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GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death

Lorenzo Sborgi, Sebastian Rühl, Estefania Mulvihill, Joka Pipercevic, Rosalie Heilig, Henning Stahlberg, Christopher J Farady, Daniel J Müller, Petr Broz, Sebastian Hiller

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Transaction Report:

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

1st Editorial Decision 02 June 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below. I am still waiting for input from a 4th referee but given the positive and constructive comments provided by the referees I would like to invite a revision. I will pass on referee's comments as soon as I receive them.

As you can see below, the referees find the analysis interesting and timely. They raise a number of specific concerns that I anticipate that you should be able to resolve in a good manner. Please note that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

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

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

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

Don't hesitate to contact me if you need further input from me.

REFEREE REPORTS

Referee #1:

In this paper the authors propose the mechanism of action of Gasdermin D, the effector protein for caspase 1 and caspase 11 driven pyroptosis, is to form a pore within the membrane. Using structural techniques they show that caspase 1-cleaved Gasdermin D forms pore-like structures and, when combined with their work using different pore-blocking molecules to prevent inflammasome driven-cell death, they present strong evidence to support their hypothesis.
There are some minor points to be addressed by the authors
1. The authors present a rather circumstantial argument to suggest the stoichiometry of the number of proteins in the Gasdermin D pore structure. This small section of the paper is currently unconvincing and either needs to be removed altogether (which will have little impact on the paper) or supported with quantitative biological data (structural or microscopic). It is very important, in the long term, to understand the stoichiometry of the Gasdermin D oligomeric structure (this relates to point 2 as well), but is not critical for this MS given the other data sets presented.
2. Why is there such a variation in the "pore" structures seen in the structural work? Is it artefactual given liposomes were used for this work? Will this be retained in cell membranes? The authors need to discuss this further in the MS.

Referee #2:

Pyroptosis, a form of necrotic death, plays an important role in the innate immune response. Recent landmark studies found that Gasdermin D [GSDMD] is required for pyroptosis, but the exact mechanism by which it mediates necrotic death was left unanswered. The manuscript by Sborgi et al. addresses this important question. Using biochemical studies, including subcellular fractionation and liposome-based pore forming assays, they conclude that GSDMD forms pores in the plasma membrane. This event leads to necrotic cell death. Although this is a very important and interesting finding, the manuscript is missing many essential controls.

Fig. 1: Sborgi et al. report that PEG3000 inhibits LDH release, but does not affect PI-influx (Fig. 1C and D), indicating that PEG3000 can block osmotic lysis-induced membrane rupture (secondary event), but not GSDMD pore formation (initial event). Indeed, the authors conclude that PEG3000 inhibited LDH release without blocking GSDMD-pore formation (p. 5). The rationale for the study and resulting conclusions regarding the release of IL-1b/LDH and PEG3000 are difficult to follow. It'll be most helpful to include a cartoon model that addresses which event: GSDMD mediated pore formation or membrane permeabilization by osmotic lysis? - is responsible for PI-Influx, LDH-, and IL-1b-release.

Fig 1C: Does the effect of PEG3000 reach statistical significance? It doesn't look like it, but in order for the reader to judge this, the number of independent experiments (n) and whether the error bars represent standard deviation or standard error of the mean need to be indicated in the figure legend. Fig 1C/D indicate that the molecular size of PEG3000 is 3.5 nm, while the text (P. 5) suggests 3.2 nm. These numbers should be reconciled.

In addition, Fig. 1C and D are missing PEG treatment alone (without dox) controls. Fig. 1E and F also lack the PEG treatment alone controls (without Salmonella). Hence, one is not sure if the signals (PI-influx and LDH release) are arising from pyroptosis or non-specific toxicity of PEGs.

Fig. 2: The authors need to provide much better evidence that the 30 kD band represents the N-terminus of GSDMD. The mere presence of 30 kD bands do not necessarily equate to the presence of the N-terminus of GSDMD. The bands could be Salmonella-derived protein that is nonspecifically stained by the GSDMD antibody. Western blotting Salmonella-infected GSDMD

KO macrophages (used in Fig S1) can easily address this significant concern. It is also important to show that the GSDMD antibody does not cross-react with a bacterial 30 kD protein by immune-blotting several ug of Salmonella extract in a single lane. Both controls, (i) Salmonella-infected GSDMD KO macrophages and (ii) Salmonella extracts, should be analyzed on the same blot with wild type macrophages functioning as a reference control. As the authors only rely on the presence of "30 kDa bands" to identify the N-terminus of GSDMD, the requested data are important. Also the catalogue number of the GSDMD antibody (Santa Cruz Biotechnology) should be provided.

Band patterns in Fig. 2B GSDMD WB (total lysate NS, 10, 20) are disturbingly different from a seemingly identical experiment: Fig. 2A (NS, 10, 20). Can the authors please provide an explanation as to why the banding patterns are different? In Fig. 2B, full-length GSDMD disappears. This result is quite unexpected, as so little of the N-terminal fragment of GSDMD is usually sufficient to kill cells. In other words, it seems unlikely that all of GSDMD would be cleaved prior to death. It is also improbable that cells selectively release full-length GSDMD into the supernatant without releasing the N-terminal fragment.

Caspase-1 WB panel (Fig. 2A) is missing a 37 kDa marker. Can the authors please check the original film and confirm that "37" is not in fact mislabeled as "28"?

In the fractionation study (Fig. 2B), please indicate how many cells were used per lane? If different numbers of cells were used for each fraction, is it fair to compare 30 kDa protein levels in different fractions?

I found the following conclusion particularly baffling: "Instead the majority of GSDMDNterm was found in the P150 fraction and partially also in the P10 fraction, correlating with the presence of the plasma membrane marker Na+K+ ATPase. (p. 6)" In Fig. 2B, however, the 30 kD band did not predominantly co-fractionate with Na+K+ ATPase (plasma membrane marker). Rather, the majority of the Na+K+ ATPase fractionated in P10, and the majority of p30, in the P150 fraction. This clearly indicates that p30 predominantly exists in a non-plasma membrane fraction, and contradicts the conclusion reached by the authors. Complicating matters is the fact that the mitochondrial membrane marker (VDAC) is present in the same fraction as the plasma membrane marker, Na+K+ ATPase (in P10).
If the authors want to definitely state that GSDMD pores are present in the plasma membrane, as indicated in the title and abstract, more precise localization assays are required. It may be best, at this stage to modify the claim of plasma membrane localization to membrane localization. This is more in keeping with the data.

The title of Figure 2 reads "localizes to plasma after inflammasome activation", rather than plasma membrane. Also, the legend for Fig. 2 has numerous mistakes and does not match the figure. For example, Fig. 2B is listed as the schematic for fractionation, but that is Fig. 2C. Fig. 2D states the presence of markers for HDAC1, GAPDH, etc, but these are only present in Fig. 2B

Fig. 3: The liposome pull down study (Fig. 3C) is missing an essential "no liposome" control for GSDMD+Casp1.

The gel in Fig 3B is cropped such that it is difficult to see the corresponding lanes that could contain "aggregated GSDMD-p30". The authors should include a less cropped gel that allows all lanes to be compared.

Fig. 4: Similar kinetics of WT and I104N processing should be confirmed. It is difficult to see the difference between red and orange in Fig. 4, I would suggest using very different colors for publication (for example, red for WT and blue for I104N).

Fig.5: Figure 5 A/B: Given that the dye release assays reveal saturation in as little as 3 minutes at GSDM concentrations of ~ 0.5 uM, it seems odd that the authors have incubated liposomes with 2.6 uM GSDM for 2 hours prior to acquiring cryo-EM micrographs. As stated, "large ruptures of the liposome structure were observed". It is not clear that the "assemblies" observed by cryo-EM are GSDM pores. To give the best chance of observing the pores the authors should repeat this study with shorter incubation times and perhaps lower GSDM concentrations.

The liposomes used in this study were derived from E. coli. Do the authors get the same results with liposomes that better mimic mammalian membranes?

Referee #4:

The authors have investigated the role of GSDMD in the disruption of the plasma membrane during pyroptosis. They show that ectopic expression of the cleaved form of the protein induces plasma membrane permeabilization and pyroptotic-like cell death. They also produce recombinant GSDMD and show that upon cleavage by caspase 1, the protein induces pore formation in liposomes and in supported lipid bilayers. Their findings are very interesting and contribute to progress in the field, but a number of issues should be addressed.

The association of N-terminal GSDMD with the plasma membrane is a key aspect of the model proposed. The fractionation experiments are not fully conclusive. There is no clear protein marker for the P150 fraction, as the Na+K+ATPase is as much there as in P0.7, and it is most abundant in P15. Binding of GSDMD to the plasma membrane upon cleavage should be verified by additional methods, like fluorescence microscopy of immunostained protein or GFP-fusion proteins.

In the experiments with PEGs, the authors should be able to test larger molecules if they decrease the concentration. In general, authors should be cautious with indirect evidences about the size of the pores. In the experiment with PEGs of different sizes, how did they estimate a pore size of about 3.2 nm based on the size of PEG3000? It seems they just took it from a reference, which took it from a reference... PEG sizes are hard to estimate since they (mainly bigger ones) can coil, fold and twist. Also the hydration degree could affect the size estimation.

The authors show that cleavage of GSDMD by caspase 1 induces the permeabization of large unilamellar vesicles. However, these experiments are performed at maximum concentrations of around 500nM and go down to 65nm, as estimated for the authors (Fig 4A). How do these concentrations relate to the intracellular abundance of cleaved GSDMD?

To clearly see the concentration dependent effect of GSDMD on liposome permeabilization, the authors should plot the % calcein release (at a given time point) as a function of protein concentration and include the corresponding error bars for each concentration point, to show statistics and reproducibility. This also applies to Fig4B and will help visualizing the differences in Fig 4C. In the experiments with dextrans, the authors should try with bigger dextrans that are not able to cross the pore. It is a pity that the liposome experiments are performed with lipid compositions that have nothing to do with the inner leaflet of the plasma membrane.

The whole calculations about the number of molecules required from a pore related to Fig 4A lack any kind of solid basis. First, the population of 100nm liposomes is a distribution of sizes with different amounts of lipids, so that an estimation of the concentration of liposomes is very rough. Second, the authors do not show if, for the 50% permeabilization they calculate at 65nM protein, only half of the vesicles are fully permeabilized (all-or-none mechanism) or all of them are permeabilized only to 50% (graded mechanism). Related to this, they do not show if the binding of the protein to the vesicles is homogeneous, or which fraction of the vesicles actually contains protein. They also do not know how many pores there are per vesicle. With such a simple analysis of the pore activity and so many unknowns, they should decide whether to perform the experiments required for such calculations or fully remove this part.

The EM images look strange and are difficult to interpret. It seems as it the membranes would have been fully destroyed. The authors should control that in those samples the vesicles have not been dissolved by dynamic light scattering. This control should also be made under the conditions of the calcein experiments, as this is the first time that the pore activity of cleaved GSDMD is reported.

Also, the rings that the authors are referring to should be better highlighted in the images. Are the membrane remnants completely covered with rings? Would it be possible to perform these experiments to a lower protein to lipid ratio where it is possible to detect liposomes?

The AFM images show nice rings and arc-like assemblies. The authors should perform a quantitative, statistical analysis of their size distribution and also quantify how many of these structures are associated with membrane defects. The size of the resulting pores should also be indicated. How do they explain that some rings are not associated with membrane pores? Are the membrane defects usually observed in supported bilayers covered by protein? How do they look?

The authors discuss the pore properties of cleaved GSDMD in the context of other pore forming toxins. While they highlight the differences with Bax, they forget to mention the similarities with this protein, as well as with other toxins like CDCs. For example, these arc structures have been now described for a number of pore forming proteins, as well as the formation of pores of variable sizes and the participation of lipids together with protein on the pore rims. All these evidences, as well as additional details about the mechanism of pore formation that can be driven from the experimental evidences reported in the manuscript, should be included in the discussion.

The authors show compelling evidence that cleaved GSDMD can form pores in model membranes. However, as long as there is no direct microscopic evidence of GSDMD pores on the plasma membrane of pyroptotic cells, the authors should tone down their statements throughout the manuscript regarding the functional role of GSDMD.

Minor points:
-On p6, references to Fig2B and 2C are mixed.
- In the caspase 1 cleavage experiments, a third band of lower molecular weight than 28 kDa appears in the SDS-PAGE gels that the authors associate with the C-terminal fragment of GSDMD. Is this band also present in the WB of Fig 2? The corresponding region should included in the figure.
-All experiments in Fig4 need corresponding negative controls as a reference.
-It is strange that the kinetics of release of FD-40 is faster than FD-20, is there an explanation for that?

Additional Correspondence 9 June 2016

I have not heard back from the 4th referee and at this stage don't think that I will. So lets go ahead with the three referee reports that we have on hand. Let me know if you have any questions regarding the revisions needed.

1st Revision - authors' response 19 June 2016

Referee #1:

In this paper the authors propose the mechanism of action of Gasdermin D, the effector protein for caspase 1 and caspase 11 driven pyroptosis, is to form a pore within the membrane. Using structural techniques they show that caspase 1-cleaved Gasdermin D forms pore-like structures and, when combined with their work using different pore-blocking molecules to prevent inflammasome driven-cell death, they present strong evidence to support their hypothesis.

There are some minor points to be addressed by the authors:

1. The authors present a rather circumstantial argument to suggest the stoichiometry of the number of proteins in the Gasdermin D pore structure. This small section of the paper is currently unconvincing and either needs to be removed altogether (which will have little impact on the paper) or supported with quantitative biological data (structural or microscopic). It is very important, in the long term, to understand the stoichiometry of the Gasdermin D oligomeric structure (this relates to point 2 as well), but is not critical for this MS given the other data sets presented.

We agree that this small section is not critical for the manuscript and have removed it as suggested.

2. Why is there such a variation in the "pore" structures seen in the structural work? Is it artefactual given liposomes were used for this work? Will this be retained in cell membranes? The authors need to discuss this further in the MS.

There is indeed substantial structural variation of the pore, including different observed states that represent assembly intermediates. Following the experience with other pore forming toxins, such variability is usually an intrinsic property of the pore-forming protein that is observed under many different lipid compositions, whereby the occurrences of different substates can additionally be modulated by the lipid content. In the revised manuscript, we describe the structural variability quantitatively by new AFM experiments and discuss the results, also in the light of other pore forming toxins.

Referee #2:

Pyroptosis, a form of necrotic death, plays an important role in the innate immune response. Recent landmark studies found that Gasdermin D [GSDMD] is required for pyroptosis, but the exact mechanism by which it mediates necrotic death was left unanswered. The manuscript by Sborgi et al. addresses this important question. Using biochemical studies, including subcellular fractionation and liposome-based pore forming assays, they conclude that GSDMD forms pores in the plasma membrane. This event leads to necrotic cell death. Although this is a very important and interesting finding, the manuscript is missing many essential controls.

We thank you the appreciation of the importance of the work. We have added the requested controls (see below).

Fig. 1: Sborgi et al. report that PEG3000 inhibits LDH release, but does not affect PI-influx (Fig. 1C and D), indicating that PEG3000 can block osmotic lysis-induced membrane rupture (secondary event), but not GSDMD pore formation (initial event). Indeed, the authors conclude that PEG3000 inhibited LDH release without blocking GSDMD-pore formation (p. 5). The rationale for the study and resulting conclusions regarding the release of IL-1b/LDH and PEG3000 are difficult to follow. It'll be most helpful to include a cartoon model that addresses which event: GSDMD mediated pore formation or membrane permeabilization by osmotic lysis? - is responsible for PI-Influx, LDH-, and IL-1b-release.

This is a very helpful suggestion. We have added such a cartoon model in Appendix Figure S1.

Fig 1C: Does the effect of PEG3000 reach statistical significance? It doesn't look like it, but in order for the reader to judge this, the number of independent experiments (n) and whether the error bars represent standard deviation or standard error of the mean need to be indicated in the figure legend.
Fig 1C/D indicate that the molecular size of PEG3000 is 3.5 nm, while the text (P. 5) suggests 3.2 nm. These numbers should be reconciled.

We have added the statistic information to the legend of Fig. 1 and corrected the typo in the text (to 3.5 nm).

In addition, Fig. 1C and D are missing PEG treatment alone (without dox) controls. Fig. 1E and F also lack the PEG treatment alone controls (without Salmonella). Hence, one is not sure if the signals (PI-influx and LDH release) are arising from pyroptosis or non-specific toxicity of PEGs.

Prior to our experiments, we have tested the toxicity of PEGs as a function their size. We found that PEG3000 and smaller PEGs were non-toxic to cells, while PEG6000 was cytotoxic. Therefore, only PEG600–3000 were used in our experiments. We now show these control data in Appendix Figure S1.

Fig. 2: The authors need to provide much better evidence that the 30 kD band represents the N-terminus of GSDMD. The mere presence of 30 kD bands do not necessarily equate to the presence of the N-terminus of GSDMD. The bands could be Salmonella-derived protein that is nonspecifically stained by the GSDMD antibody. Western blotting Salmonella-infected GSDMD KO macrophages

(used in Fig S1) can easily address this significant concern. It is also important to show that the GSDMD antibody does not cross-react with a bacterial 30 kD protein by immune-blotting several ug of Salmonella extract in a single lane. Both controls, (i) Salmonella-infected GSDMD KO macrophages and (ii) Salmonella extracts, should be analyzed on the same blot with wild type macrophages functioning as a reference control. As the authors only rely on the presence of "30 kDa bands" to identify the N-terminus of GSDMD, the requested data are important. Also the catalogue number of the GSDMD antibody (Santa Cruz Biotechnology) should be provided.

We fully agree that this is an important and necessary control experiment, which we have done before the outset of our experiments. We have added these control data (WT and GSDMD Kos infected with *Salmonella*) to Appendix Figure S2.
Indeed, we and others (Dick et al. Nat. Commun. 2016, Kayagaki et al. Nature 2015) have observed that the anti-GSDMD antibody from Sigma shows a strong cross-reactive band close to the size of the GSDMD-Nterm in samples treated with Gram-negative bacteria (*Salmonella* etc.). No such band occurs however for the antibody used in this work (Santa Cruz Biotech sc393656), as evidenced in Appendix Figure S2.

Band patterns in Fig. 2B GSDMD WB (total lysate NS, 10, 20) are disturbingly different from a seemingly identical experiment: Fig. 2A (NS, 10, 20). Can the authors please provide an explanation as to why the banding patterns are different? In Fig. 2B, full-length GSDMD disappears. This result is quite unexpected, as so little of the N-terminal fragment of GSDMD is usually sufficient to kill cells. In other words, it seems unlikely that all of GSDMD would be cleaved prior to death. It is also improbable that cells selectively release full-length GSDMD into the supernatant without releasing the N-terminal fragment.

We have also noted the difference in the extent of GSDMD processing between Figures 2A and 2B. However, although cells are infected with *Salmonella* in both cases, the sample processing is very different: In the experiment shown in Figure 2A, cells are taken up in a strong denaturing buffer (RIPA) which immediately denatures all proteins and prevents further caspase-1 activity. In contrast, for samples shown in Figure 2B cells are taken up in a mild non-denaturing buffer and processed for longer periods of time during the fractionation. Although protease inhibitors are added to this buffer, residual caspase-1 activity could account for additional processing of GSDMD in this procedure, leading to the observed differences in the level of GSDM processing.

Caspase-1 WB panel (Fig. 2A) is missing a 37 kDa marker. Can the authors please check the original film and confirm that "37" is not in fact mislabeled as "28"?

We double-checked that the 28 kDa marker was correctly labeled. We have now also added the 37 kDa marker.

In the fractionation study (Fig. 2B), please indicate how many cells were used per lane? If different numbers of cells were used for each fraction, is it fair to compare 30 kDa protein levels in different fractions?

In the fractionation experiments we have loaded equal protein amounts per lane. To allow for a comparison at equal cell number per lane as suggested, we have repeated the fractionation with equal amounts of cells ($2.8x10^6$ cells/lane) and show this now in Appendix Figure S2. This fractionation looks comparable and does not change the conclusion that the GSDMD-Nterm targets cellular membranes.
We would like to emphasize that we do not compare the amount of $GSDMD^{Nterm}$ across different fractions, but only between not treated and treated conditions within each fractionation.

I found the following conclusion particularly baffling: "Instead the majority of GSDMDNterm was found in the P150 fraction and partially also in the P10 fraction, correlating with the presence of the plasma membrane marker Na+K+ ATPase. (p. 6)" In Fig. 2B, however, the 30 kD band did not predominantly co-fractionate with Na+K+ ATPase (plasma membrane marker). Rather, the majority of the Na+K+ ATPase fractionated in P10, and the majority of p30, in the P150 fraction. This clearly indicates that p30 predominantly exists in a non-plasma membrane fraction, and contradicts the conclusion reached by the authors. Complicating matters is the fact that the

mitochondrial membrane marker (VDAC) is present in the same fraction as the plasma membrane marker, Na+K+ ATPase (in P10).
If the authors want to definitely state that GSDMD pores are present in the plasma membrane, as indicated in the title and abstract, more precise localization assays are required. It may be best, at this stage to modify the claim of plasma membrane localization to membrane localization. This is more in keeping with the data.

As noted in the manuscript, the Na+K+ ATPase can also be found to a large part in the p10 fraction since it is also present in the ER/Golgi. This has also been observed in other publications that have assessed the localization of the GSDMD-Nterm after pyroptosis (Ding et al. Nature 2016).
We have tried different methods to further confirm plasma membrane localization of the GSDMD-Nterm, such as microscopy. But these approaches proved to be experimentally difficult due to the fact that the GSDMD-Nterm permeabilizes the plasma membrane and this results in rapid lysis of the host cell.
Overall, we agree with the referee and have toned down our claims to state that GSDMD localized to “cellular membranes”.

The title of Figure 2 reads "localizes to plasma after inflammasome activation", rather than plasma membrane. Also, the legend for Fig. 2 has numerous mistakes and does not match the figure. For example, Fig. 2B is listed as the schematic for fractionation, but that is Fig. 2C. Fig. 2D states the presence of markers for HDAC1, GAPDH, etc, but these are only present in Fig. 2B

Thank you for pointing out this mislabeling and the typos, which we have corrected.

Fig. 3: The liposome pull down study (Fig. 3C) is missing an essential "no liposome" control for GSDMD+Casp1.

This control is present in Fig 3B (2nd lane).

The gel in Fig 3B is cropped such that it is difficult to see the corresponding lanes that could contain "aggregated GSDMD-p30". The authors should include a less cropped gel that allows all lanes to be compared.

We have decreased the cropping of Fig 3B.

Fig. 4: Similar kinetics of WT and I104N processing should be confirmed. It is difficult to see the difference between red and orange in Fig. 4, I would suggest using very different colors for publication (for example, red for WT and blue for I104N).

We have changed the coloring as suggested and show the comparison of caspase cleavage kinetics of GSDMD wild type vs. GSDMD I104N in Appendix Fig S4A-B.

Fig.5: Figure 5 A/B: Given that the dye release assays reveal saturation in as little as 3 minutes at GSDM concentrations of ~ 0.5 uM, it seems odd that the authors have incubated liposomes with 2.6 uM GSDM for 2 hours prior to acquiring cryo-EM micrographs. As stated, "large ruptures of the liposome structure were observed". It is not clear that the "assemblies" observed by cryo-EM are GSDM pores. To give the best chance of observing the pores the authors should repeat this study with shorter incubation times and perhaps lower GSDM concentrations.

We initially acquired cryo-EM micrographs at the same protein/lipid molar concentrations as we had used in the liposome leakage assay, however even at the higher GSDMD concentrations of ~ 0.5 uM, the number of GSDMD pores formed in the liposome membrane was not sufficient for a clear visualization of pores by Cryo-EM. Note that a single pore per liposome is sufficient to allow full dye release in the experiments of Fig. 4, whereas for reliable observation in cryo-EM, a substantially larger number of pores per liposome is necessary.
Following the referee’s suggestion we have now acquired additional cryo-EM micrographs with low (1/1000), medium (1/500) and high (1/100) protein/lipid molar ratios (Fig 5A-D). We have also visualized the formation of GSDMD pores in liposome membranes over time at a molar ratio of 1/100 protein/lipid (Appendix Fig S6A-F).

The liposomes used in this study were derived from E. coli. Do the authors get the same results with liposomes that better mimic mammalian membranes?

Following the referee suggestion, we performed additional liposome leakage experiments using a mammalian lipid extract for liposome preparation (total lipid extract from rat brain). In full agreement with the experiments in E. coli lipids, GSDMD is also found to form membrane pores in a concentration-dependent manner in these liposomes. The new results are reported in Fig 4C.

Referee #4:

The authors have investigated the role of GSDMD in the disruption of the plasma membrane during pyroptosis. They show that ectopic expression of the cleaved form of the protein induces plasma membrane permeabilization and pyroptotic-like cell death. They also produce recombinant GSDMD and show that upon cleavage by caspase 1, the protein induces pore formation in liposomes and in supported lipid bilayers. Their findings are very interesting and contribute to progress in the field, but a number of issues should be addressed.

Thank you for your encouraging comments.

The association of N-terminal GSDMD with the plasma membrane is a key aspect of the model proposed. The fractionation experiments are not fully conclusive. There is no clear protein marker for the P150 fraction, as the Na+K+ATPase is as much there as in P0.7, and it is most abundant in P15.

Please see our response to similar comments by referee #2 above.

Binding of GSDMD to the plasma membrane upon cleavage should be verified by additional methods, like fluorescence microscopy of immunostained protein or GFP-fusion proteins.

We have intensely tried fluorescence microscopy as suggested, either with antibodies for the endogenous GSDMD or with cell lines expressing HA-tagged versions. Below we show an example of these experiments, in which we have used *Gsdmd*-deficient macrophages expressing HA-hGSDMD that we either left uninfected or infected with *S. typhimurium* to induce inflammasome activation. Cells were stained with anti-HA antibodies to visualize HA-hGSDMD and with fluorescently-labelled Wheat Germ Agglutinin (WGA) to visualize the plasma membrane. Our data show that WT GSDMD cannot be detected in sufficient concentrations at the PM to allow a definitive conclusion about its localization, even though we can occasionally find cells in which HA staining co-localizes with WGA.
The low levels of GSDMD at the PM might be due to cell death/lysis, which most likely prevents further recruitment of GSDMD-Nterm to the PM. Indeed a recent publication by Feng Shao's lab confirms these results for the WT protein (Ding et al. Nature 2016).

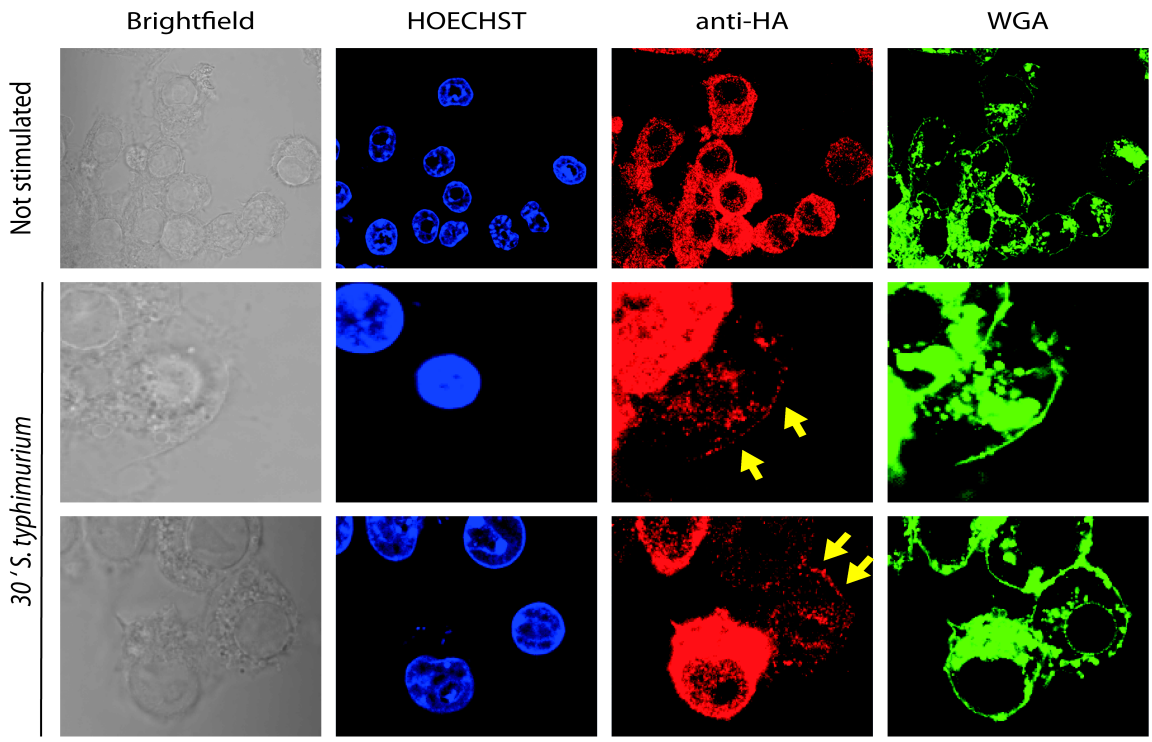

Figure: Mouse immortalized macrophages expressing HA-hGSDMD were left untreated or infected with *S. typhimurium* as indicated. Cells are stained with Hoechst (blue), anti-HA (red) and WGA (green). Arrows indicate co-localization of WGA signal with anti-HA signal.

In the experiments with PEGs, the authors should be able to test larger molecules if they decrease the concentration. In general, authors should be cautious with indirect evidences about the size of the pores. In the experiment with PEGs of different sizes, how did they estimate a pore size of about 3.2 nm based on the size of PEG3000? It seems they just took it from a reference, which took it from a reference... PEG sizes are hard to estimate since they (mainly bigger ones) can coil, fold and twist. Also the hydration degree could affect the size estimation.

Thank you. We now cite the original reference, which describes how the pore size can be estimated.

The authors show that cleavage of GSDMD by caspase 1 induces the permeabilization of large unilamellar vesicles. However, these experiments are performed at maximum concentrations of around 500nM and go down to 65nm, as estimated for the authors (Fig 4A). How do these concentrations relate to the intracellular abundance of cleaved GSDMD?

Currently, we do not have a quantitative estimate of the intracellular abundance of cleaved GSDMD. Although it should be technically possible to estimate the concentration of full-length GSDMD before Caspase-mediated activation, it seems highly challenging to reliable quantify the cellular concentration of cleaved GSDMD, since upon GSDMD cleavage cells lyse rapidly and loose their cytosolic content. For sure, this is beyond our technical scope for this work.

To clearly see the concentration dependent effect of GSDMD on liposome permeabilization, the authors should plot the % calcein release (at a given time point) as a function of protein concentration and include the corresponding error bars for each concentration point, to show statistics and reproducibility. This also applies to Fig4B and will help visualizing the differences in Fig 4C.

This is a good suggestion and we have added these plots (Fig 4D and 4F).

In the experiments with dextrans, the authors should try with bigger dextrans that are not able to cross the pore.

We have done the experiments with dextran-linked dye in all sizes that were available to us.

It is a pity that the liposome experiments are performed with lipid compositions that have nothing to do with the inner leaflet of the plasma membrane.

Thanks for pointing this out (see also a similar comment by referee #2 above). We have now performed additional liposome leakage assays using total lipid extracts from rat brain that better mimics the composition of the mammalian plasma membrane. As with the other lipid compositions that we have tested, GSDMD is forms membrane pores in a concentration-dependent manner. The results are reported in the new Fig. 4C.

The whole calculations about the number of molecules required from a pore related to Fig 4A lack any kind of solid basis. First, the population of 100nm liposomes is a distribution of sizes with different amounts of lipids, so that an estimation of the concentration of liposomes is very rough. Second, the authors do not show if, for the 50% permeabilization they calculate at 65nM protein, only half of the vesicles are fully permeabilized (all-or-none mechanism) or all of them are permeabilized only to 50% (graded mechanism). Related to this, they do not show if the binding of the protein to the vesicles is homogeneous, or which fraction of the vesicles actually contains protein. They also do not know how many pores there are per vesicle. With such a simple analysis of the pore activity and so many unknowns, they should decide whether to perform the experiments required for such calculations or fully remove this part.

See also a similar comment by referee #1. Since this part is not critical for the manuscript, we have removed it from the revised manuscript.

The EM images look strange and are difficult to interpret. It seems as it the membranes would have been fully destroyed. The authors should control that in those samples the vesicles have not been dissolved by dynamic light scattering. This control should also be made under the conditions of the calcein experiments, as this is the first time that the pore activity of cleaved GSDMD is reported.

We performed time-course dynamic light scattering measurements to assess the liposome diameter for the two suggested conditions. The results are reported in Appendix Fig S6F, showing that in both cases liposomes are still intact after two hours of incubation. Interestingly, in conditions with high protein/lipid molar ratios the average diameter slightly increases with time, probably due to the high number of pores on the liposome surface.

Also, the rings that the authors are referring to should be better highlighted in the images. Are the membrane remnants completely covered with rings? Would it be possible to perform these experiments to a lower protein to lipid ratio where it is possible to detect liposomes?

We have highlighted the rings better by arrows. We have also performed the cryo-EM experiments at a lower protein to lipid ratio (Fig 5A-D).

The AFM images show nice rings and arc-like assemblies. The authors should perform a quantitative, statistical analysis of their size distribution and also quantify how many of these structures are associated with membrane defects. The size of the resulting pores should also be indicated.

We have performed additional AFM experiments to provide quantitative and qualitative measurements of the GSDMD assemblies. The analysis is reported in the new Fig 6F-H. It shows that GSDMD-Nterm oligomers assembling either arcs, slits or rings can form transmembrane pores, but that not all do so (Fig 6F,G). The distribution of the diameter of the rings formed by GSDMD-Nterm oligomers shows a variability of the size of the rings forming transmembrane pores (Fig 6H).

How do they explain that some rings are not associated with membrane pores? Are the membrane defects usually observed in supported bilayers covered by protein? How do they look?

Membrane-associated states without pore formation are typically observed for pore-forming toxins (see answer to next question below). The reviewer also asks how membrane defects observed in

supported lipid membrane (SLM) usually look. If we adsorb liposomes made from *E. coli* polar lipids extract to the mica support they show no arc-, slit- or ring-like structures (Mulvihill, van Pee et al., 2015). Only if we incubate the liposomes with GSDMD we could observe arc-, slit- or ring-like structures. We have now added this information to the manuscript (see revised Methods).

The authors discuss the pore properties of cleaved GSDMD in the context of other pore forming toxins. While they highlight the differences with Bax, they forget to mention the similarities with this protein, as well as with other toxins like CDCs. For example, these arc structures have been now described for a number of pore forming proteins, as well as the formation of pores of variable sizes and the participation of lipids together with protein on the pore rims. All these evidences, as well as additional details about the mechanism of pore formation that can be driven from the experimental evidences reported in the manuscript, should be included in the discussion.

We fully agree with the referee and have expanded our discussion accordingly. Indeed, some of the rings (or GSDMDNterm oligomers in general) are not associated with the formation of transmembrane pores, as shown and statistically analyzed in Fig 6, and this observation may appear surprising at first glance. For several different cholesterol-dependent cytolysins (CDCs) it has however been reported that these pore forming toxins (PFTs) bind to lipid membranes as oligomers forming arcs, slits, and pores and that each of these oligomeric structures has a certain probability to form transmembrane pores (Leung, Dudkina et al. 2014, Mulvihill, van Pee et al. 2015, Podobnik, Marchioretto et al. 2015, Sonnen, Plitzko et al. 2014, reviewed in Hodel, Leung et al. 2016). Furthermore, it has been reported that oligomeric CDCs (arcs, slits or rings) can dynamically reassemble on the membrane surface to form larger pores. By time-lapse AFM it has been also observed that with the incubation time (can last up to 24 hours and longer in some systems) these oligomers transit from the prepore state to the pore state, which form transmembrane pores. In our revision we now discuss these issues in more detail and cite the relevant publications reporting these transitions from the prepore to the pore state.

The authors show compelling evidence that cleaved GSDMD can form pores in model membranes. However, as long as there is no direct microscopic evidence of GSDMD pores on the plasma membrane of pyroptotic cells, the authors should tone down their statements throughout the manuscript regarding the functional role of GSDMD.

We have modified the text accordingly.

Minor points:
-On p6, references to Fig2B and 2C are mixed.

This mistake has been corrected

- In the caspase 1 cleavage experiments, a third band of lower molecular weight than 28 kDa appears in the SDS-PAGE gels that the authors associate with the C-terminal fragment of GSDMD. Is this band also present in the WB of Fig 2? The corresponding region should included in the figure.

The C-terminal fragment of GSDMD is also expected to be present in pyroptotic cells (Figures 1+2). The antibody (sc-393656) was raised against an epitope mapping within the N-terminal domain (amino acids 169–188) of GSDMD. The Western Blots thus detect only full-length GSDMD and the cleaved N-terminal fragment.

-All experiments in Fig4 need corresponding negative controls as a reference.

For each of the experiments, three negative controls are shown in the corresponding Appendix Figure 5A-I.

-It is strange that the kinetics of release of FD-40 is faster than FD-20, is there an explanation for that?

It is reported on the product specification of the Fluorescein isothiocyanate–dextran (Sigma) that dextrans with MW greater than 10 kDa behave as highly branched molecules with very different

shape and symmetry. Those properties could affect the kinetics of the dye release from a higher concentrated compartment and explain this inconsistency.

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Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #2 and #4 and as you can see below they appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

REFEREE REPORTS

Referee #2:

All concerns have been adequately addressed.

Referee #4:

The authors have addressed the reviewer's comments adequately.

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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The data shown in figures should satisfy the following conditions:

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- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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