THE EMBO JOURNAL

Manuscript EMBO-2010-75207

Metalloprotease Type III effectors that specifically cleave JNK and NF-κB

Kobi Baruch, Lihi Gur-Arie, Chen Nadler, Simi Koby, Gal Yerushalmi, Yinon Ben-Neriah, Orli Yogev, Eitan Shaulian, Chen Guttman, Raz Zarivach and Ilan Rosenshine

Corresponding author: Ilan Rosenshine, Hebrew University

Review timeline:

Submission date:	29 June 2010
Editorial Decision:	28 July 2010
Revision received:	19 September 2010
Editorial Decision:	06 October 2010
Revision received:	27 October 2010
Accepted:	29 October 2010

Transaction Report:

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

1st Editorial Decision 28 July 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees express interest in your work, although they vary widely in their overall enthusiasm - with referees 1 and 3 being more positive, while referee 2 does not support publication. Still, despite these varying recommendations, their concerns are very consistent, and I would like to lay out here what we see to be the most important points.

- Firstly, all three reviewers comment that the functional relevance of NleD-mediated JNK clipping remains largely untested, in terms of looking at downstream conseqeuences on e.g. cytokine production (ref1 point 1, ref2 point 3, ref3 point 1).
- Secondly, both referees 2 and 3 point out that you have not tested whether NleD might mediate cleavage of p38 MAPKs, which could be relevant here (ref2 point 2, ref3 point 1).
- Thirdly, ref3 (point 2) questions whether you have definitively demonstrated that the effects of NleC on IL8 production are mediated by inhibition of p65, and makes constructive suggestions as to how you could address this.
- Fourthly, and perhaps most critically, you do not test the importance of NleC and NleD in EPEC infection, ideally in vivo (ref1 point 1, ref2 point 5).

Addressing these critical points to the satisfaction of the referees would be essential for a positive outcome on your manuscript. I would in addition draw your attention to some of the more technical concerns raised, particularly ref2 point 1, which clearly have implications for the conclusions of your work.

I do realise that addressing these concerns will require a lot of work, but given the interest expressed by all three referees, and the overall positive recommendation of the majority of them, I would like

to give you the opportunity to submit a revised version of your manuscript. However, I would only encourage you to do so if you feel you are able to respond adequately to the major points outlined above, and I do understand that you may instead wish to take your manuscript elsewhere at this point without undertaking such extensive revision (in which case, please let me know). I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on 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.

Please don't hesitate to get in touch if you have any questions or comments regarding this revision. Thank you for the opportunity to consider your work for publication, and I look forward to your receiving the revised version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Baruch et al., describe the anti-inflammatory functions of two EPEC type III secretion system effectors, NleD and NleC. Through a series of experiments they show that NleD and NleC block the JNK and NF-kB signaling pathways respectively. The manuscript is extremely well written and the experiments are very convincing, The study is interesting and significant to the field, adding to our understanding of how EPEC alters inflammatory signaling. Given the multitude of ways that EPEC accomplishes this, the study could be strengthened by some demonstration of an important biologic effect of NleD and/or an effect in vivo of either NleD or NleC (see point 1).

Comments:

1. Figure 5 provides a nice summary of the known effects of EPEC effectors that alter inflammatory signaling. The manuscript would be strengthened by any experiment that helped put the function of individual effectors into perspective. This might be in the form of animal infection experiments with an AE pathogen, e.g., C. rodentium, which the authors show encodes two NleD homologs that also function to cleave JNK (Fig. S1). (The potential redundancy of the two effectors could prove to be a technical challenge.) Alternatively, the authors could address the biological function of NleD. Whereas the anti inflammatory effect of NF-kB blocking by NleC is demonstrated in the paper (i.e. it represses TNF-alpha induced IL-8 expression), there is no follow up for the functional effect of NleD cleavage of JNK and reduction of c-Jun phosphorylation. NleD did not have an effect on the proinflammatory cytokine production. Demonstrating that NleD has a negative effect on one or more of the functions of the JNK-c-Jun signaling (e.g. apoptosis) would strengthen the conclusions drawn by the authors.

Other comments:

2. Based on the transfection experiments and fluorescent microscopy the authors conclude that NleC clips p65 both in the cytoplasm and the nucleus. (See also Fig. 5.) However, in a natural infection does NleC gain access into the nucleus? Is there enough NleC translocated to follow an epitope

tagged version by microscopy? Does nuclear vs. cytoplasmic fractionation provide support for this model? Is there an apparent nuclear localization sequence in NleC?

3. The authors should demonstrate that the point mutants of NleC and NleD are as stable as wild type.

4. p. 7 and Fig. 2. The authors should include an untransfected control, either in figure or supplemental data or as data not shown.

5. Fig. 2C and p. 8. I am not aware of previous evidence that the N- and C-termini of JNK remain associated after cleavage of the central loop. The authors could provide better evidence of this by immunoprecipitating with anti JNK (C-terminus) and probing with either anti-JNK or anti-HA.

6. p. 10 second paragraph. The authors should provide some measure of transfection efficiency of the different plasmids, and assess the statistical significance of any differences in p65 staining.

7. While explaining Fig. 1 in the text, mention that ΔescV is the TTSS mutant.

8. Fig. 2. In the text, it is not clear how the authors arrived at the conclusion that the C-terminal portion of the clipped JNK remains in complex with the N-terminal fragment. Explain it in more detail.

9. The authors use both JNK and JNK2 in their experiments. Does JNK means JNK1 or the whole JNK family? Clarify.

10. Abstract. Add "provide" to line 4.

Referee #2 (Remarks to the Author):

Baruch et al. show that infection of human cells (HeLa for the most part) with enteropathogenic E coli (EPEC) leads to cleavage of JNK and NF-kB-p65 at specific sites. These phenomena are linked, respectively, to the NleD and NleC Zn metalloproteases and mutagenesis of the Zn metalloprotease active site (E143A in NleD, for example) abolishes target protein cleavage. Overexpression of NleD leads to reduced subsequent UV-stimulated JNK activity and infection with EPEC expressing wt, but not mutant NleC (but not NleD) reduces subsequent TNF-stimulated IL-8 production coincident with clipping of p65.

The results imply that these two proteases, by attenuating JNK and NF-kB activation, impair the host defense response to EPEC.

While this is an interesting hypothesis, the results are far too premature to support this idea, and many of the experiments presented are flawed. The following are major concerns.

1) The EPEC infection shows JNK clipping as barely detectable by 48 min. How does this compare to EPEC-stimulated JNK activation? Most JNK is activated and produces a functional signal well before 48 min. Thus, at most, this cleavage might only impair second stage cytokine action rather than initial PAMP-stimulated responses.

2) Although ERK is unaffected, the authors did not look at cleavage of the p38 MAPKs, all four of which are critical to inflammation. This is particularly important since IL-8 production is regulated strongly by post-transcriptional mechanisms that are p38-dependent.

3) NleD appears not to be relevant to IL-8 secretion. What, then, is the relevance of NleD-mediated JNK inhibition?

4) Does NleC affect ERK or p38?

5) There are no in vivo studies, or studies with relevant inflammatory cells (macrophages, dendritic cells, lymphocytes, etc. or even gut epithelial cells which can perform a sentinel function) with

which to gauge the relative importance of NleD or NleC-mediated JNK or p65 clipping to EPEC pathogenesis.

Referee #3 (Remarks to the Author):

The manuscript by Baruch et al. reports that two type III secretion system effector proteins from enteropathogenic E. coli (EPEC), NleC and NleD, possess metalloprotease activities that directly act on host p65 and JNK, thereby inactivating NF-κB and JNK signaling pathways, respectively. For NleD, the authors convincingly demonstrate that JNK is cleaved into two halves both in vitro and in EPEC-infected cells. Cleavage of JNK appears to correlate with deficient phosphorylation of its substrate c-Jun in response to UV stress, at least in cells ectopically expressing NleD. The NleD cleavage site in JNK is mapped to R174 in the kinase activation by mass spectrometry using a reconstituted in vitro assay. When examining the role of effectors in inhibiting EPEC infection-induced cytokine (IL-8) induction, the authors further discover that the lack of another metalloprotease effector known as NleC, rather than the expected NleD effector, attenuates the inhibitory effects on IL-8 expression. They then demonstrate that NleC indeed can cleave p65 in the NF-κB signaling pathway at the very N-terminus, which correlates with the decreased p65 staining in NleC-transfected cells and a diminished level of nuclear p65 in EPEC-infected cells. Overall, the work describes two remarkable examples of how enteric pathogens employ effectors with the proteolytic activity to attack two important innate immunity-related host signal transduction pathways. These new findings are of significant interest in the field of bacteria-host interaction and shall shed new lights into the mechanism of bacterial virulence. However, while biochemical illustration of JNK and p65 cleavage by NleD and NleC, respectively, are quite demonstrative in the present manuscript, several concerns remain and shall be addressed or clarified to make the study an elegant one.

Major concerns:

1. As no biological/physiological functions of JNK cleavage by NleD are assigned at this point, it is necessary to more extensively probe the substrate specificity of NleD. In another word, the authors can test whether p65 and also other MAPKs including the closely related p38 and Erk are cleaved by NleD or not. The authors should have all the reagents ready to do these experiments under both infection and in vitro conditions. Moreover, it is much desirable if the authors can show that type III-delivered NleD can block stress-induced phosphorylation of c-Jun. This will strengthen the conclusion that JNK cleavage by NleD has a direct functional consequence on the host JNK pathway during infection.

2. The authors have nicely demonstrated that NleC can cleave p65 and NleC genetically contributes to inhibition of IL-8 expression by EPEC infection. However, evidences supporting the claim that the latter is a consequence of p65 cleavage as well as p65 cleavage can inhibit NF-κB-dependent transcription are largely lacking. The concern arises from two facts. 1) NleC cleaves the very N-terminus of p65; it is not known whether the cleavage affects IκBα association (but presumably should not affect p65 nuclear translocation). 2) The authors did not investigate whether the cleaved p65 still maintains transcriptional activities or not. To address this concern, the authors can the effects of NleC on p65/IκBα association in unstimulated cells. They can further examine p65 cleavage and measure NF-κB-luciferase activation and IL-8 transcription in response to TNF treatment in cells expressing NleC or its catalytic mutant. As the total level of p65 following NleC cleavage is significantly decreased (Fig. 4A), data from these experiments shall help to clarify whether inhibition of NF-κB activation by NleC cleavage of p65 is a result of lower transcriptional activity or instead the instability of the cleaved form of p65 as they have mentioned in the discussion.

Minor points:

The JNK blots shown in Fig. 1 and Fig. 2 are not the highest quality and sometimes ambiguous. For example, the pattern of JNK signals in Fig. 1A and 1C seem to be quite different. The JNK antibody used should detect both JNK1 (46 kD) and JNK2 (54 kD). Therefore, it will be nice if the authors can clearly annotate the bands in these blots along with molecular weight markers so that the readers can tell which band is which cleaved portion from which particular JNK isoform.

The monoclonal JNK antibody from BD (554285) is generated with bacterially expressed JNK protein. The authors claim that the epitope this antibody recognizes is within a small region in the C-terminus of JNK (Fig. 2A). The source or the rationale of this claim should be indicated.

The authors suggest that NleD might have multiple cleavage sites in the activation loop of JNK, and using mass spectrometry they have mapped one of them (R174). The mass spec data should be provided and explanation of failing to identify other cleavage sites should be discussed.

It is intriguing that the two halves of JNK still associate together following NleD cleavage (Fig. 2B and 2C). However, c-Jun phosphorylation in response to UV radiation is blocked by NleD (Fig. 1D). These presumably suggest that the cleaved JNK, though stay together, is resistant to phosphorylation/activation by upstream MAPKKs. Experimental data are needed to clarify this.

In Fig. 3A and 3B, relative levels of inhibited IL-8 expression by WT EPEC infection are inconsistent (0.01 vs 0.001). The NleD deletion mutant can also be included in this assay.

p65 staining image in Fig. 3C are of poor quality and seems to be inconsistent with established facts that p65 is distributed predominantly in the cytoplasm in unstimulated cells and translocates into the nucleus upon TNF stimulation. Additional antibody specifically recognizing the N-terminus of p65 (SC-109, Santa Cruz) might be useful in this regard.

More controls such as other Rel homologous proteins and NleD-substrate JNK can be included to demonstrate that NleC specifically cleaves p65.

The HEXXH Zn-binding motif and the hypothetic metalloprotease activity of NleC and NleD have been noted in the previous study (Marchés O et al., Infect Immun. v73, p8411). The study also shows that deletion of NleC and NleD have no effects on bacterial colonization in the animal infection model. The authors should refer this study and discuss it.

Many of the descriptions and data interpretations are not accurate. The subtitle 'Ecotopically expressed NleD cuts and inactivates native JNK' in the results session is not accurate as there are no direct evidences demonstrating cleavage in fact inactivates JNK. Also in the results session, the last sentence in the third paragraph from the end, "p65 clipping leads to reduced nuclear p65 levels", should be "p65 clipping correlates with...". The caption of Fig. S1 "JNK degradation by NleD" should read as "JNK cleavage by NleD". For the caption of Fig. S5 "NleC and NleBE cooperate to achieve .....", there are indeed no data showing "cooperate' in this figure.

Cleavage of JNK by NleD is very much similar to the cysteine-protease type III effector AvrPphB from P. syringae, which also cleaves a host Ser/Thr kinase (PBS1) at the kinase activation loop (Shao F, Science. v301, p1230). The authors can discuss the interesting biochemical similarities between animal and plant bacterial pathogens.

There are no page numbers that make the manuscript a little difficult to follow.

1st Revision - authors' response 19 September 2010

Referee #1 (Remarks to the Author):

Comments:

1. Figure 5 provides a nice summary of the known effects of EPEC effectors that alter inflammatory signaling. The manuscript would be strengthened by any experiment that helped put the function of individual effectors into perspective. This might be in the form of animal infection experiments with an AE pathogen, e.g., C. rodentium, which the authors show encodes two NleD homolog's that also function to cleave JNK (Fig. S1). (The potential redundancy of the two effectors could prove to be a

technical challenge.) Alternatively, the authors could address the biological function of NleD. Whereas the anti inflammatory effect of NF-kobo blocking by NleC is demonstrated in the paper (i.e. it represses TNF-alpha induced IL-8 expression), there is no follow up for the functional effect of NleD cleavage of JNK and reduction of c-Jun phosphorylation. NleD did not have an effect on the proinflammatory cytokine production. Demonstrating that NleD has a negative effect on one or more of the functions of the JNK-c-Jun signaling (e.g. apoptosis) would strengthen the conclusions drawn by the authors.

We leave studies involving experimental infection of animal models for follow-up work for several reasons. First, a good animal model for EPEC infection is not yet available. Using *Citrobacter rodentium* instead of *E. coli* is a long project of setting up the system. Furthermore, the *C. rodentium* system was already used to analyze the role of NleD, without success (Kelly et al, 2006, *Infect Immun*, **74**, 2328 and Marches et al, 2005, *Infect Immun*, **73**, 8411). This is not a complete surprise since the *C. rodentium* model do not always reflects the reality of *E. coli* interaction with human intestine. For instance, EPEC infect the small intestine while *C. rodentium* is infecting the colon. We are in the process of establishing the human intestinal xenograft (Goaln et al JID, 2009, 199:350 and Golan et al., in press) as a new, and perhaps in some aspects better, model for EPEC and plan to use it to test the mutants that we generated. Nevertheless, we adopted the alternative suggestion of the reviewer in order to address this comment and we provide in the new version extensive follow-up on the biological function of NleD. Firstly we already showed that JNK cleavage correlated with its inability to phosphorylate c-Jun (Fig 1E). We further show that NleD is inhibiting JNK-dependent apoptosis (Fig 3). In addition, we demonstrate that NleD is contributing to the inhibition of IL8 production/secretion (and thus inflammation) by a post-transcriptional mechanism (Fig 7). Moreover, we show that to achieve maximal inhibition of IL8 production by EPEC, four effectors are required: NleE, NleB, NleC and NleD (Fig 7).

Other comments:

2. Based on the transfection experiments and fluorescent microscopy the authors conclude that NleC clips p65 both in the cytoplasm and the nucleus. (See also Fig. 5.) However, in a natural infection does NleC gain access into the nucleus? Is there enough NleC translocated to follow an epitope tagged version by microscopy? Does nuclear vs. cytoplasmic fractionation provide support for this model? Is there an apparent nuclear localization sequence in NleC?

We could not detect NLS in the NleC sequence, but we localized NleC fused to mCherry predominantly to the nucleus of cells transiently expressing mCherry-NleC (Fig. S10). Some mCherry-NleC staining was seen also in the cytoplasm. Unfortunately, we could not detect by microscopy natively injected tagged NleC. Using highly sensitive translocation assay (Mills et al 2008) we found that under the conditions used in our study, upon infection both expression and translocation of NleD and NleC are very low. In these experiments we tested the native expression and translocation of the chromosomal encoded proteins, fused to the TEM reporter gene, and expressed from their native promoter. For comparison, the Tir effector is translocated at an

efficiency ~100 fold higher than that of NleC. This might explain why attempts to localize the natively expressed and injected NleC were unsuccessful. Data about expression and translocation of NleC and NleD is out of the scope of this report and thus not included.

3. The authors should demonstrate that the point mutants of NleC and NleD are as stable as wild type.
Done: see Fig S6. We show that both wild type and mutant proteins are similarly stable in the bacteria (by western blot analysis) and in the host cells (by translocation assay).

4. p. 7 and Fig. 2. The authors should include an untransfected control, either in figure or supplemental data or as data not shown.
This control was preformed and the results are indicated as data not shown (see the legend of Fig. 2).

5. Fig. 2C and p. 8. I am not aware of previous evidence that the N- and C-termini of JNK remain associated after cleavage of the central loop. The authors could provide better evidence of this by immunoprecipitating with anti JNK (C-terminus) and probing with either anti-JNK or anti-HA.
We re-confirm the findings. To this end we subjected purified 6His-taged JNK2 to digestion by purified NleD. The reaction products were further purified by gel filtration and fractions containing the cleaved JNK were precipitated with Ni-affinity. Again we show that the two parts of the cleaved JNK remain in complex (see Fig S4 in the new version).

6. p. 10 second paragraph. The authors should provide some measure of transfection efficiency of the different plasmids, and assess the statistical significance of any differences in p65 staining.
These were included in the revised manuscript

7. While explaining Fig. 1 in the text, mention that DescV is the TTSS mutant.
This was corrected

8. Fig. 2. In the text, it is not clear how the authors arrived at the conclusion that the C-terminal portion of the clipped JNK remains in complex with the N-terminal fragment. Explain it in more detail.
The explanation was corrected in the new version

9. The authors use both JNK and JNK2 in their experiments. Does JNK means JNK1 or the whole JNK family? Clarify.
JNK is encoded by three genes (1, 2 and 3) and each of these genes is producing several splice forms. Thus, together the cells can produce 5 to 10 different JNK proteins. These were labeled in the manuscript as "JNK". When looking at native proteins the antibody could not distinguish between the different JNK proteins, only when using recombinant proteins we could know for certain its

identity. Thus the terms JNK1 and JNK2 were used to indicate to specific recombinant proteins transcribed from specific cDNA in either bacteria or cells.

10. Abstract. Add "provide" to line 4.
Done

Referee #2 (Remarks to the Author):

Comment #1 and the response were divided to two parts
1) The EPEC infection shows JNK clipping as barely detectable by 48 min. How does this compare to EPEC-stimulated JNK activation? Most JNK is activated and produces a functional signal well before 48 min.

The relation between stimulation (by PAMPS) and repression (by the effectors) of the inflammatory response depends on the specific used conditions. Upon using overnight grown culture of EPEC for infection it would take >2.5 h to detect JNK clipping. During this time the bacteria activate the expression of the TTSS genes, assemble the TTSS apparatus, attach to the host cells and initiate injection of effectors. To these stages one should add the activity kinetics of injected NleD. Under these conditions PAMP-stimulated response was detected only after more than 4 h infection (Ruchaud-Sparagano et al 2007, Cell Microbiol. 9:1909 and our unpublished results). This is in good correlation with repression of flagella expression at early time points and induction of flagella expression after more than 4 h infection (Yona-Nadler et al, 2003, Microbiol 149:877). In the experiment shown in Fig 2B, we used culture which was primed to express the TTSS prior to infection. It should be noted that these condition do not induced NleD expression, which remain very low but constant (data not shown). Thus the 48 min represent the time that it takes the bacteria to attach to the cells to connect the TTSS to the host cell membrane, to inject NleD and the kinetic of the activity of the injected NleD. During this time PAMP-stimulated response could not be detected. This is probably since the bacteria is releasing only very little LPS (and HeLa cells are not very sensitive to LPS) and also flagella expression is suppressed.
Thus, at most, this cleavage might only impair second stage cytokine action rather than initial PAMP-stimulated responses.
This part of the comment suggests that the reviewer is assuming that the physiological function of NleD (and the other anti-inflammatory effectors) is to block the so called "**initial** PAMP-stimulated responses". This is very simplistic view of the system. First, it should be stressed that EPEC is not invasive and remain in the intestine lumen through the infection process. Needless to say that the intestine lumen is saturated with PAMPs, generated by the micrbiota, well before exposure to EPEC. So what is the function of the effectors? In bovine, EHEC colonizes the colon without casing symptoms and this colonization, which can last for very long periods, is TTSS dependent and perhaps also NleD dependent (Dziva et al 2004). Also, it was reported that sub-population of

humans are chronically and asymptomatically colonized with EPEC for very long periods (Maddocks et al 2009 PLoS One 4:e5517 and references within). It is therefore possible that NleD (as well as other effectors) functions not to block the initial PAMP-stimulated responses, but to modulate the signaling networks in the host in order to reach long term homeostasis with the host tissue. Indeed, the next challenge after elucidating the effectors function would be to solve how they work together. Nevertheless, the question of what come first NleD or the PAMPs is clear: in the context of the intestine the PAMPs come first.

2) Although ERK is unaffected, the authors did not look at cleavage of the p38 MAPKs, all four of which are critical to inflammation. This is particularly important since IL-8 production is regulated strongly by post-transcriptional mechanisms that are p38-dependent.
This comment was particularly useful and we thank the reviewer for highlighting the possible post-transcription effect of MAPKs on IL8 secretion. In response to this comment we re-confirmed that NleD did not cleaved ERK, but we found that NleD cut p38, although at efficiency lower then that of JNK cleavage (Fig. 1C and corresponding text). We also found that NleD cleaves p38 in vitro and this data is mentioned, but not shown, as it is redundant to that shown for JNK. Finally, as predicted by the reviewer, we found that NleD inhibits IL-8 secretion posttranscriptionally (Fig 7).

3) NleD appears not to be relevant to IL-8 secretion. What, then, is the relevance of NleD-mediated JNK inhibition?
In the original manuscript we tested the relevance of NleD for IL-8 transcription (but not for secretion) and we showed that NleD was not affecting IL-8 transcription. In response this comment, comment #2 of this reviewer, and other comments of other reviewers related to the biological relevance of NleD activity, we show that NleD is inhibiting IL8 secretion presumably by inactivation of JNK and\or p38, to inhibit their post-transcriptionally activation of IL-8 production. In addition, we show that NleD blocks JNK-dependent apoptosis. The new data is included in two new figures in the revised version; Fig 3 and Fig 7 and in associated text sections.

4) Does NleC affect ERK or p38? No, NleC is not cleaving ERK or p38. This is shown in the new figure 1C: notice that the *nleD* mutant, which expresses NleC, has no effect on ERK or p38 stability. See also Fig S3.

5) There are no in vivo studies, or studies with relevant inflammatory cells (macrophages, dendritic cells, lymphocytes, etc. or even gut epithelial cells which can perform a sentinel function) with which to gauge the relative importance of NleD or NleC-mediated JNK or p65 clipping to EPEC pathogenesis.
Please see our response to comment #1 of reviewer 1: Also, the primary physiological targets of EPEC are not macrophages, dendritic cells, or lymphocytes, but the gut epithelial cells. Therefore to address this comment we tested the function of NleD and NleC using the human intestinal cell-line

Caco2. These cells are closely related to native entrocyts; the physiological target of EPEC. We show that both NleC and NleD are active also in the context of these cells (Fig S3 and S11).

Referee #3 (Remarks to the Author):

Major concerns:

1. As no biological/physiological functions of JNK cleavage by NleD are assigned at this point, it is necessary to more extensively probe the substrate specificity of NleD. In another word, the authors can test whether p65 and also other MAPKs including the closely related p38 and Erk are cleaved by NleD or not. The authors should have all the reagents ready to do these experiments under both infection and in vitro conditions. Moreover, it is much desirable if the authors can show that type III-delivered NleD can block stress-induced phosphorylation of c-Jun. This will strengthen the conclusion that JNK cleavage by NleD has a direct functional consequence on the host JNK pathway during infection.

These concerns of the reviewer were addressed in the revised version (Figures 1C, 3, 7, S3 and S11, and associated texts). Please see also the responses to comment #1 of reviewer 1 and comments #2, #3, and #4 of reviewer 2.

2. The authors have nicely demonstrated that NleC can cleave p65 and NleC genetically contributes to inhibition of IL-8 expression by EPEC infection. However, evidences supporting the claim that the latter is a consequence of p65 cleavage as well as p65 cleavage can inhibit NF-kB-dependent transcription are largely lacking. The concern arises from two facts. 1) NleC cleaves the very N-terminus of p65; it is not known whether the cleavage affects IkB association (but presumably should not affect p65 nuclear translocation). 2) The authors did not investigate whether the cleaved p65 still maintains transcriptional activities or not. To address this concern, the authors can test the effects of NleC on p65/IkB1; association in unstimulated cells. They can further examine p65 cleavage and measure NF-kB-luciferase activation and IL-8 transcription in response to TNF treatment in cells expressing NleC or its catalytic mutant. As the total level of p65 following NleC cleavage is significantly decreased (Fig. 4A), data from these experiments shall help to clarify whether inhibition of NF-kB activation by NleC cleavage of p65 is a result of lower transcriptional activity or instead the instability of the cleaved form of p65 as they have mentioned in the discussion.

To address the concern mentioned in this comment we first established an *in vitro* system for p65 cleavage by NleC and mapped the cleavage site precisely. This new data is included in the new version (Fig 5D and related text). Having the model of p65 bound to DNA, and based on previous reports, we concluded that NleC clips of 4 AA critical for DNA binding and thus we predicted the consequences of the cleavage by NleC (Fig S12, S14 and related texts). In addition, we adapted the suggestions made in the above comment and experimentally tested the effects of NleC on NF-κB promoter activity using the lucifrase reporting system (Figure 6A and associated text in the new version). We also tested, as suggested, whether association of p65 with IkB would influence p65

cleavage. To this end we took advantage of strains expressing NleBE which protects IkB from degradation and strains that cannot protect IkB from degradation. These new findings are described in Fig 6B and associated text.

Minor points:

The JNK blots shown in Fig. 1 and Fig. 2 are not the highest quality and sometimes ambiguous. For example, the pattern of JNK signals in Fig. 1A and 1C seem to be quite different. The JNK antibody used should detect both JNK1 (46 kD) and JNK2 (54 kD). Therefore, it will be nice if the authors can clearly annotate the bands in these blots along with molecular weight markers so that the readers can tell which band is which cleaved portion from which particular JNK isoform.
Blots appear different upon using different gel percentage and detection systems. In HeLa cells both JNK1 and JNK2 genes are expressed and each of them is producing several splice forms. Thus, together HeLa cells can produce >5 different JNK proteins of various sizes and the used antibody react with all of them. Consequently, in the blot we cannot confidently distinguish between them and therefore we used the term "JNK" to describe the undefined collection of JNK proteins. The terms JNK1 and JNK2 were used to describe specific **recombinant** proteins transcribed from specific cDNA in either bacteria or cells. Only in the latter case we can be sure that the antibody reacted with JNK1 or JNK2.

The monoclonal JNK antibody from BD (554285) is generated with bacterially expressed JNK protein. The authors claim that the epitope this antibody recognizes is within a small region in the C-terminus of JNK (Fig. 2A). The source or the rationale of this claim should be indicated.
This is a misunderstanding, we are not claiming that the antibody is reacting with a small defined epitope and the indication in Fig 2A is just a schematic representation. We do know, however, that the antibody reacts with the C-terminal portion of the protein. This point is better explained in the revised version.

The authors suggest that NleD might have multiple cleavage sites in the activation loop of JNK, and using mass spectrometry they have mapped one of them (R174). The mass spec data should be provided and explanation of failing to identify other cleavage sites should be discussed.
We re-examined this point carefully. We are now confident that NleD cut JNK predominantly only at one point. This is based on the in-vitro reaction using purified NleD (Fig 1G). We also re-examined to cleavage point. Initially we submitted the C-terminal of the cleaved JNK to mass spectrometry. The fragment was trypsine-digested and the peptides were sequenced. Base on the sequence data we determine the cleavage site as the most proximal sequence. However, upon re-thinking about it we realized that this method will indicate on close proximity to the cleavage site but not necessarily the precise cleavage point. Therefore we re-submitted the purified C-terminal fragment and this time for N-terminus sequencing using the Edman degradation method. This procedure provides unambiguous identification of a single cleavage point and the new data is

included in the new version. Both NleD and NleC appear to cleave their targets predominantly at one specific point (Fig S7 and S12).

It is intriguing that the two halves of JNK still associate together following NleD cleavage (Fig. 2B and 2C). However, c-Jun phosphorylation in response to UV radiation is blocked by NleD (Fig. 1D). These presumably suggest that the cleaved JNK, though stay together, is resistant to phosphorylation/activation by upstream MAPKKs. Experimental data are needed to clarify this.
We re-confirmed this association (Fig S3). We also show that NleD cut within the TPY motif since the phosphorylation of the T and Y in this motif result in JNK activation it would not be very speculative to assume that such cleavage would inactivate JNK. We added in the revised version modeling showing this point (Fig S8). In addition we experimentally demonstrate that NleD blocks JNK/p38-dependent processes, including c-Jun phosphorylation, JNK-dependent apoptosis and IL-8 secretion (Fig 1E, Fig 3 and Fig 7).

In Fig. 3A and 3B, relative levels of inhibited IL-8 expression by WT EPEC infection are inconsistent (0.01 vs 0.001). The NleD deletion mutant can also be included in this assay.
The differences between Fig 3A and 3B are due to differences in the lower values (due to repression of IL-8 expression). When very small amounts of RNA are involved, variations are more apparent (these are due to differences from experiment to experiments in the amounts of cells, bacteria and total RNA used for the qPCR reaction). However, the results are similar and highly significant.
We indicated that deletion of *nleD* did not influence IL-8 expression as (data not shown) and show that even overexpression of NleD (and thus its over-injection) cannot mediate inhibition of IL8 transcription (Fig 4A).

p65 staining image in Fig. 3C are of poor quality and seems to be inconsistent with established facts that p65 is distributed predominantly in the cytoplasm in unstimulated cells and translocates into the nucleus upon TNF stimulation. Additional antibody specifically recognizing the N-terminus of p65 (SC-109, Santa Cruz) might be useful in this regard.
We disagree that the figure quality is not good; perhaps the copy used by the reviewer is of lower resolution. In the new version we are using an image with higher resolution. Also, it should be noted that these images were not taken by confocal microscope and thus representing multiple focal plans. This was done to best show the phenotype of reduced p65 staining.

More controls such as other Rel homologous proteins and NleD-substrate JNK can be included to demonstrate that NleC specifically cleaves p65.
We show that NleC is not cleaving JNK, p38 or Erk (Fig 2A 2C and S3). We also show that NleC cleaved p65 in its REL homology domain (Fig S12) and also other NF-kB family members containing the same conserved domain including RelB (Fig S13) and p50 (indicated as data not shown)

The HEXXH Zn-binding motif and the hypothetic metalloprotease activity of NleC and NleD have been noted in the previous study (Marchés O et al., Infect Immun. v73, p8411). The study also shows that deletion of NleC and NleD have no effects on bacterial colonization in the animal infection model. The authors should refer this study and discuss it.
As suggested, this was added and discussed in the new version.

Many of the descriptions and data interpretations are not accurate. The subtitle 'Ecotopically expressed NleD cuts and inactivates native JNK' in the results session is not accurate as there are no direct evidences demonstrating cleavage in fact inactivates JNK. Also in the results session, the last sentence in the third paragraph from the end, "p65 clipping leads to reduced nuclear p65 levels", should be "p65 clipping correlates with...". The caption of Fig. S1 "JNK degradation by NleD" should read as "JNK cleavage by NleD". For the caption of Fig. S5 "NleC and NleBE cooperate to achieve .....", there are indeed no data showing "cooperate' in this figure.
The text was corrected through the manuscript for better accuracy.

Cleavage of JNK by NleD is very much similar to the cysteine-protease type III effector AvrPphB from P. syringae, which also cleaves a host Ser/Thr kinase (PBS1) at the kinase activation loop (Shao F, Science. v301, p1230). The authors can discuss the interesting biochemical similarities between animal and plant bacterial pathogens.
Done

There are no page numbers that make the manuscript a little difficult to follow.
Sorry about it. It was corrected in the new version

2nd Editorial Decision 06 October 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-75207R to the EMBO Journal. It has now been seen again by all three referees, whose comments are enclosed below. As you will see, all three find the manuscript to be significantly improved and are supportive of publication. However, referee 3 has a couple of remaining concerns that I would ask you to address before we can accept the paper.

Firstly, the referee still finds that some issues regarding the modulation of IL8 expression have not been adequately resolved. In this context, I note that you refer to the effects of NleD on IL8 mRNA levels as "data not shown". Given the referee's comments, I would ask you to include these data in the manuscript. I also agree that the data arguing for a role of NleD in post-transcriptional regulation of IL8 is somewhat weak, and would ask you minimally to tone down these conclusions if you can not provide better evidence for this.

Secondly, the referee points out that, while you state in your point-by-point response that you have provided data to show that NleC does not cleave JNK, p38 or ERK and refer to figures 2 and S3, these data are not present. Please can you include these data and ensure they are appropriately mentioned in the text.

In addition, I have a few points from an editorial side. I note that, while you do show error bars and

statistical significance for the graphs presented, there is no indication of how many times the experiments were replicated. Please can you state the n number in the figure legends? It also appears that some of the blots - particularly those in figure 2 -have been subjected to very high contrast adjustments. I would recommend that you replace these panels with less highly contrasted images that better reflect the original data. Can I also ask you to send us original copies of these blots for our records? This is something we routinely ask for in cases where we have requested changes in image presentation. Finally, we are currently implementing a policy of requiring a statement of author contributions for all accepted manuscripts: please can you include such a statement after the acknowledgments section in your manuscript text?

I hope that these final revisions should be straightforward; please do not hesitate to get in touch should you have any questions or comments regarding these changes.

Many thanks and best wishes,

Editor

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors have addressed all of the issues raised in the previous review. It is a very nice paper.

Referee #2 (Remarks to the Author):

Additional experiments have improved the paper.

Referee #3 (Remarks to the Author):

The authors have done a reasonably good job in addressing my previous concerns, and the revised manuscript has been significantly improved. Two concerns remain to be addressed:

1) The revised version fails to address my previous comments regarding to measure IL-8 mRNA level in infected cells. In Figure 4A and 4B (previously 3A and 3B), I still do not understand why the two panels of assays cannot be performed in a single experiment and thus the results can be compared on the same scale. This is particularly important given the authors' new claim that NleD does not affect IL-8 transcription, but inhibits IL-8 secretion in a posttranscriptionally manner. I would suggest that the authors be more cautious in making such a conclusion. Firstly, both conclusions (no transcriptional inhibition and IL-8 ELISA assay of infected cells) are based on a single piece of data that are usually highly variable (as the authors themselves agree). Secondly, posttranscriptional inhibition is an unusual mechanism, and here particularly hard to understand given that NleD proteolytically inactivates JNK and p38. I would suggest to either perform more extensive analyses to solidify these points or simply remove the part of NleD effects on IL-8 transcription and secretion.

2) I previously suggest the authors to examine whether NleC specially cleaves p65. Following their response "We show that NleC is not cleaving JNK, p38 or Erk (Fig 2A 2C and S3)", however, I could not find any relevant data in Fig 2A and 2C. The authors need to clarify this and address the comments. It is probably still much desirable to perform an in vitro assay showing that NleC, even at high dosages, has no effects on JNK and/or other MAPKs (as the title of the manuscript is particularly emphasizing the cleavage specificity).

2nd Revision - authors' response 27 October 2010

Please find the revised version. It is almost identical to the previous one except several points as requested by you and reviewer #3. These include:

1. In the new version we added the information showing that NleD is not influencing IL8 expression. In the previous version it was indicated as “data not shown” and now it is in the supplementary data (Fig. S9).

2. We also toned down in the manuscript the suggestion that NleD influences IL8 production by post transcriptional regulation.

3. We indicate that the data in Fig 1A and 1C and S3 do show that NleC is not targeting Erk, JNK or p38 upon infection (i.e. the nleD mutant is not able to cleaved Erk, JNK or p38 although it has functional NleC gene). To better demonstrate this point we added below the blot in Fig S3 a table that emphasize it. Related to this comment; in the previous letter we made a mistake and indicate on Fig 2A and 2C instead of Fig. 1A and 1C. We are sorry about the confusion. In addition, as suggested by reviewer #3, we further demonstrate the NleD/NleC specificity using in vitro assay showing that NleC, even at high dosages, has no effects on JNK. This new data is mentioned in the text and added in the supplementary (Fig S15).

4. Since we added two new figures to the supplementary, we adjusted the numbering in the text.

5. We included in the relevant figure legends indication of how many times the experiments were replicated and n.

6. We replaced Fig. 2D with the same, yet uneditted, image.

7. We added a statement of author contributions after the acknowledgments

