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Caspase-2 is an initiator caspase responsible for pore-forming toxin-mediated apoptosis

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1st Editorial Decision 21 October 2011

Thank you very much for submitting your manuscript on caspase-2 as initiator caspase in PFT-mediated apoptosis for consideration to The EMBO Journal editorial office. As you will recognize from the comments, three scientists assessed the study in some detail. Though appreciating potential interest, it still became obvious that the mostly descriptive study lacks necessary molecular insight, mechanistic underpinnings and sufficient physiological support. As these are essential criteria at least for consideration at our rather general and at the same time molecular-oriented title, I am sorry to have to conclude that we are unable to offer further proceedings.

I do apologize for the delay that was caused by one of the scientists traveling and thus returning comments rather late. I still hope that the rather constructive comments will guide your decision on how to proceed with the work.

I also hope that you continue to consider The EMBO Journal as venue for future, more suitable studies.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

This paper attempts to make the case that Casp2 is the critical caspase responsible for pore-forming toxin mediated apoptosis. They mainly use to demonstrate their findings, but some later experiments are conducted with aerolysin. They show that Casp2 is activated independently of the PIDDosome but appears to be associated with another high molecular weight complex. They argue that the activation of C2 requires K+ efflux.
Overall, the authors do an incomplete job demonstrating that the observed cell death is truly Casp2-dependent apoptosis. They fail to show or explain key apoptotic events resulting from Casp2 activation, including Bid cleavage and cytochrome C release. Furthermore, they show that the cell death is Casp9-independent, suggesting that Casp2 is not working through the canonical pathway to promote cell death.

The authors fail to discuss how apoptosis may be occurring downstream of Casp2 activation. I think these critical points need to be addressed before the authors can conclusively say that the observed cell death is apoptosis promoted by Casp2 activation.
Figure 1: Throughout the paper the authors use 300ng/mL of α-toxin, but only use 5ng/mL of aerolysin.
It would be helpful if the physiological relevance of toxin concentrations used in the experiments were discussed in the text.
1F: The authors should examine Bid cleavage and cytochrome C release here. Since the authors are claiming that the cell death is caspase dependent apoptosis, they need to do a better job of characterizing/discussing the downstream events following Casp2 activation. Typically Casp2 activation results in activation of the mitochondrial apoptotic pathway, but the authors fail to show if this is the case with α-toxin. In fact, in a later figure they show that the cell death is Casp9 independent, which suggests that Casp2 is not promoting cell death through a canonical activation of mitochondrial apoptosis. If this is the case, the authors need to further investigate/discuss the downstream events following Casp2 activation. Once a more defined apoptotic pathway has been presented, the authors should include additional measures of apoptosis (i.e. C3, C8, or PARP cleavage) in addition to PI and Annexin V staining to reflect this pathway throughout the paper.

1F: zVAD treated samples show loss of C8 with α-toxin treatment (Neither FL and nor cleavage product are present). What do the authors make of this? Perhaps they can discuss this.

Figure 2: To make the claim that Casp2 is the initiator caspase required during α-toxin α-toxin α-toxin mediated apoptosis, the authors should show activation of Casp2 prior to or in the absence of Casp3 (or any other relevant downstream apoptotic factors since feedback activation could be occurring). In addition, only the Casp2 blot is shown after biotin-VAD pull-down. Some additional caspases should be immunoblotted to exclude the involvement of other intiator caspases that might not have been detected by mass spectrometry.

Figure 4: Again, it would be nice to see bid cleavage and cyto c release here, as well as, cleaved C3 as a cell death measurement since they are using a different toxin. It is important to know if the cell death pathway is the same, since both seem to rely on Casp2.

Figure 5: The goal of this figure is to show that induced cell death is not dependent on Casp8 or Casp9. This figure is a little perplexing and a little concerning. Additional experiments would help clarify what might be going on downstream of casp2 activation in treatment.

Interestingly, they do show that the observed Casp8 cleavage does seem to be dependent on Casp2, suggesting that Casp8 could play a role downstream of Casp2 in cell death(5C). However, they then show that the cell death is not dependent on C8, more or less negating this possibility (5G).

Additionally, they show that Casp2 is required for C9 processing in response to the toxin (5D) but then say that Casp9 is not required for cell death (5H), which if Casp2 were promoting cell death through activation of C3 via the apoptosome, it would be.

These considerations suggest either that one of their results is incorrect, or Casp2 can promote cell death by engaging either C8 or C9 (whichever is available; this would be an unprecedented mechanism). To test this possibility, the authors should repeat their experiments in cells with both C8 and C9 knockeddown simultaneously to see if Casp2 can still promote cell death.

Figure 6: The gel filtration results presented in H are not clear; there is a lot of processing of C2 that is occurring both in control and toxin treated cells, however, I am not sure this can be eliminated. Additionally, the presence of C2 in the HMW fraction is intriguing. It seems like a Casp2 band around 37 kDa is all that appears in this high molecular weight and it is restricted to only one fraction. I think a longer exposure of this immunoblot would allow a determination of whether FL Casp2 is present at all or whether other fractions might contain smaller amounts of the complex. This may help in determining whether Casp2 is recruited to this complex as a FL protein and then processed or if it is recruited after the first processing step.
Figure 7: 7A-They don't discuss Nigericin treatment at all in the text, only in the figure legend. A short discussion of its importance in the text should be included.
7B, C, D- The authors should show that adding excess K to the media actually blocks the K efflux from cells. This can be done by performing the same exp as done in A, just in the presence and absence of K+.

Additionally, it would be interesting to see if Nigericin treatment promotes Casp2 activation and/or movement of Casp2 to a HMW fraction. Does triggering K+ efflux promote Casp2 activation and/or HMW complex formation on its own?

Finally, it is important for the authors to show that Casp1 is not involved in the cell death associated with α-toxin treatment in Hela cells. They mention in the final figure that Pore Forming Toxins can promote
Casp1 activation in immune cells via K+ ion efflux, and while Hela cells express Casp1, they don't demonstrate that Casp1 isn't involved in this cell death. They should perform a Casp1 k/d similar to the Casp8 and 9 k/d they do in Figure 5.
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Referee #2:

The article reports on the observation that two bacterial toxins (alpha toxin and aerolysin) cause caspase-2 mediated apoptosis, in a PIDDosome, caspase-8 and caspase-9 independent way due to potassium efflux.

The authors claim that except for heat-stress induced activation of caspase-2, caspase-2 functions remain unclear. This may be true, but also heat stress is disputed in the literature (Shelton et al. JBC 2009). However, other emerging conditions leading to caspase-2 activation are ER stress.

The major issue with this paper is that the reported data are highly descriptive and do not provide a mechanism linking the toxins to caspase-2 activation, except for the stimulus no real new data are given. Many presented data are in fact already known in connection with caspase-2 (lowering potassium leads to processing of caspase-2, PIDD and RAIDD are not implicated in caspase-2 activation, HMW complex formation - but what are the constituents). Besides a lack of a mechanism of toxin induced activation, the report also misses a functional or physiological framework of the reported findings. How do caspase-2 knockout mice, which are widely available, respond to these or similar toxins or infections. MEF cells are maybe not the best choice because they are fibroblasts and not epithelial cells. Finally, how is apoptosis exerted? The authors exclude caspase-9 and -8, but are the executioner caspases implicated in apoptosis? What is the role of the inflammasome?

Referee #3:

Here the authors provide evidence that caspase-2 is required for Staph A alpha-toxin and Aeromonas aerolysin pore-forming toxin-induced apoptosis in HeLa cells. The paper is interesting and makes a reasonable case (mainly using knockdown approaches) that caspase-2 is required for these forms of apoptosis. However, many controls are missing from the manuscript and the mechanism of caspase-2 activation utilized here remains obscure. I have the following specific comments.

1. At the outset, it is important to note that there is nothing unusual about detecting caspase-2 processing during any form of apoptosis. Caspase-2 is well known to be processed (by caspase-3) within the intrinsic pathway to apoptosis (i.e. post-cytochrome c release), which all death stimuli

ultimately engage. The authors imply that caspase-2 processing during apoptosis is unusual (e.g. bottom of page 7, leading to page 8), which is not the case. The statement (top of page 8) that "These results revealed that caspase-2 is involved in PFT-mediated apoptosis in multiple cell types" is therefore misleading. The results at this stage of the paper merely indicate that caspase-2 is processed, not that it's involved.

2. The authors use biotin-VAD to label proximally-activated caspases in HeLa cells treated with Staph A alpha-toxin (Figure 2). However, they have not blotted these pulldowns using antibodies specific for the well-established initiator caspases (caspase-8 and caspase-9) and have only looked for caspase-2 labeling in the biotin-VAD pulldowns (Fig 2D). Again, this is a circular experiment, if only one caspase is looked for, then its not surprising that one caspase is found. These experiments should be repeated as a timecourse after alpha-toxin treatment where the resulting pulldowns are probed for caspase-2, caspase-8 and caspase-9. Merely failing to detect the latter caspases by Mass spec analysis does not provide proof that they are not there. A similar comment can be made with respect to the time course analysis presented in Figure 2A, where only caspase-2 is examined, this time course should be blotted for caspases-8 and -9 for comparison. By only looking at caspase-2 the authors are biasing their analyses towards this protease. Similarly, the authors only use a single 'caspase-2-specific' tetrapeptide (FAM-VDVAD-fmk) to look for evidence for caspase-2 activity (Figure 2B). No other peptides are examined.

3. The most convincing evidence for a proximal role for caspase-2 in pore-forming toxin induced apoptosis is provided by caspase-2 knockdown experiments in Figure 3. Four different caspase-2-targeting oligos are used to knockdown caspase-2 stably (three of which are effective). It is essential that the authors examine the expression status of caspase-8 and caspase-9 in these knockdown lines (versus control cells) to ensure that expression of the latter caspases have not been affected through clonal selection effects.

4. Staying with Figure 3, the authors have not examined any other pro-apoptotic stimulus on these cells (i.e shControl versus shCasp2#2 and shCasp2#3) that should unaffected by loss of caspase-2 (e.g. cisplatin, anti-Fas, daunorubicin). Importantly, non-saturating doses of these stimuli should be used that achieve approx. 50-60% cell death in the control (saturating doses can overwhelm protective effects). It is not uncommon for subclones to emerge during selection that are resistant to many pro-apoptotic stimuli.

5. In the control versus caspase-2-depeleted cells treated with Staph A alpha-toxin (Figure 3), the authors should examine the processing status of caspase-8, caspase-9 and caspase-2 to support the idea that the former caspases are not processed under these conditions.
6. The authors use WT versus Casp-2-/- MEFs to explore whether these cells are resistant to aerolysin and provide data to suggest that they are. Is cell death restored by restoring caspase-2 into these MEFs?

7. The authors then examine whether knockdown of caspase-8 or caspase-9 has any protective effect on PFT-induced death and find no evidence for this (Figure 5). They control this with casp-2 knockdown (KD) and show that whereas KD of caspase-8 or caspase-9 did not affect alpha-toxin-induced casp-2 processing, KD of casp-2 attenuated caspase-9 processing. The authors do not examine whether KD of caspase-2 attenuated alpha-toxin-induced caspase-8 processing in the same blot series (Fig. 5D). Where they do examine this (Fig. 5C) casp-2#2 KD did not attenuate casp-8 processing, casp-2#3 KD apparently did so but this lane is underloaded (see actin loading control here). All relevant caspases should be examined on the same blot series, as it stands, examining selective caspases on different panels is puzzling and makes direct comparison difficult.

8. No controls are presented for biological impact of caspase-8 knockdown (e.g. resistance to fas-induced cell death) or caspase-9 knockkdown (e.g. resistance to cisplatin-induced cell death) to confirm that these knockdowns are functional. As it stands, the data in Figure 5 are all negative.

9. The authors rule out a role for PIDD and RAIDD in promoting caspase-2 activation in this context (Figure 6) but suggest that caspase-2 is recruited to a high molecular weight complex in response to alpha-toxin-treatment. However, the data shown are not of very high quality and only a small fraction of the total processed caspase-2 appears to be present in a high MW fraction. Moreover there is no evidence that this high MW fraction is a complex or is biologically significant.

10. The authors make an effort to explore the molecular mechanism underpinning caspase-2 processing in response to alpha-toxin and, perhaps unsurprisingly for pore-forming toxins, detect a

decrease in cellular K+ ions. No other iopn is measured. Inhibition of this efflux by culturing cells in high K+ attenuated caspase-2 processing here, as well as DNA fragmentation. No other ion is elevated in the medium as a control for the selectivity of the inhibition. Neither did the authors examine Annexin V/PI uptake here for some reason. As a mechanism of caspase-2 activation, the data presented are not very convincing.

11. It would be very useful to know whether caspase-2 processing and apoptosis are blocked by Bcl-x overexpression in response to the pore-forming toxins used here. If not, this would suggest that caspase-2 activation occurs upstream of cytochrome c release which would help to order the sequence of events. Appropriate positive control stimuli should be used to control for Bcl-x protection.

12. Overall, the mechanism underpinning caspase-2 activation in this context remains obscure. That said, if the authors can provide the suggested controls to argue convincingly that caspase-2 is the initiator caspase in response to the pore-forming toxins used herein, this would be a significant advance and would merit publication.

Author Rebuttal 21 October 2011

Note:

The authors rebutted the decision based on the notion that substantial functional support had already been provided in the initial submission and most of the constructive criticisms could be addressed in a reasonable timeframe. Though not changing the initial decision, it was agreed to re-assess the paper as a NEW SUBMISSION in the near future.

Resubmission 14 February 2012

We thank the reviewers for their constructive criticisms which have helped us to improve the manuscript. Please find our point by point response below.

Referee #1:

This paper attempts to make the case that Casp2 is the critical caspase responsible for pore-forming toxin mediated apoptosis. They mainly use a-toxin to demonstrate their findings, but some later experiments are conducted with aerolysin. They show that Casp2 is activated independently of the PIDDosome but appears to be associated with another high molecular weight complex. They argue that the activation of C2 requires K+ efflux.

Overall, the authors do an incomplete job demonstrating that the observed cell death is truly Casp2-dependent apoptosis. They fail to show or explain key apoptotic events resulting from Casp2 activation, including Bid cleavage and cytochrome C release. Furthermore, they show that the cell death is Casp9-independent, suggesting that Casp2 is not working through the canonical pathway to promote cell death.

The authors fail to discuss how apoptosis may be occurring downstream of Casp2 activation. I think these critical points need to be addressed before the authors can conclusively say that the observed celldeath is apoptosis promoted by Casp2 activation.

Response: We thank the reviewer for her/his constructive criticisms and we have duly addressed the issues raised by the reviewer. We have also characterized the events downstream of caspase-2 activation as suggested by the reviewer. In particular, we now present evidence that effector caspases are very much required for caspase-2-mediated cell death. Loss of both caspases-3 and-7 renders MEFs profoundly resistant to PFT-mediated cell death. Following reviewer's advise, we have also investigated the role of MOMP in PFT-mediated cell death. All other issues raised by this reviewer have also been addressed as detailed in our responses below.

Figure 1: Throughout the paper the authors use 300ng/mL of a-toxin, but only use 5ng/mL of aerolysin. It would be helpful if the physiological relevance of toxin concentrations used in the experiments were discussed in the text.

Although aerolysin and α-toxin of *S.aureus* both belong to the small β-barrel forming toxins sub-family of PFT, their proposed receptors and mode of binding/activation on the cell surface are thought to be quite different. Aerolysin binds to a GPI-anchored receptor and activation of bound toxin requires furin (for review of aereolysin binding and activation: Abrami et al., Trends in Microbiology 2000. 168 Vol 8 No4). In the case of a-toxin, ADAM10 has been recently proposed as a high affinity receptor (Wilke GA, Bubeck Wardenburg J. Proc Natl Acad Sci U S A. 2010 Jul 27;107(30):13473-8), but others have shown that clustered lipids are principally sufficient for binding and activation (Valeva A, Hellmann N, Walev I, Strand D, Plate M, Boukhallouk F, Brack A, Hanada K, Decker H, Bhakdi S. J Biol Chem. 2006 Sep 8;281(36):26014-21); cleavage of bound toxin is not required for activation. The density of the receptors for aerolysin and α-toxin respectively may be very different in a given cell-type, and may vary between cell-types, thus explaining different concentration requirements in experiments with these two toxins. That aerolysin is more potent than α-toxin is quite meaningful: To be effective in the comparably large volume of the gut, aerolysin ought to be of much higher affinity (and possibly specificity) than α-toxin. This is because *S. aureus* may grow to rather high densities in the confined compartment of an abscess, or on a wound where it may even create biofilms. The amounts of α-toxin that accumulate in the fluid phase of abscesses of mice infected with *S. aureus* correspond to ~ 5-50-times the lethal dose for a mouse (*Kapral et al, Infect Immun. 1980;30(1):204-11*). The lethal dose is ~ 200-600 ng (*Wiseman-GM, Bacteriol Rev. 1975 Dec;39(4):317-44*, and our own observations). Even if the average volume of the abscess was 1ml in the study by *Kapral et al*., the concentration in the abscess would thus have ranged from 1mg/ml to 30mg/ml. Therefore, α-toxin concentrations in the range used in the present work (300 ng/ml) do occur *in vivo*. Of note, Inoshima et al. have employed –50 (fifty)/mg/ml α-toxin - in their recent study "A Staphylococcus aureus pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice*"(Inoshima et al. Nat Med. 2011 Sep 18;17(10):1310-4))*. We have clarified these points in the manuscript.

1F: The authors should examine Bid cleavage and cytochrome C release here. Since the authors are claiming that the cell death is caspase dependent apoptosis, they need to do a better job of characterizing/discussing the downstream events following Casp2 activation. Typically Casp2 activation results in activation of the mitochondrial apoptotic pathway, but the authors fail to show if this is the case with a-toxin. In fact, in a later figure they show that the cell death is Casp9 independent, which suggests that Casp2 is not promoting cell death through a canonical activation of mitochondrial apoptosis. If this is the case, the authors need to further investigate/discuss the downstream events following Casp2activation. Once a more defined apoptotic pathway has been presented, the authors should include additional measures of apoptosis (i.e. C3, C8, or PARP cleavage) in addition to PI and Annexin V staining to reflect this pathway throughout the paper.

Response: We have characterized the downstream events as advised by the reviewer. In the first place, we have checked for Bid cleavage and Smac/DIABLO release in PFT treated cells. We could demonstrate both MMP loss and the release of Smac/Diablo to cytosol upon PFT treatment in HeLa cells. These data are now included in the supplement Figure S3A-B. However, we couldn't detect any significant Bid cleavage in PFT-treated cells, probably due limitations in detection. These data are now included in the revised version of the manuscript (Supplementary Figure S3C). To further evaluate the contribution of MOMP in this process, we have resorted to Bax/Bak DKO MEFs. Interestingly, loss of both Bax and Bak led to inhibition of aerolysin-mediated cell death. In the same lines, Loss of Bax can partially prevent α-toxin-mediated cell death in HeLa cells. These data are also now included in the supplement. As expected, depletion of caspase-2 prevents Smac release from the mitochondria (please see our new Supplementary Figure S9A-D). Further we would like to add that we resort not to completely rule out a role for caspase-8/9 in this process as depletion of these caspases unlike caspase-2 has led to a partial but statistically insignificant blockade in PFT-mediated cell death.

Finally, we also present evidence that effector caspases are very much required as loss of both casp-3 and -7 prevents PFT-mediated apoptosis (Figure 5I). Furthermore we could detect caspase-3 cleavage as well as PARP fragmentation upon alpha toxin treatment (Figure 1F and Figure 5D). PARP cleavage was inhibited by caspase-2 depletion, indicating the upstream role for caspase-2 in this process. Taken together, we propose that caspase-2 functions as an initiator caspase which then contributes to MOMP in a Bax-Bak dependent manner leading to Smac release and effector caspase activation during PFT-mediated apoptosis. The model previously presented in Figure 7 has now been refined following our new observations.

1F: zVAD treated samples show loss of C8 with a-toxin treatment (Neither FL and nor cleavage product are present). What do the authors make of this? Perhaps they can discuss this.

In the experiments presented as IF, the cells were treated with toxin for 24 h. At these time points post treatment we often witness degradation of the entire proteome of the cells probably due to lysis. This indicates that ZVAD-fmk (which actually only weakly inhibits caspase-2 activity compared to other caspases (Chauvierd et al, CDD, 2007 14(2), 387-91) alone was not enough to completely block cell death (see also sub G1 data- Figure 1E and S2C). And this also supports the idea that Caspase 8 and 9 (which, among others can be strongly inhibited by zVAD-fmk) are not upstream components of the pathway. We have added the following line to the text on page no 8 "The degradation of Caspase-8 even in the presence of caspase inhibitor might be due to the fact that at later time points the whole proteome degrades due to complete cell lysis"

Figure 2: To make the claim that Casp2 is the initiator caspase required during a-toxin mediated apoptosis, the authors should show activation of Casp2 prior to or in the absence of Casp3 (or any other relevant downstream apoptotic factors since feedback activation could be occurring). In addition, onlythe Casp2 blot is shown after biotin-VAD pull-down. Some additional caspases should be immunoblotted

to exclude the involvement of other intiator caspases that might not have been detected by mass spectrometry.

We appreciate this comment and we have repeated these experiments and probed the biotin-VAD precipitates with caspase-8,-9 and -2 antibodies. As expected, we could NOT detect either caspase-8 or caspase-9 in these precipitates upon toxin treatment. A new figure has now been added (Figure 2C). Further, we have also tested for VDVAD-fmk activity in caspase-3/7 double deficient MEFs and we could detect high caspase-2 activity in these cells upon toxin treatment, although cell death was significantly reduced in these cell type (see comments above and Figure 5I). These data are now presented in the supplement as Figure S5C.

Figure 4: Again, it would be nice to see bid cleavage and cyto c release here, as well as, cleaved C3 as a cell death measurement since they are using a different toxin. It is important to know if the cell death pathway is the same, since both seem to rely on Casp2.

We now present extensive data on aerolysin with Bax/Bak DKOs,caspase-3/7 DKOs to characterize the downstream signalling. As α- toxin fails to induce any cell death in the MEFs we have to resort to aerolysin to perform these MEF experiments. We have tried our best to compliment our knock down experiments with cells derived from mice deficient in various regulators to characterize the signalling pathway leading to PFT-mediated cell death.

Figure 5: The goal of this figure is to show that a-toxin induced cell death is not dependent on Casp8 or

Casp9. This figure is a little perplexing and a little concerning. Additional experiments would help clarify what might be going on downstream of casp2 activation in a-toxin treatment. Interestingly, they do show that the observed Casp8 cleavage does seem to be dependent on Casp2,suggesting that

Casp8 could play a role downstream of Casp2 in cell death(5C). However, they then show that the cell death is not dependent on C8, more or less negating this possibility (5G).

Additionally, they show that Casp2 is required for C9 processing in response to the toxin (5D) but then

say that Casp9 is not required for cell death (5H), which if Casp2 were promoting cell death through activation of C3 via the apoptosome, it would be. These considerations suggest either that one of their results is incorrect, or Casp2 can promote cell death by engaging either C8 or C9 (whichever is available; this would be an unprecedented mechanism). To test this possibility, the authors should repeat their experiments in cells with both C8 and C9 knocked down simultaneously to see if Casp2 can still promote cell death.

Our prime goal is to substantiate our biotin-VAD experiments, which support a clear role for caspase-2 as an initiator caspase in this pathway. Thus, we have tested for the processing of caspase-8 and -9 in caspase-2 depleted cells and as expected caspases-8 and -9 are processed downstream of caspase-2. However, depletion of either caspase-8 or caspase-9 failed to provide any significant protection to PFT-mediate cell death in these cell types suggesting that the processing of caspases-8 or -9 could be a secondary event. We also would like to remind the reviewer that depletion of potassium has also been shown to influence activation of caspase-9 (Karki P, CDD, 2007 14(12):2068-75). However, as mentioned earlier, we don't want to completely rule out a downstream role for caspase-9 or caspase-8 in this process as we do see a reduction (which is however not statistically significant) in PFT-mediated cell death in these cell types. One possibility is that in these cell types Caspase-2 might directly activate effector caspases to elicit apoptosis. Further the lack of a significant role for these caspases during PFT –mediated cell death could also be cell type dependent as they might be very much required to amplify the cascade if the cells happen to express more anti-apoptotic molecules like IAPs. The existence of parallel or bifurcated pathways is not a unique event in cell death biology as for instance it is known that different levels of various FLIP isoforms determines the shift from apoptosis to necroptosis (Feoktistova et al, Mol Cell, 2011).

Following THE reviewer's advice, we have also performed double knock down experiments. As expected, depletion of both caspase-8 and -9 failed to provide any significant protection to PFT-mediated cell death. These data are now included in the supplement as Figure S8A and B. Thus our model bestows the initial caspase role for caspase-2 which then seems to depend on MOMP and effector caspases for accomplishing cell death. The text and the discussion have been modified accordingly.

Figure 6: The gel filtration results presented in H are not clear; there is a lot of processing of C2 that is occurring both in control and toxin treated cells, however, I am not sure this can be eliminated.
Additionally, the presence of C2 in the HMW fraction is intriguing. It seems like a Casp2 band around 37 kDa is all that appears in this high molecular weight and it is restricted to only one fraction. I think a longer exposure of this immunoblot would allow a determination of whether FL Casp2 is present at all or whether other fractions might contain smaller amounts of the complex. This may help in determining whether Casp2 is recruited to this complex as a FL protein and then processed or if it is recruited after the first processing step.

There is indeed a lot of processing of Casp-2 occuring in control cells which could be attributed to increased local concentrations of this protease owing to the lysis of cells in small volumes of buffer for gel filtration studies. However, we have examined the presence of full length caspase-2 in the HMW fractions and at higher exposures (and with more sensitive ECL), we could indeed detect full-length caspase-2 in the HMW fraction. The Figure 6H has been changed accordingly. The localization of caspase-2 to one fraction is primarily due to the improved resolution of our gel filtration analysis. For instance in Superose6 10/300 GL, we have a broad spectrum distribution of Casp-2 to various HMW fractions (Supplementary Figure S12). To further elucidate the size and biophysical properties of this complex, we have resorted to HiPrep 16/60 Sephacryl S 500 HR

column where Casp-2 is confined primarily to one fraction. Based on these observations, our interpretation is also very much in line with the reviewer: That Casp-2 is possibly recruited to this complex as a FL protein and then processed. To further, prove caspase-2 activation/dimerization/olgomerization in situ upon toxin treatment, we have also adopted BiFC approach. In collaboration with Douglas Green's lab, we have tested for the possible dimerization of Caspase-2 CARD-BIFC constructs upon toxin treatment. As expected, we have a strong BiFC of caspase-2 CARD domains upon toxin treatment in the transfected cells (Please note that the transfection efficiency is around 20-30% in these cells as checked by GFP controls) . Further, we detected that BiFC signals of CARD domains are primarily localized to the cytosol in the toxin treated cells. These data are now added to the manuscript as Figure 2D and E and supplementary Figure S5D. These results further strengthen our argument that caspase-2 is indeed dimerized and activated in response to PFT treatment. The studies so far in caspase-2 activation primarily checked for the possible recruitment of caspase-2 to HMW complexes by shifting the lysates from 4°C to 37°C. Here we define conditions under which one can detect endogenous caspase-2 complexes with a trigger where caspase-2 is activated as an initiator caspase. Thus we feel the study can provide a significant advance to characterize PIDDOsome independent activation platform (s) for Caspase-2.

Figure 7: 7A-They don't discuss Nigericin treatment at all in the text, only in the figure legend. A short discussion of its importance in the text should be included.

We have performed additional experiments as suggested by this reviewer (please see below) and an appropriate discussion has now been added to the text (see page 16). We thank the reviewer for kindly pointing this out.

7B, C, D- The authors should show that adding excess K to the media actually blocks the K efflux from cells. This can be done by performing the same exp as done in A, just in the presence and absence of K+.

We have performed this experiment and as expected adding excess K^+ ion to the media blocked potassium efflux from cells as revealed by PBFI analysis. These data are now included in the supplement as Fig S14A.

Additionally, it would be interesting to see if Nigericin treatment promotes Casp2 activation and/or movement of Casp2 to a HMW fraction. Does triggering K+ efflux promote Casp2 activation and/or HMW complex formation on its own?

This is indeed a good suggestion. We have addressed this issue by testing for the activation of caspase-2 after Nigericin treatment by flow cytometry analysis. As expected, we have detected FAM-VDVAD-fmk activity in more than 80% of the cells. These data are now included in the supplementary information (Figure S14B). Consistently, loss of caspase-2 prevented Nigericin-mediated cell death. The results and discussion has also been modified accordingly (Figure S14C).

Finally, it is important for the authors to show that Casp1 is not involved in the cell death associated with a-toxin treatment in Hela cells. They mention in the final figure that Pore Forming Toxins can promote Casp1 activation in immune cells via K+ ion efflux, and while Hela cells express Casp1, they don't demonstrate that Casp1 isn't involved in this cell death. They should perform a Casp1 k/d similar to the

Casp8 and 9 k/d they do in Figure 5.

This is indeed a very valid issue and we have addressed this concern as advised by the reviewer. First, we couldn't detect any major processing/activation of caspase-1 in PFT-treated HeLa cells

until 24 h post treatment. While other caspases (-8,-9-2) are activated quite early, the cleavage or processing of caspase-1 is not detected at these early time points (Figure 2A). We have also established stable shRNA-mediated silencing of caspase-1 expression in HeLa cells. Loss of caspase-1 failed to prevent PFT-mediated apoptosis suggesting that unique caspases are exploited for eliciting various modes of cell death in different cell types. These data are now included as supplement in Figure S10 A-C.

Referee #2:

The article reports on the observation that two bacterial toxins (alpha toxin and aerolysin) cause caspase-2 mediated apoptosis, in a PIDDosome, caspase-8 and caspase-9 independent way due to potassium efflux.

The authors claim that except for heat-stress induced activation of caspase-2, caspase-2 functions remain unclear. This may be true, but also heat stress is disputed in the literature (Shelton et al. JBC 2009). However, other emerging conditions leading to caspase-2 activation are ER stress.

We thank the reviewer for pointing this out and we have modified the sentence .

The major issue with this paper is that the reported data are highly descriptive and do not provide a mechanism linking the toxins to caspase-2 activation, except for the stimulus no real new data are given. Many presented data are in fact already known in connection with caspase-2 (lowering potassium leads to processing of caspase-2, PIDD and RAIDD are not implicated in caspase-2 activation, HMW complex formation - but what are the constituents). Besides a lack of a mechanism of toxin induced activation, the report also misses a functional or physiological framework of the reported findings. How do caspase-2 knockout mice, which are widely available, respond to these or similar toxins or infections. MEF cells are maybe not the best choice because they are fibroblasts and not epithelial cells.

We respectfully disagree with the reviewer on this evaluation. Despite being the first mammalian caspase known the role of caspase-2 remains enigmatic to date. Here we present the first host-pathogen interaction scenario where caspase-2 functions as an initiator caspase to elicit apoptotic cell death in epithelial cells, MEFs and human primary keratinocytes. Depletion of physiological levels of caspase-2 by various means confers protection to PFT-mediated cell death in a potassium ion sensitive manner. Though PIDD/RAIDD independent mode of caspase-2 activation has been presented, recruitment of caspase-2 to high molecular weight complexes in cells have not been shown before (Please see discussion in Manzl C et al JCB 185(2):291-303. Page 299). Previous studies have shown recruitment of caspase-2 to high molecular weight complexes primarily by temperature shift in vitro by shifting the lysates from 4°C to 37 °C. Our study is the first of its kind to purify a native caspase-2 containing complexes from apoptotic cells and our extensive efforts with gel filtration studies have at least defined the ways to identify and characterize these potential activation platforms for this still enigmatic caspase. Identifying the constituents of this complex and their relative contributions to regulate caspase-2 activation and cell death is far beyond the scope of this manuscript.

Finally, how is apoptosis exerted? The authors exclude caspase-9 and -8, but are the executioner caspases implicated in apoptosis?

Our new data demonstrates a clear role for effector caspases-3 and -7 in PFT-mediated cell death. Loss of both caspases-3 and-7 confers resistance to PFT-mediated cell death in MEFs. Further we

demonstrate that caspase-2 activity can be detected in 80% of caspase-3/7 DKO MEFs upon aerolysin treatment. We now demonstrate that loss of both caspase-8 and -9 failed to provide any significant protection to HeLa cells from α-toxin-mediated cell death. Please also see our detailed response with figure references to reviewer1.

What is the role of the inflammasome?

We have addressed this issue by employing caspase-1 depleted cells. Loss of caspase-1 failed to prevent PFT-mediated apoptosis in these cell types. The new data has now been added to the manuscript (Supplementary Figure S10 A-C).

Referee #3:

Here the authors provide evidence that caspase-2 is required for Staph A alpha-toxin and Aeromonas aerolysin pore-forming toxin-induced apoptosis in HeLa cells. The paper is interesting and makes a reasonable case (mainly using knockdown approaches) that caspase-2 is required for these forms of apoptosis. However, many controls are missing from the manuscript and the mechanism of caspase-2 activation utilized here remains obscure. I have the following specific comments.

We thank the reviewer for her/his interest in the study and for constructive criticisms. We have duly addressed the concerns raised by this reviewer and we hope that the reviewer continue to support us to publish these observations in EMBO J.

At the outset, it is important to note that there is nothing unusual about detecting caspase-2 processing during any form of apoptosis. Caspase-2 is well known to be processed (by caspase-3) within the intrinsic pathway to apoptosis (i.e. post-cytochrome c release), which all death stimuli ultimately engage. The authors imply that caspase-2 processing during apoptosis is unusual (e.g. bottom of page 7, leading to page 8), which is not the case. The statement (top of page 8) that "These results revealed that caspase-2 is involved in PFT-mediated apoptosis in multiple cell types" is therefore misleading. The results at this stage of the paper merely indicate that caspase-2 is processed, not that it's involved.

We agree to this comment and we have modified this statement following the advise of the reviewer. We further also present new evidence that caspase-2 activity is highly detected in cells deficient in caspase3- and 7 (Please see Supplementary Figure S5C).

The authors use biotin-VAD to label proximally-activated caspases in HeLa cells treated with Staph A alpha-toxin (Figure 2). However, they have not blotted these pulldowns using antibodies specific for the well-established initiator caspases (caspase-8 and caspase-9) and have only looked for caspase-2 labeling in the biotin-VAD pulldowns (Fig 2D). Again, this is a circular experiment, if only one caspase is looked for, then its not surprising that one caspase is found. These experiments should be repeated as a timecourse after alpha-toxin treatment where the resulting pulldowns are probed for caspase-2, caspase-8 and caspase-9. Merely failing to detect the latter caspases by Mass spec analysis does not provide proof that they are not there. A similar comment can be made with respect to the time course analysis presented in Figure 2A, where only caspase-2 is examined, this time course should be blotted for caspases-8 and -9 for comparison. By only looking at caspase-2 the authors are biasing their analyses towards this protease. Similarly, the authors only use a single 'caspase-2-specific' tetrapeptide (FAM-VDVAD-fmk) to look for evidence for caspase-2 activity (Figure 2B). No other peptides are examined.

We have followed the advise of the reviewer and have checked for the presence of caspases-8 and -9 in the biotinVAD precipitates and we have failed to detect them. These data are now included in Figure 2C. We have also checked for the processing of caspases-8,-9,and -1 in the immunoblots originally presented as Figure 2A. As expected, all three caspases seem to be processed almost at the same time which further warrants for the biotin-VAD approach to identify the proximal caspase. We have also now present new evidence to demonstrate dimerization of caspase-2 by employing newly developed Caspase-2CARD-BIFC constructs upon toxin treatment. These data are now included in Fig 2 as D and E and Supplementary Figure S5D.

We have also applied other substrates (for example caspase-3, -7 and polycaspase substrates with VAD sequence, data not shown) than FAM-VDVAD-fmk. But since these substrates are not specific to one caspase type (Please see McStay GP, et al . *Cell Death Differ,* **15,** 322-331). However biotin-VAD and cells with knock down/knock out experiments clearly showed that Caspase-2 is the main initiator caspase in this process. Our aim of applying Caspase-2 substrate was simply to show the activity of Caspase-2 by another (though not well appreciated strategy) not to exclude the activity of other caspases in the process.

The most convincing evidence for a proximal role for caspase-2 in pore-forming toxin induced apoptosis is provided by caspase-2 knockdown experiments in Figure 3. Four different caspase-2-targeting oligos are used to knockdown caspase-2 stably (three of which are effective). It is essential that the authors examine the expression status of caspase-8 and caspase-9 in these knockdown lines (versus control cells) to ensure that expression of the latter caspases have not been affected through clonal selection effects.

We have presented blots to examine the levels of caspase-8 and caspase-9 expressed in Caspase-2 depleted cells (Please see Figure 5 C and D). Further, to avoid clonal selection effects we have employed shRNA-enriched population and not single cell clones.

Staying with Figure 3, the authors have not examined any other pro-apoptotic stimulus on these cells (i.e shControl versus shCasp2#2 and shCasp2#3) that should be unaffected by loss of caspase-2 (e.g. cisplatin, anti-Fas, daunorubicin). Importantly, non-saturating doses of these stimuli should be used that achieve approx. 50-60% cell death in the control (saturating doses can overwhelm protective effects). It is not uncommon for subclones to emerge during selection that are resistant to many pro-apoptotic stimuli.

We have indeed tested for the effectiveness of the knock downs by employing positive controls. These data are now presented in the supplement as Figure S7A and B. We have employed TRAIL and staurosporine primarily as inducers. Loss of caspase-9 also partially protects TRAIL-mediated apoptosis in Hela cells (as they behave as type II cells).

In the control versus caspase-2-depeleted cells treated with Staph A alpha-toxin (Figure 3), the authors should examine the processing status of caspase-8, caspase-9 and caspase-2 to support the idea that the former caspases are not processed under these conditions.

These data were shown as Figure 5 C and D, which shows that processing of caspase-8 and -9 are occuring down stream of caspase-2.

The authors use WT versus Casp-2-/- MEFs to explore whether these cells are resistant to aerolysin and provide data to suggest that they are. Is cell death restored by restoring caspase-2 into these MEFs?

We have attempted these experiments and we could not achieve any significant transfection

efficiency in these MEFs. Further, different levels of expression often leads to different effects and most of the cells have died following transfection of full length caspase. We appreciate the concern of the reviewer on specificity but complementation cannot always be a standard to prove the specificity of the phenotype. That being the case, we have employed multiple shRNAs to confirm the specificity of the phenotype. Our data with MEFs compliments and further confirms the results obtained in HeLa cells after depleting various regulators by employing shRNAs.

The authors then examine whether knockdown of caspase-8 or caspase-9 has any protective effect on PFT-induced death and find no evidence for this (Figure 5). They control this with casp-2 knockdown (KD) and show that whereas KD of caspase-8 or caspase-9 did not affect alpha-toxin-induced casp-2 processing, KD of casp-2 attenuated caspase-9 processing. The authors do not examine whether KD of caspase-2 attenuated alpha-toxin-induced caspase-8 processing in the same blot series (Fig. 5D). Where they do examine this (Fig. 5C) casp-2#2 KD did not attenuate casp-8 processing, casp-2#3 KD apparently did so but this lane is underloaded (see actin loading control here). All relevant caspases should be examined on the same blot series, as it stands, examining selective caspases on different panels is puzzling and makes direct comparison difficult.

I hope the reviewer appreciates the fact that it is almost impossible to probe the same membrane more than twice. We have reloaded the samples in most cases to check for the processing of various caspases. In both cases (Figure 5C and 5D) the controls are very much included for comparison. We have obtained 4 different shRNAs for casapse-2 and most of them elicit efficient knock down. Despite being transduced with lentiviruses, the knock down efficiency is reduced after few passages despite antibiotics. In this particular experiment (Figure 5C), sh Casp-2 #2 is not providing the best knock down efficiency, while shRNAs #3 and # 4 does. In cells expressing shRNA#4, caspase-8 activation/processing is completely prevented. Agreed, lane 8 is underloaded , but even in lane 7, the processing of casapse-8 is reduced (see quantification in Figure 5C). We would like to present this figure as it shows the effect with various levels of knock downs with multiple shRNAs. We have however included the quantification of the caspase-8 processing (normalized to loading controls) under the blots for interpretation.

No controls are presented for biological impact of caspase-8 knockdown (e.g. resistance to fas-induced cell death) or caspase-9 knockkdown (e.g. resistance to cisplatin-induced cell death) to confirm that these knockdowns are functional. As it stands, the data in Figure 5 are all negative.

The positive control has now been included in the supplement and loss of either caspase-8 or caspase-9 led to reduction in TRAIL and staurosporine-mediated cell death. In the same lines, loss of caspase-2 fails to provide any significant protection from TRAIL-mediated apoptosis (Supplementary Figure S7 A and B).

The authors rule out a role for PIDD and RAIDD in promoting caspase-2 activation in this context (Figure 6) but suggest that caspase-2 is recruited to a high molecular weight complex in response to alpha-toxin-treatment. However, the data shown are not of very high quality and only a small fraction of the total processed caspase-2 appears to be present in a high MW fraction. Moreover there is no evidence that this high MW fraction is a complex or is biologically significant.

We have tried our best to detect the recruitment of endogenous caspase-2 to HMW fractions by applying high-resolution gel filtration chromatography. Previous studies have shown the recruitment of caspase-2 to HMW complexes only upon shifting the lysates from 4C to 37 C. So far, no evidence has been presented whether caspase-2 can be recruited to any HMW complexes in vivo in apoptotic cells (Please see discussion in Manzl C et al JCB 185(2):291-303, page 299). Our efforts attempted to characterize such a complex, which could possibly serve as an activation platform. Our analysis suggests that it is indeed a complex and not formed as a result of aggregation. Please note that the fraction where the complex is detected is far from the range of the void volume of the column and for additional explanation also see our response to reviewer 1.

We could further prove that the complex formation can be inhibited by prevention of potassium efflux. Currently, the constituents of this complex are not clear and only future studies can reveal the biological role and significance of the components of this complex in regulating PFT-or other stress –mediated apoptosis. Following the advise of reviewer1 we have checked for the presence of full length caspase-2 in the HMW fraction and we could indeed detect FL-caspase-2 in these fraction suggesting that caspase-2 is probably recruited for its activation (Figure 6H). By employing BIFC, we could further demonstrate that CARD-Caspase-2 dimerization is induced by pore forming toxins (Figure 2D and E and Supplementary Figure S5D). Taken together, we believe that our data for the first time attempted to identify and partially characterize a caspase-2 activation platform devoid of PIDD and RAIDD.

The authors make an effort to explore the molecular mechanism underpinning caspase-2 processing in response to alpha-toxin and, perhaps unsurprisingly for pore-forming toxins, detect a decrease in cellular K+ ions. No other ion is measured. Inhibition of this efflux by culturing cells in high K+ attenuated caspase-2 processing here, as well as DNA fragmentation. No other ion is elevated in the medium as a control for the selectivity of the inhibition. Neither did the authors examine Annexin V/PI uptake here for some reason. As a mechanism of caspase-2 activation, the data presented are not very convincing.

The reduction in potassium ion concentration is always compensated with other positively charged ions like sodium. Following the advise of reviewer1 we have also confirmed that increasing extracellular potassium levels prevents K^+ efflux from these cells when treated with α-toxin. We have also attempted to increase the concentrations of other ions like Calcium which led to strong cell death induction in the control cells. Further our new data reveal that potassion ionophore Nigericin treatment also leads to an increase in caspase-2 activity in cells and that cell death mediated by Nigericin can be prevented by the loss of caspase-2 (Supplementary Figure S14B and C). Data from annexin-V-PI assays from three independent experiments have now been presented as Figure 7E.

It would be very useful to know whether caspase-2 processing and apoptosis are blocked by Bcl-x overexpression in response to the pore-forming toxins used here. If not, this would suggest that caspase-2 activation occurs upstream of cytochrome c release which would help to order the sequence of events. Appropriate positive control stimuli should be used to control for Bcl-x protection.

We appreciate the comment of the reviewer to unveil the role of mitochondria in regulating PFT-mediated cell death. We have failed to achieve significant transfection efficiency in HeLa or other cell types with various Bcl-2 constructs. However, we have addressed this issue by employing BAX/BAK DKO MEFs and by employing BAX depleted HeLa cells. Loss of both BAX and BAK provided strong protection from aerolysin-mediated apoptosis. Similarly, loss of BAX partially blocked alpha toxin-mediated cell death in HeLa cells. As expected, caspase-2 depletion led to a decrease in the cytosolic Smac levels in PFT treated cells. All these data suggests that caspase-2 activation is occurring upstream of MOMP and that MOMP and effector caspases are required to accomplish PFT-mediated cell death. We have employed staurosporine as positive control to test for the efficiency of inhibition in BAX/BAK DKO MEFs. These data are now presented in Supplementary Figure S9 A-D.

Overall, the mechanism underpinning caspase-2 activation in this context remains obscure. That said, if the authors can provide the suggested controls to argue convincingly that caspase-2 is the initiator caspase in response to the pore-forming toxins used herein, this would be a significant advance and would merit publication.

We hope that the reviewer is now convinced with our new data revealing the downstream events following caspase-2 activation in response to PFT in various cell types. The requested controls have also been provided and we hope that the reviewer now support us to publish these interesting observations in EMBO J.

2nd Editorial Decision 01 March 2012

I finally received comments from one of the original referees being satisfied with the amount of revisions provided and thus supporting publication (see below).

Thus, the editorial office will soon be in touch with an official acceptance letter and necessary post-acceptance paperwork.

Please allow me to congratulate to your study.

P.S. As before, we would be grateful for the provision of original source data, (particularly electrophoretic gels and blots), to present un-cropped/unprocessed scans for the key data of published work. We would be grateful for one file per figure that combines this information. These will be linked online as supplementary "Source Data" files.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT:

Ref#1 remarks:

Our comments have been adequately addressed and I now can recommend publication.