THE EMBO JOURNAL

Neutrophils discriminate live from dead bacteria by integrating signals initiated by Fprs and TLRs

Giuseppe Teti, Germana Lentini, Giuseppe De Gaetano, Agata Famà, Roberta Galbo, Francesco Coppolino, Giuseppe Mancuso, and Concetta Beninati
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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 15th Sep 2021

Dear Prof. Teti,

Thank you again for the submission of your manuscript entitled "Neutrophils discriminate live from dead bacteria by integrating signals initiated by Fprs and TLRs" and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, all three referees are very positive towards your work but point out to some concerns that will require your attention before your manuscript can be published in The EMBO Journal. Without repeating the comments of the referees her, I would like to point out to some specific concerns that need to be addressed: Referee #1 believes that the depth of your mechanistic analysis of the role of the p38 MAPK in the process and suggests a number of experiments to strengthen the manuscript. Referee #3, in turn, raises some issues with the role of CXCL2, and particularly its activity in recruitment of neutrophils vs. elimination of bacteria.

Based on the overall interest expressed in the reports, I would like to invite you to address the comments of all referees in a revised version of the manuscript. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but we are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, so please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

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: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

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

Best regards,

David

David del Alamo, PhD.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

7) 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:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

Additional information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 14th Dec 2021:

Link Not Available

Referee #1:

The authors present a study that addresses the question of live/dead discrimination in innate immune cells: According to this concept, the magnitude of inflammatory responsiveness is adjusted to the level of infectious threat with live bacteria eliciting a

more vigorous response. Here the authors show that neutrophils produce more CXCL2 in response to live bacterial infection and this is mediated by a combination of secretion of formylated bacterial signal peptides (only produced when bacteria are viable) and Toll-like receptor stimulation. Mechanistically, the authors argue for a role of p38 MAP kinase activation in this process. Although the study is sound with respect to the identification of Fpr1/Fpr2/TLR signaling as a mechanism of sensing viable bacteria, the intracellular mechanistical study remains limited. To the reviewer, it is not clear (and not tested) why p38 MAPK shall have specific effects for CXCL2 only, as other cytokines are not dependent on live/dead sensing.
Major points:
1. The authors should provide experimental evidence that indeed p38 MAP kinase activation is crucial in the process and can explain specificity of the effects (for the different cytokines). Although a hypothesis is presented (AP1 sites in the promoter), this is not formally tested. Moreover, p38 MAP kinase inhibition is also sufficient to reduce TLR stimulation and TNF induction in other innate cells (e.g. macrophages).Thus for Fig. 7 it is mandatory to test the inhibitors (at least the active ones) with HK bacteria and TLR stimulation alone AND to test TNF and CXCL1. When the latter cytokines are affected as well, the link between p38 activity and CXCL2 specific live/dead discrimination is lost. Reporter genes assays might directly test the AP1 binding site hypothesis. Alternative explanations for the specificity for CXCL2 might include kinetic aspects, thus CXCL2 induction might be more dependent on robust and lasting p38/AP1 activation. Taken together, the data on p38/AP1 (Fig. 7/8) must be considered preliminary. I think the study has a shortcoming at this mechanistical level.
2. Fig 5C/D: TNF and CXCL1 should be presented as well - similar point as comment 1. Specificity for one cytokine is the surprising finding, yet not convincingly explained.
Minor points:
3. For the comparison of heat-killed vs. live stimulation: Does the mass of HK-bacteria (1,5,10, 20µg/ml) equals the bacterial mass of live bacteria at the used MOI (2,5,10,20)? In other words, do the authors compare equal amounts of bacteria for the two conditions?
4. Importantly, the authors stop infection with live bacteria after 1h by adding antibiotics (in order to avoid effects due to growing bacteria). Has the killing activity been checked by bacterial plating? Otherwise it might be that residual bacteria replicate and thus blur comparability (comment 3).
5. Fig 4A, B: Y-axis scale: CFU x10-6 or -7 (minus 6, minus 7?). Should be 1E6 but not 1E-6 to my understanding.
6. The authors seem to have experience with Fpr1/2 kos: Do innate cells from those mice secrete similar amounts of CXCL2 as compared to WT when stimulated by TLR agonists alone?
7. Fig 6D: None of the inhibitors has any effects. How was effectiveness controlled?
8. Fig S1 sRNA should be mentioned in the legend.

Referee #2:

The study by Lentini et al. uncovers molecular pathways that allow murine neutrophils to distinguish between viable and killed bacteria. Specifically, they found that a combination of bacterial signal peptides and TLR agonists induce is sufficient and required for the induction of high levels of the neutrophil chemoattractant CXCL2/MIP-2, which was produced in large quantities in response to live, but not to killed bacteria. In a series of elegant experiments the authors demonstrate the requirement for the formylated peptide receptors FRP1 and FRP2 for the production of CXCL2 and ROS, and for the migration of neutrophils towards a site of infection and for antibacterial immunity GBS infection model. They identify several bacterial peptides capable of activating FRP1 or FRP2 to induce CXCL2. They provide convincing evidence that the sensing of bacterial signal peptides through FRP1 and FRP2 requires p38 MAPK and AP-1 activation for the induction of CXCL2.

This is a technically sound and mechanistically interesting and convincing study. The manuscript is clear and well-written. The study adds important molecular insights into the long known phenomenon that neutrophils and other innate immune cells respond more vigorously to live compared to dead bacteria.

Overall, this is a very stringent and convincing study, which provides a significant advance over existing concepts.

I have only a few specific comments and questions, which I believe would be important to address.

1. The study was performed in murine cells. Given that this is likely a conserved mechanism of innate immunity, it would be relevant to corroborate some of the findings in human neutrophils (e.g. live vs killed bacteria, role of FRP stimulating peptides in CXCL2 induction)

2. It has been shown previously that human and mouse cells produce high amounts of IL-1b in response to live, but not in response to killed bacteria. Did the authors measure IL-1b in their experiments? Was there a role for FRPs?

3. In Figure 6, the authors show that loss of either FRP1, FRP2 or MyD88 reduces ROS production in response to live bacteria. Would FRP agonists (peptides identified in Fig. 5) together with TLR/MyD88 agonists be sufficient to drive ROS and CXCL2 production?

4. In Fig. 7 the authors use pharmacological inhibitors to demonstrate a role for TAK1, p38 and NF-kB in the induction of CXCL2.

Given the caveats associated with pharmacological inhibitors and the broad role of these pathways in innate immune responses, it would be important to show the effect of these inhibitors on CXCL11 and/or TNF production in, which were shown to be induced independently of bacterial viability and FRPs.

5. In Fig. 8 the authors use immunoblots to demonstrate p38 phosphorylation in the presence of different ligands and inhibitors in wt and KO cells. It would be important to show the total p38 levels as a control (in addition to the "housekeepers" used to control for protein loading).

Referee #3:

In this study, authors have carried out fairly comprehensive studies and make a case that neutrophil activation mechanisms are different for dead vs. live bacteria, and that live bacteria trigger release of Cxcl2 by neutrophils by a three-signal mechanism and show experimental at the level of transcription machinery. These studies are potentially interesting as it aims to capture the complexities of the in vivo mechanisms for containing infection, but falls short for the reason that the authors provide no evidence that increased expression of Cxcl2 is responsible for successful elimination of live bacteria and why Cxcl2 and not Cxcl1 expression leads to resolution.
Recent studies have shown that Cxcl2 is more potent than Cxcl1 for Cxcr2 activity but their recruitment activities are different (PMID: 32881070). For instance, these studies show that Cxcl2 at high in vivo concentrations fail to recruit neutrophils. The in vivo recruitment is dictated by haptotactic and chemotactic gradients that are governed by local concentrations, glycosaminoglycan interactions, and Cxcr2 activation. Authors need to provide evidence and also provide a model of how elevated Cxcl2 (and not Cxcl1) production by a three-signal mechanism and autocrine activation of neutrophils leads to elimination of live bacteria. In particular, authors need to show what are the consequences of autocrine activation of neutrophils by elevated Cxcl2 levels -does it lead to a more robust release of superoxide and NETs and/or does it result in higher recruitment of neutrophils to the infected site?
Authors also need to take into consideration, in the context of in vivo, LPS triggers Cxcl1 and Cxcl2 release by mast cells and macrophages that are either prestored or regulated at the transcription level (De Filippo et al., 2008, 2013; PMID: 18322244, 23645836). So the neutrophil phenotype is impacted by additional factors that are not captured in the in vitro studies.

Point-by-point response to the reviewers' comments

Referee #1:

The authors present a study that addresses the question of live/dead discrimination in innate immune cells: According to this concept, the magnitude of inflammatory responsiveness is adjusted to the level of infectious threat with live bacteria eliciting a more vigorous response. Here the authors show that neutrophils produce more CXCL2 in response to live bacterial infection and this is mediated by a combination of secretion of formylated bacterial signal peptides (only produced when bacteria are viable) and Toll-like receptor stimulation. Mechanistically, the authors argue for a role of p38 MAP kinase activation in this process.

Although the study is sound with respect to the identification of Fpr1/Fpr2/TLR signaling as a mechanism of sensing viable bacteria, the intracellular mechanistical study remains limited. To the reviewer, it is not clear (and not tested) why p38 MAPK shall have specific effects for CXCL2 only, as other cytokines are not dependent on live/dead sensing.

Major points:

1. The authors should provide experimental evidence that indeed p38 MAP kinase activation is crucial in the process and can explain specificity of the effects (for the different cytokines). Although a hypothesis is presented (AP1 sites in the promoter), this is not formally tested. Moreover, p38 MAP kinase inhibition is also sufficient to reduce TLR stimulation and TNF induction in other innate cells (e.g. macrophages).Thus for Fig. 7 it is mandatory to test the inhibitors (at least the active ones) with HK bacteria and TLR stimulation alone AND to test TNF and CXCL1. When the latter cytokines are affected as well, the link between p38 activity and CXCL2 specific live/dead discrimination is lost.

We agree with the reviewer about the importance of testing the inhibitors using heat-killed bacteria as a stimulus and of analyzing the production of TNF and Cxcl1, in addition to Cxcl2. Therefore, in the revised manuscript, we now report data on the effects of the inhibitors on the induction of Cxcl1, TNF-α, and Cxcl2 by killed bacteria and LPS. The data, shown in new figures (Appendix Fig S2C-H), show that AP1 or p38 blockade induced only slight (25-37%) inhibition, and only at the highest inhibitor doses tested. This is in sharp contrast with the marked (80-90%) reduction in Cxcl2 levels observed with low doses of p38 and AP1 inhibitors when using live bacteria as a stimulus (Fig 7).

To further demonstrate the specificity of p38/AP1 blockade in terms of Cxcl2 production, we show additional new data on Cxcl1 and TNF elevations induced by live bacteria, as suggested by reviewer 2 (see comment no. 4). Specifically, we have added two additional panels (Appendix Fig S2A and B) showing the effects of pharmacological inhibitors on the production of Cxcl1 and TNF-α after stimulation with live bacteria. The data show that p38 or AP1 blockade only slightly reduced the release of Cxcl1 and TNF-α after stimulation with live bacteria and only at high doses. Again, this is in contrast with the marked effects of low doses of p38 or AP1 inhibitors on Cxcl2 induction.

Therefore, the ability of p38 and AP1 inhibitors to almost completely prevent Cxcl2 induction seems specific for this chemokine and for stimulation with live bacteria. We believe that the newly

presented data corroborate the hypothesis that the p38/AP1 pathway is selectively involved in Cxcl2 induction by live bacteria. We would like to thank the reviewer for his/her suggestion, which encouraged us to provide more convincing evidence to support our conclusions.

- *Reporter genes assays might directly test the AP1 binding site hypothesis. Alternative explanations for the specificity for CXCL2 might include kinetic aspects, thus CXCL2 induction might be more dependent on robust and lasting p38/AP1 activation.*

We totally agree on the importance of gene reporter studies, particularly for identifying the exact sites in the Cxcl2 promoter that are involved in binding to AP1 components after stimulation with live bacteria. However, experiments of this kind are complex and require a long time to be accurately performed. Moreover, these studies would need considerable space to be adequately reported. On the other hand, identification of the mechanisms of activation of the Cxcl2 gene promoter was not among the primary objectives of the present study that dealt instead with the identification of the bacterial agonists and host receptors involved in the recognition of live bacteria. For all these reasons, we would prefer to devote soon a separate study entirely to the analysis of the mechanisms of Cxcl2 promoter activation after stimulation with live bacteria. Thus, if this reviewer agrees, we would like not to wait to perform experiments involving reporter genes, in order to avoid delays in publication of the present study.

We would like to thank the reviewer for his/he comment on the kinetics aspects of p38/AP1 activation and for the suggestion that recognition of live bacteria may result in more lasting activation. Following this comment, we have performed comparative kinetics studies and found that indeed stimulation with live bacteria results not only in more robust, but also in more prolonged p38/AP1 activation than heat-killed bacteria. These new results are reported in Fig. 8 and Appendix Fig. S3.

2. Fig 5C/D: TNF and CXCL1 should be presented as well - similar point as comment 1.

We agree with this suggestion and have now added the TNF and Cxcl1 data in a new Figure (Appendix Fig S1). The data show that HK-GBS, and the other TLR agonists tested, do not significantly synergize with Fpr agonists in the induction of Ccxl1 or TNF-α. Therefore, these synergistic effects seem to be Cxcl2-specific.

Minor points:

3. For the comparison of heat-killed vs. live stimulation: Does the mass of HK-bacteria (1,5,10, 20µg/ml) equals the bacterial mass of live bacteria at the used MOI (2,5,10,20)? In other words, do the authors compare equal amounts of bacteria for the two conditions?

We now provide the requested information in the Materials and methods section of the revised manuscript (Lines 494-495). We clarify that 1 µg of heat-killed bacteria corresponds to approximately $1x10^6$ bacteria, therefore comparable quantities (1-20 million) of live and killed bacteria were used as stimuli.

4. Importantly, the authors stop infection with live bacteria after 1h by adding antibiotics (in order to avoid effects due to growing bacteria). Has the killing activity been checked by bacterial plating? Otherwise it might be that residual bacteria replicate and thus blur comparability (comment 3).

We now state in the Materials and methods section that the killing activity of the antibiotics added was indeed checked and that no bacteria could be cultured after their addition (lines 551-553).

5. Fig 4A, B: Y-axis scale: CFU x10-6 or -7 (minus 6, minus 7?). Should be 1E6 but not 1E-6 to my understanding.

The data are now reported as CFUx10^6, CFUx10^7 … etc

6. The authors seem to have experience with Fpr1/2 kos: Do innate cells from those mice secrete similar amounts of CXCL2 as compared to WT when stimulated by TLR agonists alone?

Yes, results are shown in Figs 3B, D and F

7. Fig 6D: None of the inhibitors has any effects. How was effectiveness controlled?

In preliminary experiments we tested the ability of DMTU, MnTBAP and DPI to inhibit ROS production in GBS-stimulated neutrophils using the CellROX kit. This information is now reported in the Materials and methods section (lines 565-567).

8. Fig S1 sRNA should be mentioned in the legend.

sRNA is now mentioned. Thank you for carefully reading the paper and for detecting this omission.

Referee #2:

The study by Lentini et al. uncovers molecular pathways that allow murine neutrophils to distinguish between viable and killed bacteria. Specifically, they found that a combination of bacterial signal peptides and TLR agonists induce is sufficient and required for the induction of high levels of the neutrophil chemoattractant CXCL2/MIP-2, which was produced in large quantities in response to live, but not to killed bacteria. In a series of elegant experiments the authors demonstrate the requirement for the formylated peptide receptors FRP1 and FRP2 for the production of CXCL2 and ROS, and for the migration of neutrophils towards a site of infection and for antibacterial immunity GBS infection model. They identify several bacterial peptides capable of activating FRP1 or FRP2 to induce CXCL2. They provide convincing evidence that the sensing of bacterial signal peptides through FRP1 and FRP2 requires p38 MAPK and AP-1 activation for the induction of CXCL2.

This is a technically sound and mechanistically interesting and convincing study. The manuscript is clear and well-written. The study adds important molecular insights into the long known phenomenon that neutrophils and other innate immune cells respond more vigorously to live compared to dead bacteria.

Overall, this is a very stringent and convincing study, which provides a significant advance over existing concepts.

I have only a few specific comments and questions, which I believe would be important to address.

1. *The study was performed in murine cells. Given that this is likely a conserved mechanism of innate immunity, it would be relevant to corroborate some of the findings in human neutrophils (e.g. live vs killed bacteria, role of FRP stimulating peptides in CXCL2 induction)*

We would like to thank this reviewer for his/her appreciation of our studies using murine neutrophils. We completely agree on the importance of determining whether similar mechanisms apply to human neutrophils. Indeed, we have set out to perform experiments using neutrophils from healthy volunteers to determine if live bacteria can induce much higher levels of interleukin 8, which is functionally homologous to Cxcl1 and Cxcl2. However, we were advised to obtain a permission from the ethical committee of our institution even to obtain blood samples from authors or colleagues. Unfortunately, this process is rather long at our institution (particularly so in this period, in which the COVID emergency has not completely subsided) and further time would be needed to arrange medical visits for the donors. Therefore, unless the reviewer believes that it is absolutely necessary to include data on human neutrophils, we would be inclined to not include such studies, in order to avoid a major delay in the publication of this manuscript.

2. It has been shown previously that human and mouse cells produce high amounts of IL-1b in response to live, but not in response to killed bacteria. Did the authors measure IL-1b in their experiments? Was there a role for FRPs?

Thank you for this important comment. We measured IL-1β levels in culture supernatants of neutrophils from mice lacking Fpr1 or Fpr1 after stimulation with live bacteria and found that there were no differences in IL-1β levels in comparison with wild type neutrophils. Results are now presented in Fig EV2B of the revised manuscript. Therefore, the requirement for Fprs seems specific for Cxcl2 responses.

3. In Figure 6, the authors show that loss of either FRP1, FRP2 or MyD88 reduces ROS production in response to live bacteria. Would FRP agonists (peptides identified in Fig. 5) together with TLR/MyD88 agonists be sufficient to drive ROS and CXCL2 production?

Yes, Fig. 5C and D shows that Fpr agonists are by themselves unable to induce Cxcl2, but that they synergize with TLR agonists in inducing Cxcl2 production. In addition, similar synergistic activities are detectable in terms of ROS production (Fig EV4A and B).

4. In Fig. 7 the authors use pharmacological inhibitors to demonstrate a role for TAK1, p38 and NF-kB in the induction of CXCL2. Given the caveats associated with pharmacological inhibitors and the broad role of these pathways in innate immune responses, it would be important to show the effect of these inhibitors on CXCL11 and/or TNF production in, which were shown to be induced independently of bacterial viability and FRPs.

We agree with the reviewer. Thanks for this important suggestion. In the revised manuscript we now report data on the effects of pharmacological inhibitors on Cxcl1 and TNF-α responses to live bacteria, as suggested by this reviewer. In addition, we also report data on cytokine induction by killed bacteria after pharmacological inhibition, as requested by reviewer 1, (please see his/her major point no.1). The new data demonstrate that the ability of p38 and AP1 inhibitors to almost completely prevent Cxcl2 induction after stimulation with live bacteria is specific for this chemokine and for stimulation with live bacteria.

5. In Fig. 8 the authors use immunoblots to demonstrate p38 phosphorylation in the presence of different ligands and inhibitors in wt and KO cells. It would be important to show the total p38 levels as a control (in addition to the "housekeepers" used to control for protein loading).

We agree, thanks for this suggestion. In the revised version of the manuscript (Fig 8 and Appendix Fig. S3) we now show total p38 levels as a control, as recommended by the reviewer.

Referee #3:

In this study, authors have carried out fairly comprehensive studies and make a case that neutrophil activation mechanisms are different for dead vs. live bacteria, and that live bacteria trigger release of Cxcl2 by neutrophils by a three-signal mechanism and show experimental at the level of transcription machinery. These studies are potentially interesting as it aims to capture the complexities of the in vivo mechanisms for containing infection, but falls short for the reason that the authors provide no evidence that increased expression of Cxcl2 is responsible for successful elimination of live bacteria and why Cxcl2 and not Cxcl1 expression leads to resolution.
Recent studies have shown that Cxcl2 is more potent than Cxcl1 for Cxcr2 activity but their recruitment activities are different (j). For instance, these studies show that Cxcl2 at high in vivo concentrations fail to recruit neutrophils. The in vivo recruitment is dictated by haptotactic and chemotactic gradients that are governed by local concentrations, glycosaminoglycan interactions, and Cxcr2 activation. Authors need to provide evidence and also provide a model of how elevated Cxcl2 (and not Cxcl1) production by a three-signal mechanism and autocrine activation of neutrophils leads to elimination of live bacteria. In particular, authors need to show what are the consequences of autocrine activation of neutrophils by elevated Cxcl2 levels -does it lead to a more robust release of superoxide and NETs and/or does it result in higher recruitment of neutrophils to the infected site? Authors also need to take into consideration, in the context of in vivo, LPS triggers Cxcl1 and Cxcl2 release by mast cells and macrophages that are either prestored or regulated at the transcription level (De Filippo et al., 2008, 2013; PMID: 18322244, 23645836). So the neutrophil phenotype is impacted by additional factors that are not captured in the in vitro studies.

Thank you for these important comments. The reviewer remarks that it's unclear whether increased expression of Cxcl2 results in bacterial clearance and would like to see more evidence concerning the mechanisms whereby this might occur. We thank the reviewer for raising this issue, which induced us to perform new experiments, as detailed below. I addition, we realized that we had not sufficiently clarified the point addressed by the reviewer in the original manuscript. Indeed, most of

the questions asked by the reviewer are answered in our previous study (Lentini et al, J. Immunol. 2020) that was only briefly mentioned in the original manuscript. We now clarify in the revised manuscript (please see lines 269-282 in the Discussion section) that neutrophils are largely responsible for *in vivo* Cxcl2 responses in our bacterial infection model and that such responses are required for bacterial clearance, as shown by our previous paper. We now also cite the studies of De Filippo et *al* -mentioned by the reviewer- dealing with Cxcl2 production by macrophages and mast-cells.
According to our previous study, Cxcl2-induced bacterial clearance is linked to both increased neutrophil recruitment and increased bactericidal activities in neutrophils, including ROS production (Lentini et al J. Immunol, 2020). Indeed, autocrionous Cxcl2, but not Cxcl1, production potentiates the bactericidal activity of neutrophils, as shown in our previous paper. These points are also more clearly discussed in the revised manuscript (please see lines 269-282 in the Discussion section).
In the present study, we show that lack of Fpr1 or Fpr2 results in both reduced Cxcl2 production and impaired antibacterial defenses. Although we did not formally prove that decreased Cxcl2 production is the cause of decreased defenses in Fpr-deficient mice, this seems likely based on our previous data. Nevertheless, to further strengthen this conclusion, in the revised manuscript we added new data that were generated after submitting the paper. The new experiments (Fig. 4F and G) show that defective host defenses in Fpr-deficient mice are rescued by exogenous administration of Cxcl2, but not Cxcl1. In summary, we believe this new data strengthen the conclusion that high-level Cxcl2 production results in increased bacterial clearance, which is now discussed more in depth in the revised manuscript. As a result, the paper seems now considerably improved and we would like to thank again the referee for these very useful comments.

Dear Prof. Teti,

Thank you for the submission of your revised manuscript to The EMBO Journal and please accept my apologies for the delay in responding. We have now received the comments from former referees #1 and #3 and we are almost ready to move forward with the publication of your article. Please note that although referee #3 is rather negative on account of the relative contributions of Cxcl1 and Cxcl2, we believe in agreement with referee #1 that this point is extensively discussed in the paper and that it is in any case somewhat peripheral to the main topic, the differential response to live vs. dead bacteria. There are however a few editorial points that will need to be addressed before your study can be accepted:

- The "Summary" section after the abstract needs to be removed as it does not fit The EMBO Journal format.
- The "Declaration of Interests" section must be renamed to "Conflicts of Interest".
- Figures 3A and 3B are not mentioned in the text. Please add the figure callouts where appropriate.

Please provide the paper's synopsis composed of:
- a short 'blurb' text summarizing in two sentences the study (max. 250 characters). Add as well three to four 'bullet points' highlighting the main findings. Bullet points and standfirst text should be submitted as a separate manuscript file in LaTeX, RTF or MS Word format.
- A "synopsis image", which can be used as a "visual title" for the synopsis section of your paper. The image should be PNG or JPG format with pixel dimensions of 550 x 300-600 (width x height).

Please let me know if you have any further questions regarding any of these points. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

Yours sincerely,

David del Alamo
Editor
The EMBO Journal

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Please click on the link below to submit the revision online:

Link Not Available

Referee #1:

In the revised manuscript the authors address my main concerns appropriately. Especially my concerns on specificity of the p38 effects on CXCL2 induction by live bacteria have been addressed - applying also new experiments. Still, the conclusion are based only on the use of a pharmacological inhibitor and reporter assays, that might analyze the question in more detail, were not performed at this time. However, with the new data, I think that conclusions are well justified. Otherwise, as mentioned in my previous review, the study ist sound, novel and interesting.

Referee #3:

The authors have not addressed my main concerns whether the in vitro studies carried out using bone marrow neutrophils capture the complexities of Cxcl1 and Cxcl2 released by multiple cell types including neutrophils, that the chemokine levels vary, and their recruitment profiles and receptor activities are different. In particular, the recent JLB paper shows (PMID: 32881070) that Cxcl2 levels are negatively correlated to neutrophil recruitment, that the recruitment is almost negligible at high Cxcl2 levels though Cxcl2 is more active than Cxcl1 for receptor activities. Further, there is vast amount of literature showing phenotype of recruited neutrophils is not the same as 'naïve' bone marrow neutrophils.

I am not convinced that Cxcl2 released by recruited neutrophils (as inferred from in vitro data as there is no direct evidence that this is the case in their in vivo experiments) is responsible for the observed phenotype. Further, in their previous JI paper (Lentini, 2020), the authors show that the Cxcl2 levels are significantly lower and not higher (400 pg vs. 1500 pg - the y-axis is

not the same), in GBS-infected mice. Even the higher Cxcl2 levels are observed only for the early time points and no more evident at 6 hours. Their data are quite clear in showing that Cxcl1 levels are insensitive to dead or alive bacteria but Cxcl2 is not. However, this does not mean that Cxcl2 alone (though Cxcl1 levels are actually higher) is responsible for both neutrophil recruitment and in defining the neutrophil phenotype in bacterial killing. Maybe more meaningful insights could have been obtained using Cxcl1 and Cxcl2 KO mice.

Authors must take into consideration and integrate ALL of the extensive literature on in vitro and in vivo studies that include Cxcl1 and Cxcl2 released by ALL cell types in describing how infection is resolved in the peritoneum. In absence of that, these studies are correlative at best and do not establish a definitive mechanism on how chemokines and neutrophils resolve infection.

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 12th Jan 2022

Dear Prof. Teti,

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

Yours sincerely,

David del Alamo
Editor
The EMBO Journal

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Giuseppe Teti
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2021-109386

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 χ2 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.

USEFUL LINKS FOR COMPLETING THIS FORM

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Small sample sizes were chosen for the analysis of cytokine response based on the relatively large size of the expected effects. In this way we also kept to a minimun the number of mice used as a source of cells for the experiments, in compliance with the principle of reduction. The use of a small sample size was possible based on extensive, previous experience (derived from our own studies and from studies reported in the literature) for tests of this kind. For example, to assess the effects of gene deletion on cytokine responses to bacteria (i.e. a situation in which the expected effect is large, if an effect is truly present) previous experience indicated that sampling duplicate cultures of responding cells in three separate experiments -each conducted on cells obtained from a different animal- is sufficient to detect biologically meaningful and statistically significant differences. Using larger samples sizes would not be justified in this case.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Also in this case, sample size was kept to a minimum. Previous experience with the animal model reported in the manuscript indicated that using 4 animals per experimental group is sufficient to detect meaningful differences in in vivo bacterial burden or cytokine responses. Likewise, using 8 animals per group was deemed sufficient to detect differences in the frequency of clinical signs during infection, based on previous experience with the model.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from the data reported in the present paper.
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.	Animals were randomly allocated to treatment groups by using the RAND function in Microsoft Excel
For animal studies, include a statement about randomization even if no randomization was used.	Care was taken to allocate animals to different treatment groups by applying standard randomization procedures (e.g. assignment to computer-generated random numbers) .
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.	Investigators processed samples in a blind fashion. This was accomplished by labeling samples with randomly generated numbers (thereby avoiding the use of descriptive names), before sample processing and result analysis.processing
4.b. For animal studies, include a statement about blinding even if no blinding was done	Investigators processed samples in a blind fashion. This was accomplished by labeling samples with randomly generated numbers (thereby avoiding the use of descriptive names), before sample processing and result analysis.
5. For every figure, are statistical tests justified as appropriate?	Samples were collected from animals identified with randomly generated numbers. Samples were similarly labeled before processing. Therefore the investigators were blinded during dample processing and data analusis.

Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The data seem to meet the assumptions of the tests empolyed. For cytokine concentrations in cell cultures, we assumed a normal distribution, as judged by the histogram method applied to data of control cultures obtained from different experiments.
Is there an estimate of variation within each group of data?	Sandard deviations are reported in all figures.
Is the variance similar between the groups that are being statistically compared?	Yes. This is apparent from the standard deviations values reported in the figures.

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).	Done
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N.A.

* for all hyperlinks, please see the table at the top right of the document

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.	Done
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Done
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	ARRIVE guidelines were consulted and followed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N.A.
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.	N.A.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N.A.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N.A.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N.A.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N.A.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N.A.

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	Done
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N.A.
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).	N.A.
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.	N.A.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N.A.