immunological and hepatocyte compartments, an acute hepatic failure measured by level of transaminase and pro-inflammatory gene expression such as IL-1 $\beta$ , IL-18, IL-12 and IFN $\gamma$ . At least some quantifiable parameters of immune dysfunction need to be provided to support this claim.

Fig 5E/F/G : Conditional XIAP deficiency mice in hepatocyte confers a lack of efficient hepatocyte immune response. Again, the phenotype is not supported by quantifiable parameters and there is no information on the immunological status of the infected mice.

Fig 6A and B: Genetic ablation of Bid and SMAC restores immunity against Shigella infection

-The survival curve for the mice challenged with the WT shigella strain are totally different in Fig A and B : for ex at 30h all the WT mice died in Fig 6A while in figure 6B they all survived. How the authors explained such a discrepancy ?

Fig 6A : it is difficult to appreciate the quality of the anti-bacterial immune response from the survival curve. Some biological parameters should be provided

Fig 6b : The Bid KO mice did not suffer from liver damage and survive. The lack of liver damage needs to be shown, histologically or by providing a biological parameter showing a lack of hepatic failure.

Importantly, because Bid KO mice are resistant to Fas-induced hepatocellular apoptosis, it might indicate that hepatocyte apoptosis is the pivotal event modulating mice survival in response to Shigella infection. In this model of hepatitis, apoptosis in both immunological and hepatocyte compartments have been described and this parameter has not been investigated by the authors. Therefore, if the authors want to maintain this result in the final version of their manuscript, they have to show that the Bid KO mice resistant phenotype is in relation to the fact that these mice are able to mount an efficient inflammatory immune response- and not by preventing Bid-induced hepatocellular apoptosis.

## **Point-by-Point response (Andree et al)**

We thank the reviewers for the very helpful instructions to further strengthen our results. We here answer the points raised by the reviewers.

### **Referee #1:**

*This study, by Andree, et al, investigates how the XIAP-dependent innate immune response is modulated during infection by Shigella flexneri. The authors show that Shigella triggers cleavage of Bid, which may occur by calpains. This cleavage mechanism is distinct from the mechanism of Bid truncation in apoptosis, which is Caspase-8 dependent. Calpain-cleaved Bid releases SMAC, which antagonizes XIAP-dependent signaling in the absence of overt mitochondrial damage.*

*The role of XIAP in an in vivo inflammatory response is still not fully appreciated. The authors provide intriguing evidence that Shigella targets XIAP-dependent immune signaling by an alternative pathway to release SMAC, which antagonizes XIAP. The authors' model is somewhat counterintuitive in that they show that XIAP-deficient animals are more susceptible to Shigella, but also that Shigella is effectively antagonizing XIAP-mediated immune signaling. If Shigella was strongly antagonizing XIAP-dependent immune signaling in vivo, one would not predict a strong phenotype in XIAP-deficient animals. These data may point to more complex roles for XIAP in Shigella infection, which are only partially blocked by microbial interference. However, the experiments overall are well designed, well executed and the text clearly written. The points below should be addressed to better support the conclusions of the study.*

### **Response:**

We thank this reviewer for his/her insightful comments. As mentioned by this reviewer the *Shigella*-induced immune signaling indeed represents a dynamic and highly complex process. Previous data conclusively showed that the immediate early immune signaling upon recognition of intracellular *Shigella* is detrimental to bacterial colonization (Ashida et al., 2011; Ray et al., 2009). Successful colonization by *Shigella*, in turn, requires an efficient response mechanism to disrupt this early immune signaling. Our *in vitro* and *in vivo* data clearly show that XIAP plays an important role during the initial anti-bacterial immune signaling (within the first hour *p.i.*, Fig 1). The absence of XIAP in XIAP<sup>-/-</sup> mice resulted in an increased susceptibility of mice (when infected with  $1 \times 10^7$  bacteria), whereas bacterial propagation in wildtype mice was efficiently controlled when mice were infected with the same bacterial count (Fig 6). In contrast to bacterial recognition at early time points, the BID/SMAC-mediated antagonization of XIAP occurred only 3 h *p.i.* (Fig 2)

-presumably upon intracellular bacterial propagation- and in fact safeguards bacterial colonization. Indeed, lack of XIAP antagonization, in particular, in  $BID^{-/-}$  mice prevented colonization with *Shigella* even after infection with higher load of bacteria ( $1 \times 10^8$ ) (Fig. 7). The elevated number of bacteria used for infection in these experiments helps to overcome the initial immune barrier to bacterial infection in wild type mice.

Furthermore, while our data conclusively show that XIAP is involved in the immediate early immune response against *Shigella*, the evidence by no means excludes the involvement of other cellular regulatory circuits and their crosstalk with XIAP-mediated signaling. This issue is now more clearly discussed in our revised manuscript.

#### Major points, Referee #1.

*1. In Fig. 2B, the authors do show that at 6h, there is less IL-8 produced in SMAC kd cells, but the effect is quite modest. This may be because there has not been sufficient accumulation of cytokine in the supernatant after the affect of Shigella on SMAC is observed by 6h. A 24h time point should also be included.*

#### **Response:**

As requested, IL-8 and IL-6 analyses after 24 h have now been conducted. These data are presented in Fig. 2C and demonstrate the involvement of SMAC more clearly.

*2. The overexpression experiments in Fig. 2C may not represent a physiological signaling response since both SMAC and the signaling components (ie., NOD1 and RIP2) were overexpressed. To better support these data, expression of NFkB-dependent cytokines should be assessed in hepatocytes from infected WT and XIAP-deficient mice (in the hepatocyte specific knockdown).*

#### **Response:**

As requested by this reviewer, in the revised manuscript we now include the analysis of the cytokines IL-6, IL-1 $\alpha$  and RANTES in livers derived from infected wildtype and XIAP $^{\Delta\text{hep}}$  mice (Fig. 6H). These analyses clearly show that the lack of XIAP in hepatocytes significantly reduces the production of inflammatory cytokines IL-6 and IL-1 $\alpha$ .

3. In Fig. 4B, the authors provide evidence that knockdown of Bid decreases release of SMAC into the cytosol. However, the accompanying immunoblot of Bid shows very poor knockdown efficiency. Moreover, in the control blot shown, the Bid kd sample appeared to have less actin signal. Thus, the data to support that Bid kd actually occurs is not convincing. This is a major concern since it is a significant point of the paper.

**Response:**

We thank this reviewer for his/her critical comment. As requested we now include an extensive BID knockdown analysis using several BID-specific siRNAs (Fig. 4B-C) with different BID-knockdown efficiency. These data now clearly show that efficient BID knockdown is accompanied by decreased release of SMAC upon *Shigella* infection (Fig. 4D and S4D).

4. Fig. 4C did not show statistical analysis.

**Response:**

The statistical analyses are now included and the data appears in Fig. 4F of the revised manuscript.

5. In Fig. 4E, the authors use what they term "specific calpain inhibitors". Unfortunately, both of these inhibitors are also known to inhibit the proteasome. The authors need to take care to acknowledge this ambiguity when making their conclusions. As far as I can see, these inhibitors are the only evidence to implicate calpains. To better support their conclusions, the truncated Bid protein could be analyzed by mass spectrometry to determine if the cleavage site is indeed a calpain cleavage site.

**Response:**

As suggested by this reviewer we have now conducted mass spectrometry analysis to determine the cleavage site of BID (Fig. 5B and S5C-D). Confirming our previous data, the truncated Bid fragment identified in this analysis indeed contains the very first amino acids corresponding to the calpain cleavage site, whereas the N-terminal residues that would appear upon caspase 8 cleavage were not present.

Further detailed analyses using calpeptin as well as a cell permeable peptide (27 aa) corresponding to the calpain inhibitory sequence of calpastatin (the endogenous specific calpain inhibitor) now clearly demonstrate, that the specific inhibition of calpain significantly reduces the release of SMAC upon *Shigella* infection (Fig. 5D). In line with this evidence, overexpression of VirA, which is the *Shigella* effector



protein that is responsible for calpain activation (Bergounioux et al., 2012), resulted in calpain activation and SMAC release in the absence of *Shigella* infection (Fig. 5E).

Minor points, Referee #1.

*1. The manuscript could benefit from additional editing.*

**Response:**

As suggested, the current version of our manuscript has been reedited by the authors.

*2. Labeling of Fig. 2C should indicate that an engineered form of SMAC was used, not WT SMAC.*

**Response:**

The labeling of former Fig 2C (now Fig 2D) was modified as suggested by this reviewer.

**Referee #2:**

*Andree et al. report an intriguing novel anti-inflammatory mechanism in epithelial cells, which is executed during Shigella infection. Recent studies have indicated that invasive bacterial pathogens such as Shigella have multiple evolved mechanisms to modulate host inflammatory response and block the host cell death during bacterial multiplication. In light of these bacterial strategies, the authors focus on the role of the X-linked Inhibitor of Apoptosis Protein (XIAP) with special emphasis on its activity as an essential factor in inflammatory signaling. In the present study, they indicate that Shigella infection of HeLa cells somehow causes the Calpain activation, which leads to the BID cleavage, and that the activated BID results in the BID-mediated release of SMAC from the mitochondria. The BID-mediated SMAC releasing into the host cytosol can prevent the XIAP activity, by which Shigella antagonizes the NF- $\kappa$ B-mediated inflammatory response without inducing apoptotic cell death. The authors propose how the bacterial pathogen hijacks the BID-SMAC-XIAP-Caspase-apoptosis circuit to ensure the safer replicative niche. The findings of SMAC, which is originally defined as a pro-apoptotic agent via inhibiting XIAP activation, playing important role in regulating host inflammatory response to bacterial infection of epithelial cells is also intriguing. On the other hand, the in vivo study using XIAP-deficient mice infected with Shigella via intravenous would not be ideal in this case, rather the author should consider another well-established mice model, such as mice lung model infected with Shigella via the nasal route. Since the route of Shigella infection via intravenous skips epithelial barriers, the host inflammatory and defense responses would not be reflected by Shigella infection of epithelial cells. I very much appreciate their findings with HeLa cells, while I am afraid of the results with mice, those might be mostly reflected by myeloid cell responses to the bacterial injection directly into the blood vessels, which seems to mimic systemic infection. The followings are my concerns and comments to improve the manuscript.*

**Response:**

We thank this reviewer for his/her very constructive criticisms. For more information about his/her general concern about the use of an additional infection model please refer to our response to the Major comment #13.

**Major comments, Referee #2;**

*1. Fig. 2. Previous studies indicated that SMAC contributes to dampen the NOD1-RIP2-NF- $\kappa$ B activation, and XIAP mediates the NOD signaling via interaction with RIP2. After 3 h infection of cells by Shigella, is it possible to show the decrease in XIAP-RIP2 interaction and the ubiquitination of RIP2?*

**Response:**

As requested by this reviewer we performed a number of co-IP analyses that turned out to be extremely challenging as the presence of bacteria and bacterial proteins in the cellular lysates derived from the infected cells induced a number of unspecific interactions between antibodies (anti-XIAP, anti-RIPK2 and anti-SMAC antibodies)

and cellular/bacterial proteins. We therefore performed the requested pull down analyses in a stable mycXIAP-expressing HeLa cell line lacking endogenous XIAP (HeLa-shXIAP-myc-XIAP). In complete agreement with previously published data we can show an interaction between XIAP and RIPK2 3 h *p.i.*, that is disrupted 6 h *p.i.* (Fig S1H). Furthermore, the dissociation of the XIAP/RIPK2 complex was accompanied by the co-IP of SMAC with mycXIAP (Fig S2C) indicating that the released SMAC binds XIAP and interrupts the interaction between XIAP and RIPK2 as previously shown.

Most previous studies demonstrating the ubiquitylation of RIPK2 in response to NOD1/2 ligation have been performed using transient expression of tagged, ectopically expressed proteins or the use of synthetic NOD1/2 ligands. The demonstration of the endogenous RIPK2 ubiquitylation during bacterial infection has always been a difficult technical issue. Similarly, we were unable to precisely demonstrate the ubiquitylation of endogenous RIPK2 after *Shigella* infection presumably due to unspecific antibody interactions or the association of the ubiquitylated RIPK2 with insoluble cellular fractions after infection. However, the data presented by the XIAP pull-down and the specific detection of the co-IP'ed RIPK2 in the Western blot analysis (Fig S1H) shows additional bands with higher molecular mass presumably corresponding to a ubiquitylated/modified form of RIPK2. Notably, in collaboration with some experts in the field of ubiquitylation and NOD signaling we are currently trying to establish a novel biochemical approach to access the modification of RIPK2 by XIAP during infection. We hope that this reviewer will appreciate our efforts to properly address this important issue, which unfortunately was not possible during the course of the revision of this manuscript.

*2. The NOD1-RIP2 pathway is not the sole means to activate the downstream NF- $\kappa$ B. How about MAPK contribution to the pathway during infection?*

**Response:**

As requested we now include additional data concerning the role of XIAP in the activation of MEK1/2 and JNK upon *Shigella* infection. Our analyses of the phosphorylation state of MEK1/2 and JNK do not indicate any involvement of XIAP on MEK1/2 and JNK activation upon infection in our experimental setting (Fig S1E). Furthermore, the inhibition of MAPK by PD98059 (inhibition of MEK1/2 activation) did not impact on SMAC release upon *Shigella* infection (Fig 1, this document).

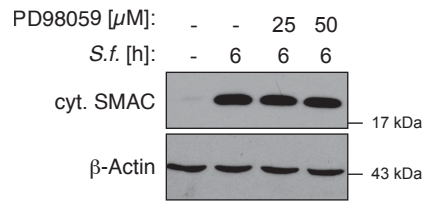


Fig 1: HeLa wt cells were left untreated (-) or were infected with *Shigella* M90T (MOI 30) in combination with the indicated concentrations of the MEK1/2 inhibitor PD98059. Cytosolic extracts were analyzed by Western blotting 6 h *p.i.*

3. Fig. 2B. The levels of IL-8 in between *shScr* and *shSMAC* look marginal, though the difference is statistically significant. To make clear the SMAC suppressed XIAP-mediated NF- $\kappa$ B activation, I recommend to look at also other NF- $\kappa$ B targeting genes, such as IL-6.

**Response:**

As recommended by this reviewer we now also performed analyses of IL-8 and IL-6 secretion at 24h *p.i.*. These data are now presented in Fig. 2C and now more clearly demonstrate the involvement of SMAC in suppressing XIAP mediated NF- $\kappa$ B activation.

4. Fig. 3A. BID undergoes Caspase-8 processing. BID is cleaved by Calpain during *Shigella* infection. In this context, Caspase-8 activation should also be included.

**Response:**

As requested, we now show additional Western blot analyses concerning the proteolytic activation of caspase-8 (Fig 3A). These new data demonstrate that caspase 8, while being activated upon exposure to UV or STS, is not activated following infection with *Shigella*.

5. Fig. 4A shows the released SMAC from mitochondria upon *Shigella* infection, which is also accompanied by BAX accumulation. But there is no statement on the BAX involvement in the text.

**Response:**

We would like to ask this reviewer to reevaluate the data in Fig. 4A. Our data do not show any Bax accumulation at the mitochondria upon infection with *Shigella*.

6. As pointed at item 3, I would like to see in Fig. 4D with Caspase-8.

**Response:**

As requested we now include additional Western blot analyses concerning the proteolytic activation of caspase 8. These analyses appear now in Fig 5A. Our data show, that caspase 8 is activated upon exposure to TRAIL, but not following *Shigella* infection.

7. Fig. 4E indicates the effect of the two Calpain inhibitors, but the data look unclear to me.

**Response:**

We apologize for the incomplete description/presentation of these analyses. The demonstration of the cleaved BID fragment has always been a very trying issue. Particularly, the detection of tBID lacking the adjacent amino acids to the caspase 8 cleavage site (BID cleaved by calpain or cathepsin) is almost impossible with commercially available anti-BID antibodies (Fig 2, this document). The present Western blot analyses of BID cleavage upon *Shigella* infection (presented in old Fig. 4, and revised Fig. 5C) were performed using an anti-BID antibody purchased from BD Biosciences, Cat. No. 550365. Unfortunately, this antibody is not available for purchase anymore. In the revised version, we therefore focused on the effect of calpain inhibition on SMAC release.

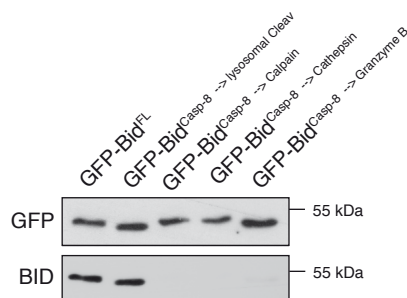


Fig 2. Anti-BID antibodies are not able to detect BID lacking the caspase 8 cleavage site. GFP-BID constructs were generated in which the immediate down-stream amino acid sequence of caspase 8 cleavage site in BID (N-terminal sequence of tBID) was replaced by the amino acid sequences of BID corresponding to the cleavage sites for lysosomal protease, calpain, cathepsin or granzyme B, respectively. Whole cell lysates were stained with an anti-BID antibody purchased from Cell Signalling (Cat. No. 2002) or with GFP antibody.

8. Fig. 4F. The authors point that Calpain inhibitors suppress BID cleavage. In Fig. 4F, the Calpeptin inhibition looks more efficient than ALLN. In this connection, I would like to see whether Calpeptin also could suppress SMAC releasing into the cytosol.

**Response:**

We apologize for these discrepancies. Addressing the reviewers concerns, further detailed analyses using calpeptin and a cell permeable peptide (27 aa) corresponding to the calpain inhibitory sequence of calpastatin (the endogenous, specific calpain inhibitor) now clearly demonstrate, that the specific inhibition of calpain significantly reduces the release of SMAC upon *Shigella* infection (Fig. 5D).

9. In Fig. 4. Bergouniux et al. have recently reported the *Shigella* VirA involving in Calpain activation via degrading Calpastatin, a Calpain inhibitor (see *Cell Host Microbe* 2012). Do the authors demonstrate whether the *Shigella* VirA involves in the BID cleavage and SMAC production?

**Response:**

As requested by this reviewer we cloned and transiently expressed GFP-tagged VirA protein. In agreement with our previous conclusions, overexpression of VirA, the *Shigella* effector protein, which is responsible for calpain activation, results in calpain activation and the release of SMAC (Fig. 5E).

10. Figs 5E-G. The authors should show the basal levels of chemokines and cytokines in the XIAP-deficient and the wild type mice such as using RTPCR or ELISA.

**Response:**

As requested basal levels of a number of chemokines and cytokines were monitored using the Mouse Cytokine Antibody Array (R&D Systems) to detect the relative levels of different cytokines, chemokines, and acute phase proteins in a single sample (Fig. S6E). These analyses do not show any significant alteration in basal cytokine/chemokine production in XIAP<sup>-/-</sup> mice.

11. Is there any difference in the levels of NF- $\kappa$ B in between the XIAP-deficient and the wild type mice? If not, perhaps due to multiple pathways to activate NF- $\kappa$ B, I am afraid of the validity of the mice model, which may not be reflected the data with epithelial cells.

**Response:**

We analyzed the basal NF- $\kappa$ B activity in the livers of wild type versus XIAP, SMAC and BID deficient mice. Our data do not show any significant differences in basal NF- $\kappa$ B activity (Fig S6F and S7B-C) which is in line with the data obtained using conventional XIAP knock-out mice (Winsauer *et al*, 2008; Bauler *et al*, 2008).

Regarding the validity of the model in terms of epithelial involvement of XIAP please refer to the response to Major comment #13.

*12. Fig. 5F needs some explanation on the level CD45 cells being increased at 48 h post infection.*

**Response:**

As requested additional comments on these analyses are now included in the revised manuscript.

*13. I encourage the authors to perform mice experiments using nasal route of lung infection with Shigella.*

**Response:**

As also stated by referee #3 (see below) there is no mouse model that fully mimics the physiological pathogenesis of shigellosis because of a natural resistance of rodents to *Shigella* infection (see also Phalipon & Sansonetti, 2007). There are 3 major approaches to model shigellosis in mouse including the inoculation of newborn mice (Fernandez *et al.*, 2003), murine pulmonary infection (intranasal infection) (Phalipon *et al.*, 1995) and the *Shigella* hepatitis mouse model (Martino *et al.*, 2005) used in our study. Although not reflecting the infection of the human gut, the use of the *Shigella* hepatitis mouse model provides significant progress and is an established model to study *Shigella* infection. Furthermore, shigellosis in humans is also associated with cytokine production in the liver as well as abnormal liver function (Levine *et al.*, 1974). The hepatitis mouse model substantially validated our conclusions *in vivo* (please also see the general comment of Referee #3 below). In particular, the use of the hepatocyte-specific XIAP KO mice (XIAP <sup>$\Delta$ hep</sup>) demonstrated that the deletion of XIAP only in hepatocytes results in hepatitis upon *Shigella* infection underscoring the involvement of XIAP in hepatocyte immune response to *Shigella* infection. Importantly, hepatitis was only observed in XIAP <sup>$\Delta$ hep</sup> mice although both wild type and XIAP <sup>$\Delta$ hep</sup> mice were intravenously infected. We believe that these data provide strong evidence for the role of XIAP during *Shigella* infection in

hepatocytes. However, we are not excluding a role of XIAP in other tissue compartment including myeloid cells that may indeed impact on this process.

Furthermore, based on the Directive 2010/63/EU and the recent adaptation of German regulations and administrative provisions concerning the protection of animals used in experimental and scientific purposes, we are currently not permitted to perform the requested experiments using the nasal route of lung infection with *Shigella*. In fact, following the reviewer suggestion we had already submitted an application to explore the pulmonary infection route in December 2013. Unfortunately, our application was rejected as the need for “the establishment and the use of an alternative infection methods could not be recognized” in view of the Directives of the European Union (2010/63/EU point 11). Our ongoing efforts and the submission of an appeal will definitely not achieve an early positive response. Therefore, the implementation of the requested experiments will be unlikely in 2014.

We feel that the use of an additional mouse model may not provide a substantial progress in this study. Given the fact that the execution of the requested animal experiments in near future is probably not possible, we would like to ask this reviewer to reconsider the necessity of an additional infection model.

Minor comments, Referee #2;

*14. Supplementary Fig. 1C legend needs to some statement on shRNA knockdown.*

**Response:**

The requested information is now included in the figure legend of former Fig S1C, now Fig S1D.

*15. Fig. 4B needs control siRNA for siBID.*

**Response:**

As requested additional analyses have been performed concerning the knockdown of BID and are now included in Fig. 4B-D.



### **Referee #3:**

#### **General comment:**

*How Shigella dampens inflammation to ensure its own survival is a topic of much interest. In this work, the authors interrogate the molecular mechanisms by which Shigella dampens the nod1 proinflammatory signaling pathway and show that Shigella disrupts the XIAP signaling pathway through a Bid-release of mitochondrial SMAC in a non-apoptotic context. Therefore, this study suggest that component of the death machinery can be diverted to exert an antagonistic function on the pro-inflammatory signaling mediated by nod1. This finding is interesting and novel since this study identify a new signaling pathway by which Shigella dampens inflammation. There is also a considerable effort for a relevance in vivo using a model of Shigella hepatitis, and with the development of hepatocyte-specific XIAP knockout mice. So far, there is no mice model that fully mimics the pathogenesis of shigellosis in the gut because of a natural resistance of rodents to oral infection with Shigella. Nevertheless, the hepatitis model used by the authors fully allows the investigation of their in vitro findings on the implication XIAP and its inhibition by BID/SMAC on Shigella pathogenesis.*

*While the mechanism of SMAC release through Bid is clearly demonstrated, there are some weaknesses in the in vitro demonstration that this signaling pathway crosstalks with XIAP and dampens XIAP induced NF-kB/inflammation. For example, whether SMAC interacts with XIAP upon Shigella infection remains to be demonstrated. The in vivo data suggest that XIAP deficient mice are unable to mount an immune response towards Shigella,. However some quantifiable parameters of immune dysfunction needs to be provided to support this claim. Importantly, in the experiment using genetic ablation of Bid and SMAC, it is still unclear whether mice survival results from an efficient immune response clearing the bacterium or from the prevention of hepatocellular apoptosis that occurs in this very particular Shigella model of infection.*

#### **Response:**

We thank this reviewer for his/her discerning view and the very helpful and detailed instruction. As recommended by this reviewer we performed a number of cell death and apoptosis analyses in livers of infected mice. These data clearly exclude any change in cell death in hepatocytes infected by *Shigella*, in the absence of XIAP, SMAC or BID. Furthermore we performed additional analyses of the cytokine levels after infection. For more detail please see our specific responses below.

#### **Specific comments, Referee #3:**

*Figure 2: Shigella induces the release of SMAC and inhibits XIAP-mediated inflammation*

*- While DNA binding activity seems to be slightly more induced upon SMAC depletion (Fig 2B), the impact of SMAC depletion on cytokine production seems to be low (Fig*

2A). The authors need to reinforce the demonstration that SMAC crosstalks with the inflammatory pathway by showing that *Shigella* induces the formation of a molecular complex between XIAP and SMAC (co-immunoprecipitation). Also, does Smac impairs XIAP RIP2 formation complex upon *Shigella* infection?. In other words, what is the mechanism proposed by the authors to implicate SMAC as an antagonist of XIAP signaling?

**Response:**

In line with previous data showing the interaction between XIAP and RIPK2 upon NOD1/2 overexpression or exposure to specific NOD ligands (Krieg et al., 2009), our data show a specific interaction between RIPK2 and XIAP upon *Shigella* infection. Specifically, we performed a mycXIAP-pull down analyses in a stable mycXIAP expressing HeLa cell line lacking endogenous XIAP (HeLa-shXIAP-myc-XIAP). Our data show an interaction between XIAP and RIPK2 3 h *p.i.*, which is disrupted 6 h *p.i.* (Fig S1G). The dissociation of the XIAP/RIPK2 complex was accompanied by the complex formation between SMAC and XIAP as illustrated by co-IP of SMAC with XIAP (Fig S2C) suggesting that the released SMAC binds XIAP and interrupts the interaction between XIAP and RIPK2 as previously shown (see also response to Rev.#2 Response#1). In agreement with previous analyses our data indicate that XIAP impacts on immune signaling induced by *Shigella* through direct interaction with RIPK2. These data, however, do not exclude additional molecular crosstalks between XIAP and other cellular regulatory circuits that can be activated by intracellular *Shigella*.

- An important statement is that SMAC release is not in relation to cell death but rather acts as an immunomodulator. Therefore, what is the impact of the stable shSMAC knockdown on cell death into *Shigella*-infected cells (LDH release)?

**Response:**

As requested we now include a detailed cell death analysis (trypan blue staining and LDH release) in HeLa cells with a stable knockdown of SMAC (Hela shSMAC) (Fig S2G) and in hepatocytes isolated from SMAC deficient mice (Fig 7E). These analyses conclusively support our notion that SMAC is not interfering with cell death upon *Shigella* infection.

*Figure 3: Intracellular Shigella induces the release of mitochondrial SMAC without mitochondrial damage*

- The statement "intra-cellular" is unclear: does it mean that *Shigella* must enter into the cells for SMAC release? Is this is the case, it has to be demonstrated by impairing *Shigella* entry.

**Response:**

New data obtained using the non-invasive *Shigella* strain BS176 show that SMAC is only released upon infection with the invasive *Shigella* strain M90T (Fig S2A).

- *The analysis of SMAC release should be performed in a more relevant model like enterocytic cells (like HCT116 with a non mutated p53 status), or in hepatocytic cell line since liver infection is the in vivo model of infection chosen in this study.*

**Response:**

As suggested the release of SMAC (and OMI) is now additionally demonstrated in the HCT116 cell line with wt p53 status (Fig S2B).

*Figure 4: Calpain-cleaved BID induces mitochondrial release of SMAC*

- *The statement that "nod1 is not involved in SMAC by SMAC release" is not at all demonstrated. The fact that nod1 overexpression or TRIDAP stimulation does not induce SMAC release (FigS4A) is not excluding the possibility that in the context of infection, nod1 could be a trigger, either through calpain or caspase 8 activations (since nod1 actually can induce caspase 8 activation- see the paper from da Silva Correia J, et al, . Cell Death Differ. 2006;14:830-839). This is a very important issue since the authors propose a model in which calpain signaling and nod signaling are somehow disconnected (Fig 6A). The impact of nod1 invalidation on SMAC release needs to be experimentally addressed.*

**Response:**

We thank the reviewer for his/her critical view and advice. As suggested we performed NOD1 knockdown to show that the down-regulation of NOD1 is not impacting on SMAC release upon *Shigella* infection (Fig S4B). The new data obtained by analyzing SMAC release in XIAP deficient cells (Fig S4C) (lacking NFκB activity after infection) confirmed this notion and suggest that the involvement of NOD signaling does not impact on SMAC release.

Our additional analyses of the proteolytic activation of Caspase 8 (Fig 3A and 5A) and its specific inhibition (Fig S5E-F) did not indicate any involvement of Caspase 8 in this process for the experimental setup used. However additional detailed analysis of the specific inhibition of calpain using calpeptin and calpastatin (Fig 5C) show a decrease in SMAC release upon calpain inhibition, clearly demonstrating the pivotal role of calpain in this process.

*Fig4C: The impact of BID invalidation of NFkB activation is poorly demonstrated, which might be due to an incomplete BID gene inactivation. In the MEF BID KO, the release of SMAC is totally abrogated, does NF KB activity is increased in gel shift? What is the impact of BID inactivation on cytokine expression? This is an important statement that would support the in vivo results obtained using the BID KO Shigella model of infection.*

**Response:**

We thank again this reviewer for his/her helpful comment. As suggested we now include the gel shift analysis for MEFs derived from BID<sup>-/-</sup> mice. The data provided support the role of BID in regulating NF-κB activity upon infection (Fig 4G). Further analyses of NF-κB activity in the livers of BID<sup>-/-</sup> mice also show that BID reduces NF-κB activity upon *Shigella* infection (Fig S7C). Our analysis further show, that BID deficiency results in significantly increased IL-6 production upon *Shigella* infection (Fig 4H).

*Fig 4E-F: implication of calpain activation on Bid cleavage*

*- Because calpain is important for Shigella entry, the authors needs to check whether the calpain inhibitors did not affect this initial step.*

**Response:**

As this reviewer mentions, the pre-treatment with calpain inhibitor may indeed interfere with *Shigella* entry (data not shown). Therefore calpain inhibition in our analyses was only performed 30 min after infection (time point zero in our analysis, see Methods), after bacterial entry had occurred. The intracellular load of *Shigella* was not altered as examined by colony forming assay (Fig 3, this document).

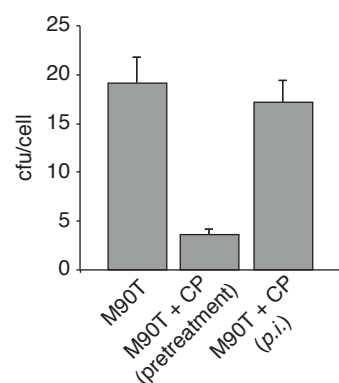


Fig 3. HeLa wt cells were infected with *Shigella* M90T (MOI 30) in combination with Calpeptin (CP), added to the cells 1 h before (pretreatment) or 30 min after infection (*p.i.*). A gentamycin protection assay was performed and colony forming units (cfu) per cell are presented.

*-The impact of calpain inhibitors on Bid cleavage is not convincing because of the poor quality of the blot (Fig 4E). Idem for the use of ZVAD that seems to slightly decrease the release of SMAC (Fig 4F). Caspase 8 has been shown to be induced upon Shigella infection in different cell types. The experiment showing the impact of calpain inhibition on bid cleavage has to be readdressed and to include the use of specific caspase 8 inhibitor Z-IETD-FMK.*

**Response:**

We apologize for the shortcomings in the presentation of these experiments. The identification of the calpain cleaved BID fragment has always been a very difficult issue. Particularly, the detection of tBID lacking the amino acids adjacent to the caspase-8 cleavage site (BID cleaved by calpain or cathepsin) is almost impossible using the commercially available anti-BID antibodies (see also Response #7 to Referee#2). The Western blot analyses of BID cleavage upon *Shigella* infection that was presented in the manuscript (former Fig 4, now Fig 5) were performed using an anti-BID antibody purchased from BD Biosciences, Cat. No. 550365. Unfortunately, this antibody is no longer available for purchase.

In order to evaluate our data, we performed mass spectrometry analysis to identify the cleavage site of BID (Fig. 5B and S5C-D). The truncated Bid fragment identified in this analysis contains the very first amino acids corresponding to the calpain cleavage site. In contrast, the N-terminal residues of BID upstream to this site, which would appear upon caspase cleavage were not found in the analysis. We now also provide additional data concerning the release of SMAC in the presence of specific inhibitors of caspase 8 and calpain that confirm the involvement of calpain in this process (Fig 5D and S5E-F).

*Figure 5: XIAP confers immunity against Shigella infection*

*Fig 5-C: XIAP deficient mice were previously shown to be more sensitive to infection in response to bacteria such as listeria and chlamydiae. Thus, XIAP deficiency sensitizes mice to various bacterial challenge. In the experimental design shown in figure 5C, we don't know whether the difference in survival between WT and XIAP-/- relies on Shigella 's invasiveness per se since there is no information provided on the sensitivity of XIAP-/- upon non invasive bacterial challenge such as BS176 or the M90T delta IpaB strain. Because XIAP confer sensitivity to Shigella is a major finding of this paper, the control experiment with the use of non invasive strain should be done, with the expectation that XIAP deficiency did not impact mice survival upon non invasive bacterial challenge, as compared to the WT mice.*

**Response:**

As requested we performed the infection of XIAP deficient mice with the non-invasive *Shigella* strain BS176 (Fig S6B). As expected, XIAP deficiency did not impact on mice survival after infection. All of the infected XIAP-deficient and wild-type mice survived more than 48 hours *p.i.* and no liver injury was detectable.

*Fig 5D : XIAP deficient mice are unable to mount an immune response towards Shigella*

*We have no information on the immunological status of the infected mice. The parameters have been well described in the Martino paper and include apoptosis in both immunological and hepatocyte compartments, an acute hepatic failure measured by level of transaminase and pro-inflammatory gene expression such as IL-1 b IL-18, IL-12 and IFN $\gamma$ . At least some quantifiable parameters of immune dysfunction need to be provided to support this claim.*

**Response:**

One important issue is that unlike the analyses provided by Martino *et al*, (2005), our data presented here indicate the changes in inflammatory signaling during the early infection phase (up to 6h *p.i.*). The analyses of cytokines or chemokines at later time points (24 *p.i.* in Martino et al) is therefore unlikely to help in elucidating the role of XIAP in this process, given that in XIAP-deficient mice severe tissue damage occurs at the later time points (48 h *p.i.*) due to uncontrolled bacterial propagation (Fig 6D and S6D). Our data clearly show that the lack of XIAP results in a reduced immune infiltration of the liver indicating changes during early immune signaling and cytokine production upon infection (6 h *p.i.*) (Fig 6). However, extensive analysis of the levels of a number of chemokines and cytokines monitored using the mouse Cytokine Antibody Array (R&D Systems) in the liver of mice infected with either invasive (M90T) or non-invasive (BS176) *Shigella* strains, did not show any significant alteration of cytokine or chemokine production when mice were treated with M90T versus BS176, presumably reflecting insufficient sensitivity of the selected approach at early phases of infection (Fig 4, this document).

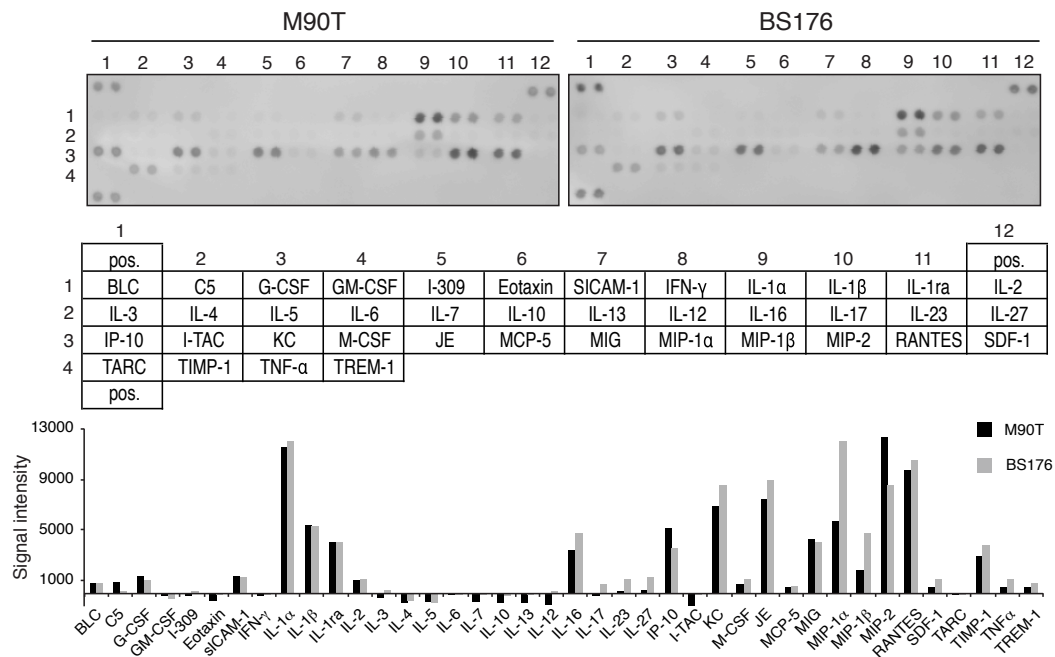


Fig 4: Wt mice were *i.v.* infected with the invasive *Shigella* strain M90T or the non-invasive strain BS176 ( $1 \times 10^7$ ). After 6 h *p.i.* mice were sacrificed and liver homogenates were analyzed for cytokine expression using the cytokine proteome profiler array. Representative dot blot (upper panel), schematic distribution pattern of cytokines (middle panel), Quantification of dot signal intensity (lower panel).

We therefore carried out additional RT-PCR analyses of a number of NF- $\kappa$ B-induced inflammatory cytokines including IL-6 and IL-1 $\alpha$ . These analyses show that the lack of XIAP results in significantly decreased production of these inflammatory cytokines leading to impaired immune infiltration of the infected livers at early stages of infection and the increase in bacterial burden (Fig. 6H).

*Fig 5E/F/G: Conditional XIAP deficiency mice in hepatocyte confers a lack of efficient hepatocyte immune response. Again, the phenotype is not supported by quantifiable parameters and there is no information on the immunological status of the infected mice.*

### Response:

Please refer to the response relating to the previous comment.

*Fig 6A and B: Genetic ablation of Bid and SMAC restores immunity against Shigella infection*

*- The survival curve for the mice challenged with the WT Shigella strain are totally*

*different in Fig A and B : for ex at 30h all the WT mice died in Fig 6A while in figure 6B they all survived. How the authors explained such a discrepancy?*

**Response:**

We apologize for the shortcomings in describing the detailed experimental procedure. The fact is, that mice derived from different genetic backgrounds are variably susceptible toward *Shigella* infection. To account for this, infection analyses of wild type and mutant mice were always performed using littermates of the same specific genetic background.

*Fig 6A : it is to difficult to appreciate the quality of the anti-bacterial immune response from, the survival curve. Some biological parameters should be provided*

**Response:**

In the revised manuscript we include additional analyses of caspase activation in the liver of the infected mice (Fig S7A). Furthermore, *Shigella*-induced cell death in isolated hepatocytes of XIAP, SMAC and BID knockout mice was examined (Fig. 6I and 7E-F) and histological staining of the infected livers of SMAC and BID deficient mice is now provided (Fig. 7B and D).

*Fig 6b : The Bid KO mice did not suffer from liver damage and survive. The lack of liver damage needs to be shown, histologically or by providing a biological parameter showing a lack of hepatic failure.*

**Response:**

In response to the reviewer comments we now provide additional histological analyses (Fig 7D). Furthermore, measurements of NF- $\kappa$ B activity in the liver of BID deficient mice after infection with *Shigella* are included in the revised manuscript. These data show that the lack of BID results in an increased NF- $\kappa$ B activity after infection (Fig S7C).

*Importantly, because Bid KO mice are resistant to Fas-induced hepatocellular apoptosis, it might indicate that hepatocyte apoptosis is the pivotal event modulating mice survival in response to Shigella infection. In this model of hepatitis, apoptosis in both immunological and hepatocyte compartments have been described and this*



*parameter has not been investigated by the authors. Therefore, if the authors want to maintain this result in the final version of their manuscript, they have to show that the Bid KO mice resistant phenotype is in relation to the fact that these mice are able to mount an efficient inflammatory immune response- and not by preventing Bid-induced hepatocellular apoptosis.*

**Response:**

We agree that our analyses can not formally exclude the impact of cell death and the role of BID in cell death which may in turn impact on immunity in response to bacterial infection. As mentioned in the previous point we could not detect any alteration in caspase activation in liver homogenates or in the level of cell death in isolated hepatocytes after *Shigella* infection (Fig S7A and 7F). Our data by no means exclude the role of BID in the potential death of other tissue compartments (e.g. myelocytes) under the experimental setting used in our study.

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Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the three referees.

As you can see below, the referees appreciate the introduced changes and support publication here. I am therefore very pleased to accept the paper. Before doing so, the referees raise a few minor issues that should be resolved in a final revision. It is just a matter of fine-tuning the text.

#### REFeree REPORTS

##### Referee #1:

The authors have done a commendable job responding to reviewer concerns. The revised manuscript describes an important finding and provides substantial data to support their model.

##### Referee #2:

The authors have provided additional convincing data and greatly improved the MS, which I believe will be sufficient to be published.

There two minor points to be checked;

1.p. 10, line 4 from the bottom. Fig. 5D should be 5E.

2. There is no statement on Fig. S7B in the text.

##### Referee #3:

##### Major comment:

The revised manuscript has adequately addressed the issues raised by the reviewer. The crosstalk between calpain activation/ bid cleavage/SMAC release and the XIAP signaling has been nicely addressed, notably in vitro. The in vivo data are not as strong since Figure S6E shows that that XIAP invalidation does not affect the cytokine proteome upon Shigella infection (Supplementary figure S6E), and I don't see any comment on this supplementary figure in the manuscript. Conversely, at the mRNA levels, Fig 6F shows that that XIAP invalidation reduces gene expression on on IL6, IL1 and rantes.

Therefore, the sentence p12 of the manuscript « Reflecting the impaired immune response in the XIAP $\Delta$ hep mice compared with control animals, the inflammatory cytokines IL-6 and IL-1 $\alpha$  were significantly reduced after 6 h p.i., » , which claimed that cytokine expression is somehow impaired by XIAP invalidation , thereby ignoring the data obtained from the cytokine proteome, should be much more cautious. May be the impact can only be seen at the transcriptional level, which is fine, but Fig S6E can't be ignored. This will make the reader more comfortable with the reading of the manuscript.

##### Minor comment:

Figure 4 D: the legend is very unclear. What are siBID1, 2, 3 and 4? Are they different siRNA against BID? (only siBid4 seems to work)

##### Referee #2:

1. p. 10, line 4 from the bottom. Fig. 5D should be 5E.

We thank this reviewer for his/her advice. The numeration is now adapted as indicated.

2. There is no statement on Fig. S7B in the text.

A statement concerning Fig. S7B is now included in the manuscript.

**Referee #3:**

1. The revised manuscript has adequately addressed the issues raised by the reviewer. The crosstalk between calpain activation/ bid cleavage/SMAC release and the XIAP signaling has been nicely addressed, notably in vitro. The in vivo data are not as strong since Figure S6E shows that XIAP invalidation does not affect the cytokine proteome upon *Shigella* infection (Supplementary figure S6E), and I don't see any comment on this supplementary figure in the manuscript. Conversely, at the mRNA levels, Fig 6F shows that XIAP invalidation reduces gene expression on IL6, IL1 and rantes. Therefore, the sentence p12 of the manuscript « Reflecting the impaired immune response in the XIAP $\Delta$ hep mice compared with control animals, the inflammatory cytokines IL-6 and IL-1 $\alpha$  were significantly reduced after 6 h p.i., », which claimed that cytokine expression is somehow impaired by XIAP invalidation, thereby ignoring the data obtained from the cytokine proteome, should be much more cautious. May be the impact can only be seen at the transcriptional level, which is fine, but Fig S6E can't be ignored. This will make the reader more comfortable with the reading of the manuscript.

We thank this reviewer for the careful consideration of the manuscript and apologize for our mistake describing Fig S6E. The data presented demonstrate the basal cytokine proteome, which was similar in wild type vs XIAP $\Delta$ hep mice. We could indeed not detect alteration of cytokine production by proteome based assays between wild type and XIAP $\Delta$ hep mice after *Shigella* infection as we explained in detail in our previous point-by-point response. Based on the insufficient sensitivity of this approach these data were not included into the revised manuscript. We now correct the description of Fig S6E and rephrased the text more specifically concerning our findings using RT-PCR analyses (Fig 6H).

2. Figure 4 D: the legend is very unclear. What are siBID1, 2, 3 and 4? Are they different siRNA against BID? (only siBid4 seems to work)

The description of the used four different siRNAs against BID appear now in more detailed in the respective figure legend.