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Direct binding of polymeric GBP1 to LPS disrupts bacterial cell envelope functions

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Authors' Response to Reviewers 6th Mar 2020

Dear Jörn,

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

As you can see from the comments the referees find the analysis interesting and are supportive of publication in The EMBO Journal. They raise a number of good points that I would like to ask you to address in a revised version. I am happy to discuss the raised points further and maybe it would be most helpful to do so via phone or skype. I will contact you in the next few days to discuss this further. I am also aware that with the current Covid-19 situation that and lab closures that carrying out experimental revisions are not so straightforward. We can discuss this further in the call.

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:
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Thank you for the opportunity to consider your work for publication. I look forward to discussing the revisions further with you

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

In this manuscript, Kutsch and colleagues, by using in-vitro binding assay, found that farnesylated human GBP1 could bind directly to LPS and transform into a protein coat encapsulating gram-negative bacteria, using intracellular Shigella flexneri as a model. In this process, human GBP1 first assembles into supramolecular polymers in a GTP-hydrolysis dependent manner. The GBP1 polymer, appearing as granular structures, then bind to LPS on the bacterial surface, and the O-antigen units in LPS further drives the transition of surface-docked GBP1 polymer into bacteria-encasing protein coats. As a result of this, the hGBP1 protein coat disrupts the O-antigen barrier function and renders lipid A more accessible to the LPS cytosolic receptor caspase-4. In addition, they also show that disruption of the O-antigen functioning by hGBP1 coating of intracellular S. flexneri leads to inhibition of bacterial motility. Overall, this is a high-quality study; the experiments are well performed and the data are solid and robust, which provides a new perspective to understand the mechanism of action for the antimicrobial function of human GBP1. However, there are still a few gaps and shortcomings that need to be addressed in order to firmly establish the model proposed by the authors.

A previous study (Ref 20) published in EMBO Journal has already suggested that LPS is the direct target of GBP and the action of GBP could promote LPS activation of caspase-11 during bacterial infection. The authors should credit more the previous study and explains in more details about the background of the current study in the introduction session. Also, "Our studies assign the first biological function to hGBP1 polymerization by demonstrating that this process is essential for hGBP1 binding to LPS and attachment to the surface of gram-negative bacteria" on Page 16 in the discussion session should be modified as it is not the first time to indicate LPS as the target.

The authors seem to indicate that hGBP1 on its own, among the GBP family, uniquely targets LPS on the bacterial surface. One major issue I have is that why hGBP1 is so special? hGBP1, 2 and 5 all bear the prenylation modification and share the similar GTP-binding/hydrolysis properties. The authors should address why other GBPs do not possess the biochemical function demonstrated with hGBP1 in the manuscript. In murine GBP2, the hGBP1 homolog, there is no polybasic motif adjacent to the C-terminal farnesyl site. Does mGBP2 share a similar function of hGBP1 in LPS binding-mediated coating of intracellular S. flexneri?

The authors set up a robust in vitro assay to mimic the GBP recruitment to cytosolic bacteria. We can clearly see the colocalization of GBP and bacteria or LPS in this system. To prove the direct binding between GBP and LPS, the authors also showed a dot-blot assay (Fig. S4B) and a competition experiment (Fig. 4E). These data are of suggestive nature to demonstrate the direct interaction between hGBP1 and LPS. Can the authors purify the supramolecular form of hGBP1 and then perform the in vitro Pull-down and SPR assays to show the direction interaction? At the LPS side, is the monomer or micelle form of LPS that can bind to hGBP1?

There is no evidence to suggest that direct binding of hGBP1 to LPS is responsible to its recruitment to the bacteria. In fact, we still don't know how the hGBP1 supramolecule is docked onto the surface of S. flexneri. Apparently, O-antigen of LPS is not responsible for the initial binding of hGBP1 to the bacteria because hGBP1 was docked to the rfaL mutant as efficiently as to wild-type S. flexneri (Fig. 5B). To illustrate this, a series of S. flexneri mutants in LPS biosynthesis pathway should be assayed in the in vitro GBP recruitment assay.

In Fig. 3B, the authors indicate that asterisks mark polymeric structures of hGBP1 that will fuse with bacterial surfaces, but to me, I can only see the protruding buds on the bacterial surface. How do the authors know that those protruding structures are indeed hGBP1 polymers? Can the authors do an immunogold antibody staining or other assays to confirm this? Ideally, a Cryo-EM assay will be really cool as it may reveal further structure information about the hGBP1 polymer.

In Fig. 5B, hGBP1 protein is docked to the rfaL mutant of S. flexneri at 10 min but disappeared at late time (60 min). Why is that? Also,

Is there any difference between the wild-type and R584-586A mutant hGBP1 in promoting the non-canonical inflammasome during bacterial infection?

The original study (PMID: 21551061) shows that many GBPs can also target Listeria monocytogenes and Mycobacterium bovis BCG (Mb BCG) that do not have LPS. What is the authors' explanation for this discrepancy?

By a critical attitude, the current data have not firmly established that LPS is directly and fully responsible for recruiting hGBP1 to the bacteria. It can not be ruled out that other bacterial molecules may mediate initial binding of hGBP1 polymer to the bacteria or contribute to the subsequent encapsulating process. The authors should weaken their statement and discuss the alternative possibilities.

What is the difference between LPS (O111:B4) and LPS (O55:B5) and why the former is weaker than the latter in hGBP1 anchoring S. flexneri?

Referee #2:

hGBP1 play important role in Cell-autonomous response during infection. It co-localizes with intracellular gram-negative bacterial pathogens, facilitates bacterial killing, promotes activation of the lipid A sensor caspase-4, and blocks actin-driven dissemination of the enteric pathogen Shigella. In this study, Kutsch et al explore how hGBP1 achieve all these functions. They show that hGBP1 Polymerize and binds directly to LPS and to gram-negative bacteria. Then, form a stable hGBP1 protein coat on bacteria expressing O-antigen. This disrupts the O antigen barrier, thereby unmasking lipid A, eliciting caspase-4 recruitment, enhancing antibacterial activity of polymyxin B, and disturbing O-antigen-dependent function of the Shigella virulence protein IcsA. This study of great importance expands our knowledge about the mechanism of function of GBPs in defense, and warrant a publication. Experiments are well executed, and I don't see need for further experiment. However, a have a few comments that I was hoping the authors could include to solidify some of the key point in the study.
1. The authors report based on microscopy examination that hGBP1 polymerize in presence of GTP. It will be nice if the authors show that by running a Native gel to determine the size and the different oligomeric hGBP1 formed.
2. The authors report that hGBP1 directly bind LPS. If it is possible to quantify the Specific binding affinity of hGBP to LPS by surface plasmon
3. Shigella Flexeneri ospc3 antagonize the function of hGBP1 to allow IcsA driven motility. yet other cytosolic bacteria such as Salmomella Sifa or Burkholderia don't secrete similar virulence factor. Does actin motility is disrupted in these species as well by the action of hGBP1. It will be very interesting if the author would show if hgbp1coating each of these bacteria disrupt their virulence factor that mediate actin motility.

Referee #3:

It has previously been shown that hGBP1 facilitates bacterial killing, promotes activation of the LPS sensor Caspase-4, and blocks actin-based bacterial motility. However, the mechanism by which a single protein can have such a variety of effects was unknown. This manuscript provides a compelling mechanism by which hGBP1 can have such diverse effects.
Kutsch et al. provide evidence that hGBP1 polymers functions as an LPS binding and LPS clustering surfactant thereby disrupting the outward-facing LPS layer. The initial binding of hGBP1 polymers to LPS was largely independent of O-antigen. However, the transition from bound polymer to evenly coated bacteria was dependent on O-antigen. hGBP1 disruption of the O-antigen layer made lipid A more exposed thereby promoting the recruitment of caspase-4 and enhancing the efficacy of the antimicrobial polymyxin B. The likely increased membrane fluidity in the presence of hGBP1 also affected unipolar IcsA localization and thereby actin motility. A convincing model describing these results was presented in Figure S7
Overall, the manuscript was well written, the data was convincing, and experiments well performed.

Major comment:
For consistency it would be good if the authors can quantify the data in all their images. For example, in Fig1C and 2C (and several other figures) no quantification is provided while in Fig. 3C and other figures the data is quantified.

Minor comments:
In Figure 4 the results with the smooth vs. rough LPS were not discussed in the result section discussing this figure but only mentioned later when results of Figure 5 are discussed.

1st Authors' Response to Reviewers 9th Apr 2020

Referee #1:

In this manuscript, Kutsch and colleagues, by using in-vitro binding assay, found that farnesylated human GBP1 could bind directly to LPS and transform into a protein coat encapsulating gram-negative bacteria, using intracellular Shigella flexneri as a model. In this process, human GBP1 first assembles into supramolecular polymers in a GTP-hydrolysis dependent manner. The GBP1 polymer, appearing as granular structures, then bind to LPS on the bacterial surface, and the O-antigen units in LPS further drives the transition of surface-docked GBP1 polymer into bacteria-encasing protein coats. As a result of this, the hGBP1 protein coat disrupts the O-antigen barrier function and renders lipid A more accessible to the LPS cytosolic receptor caspase-4. In addition, they also show that disruption of the O-antigen functioning by hGBP1 coating of intracellular S. flexneri leads to inhibition of bacterial motility. Overall, this is a high-quality study; the experiments are well performed and the data are solid and robust, which provides a new perspective to understand the mechanism of action for the antimicrobial function of human GBP1. However, there are still a few gaps and shortcomings that need to be addressed in order to firmly establish the model proposed by the authors.

We would like to thank the reviewer for their very positive assessment of our work and the nice summary confirming that our data demonstrate direct binding of hGBP1 to LPS and to bacteria, which results in the breakdown of the O-antigen barrier and thus provides a molecular mechanism for the numerous biological functions previously attributed to hGBP1. Our extensive dissection of the hGBP1-LPS binding reaction and the transition from docked hGBP1 polymers to a stable hGBP1 protein coat encapsulating bacteria enabled us to create a model for the different molecular events that are taking place during this process. Of course, no model is ever perfect and we are sure adjustments to this model will have to be made as we and other investigators explore this binding / coating process further. Indeed, our studies raise many interesting questions, e.g. how LPS modifications impact this binding / coating reaction. Therefore, we believe there is much that needs to be done in the future. However, we affirm our conviction that the data presented in this MS strongly support all the cornerstones of the model that we have laid out here.

A previous study (Ref 20) published in EMBO Journal has already suggested that LPS is the direct target of GBP and the action of GBP could promote LPS activation of caspase-11 during bacterial infection. The authors should credit more the previous study and explains in more details about the background of the current study in the introduction session.

The reviewer raises an excellent point in that several previous studies implicated a potential role for GBPs in direct LPS sensing. The first study to suggest direct sensing of LPS by GBPs was conducted by our own group (Pilla et al. 2014 PMID: 24715728). Pilla et al. demonstrated that mouse GBPs promote caspase-11 activation in response to LPS transfection. A second study published at the same time by the Broz group argued that GBPs had no impact on caspase-11 activation in response to LPS transfection (Meunier et al. 2014, Fig. 1e). However, subsequent studies including work from the Broz group itself (Ref 20: Santos et al. 2018 PMID: 29459437) confirmed the original observation made by Pilla et al. (Lagrange et al. 2018 PMID: 29339744; Santos et al. 2018 PMID: 29459437). A follow-up study from our own lab (Finethy et al. 2017 PMID: 28974614) demonstrated that GBPs promoted caspase-11 activation in response to bacterial outer membrane vesicles (OMVs) – LPS-studded vesicles released by bacteria – and Finethy et al. further demonstrated that mouse Gbp2 and LPS co-localized inside OMV-treated macrophages (Finethy et al. 2017 PMID: 28974614, Fig. S3). Santos et al. 2018 (ref 20) showed that mouse Gbp5 co-localized with transfected LPS. While these studies may have suggested LPS sensing by mouse Gbp2 or mouse Gbp5, they did not provide evidence for direct binding of GBPs to LPS. We are now discussing the previous studies in more detail on page 21 of the revised manuscript.

Also, "Our studies assign the first biological function to hGBP1 polymerization by demonstrating that this process is essential for hGBP1 binding to LPS and attachment to the surface of gram-negative

bacteria" on Page 16 in the discussion session should be modified as it is not the first time to indicate LPS as the target.

We altered the text so as to avoid any explicit priority claims. Nonetheless, to our knowledge no previous report assigned a biological function to GBP polymerization. Also, previous studies (Pilla et al. 2014, Finethy et al. 2017, Santos et al. 2018, etc.) did not demonstrate that GBPs bind to LPS but only provided circumstantial evidence in support of the hypothesis that we have shown here to be correct.

The authors seem to indicate that hGBP1 on its own, among the GBP family, uniquely targets LPS on the bacterial surface. One major issue I have is that why hGBP1 is so special? hGBP1, 2 and 5 all bear the prenylation modification and share the similar GTP-binding/hydrolysis properties. The authors should address why other GBPs do not possess the biochemical function demonstrated with hGBP1 in the manuscript.

The reviewer raises an excellent question that we already answered in a previous publication (Piro et al. 2017 PMID: 29233899) where we demonstrated that the transfer of the hGBP1 polybasic motif (PBM) is sufficient to equip hGBP2 with the ability to associate with bacteria inside cells (Piro et al. 2017 PMID: 29233899, Fig. 2C-D). In other words what is unique about hGBP1 (vs hGBP2 and maybe also vs hGBP5) is its PBM. We are attaching some data below that recapitulate our findings previously published by Piro et al. and we have stressed these previous observations more emphatically on page 8 of the revised manuscript. In the present study we further demonstrate the importance for the PBM in enabling sustained binding to bacteria *in vitro.*

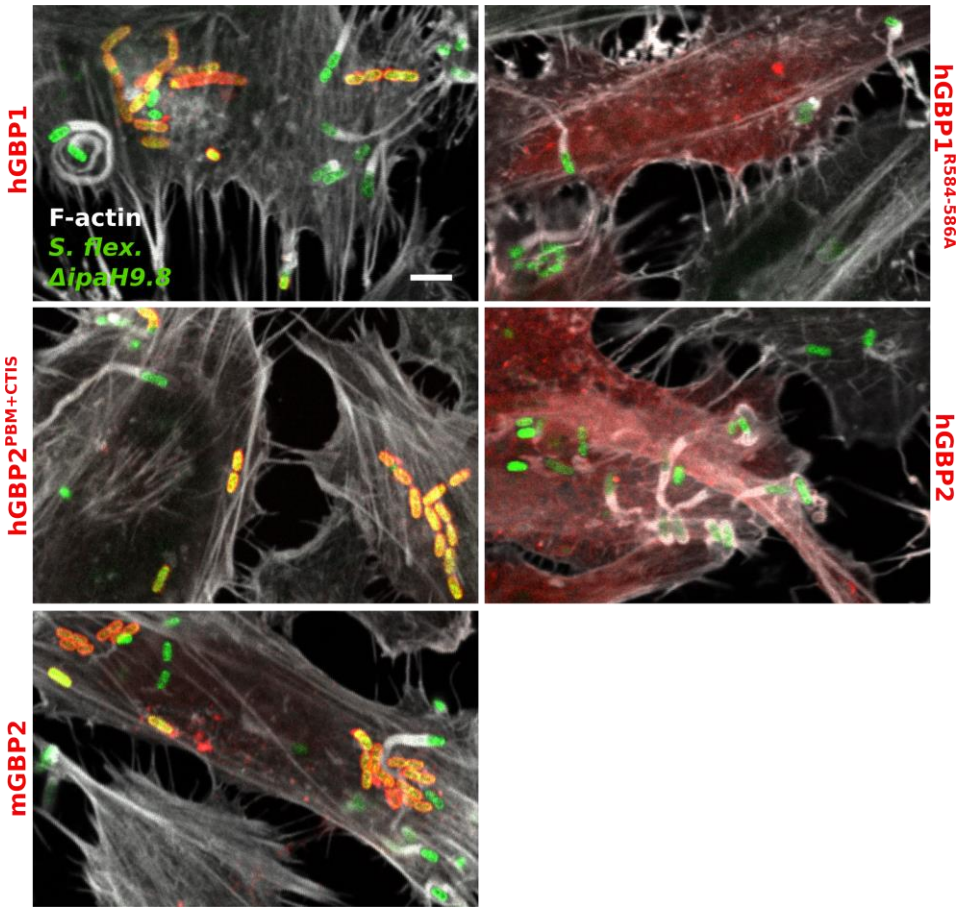
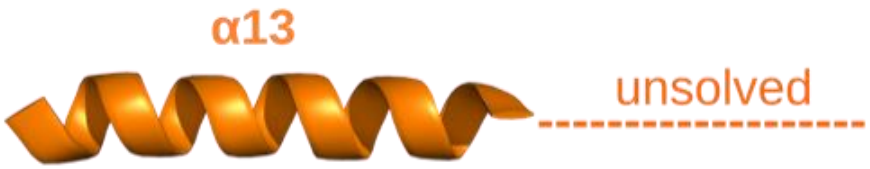


Figure Legend. Ectopically expressed hGBP1 but not hGBP2 co-localizes with *S. flexneri* (bacteria in green; proteins in red) in Hela-GBP1-KO cells. A hGBP1 mutant lacking the PBM (R584-586A) fails to co-localize with *S. flexneri*, whereas hGBP2 equipped with the hGBP1 PBM acquired the ability to bind to *S. flexneri* inside Hela-GBP1-KO cells. Collectively, these data demonstrate that the C-terminal PBM is the unique feature of hGBP1 which enables it to recognize cytosolic gram-negative bacteria (also see Piro et al. 2017 PMID: 29233899). Ectopically expressed mouse Gbp2 also co-localizes with *S. flexneri* in Hela-GBP1-KO cells, suggesting that mGBP2 has the ability to recognize gram-negative bacteria directly, similar to hGBP1. However, whether or not mGbp2 binds to bacteria directly needs to be tested further through *in vitro* assays.

In murine GBP2, the hGBP1 homolog, there is no polybasic motif adjacent to the C-terminal farnesyl site. Does mGBP2 share a similar function of hGBP1 in LPS binding-mediated coating of intracellular S. flexneri?

We agree with the reviewer that specific GBP orthologs expressed in other mammalian species including the mouse are likely to have LPS-binding properties similar to hGBP1 and we agree that mouse Gbp2 is a strong candidate to fulfill such a function. Indeed, we previously showed that mouse Gbp2 co-localizes with LPS in mouse macrophages treated with bacterial OMVs (Finethy et al. 2017 PMID: 28974614) and we can show that ectopically expressed mGbp2 co-localizes with *S. flexneri* in human HeLa GBP1-KO cells (see data embedded above in this document). However, the reviewer is mistaken in stating that mGbp2 does not have a C-terminal polybasic motif. Polybasic motifs don't have a clearly defined consensus but rather consist of a stretch of several positively charged residues. Mouse Gbp2 has 3 positively charged residues immediately adjacent to its CaaX box (whereas hGBP2 only has 2 positively charged residues at its C-terminus and also a five amino acid spacer between its CaaX box and the most proximal positively charged residue) – see alignment below. We think it is likely that the lysines in the C-terminus of mGbp2 are important for its function and will explore this further in future studies. However, such studies go beyond the scope of this work, which provides a detailed account of the mechanism by which human GBP1 binds to LPS and to gram-negative bacteria.

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hGBP1 FQKESRIMKNEIQDLQTKMRRR-KACTIS 592
hGBP2 FENESKRLQKDIWDIQMRSKSLEPICNIL 591
mGBP2 FENESKKLIREIDTLKQNK--SSGKCTIL 589
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The authors set up a robust in vitro assay to mimic the GBP recruitment to cytosolic bacteria. We can clearly see the colocalization of GBP and bacteria or LPS in this system. To prove the direct binding between GBP and LPS, the authors also showed a dot-blot assay (Fig. S4B) and a competition experiment (Fig. 4E). These data are of suggestive nature to demonstrate the direct interaction between hGBP1 and LPS. Can the authors purify the supramolecular form of hGBP1 and then perform the in vitro Pull-down and SPR assays to show the direction interaction?

As the referee stated, we do provide those two different *in vitro* assays to show the direct interaction between LPS and hGBP1 (*in vitro* binding monitored by confocal microscopy / time-lapse microscopy and dot-blot assays). As a third method to demonstrate direct interactions between LPS and hGBP1

we use turbidity assays which demonstrate that LPS accelerates hGBP1 polymerization kinetics, an observation that is line with other dynamin-like proteins that polymerize when complexed with specific lipid substrates. We don't see how pull-down experiments would provide much additional information since the dot-blot assay used by us is biochemically very similar to a pull down assay. We agree that SPR and other methods to define binding affinities are excellent follow-up studies (see also comments to referee 2). Unfortunately, we are currently not able to conduct these studies due to the shutdown of our laboratories during this worldwide epidemic. Moreover, these affinity measurements, although desirable, are not required to support the conclusions of our study. Overall, we provide overwhelming evidence that hGBP1 binds to LPS directly.

At the LPS side, is the monomer or micelle form of LPS that can bind to hGBP1?
The reviewer raises an interesting point. LPS is a molecule with a soluble moiety (polysaccharide of O-antigen, inner- and outer core) and a hydrophobic moiety (fatty acids of lipid A). Because of its amphipathic properties LPS in aqueous solutions exists in the form of micelles unless deaggregating LPS-binding proteins extract LPS monomers. We can therefore safely assume that LPS in our experiments exists initially as micelles. It is interesting to speculate that hGBP1 is able to disrupt the integrity of these micelles as it does with bacterial membranes and it might as a consequence bind to monomeric LPS once integrated into the micelles. We now discuss this interesting point raised by the reviewer further on page 20 and emphasize the amphipathic nature of LPS and its propensity to from micelles in aqueous solutions on page 11.

There is no evidence to suggest that direct binding of hGBP1 to LPS is responsible to its recruitment to the bacteria. In fact, we still don't know how the hGBP1 supramolecule is docked onto the surface of S. flexneri. Apparently, O-antigen of LPS is not responsible for the initial binding of hGBP1 to the bacteria because hGBP1 was docked to the rfaL mutant as efficiently as to wild-type S. flexneri (Fig. 5B). To illustrate this, a series of S. flexneri mutants in LPS biosynthesis pathway should be assayed in the in vitro GBP recruitment assay.
We politely but firmly disagree with the reviewer's assessment – the evidence in favor of a model, in which GBP1 attaches to bacteria through LPS binding seems rather overwhelming:

1) we demonstrate that hGBP1 binds directly to LPS using three distinct assays
2) we demonstrate that hGBP1 binds to various gram-negative bacteria directly but not to gram-positive bacteria
3) we show that LPS serves as a competitive inhibitor for hGBP1 binding to bacteria
4) we show that the transition into the hGBP1 coat encapsulating bacteria requires the O-antigen moiety of LPS using a bacterial mutant

We agree with the reviewer that we will be able to use additional bacterial mutants in the future to gain a more detailed understanding of the precise biochemical features of LPS that are being recognized by hGBP1 but such studies clearly go beyond the scope of the present work. The reviewer is absolutely correct that initial binding of hGBP1 polymers to the bacterial surface is independent of O-antigen. However, even in the absence of O-antigen the surface of the bacteria still largely consists of sugars that are derived from LPS. Although speculative at this point, we favor a model in which the initial docking of the GBP1 polymer occurs through broad specificity/ low affinity interactions with sugars. This model is supported by our previous studies indicating interactions between hGBP1 and host sugars (Piro et al. 2017 PMID: 29233899 and Feeley et al. PMID:28193861 – see also our response to the reviewer's comments regarding the recognition of vacuoles containing bacterial pathogens such as Mb BCG below) and our revised discussion more explicitly discusses the issue raised by the reviewer on page 20 of our revised manuscript.

In Fig. 3B, the authors indicate that asterisks mark polymeric structures of hGBP1 that will fuse with

bacterial surfaces, but to me, I can only see the protruding buds on the bacterial surface. How do the authors know that those protruding structures are indeed hGBP1 polymers?

The structures fail to form in the presence of the R48A mutant, which lacks the catalytic arginine required for GTP hydrolysis and cannot form polymers. There are no protruding buds neither on the surface of *Shigella* (same panel, upper right electron-micrograph) or on the surface of UPEC (Fig EV4) in the presence of the R48A mutant, demonstrating that the structures observed with WT hGBP1 are polymers.

Can the authors do an immunogold antibody staining or other assays to confirm this? Ideally, a Cryo-EM assay will be really cool as it may reveal further structure information about the hGBP1 polymer.

We agree that immunogold antibody staining would further strengthen our argument – unfortunately, we are not currently allowed to conduct any experimental work and furthermore believe that the use of negative controls in our experiments provide sufficient support for our conclusions. We also agree with the reviewer that cryo-EM assays would be 'cool' and indeed are planning to conduct such studies in the future – however, these studies are extensive, time-consuming and go far beyond the scope of this study**.**

In Fig. 5B, hGBP1 protein is docked to the rfaL mutant of S. flexneri at 10 min but disappeared at late time (60 min). Why is that?
As described in the manuscript, we show that O-antigen is required for a sustained binding of hGBP1 to bacteria. We show that hGBP1 undergoes unstable/ reversible interactions not only with a *Shigella* rough mutant (*rfaL* mutant, lacking the O-antigen) but also with *E. coli* rough mutants (Fig. S2B). We propose that O-antigen promotes the transition from the docked hGBP1 polymer into a smooth and stable protein coat and may also stabilize hGBP1 molecules once integrated into the bacterial outer membrane, as discussed in the last paragraph of page 18 and in the following paragraph on page 19.

Also,Is there any difference between the wild-type and R584-586A mutant hGBP1 in promoting the non-canonical inflammasome during bacterial infection?
We agree with the reviewer that the R584-586A mutant will be useful to dissect the mechanism by which hGBP1 promotes inflammasome activation in response to bacterial infections, or in response to LPS delivery by other means such as OMV exposure or LPS transfection. We are planning to conduct such studies in the future. However, such studies address questions that go beyond the current work and are not required to support the conclusions of the current study.

The original study (PMID: 21551061) shows that many GBPs can also target Listeria monocytogenes and Mycobacterium bovis BCG (Mb BCG) that do not have LPS. What is the authors' explanation for this discrepancy?

This is not a discrepancy. We had previously shown that hGBP1 fails to localize with *Listeria* in the host cell cytosol of human epithelial cells (Piro et al. 2017 PMID: 29233899) – therefore, our *in vitro* data are in agreement with the cell culture data. The studied mentioned by the reviewer - Kim et al 2011 (PMID: 21551061) - showed that mouse Gbps co-localized with Mb BCG and Listeria in mouse macrophages. Mb BCG resides in vacuoles and so does *Listeria* when ingested by macrophages. Thus, the study by Kim et al. monitored localization of Gbps to pathogen-containing vacuoles (PVs). In addition to Kim et al., we and others demonstrated that Gbps localize to PVs formed by many different bacterial pathogens. In a previous study we demonstrated that the presence of bacterial secretion systems disrupts the integrity of PVs and thereby promotes Gbp recruitment to PVs (Feeley et al.

PMID:28193861). We also specifically demonstrated in the same study and in Piro et al. that hGBP1 associates with sterilely disrupted vesicles, possibly through the direct recognition of host sugars. Additionally, we demonstrated that GBPs detect ubiquitinated PVs through interactions with ubiquitin-binding proteins (Haldar et al. 2017 PMID: 26417105). In other words GBPs can recognize PVs containing gram-positive bacteria but there is no evidence so far that hGBP1 or any other GBP binds to gram-positive bacteria directly. Indeed, our data show that hGBP1 fails to bind directly to the gram-positive bacteria that we tested. We expanded our discussion of hGBP1 recognition of PVs on page 20 of the revised manuscript.

By a critical attitude, the current data have not firmly established that LPS is directly and fully responsible for recruiting hGBP1 to the bacteria. It can not be ruled out that other bacterial molecules may mediate initial binding of hGBP1 polymer to the bacteria or contribute to the subsequent encapsulating process. The authors should weaken their statement and discuss the alternative possibilities.

Again, we politely but firmly disagree with the reviewer's assessment – the evidence in favor of the model in which GBP1 attaches to bacteria through LPS binding seems rather overwhelming:

1) we demonstrate that hGBP1 binds directly to LPS using three distinct assays
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3) we show that LPS serves as a competitive inhibitor for hGBP1 binding to bacteria
4) we show that the transition into the hGBP1 coat encapsulating bacteria requires the O-antigen moiety of LPS using a bacterial mutant

We agree with the reviewer that it is certainly possible that other microbial molecules could aid in the recruitment or retention of hGBP1 and/or other GBPs to the bacterial surface, something we now briefly discucss on page 20 of our revised manuscript. However, the essential role for LPS in the recognition of gram-negative bacteria by hGBP1 is fully supported by the data presented in our manuscript.

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What is the difference between LPS (O111:B4) and LPS (O55:B5) and why the former is weaker than the latter in hGBP1 anchoring S. flexneri?

Although the sugar composition between O55:B5 and O111:B4 is nearly identical, the linkage between the O-antigen sugars are distinct which will lead to different three dimensional structures of the O-antigen polymers which are likely to influence their aggregation behavior. Previous studies reported that the normal radius of O55:B5 aggregates is 9-19 nm, whereas O111:B4 aggregates are 20-50 nm in size. A smaller aggregate (O55:B5) has a larger effective surface area per mass to fit more hGBP1 molecules than a larger aggregate (O111:B4) (Bergstrand, Svanberg et al., 2006, Risco, Carrascosa et al., 1993, Stenutz, Weintraub et al., 2006). These physical properties of the different LPS molecules likely explain why O55:B5 is a better competitive inhibitor for LPS-dependent binding of hGBP1 to bacteria than O111:B4, as now stated on page 12 of the revised manuscript.

Referee #2:

hGBP1 play important role in Cell-autonomous response during infection. It co-localizes with intracellular gram-negative bacterial pathogens, facilitates bacterial killing, promotes activation of the lipid A sensor caspase-4, and blocks actin-driven dissemination of the enteric pathogen Shigella. In this study, Kutsch et al explore how hGBP1 achieve all these functions. They show that hGBP1 Polymerize and binds directly to LPS and to gram-negative bacteria. Then, form a stable hGBP1

protein coat on bacteria expressing O-antigen. This disrupts the O antigen barrier, thereby unmasking lipid A, eliciting caspase-4 recruitment, enhancing antibacterial activity of polymyxin B, and disturbing O-antigen-dependent function of the Shigella virulence protein IcsA. **This study of great importance expands our knowledge about the mechanism of function of GBPs in defense**, and warrant a publication. Experiments are well executed, and **I don't see need for further experiment**. However, a have a few comments that I was hoping the authors could include to solidify some of the key point in the study.

We are grateful to the reviewer for their very supportive assessment of the quality and importance of our study.

1. The authors report based on microscopy examination that hGBP1 polymerize in presence of GTP. It will be nice if the authors show that by running a Native gel to determine the size and the different oligomeric hGBP1 formed.
We conducted these studies in a previous publication in which we showed for the first time that hGBP1 polymerizes (Shydlovskyi et al. 2017 PMID: 28645896) without providing a biological function for these polymers. Fig. S5e of Shydlovskyi et al. provides a SDS gel that resolves different oligomeric states of hGBP1 during polymerization in the presence of GTP. Recent work of ours (Sistemich et al. 2020 PMID: 32087202) provides a more detailed description of the polymerization mechanism and is cited on page 17 of the revised manuscript.

2. The authors report that hGBP1 directly bind LPS. If it is possible to quantify the Specific binding affinity of hGBP to LPS by surface plasmon.
Although SPR, and moreover ITC, stopped-flow and FRET are well established assays in our labs and we are planning to address affinities of the hGBP1-LPS complex in future studies, the COVID-19 epidemic has ceased all experimental work in our laboratories. Because measurement of binding affinities is not essential to support the conclusions of our studies, we are planning to conduct these studies at a later time and to report these measurements in a future manuscript.

3. Shigella Flexeneri ospc3 (the reviewer is probably referring to IpaH9.8) antagonize the function of hGBP1 to allow IcsA driven motility yet other cytosolic bacteria such as Salmomella Sifa or Burkholderia don't secrete similar virulence factor. Does actin motility is disrupted in these species as well by the action of hGBP1. It will be very interesting if the author would show if hgbp1coating each of these bacteria disrupt their virulence factor that mediate actin motility.

The reviewer is absolutely right that antimicrobial effect of hGBP1 binding needs to be investigated in greater detail in the context of pathogens other than *Shigella* and UPEC. We are planning to follow up the suggestion made by the reviewer and will test whether hGBP1 blocks actin motility of *Burkholderia* in the future. However, we are not able to provide a comprehensive set of such experiments at this time.

Referee #3:

It has previously been shown that hGBP1 facilitates bacterial killing, promotes activation of the LPS sensor Caspase-4, and blocks actin-based bacterial motility. However, the mechanism by which a single protein can have such a variety of effects was unknown. This manuscript provides a compelling mechanism by which hGBP1 can have such diverse effects. Kutsch et al. provide evidence that hGBP1 polymers functions as an LPS binding and LPS clustering surfactant thereby disrupting the outward-facing LPS layer. The initial binding of hGBP1 polymers to LPS was largely independent of O-antigen. However, the transition from bound polymer to evenly coated bacteria was dependent on O-antigen. hGBP1 disruption of the O-antigen layer made lipid A more exposed thereby promoting the recruitment of caspase-4 and enhancing the efficacy of the antimicrobial polymyxin B. The likely

increased membrane fluidity in the presence of hGBP1 also affected unipolar lcsA localization and thereby actin motility. A convincing model describing these results was presented in FigureS7. Overall, the manuscript was well written, the data was convincing, and experiments well performed.

We would like to thank the reviewer for their very positive assessment of our work.

Major comment:

For consistency it would be good if the authors can quantify the data in all their images. For example, in Fig1C and 2C (and several other figures) no quantification is provided while in Fig. 3C and other figures the data is quantified.

We now provide quantification for the data shown in Fig1C and 2C.

Minor comments:

In Figure 4 the results with the smooth vs. rough LPS were not discussed in the result section discussing this figure but only mentioned later when results of Figure 5 are discussed.

We thank the reviewer for pointing out this omission. The data from Figure 4B and EV3A (smooth vs rough LPS) are now discussed in the Results section.

1st Revision - Editorial Decision 21st Apr 2020

Dear Jörn,

Thanks for sending me the revised manuscript. I have now had a chance to take a careful look at everything and I appreciate the introduced changes. I am therefore very pleased to let you know that we can move forward and accept the manuscript for publication here.

Before sending you the formal acceptance letter there are just a few things to sort out. You can use the link below to upload the revised version.

- For figure 1A inserts a + b maybe best to label as i and ii to avoid possible confusions.

- Thanks for adding source data. For source data for main figures => please assemble into one file/per figure. Source data for appendix and EV figures should be provided in one file.

- Can you please check Figure 4B the hGBP1f / LPS-055:B5 panel - it looks completely empty.

- Please fix callouts to appendix figures in main text to Appendix fig 1S etc

- Each movie needs to be zipped together with its legend.

- Please remove highlights from title page, relabel Summary as Abstract, Methods as Materials and Methods.

- Please upload a file with the highlights and a general synopsis blurb - we include a synopsis of the paper on our website (http://emboj.embopress.org/)

- We also need a summary figure for the synopsis. The size should be 550 wide by 400 high (pixels). You can also use something from the figures if that is easier.

-The manuscript should also have a Data Availability Section please state "This study includes no data deposited in external repositories"

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That should be all - let me know if you any questions

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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- For figure 1A inserts a + b maybe best to label as i and ii to avoid possible confusions.
done

- Thanks for adding source data. For source data for main figures => please assemble into one file/per figure. Source data for appendix and EV figures should be provided in one file.
done

- Can you please check Figure 4B the hGBP1f / LPS-055:B5 panel - it looks completely empty.

checked- it's correct as it but I think I may see the cause for the confusion. Top row of the bottom panel of Fig. 4B has no added LPS and therefore there's no signal - relabeled the top row as "—, hGBP1f" to distinguish it from the next row " LPS, hGBP1f";

- Please fix callouts to appendix figures in main text to Appendix fig 1S etc
done

- Each movie needs to be zipped together with its legend.
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- Please remove highlights from title page, relabel Summary as Abstract, Methods as Materials and Methods.
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2nd Revision - Editorial Decision 29th Apr 2020

Dear Jörn,

Thanks for sending us your revised manuscript. I have now had a chance to take a look at it and I appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication.

Congratulations on a nice study

best Karin

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Corresponding Author Name: Jörn Coers
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified

- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;

  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).
We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigator was blinded when assessing quantifications.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. We used unpaired two tailed t-tests when comparing two samples, one-way ANOVA with Tukey's multiple comparison test for a goup larger than two when comparing one treatment, and two-way ANOVA with Tukey's multiple comparison test for a goup larger than two when comparing two treatments.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Yes, we show for each sample the data points of independent repeats and the standard error of the mean (SEM) to communicate the variation within one sample.

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Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Please see Reagent and Tool Table in Methods where we provide for each antibody the source and identifier.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Please see Reagent and Tool Table in Methods where we provide for each cell line the source and identifier. We reguarly check our cell lines for mycoplasma contamination.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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