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A non-redundant role for MKP5 in limiting ROS production and preventing LPS-induced vascular injury

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 January 2009

Thank you for submitting your manuscript for consideration to The EMBO Journal editorial office. I have now had the opportunity to read the manuscript carefully and also to discuss it in detail with the other members of our editorial team. Furthermore, I kindly asked an external advisor to independently assess your manuscript for its suitability here, which might explain the slight delay in getting back to you with an initial editorial decision. I am sorry to say that this evaluation was not a positive one, as we all had to conclude that we are unable to offer publication.

We do understand that you provide genetic and some molecular evidence that MAPkinase phosphatase 5 limits ROS-production in neutrophils as revealed in an LPS-induced vascular injury model. Molecularly, this phosphatase might act at the level of p38 as this had been shown to phosphorylate p47phox in oxidase priming at inflammatory sites (Dang_PM JCI 2006). Consistent with such a proposal, p47phox deletion rescues the vascular responses after LPS-challenge. We certainly do not question the potential in-vivo relevance of these findings and also appreciate that this does reveal a potential new and specific function for MKP5. However, we also had to notice that beside the earlier mechanistic precedence, Mpk-1 deletion had also been shown to sensitize mice to LPS-induced endotoxic shock (Zhao_Q JEM 2006). All in all, with these obvious precedence, we were not convinced that the current submission does still provide the conceptual and significant advance according to the aim and scope of our more general journal and highly competitive journal. I also like to add that the results are logically presented. Nevertheless, we also noticed significant conceptual shortcomings do to earlier functional work on CDK5RAP2. Further, neither the proposed mechanism(s) nor the effects of paclitaxel/doxorubicin seem molecularly defined to an extend that would enable fare well in the rather strong selection for our more general and highly competitive journal.

Consistent with our guidelines to involve external expert advice in cases of doubt, I did consult with an active scientist in the field to independently assess potential merit and general interest of your study. I am afraid to say, that this expert essentially confirmed our critical assessment although appreciating that it is a rather well written and experimentally nice study. With such an explicit assessment from a trusted referee and recognized expert in the field and also in the interest of yours and the papers time, we had no other choice than to return the manuscript to you at this point with the message that we are unable to offer further proceedings.

Please let me add that we are looking for complete papers that describe original research of general rather than specialist interest in molecular biology and we can only afford to select those manuscripts that merit urgent publication because they report novel findings of wide biological significance, sufficient level of molecular understanding and physiological relevance. This is in fact a very tall order and it means that we end up rejecting by far the majority of the very many manuscripts we receive every day at our editorial office. Further, I would like to point out that our External Advisors are experienced active scientists that provide us on a daily basis with professional advice on manuscripts in their area of expertise. The tight association of these scientists warrants the high standards of the journal, and also the timely evaluation and handling of all manuscripts received at the editorial office.
Again, I am sorry to have to disappoint you on this occasion, but I hope that you nevertheless might consider our journal for publication of your future studies.

Resubmission 30 March 2009

Thank you for reading our manuscript, which was submitted to The EMBO Journal in January. Your decision letter has prompted us to re-examine the points that we might have missed in writing the manuscript and caused misunderstanding of its novelty and significance. I would like to clarify the two major issues that you commented (in italicized font).

1 - We do understand that you provide genetic and some molecular evidence that MAP kinase phosphatase 5 limits ROS-production in neutrophils as revealed in an LPS-induced vascular injury model. Molecularly, this phosphatase might act at the level of p38 as this had been shown to phosphorylate p47phox in oxidase priming at inflammatory sites (Dang PM JCI 2006). Consistent with such a proposal, p47phox deletion rescues the vascular responses after LPS-challenge.

As you mentioned, Dang and colleagues demonstrated that p38 MAPK is one of the kinases that phosphorylate p47phox. However, there is no published report on how p38 MAPK might be controlled in neutrophil superoxide production. There are numerous kinases known to phosphorylate p47phox, including p38, ERK, Akt, PKC and IRAK4, and the relative contributions of these kinases to LPS-induced NADPH oxidase activation were unclear. To our knowledge, there has been no previous publication on the ability of a protein phosphatase to distinguish between these kinases in their phosphorylation of p47phox. This alone indicates novelty of our work, indicating that this study does not overlap with the work by Dang et al. The current work is the first to demonstrate MKP regulation of a terminal function of neutrophils.

2 - We certainly do not question the potential in-vivo relevance of these findings and also appreciate that this does reveal a potential new and specific function for MKP5. However, we also had to notice that beside the earlier mechanistic precedence, Mpk-1 deletion had also been shown to sensitize mice to LPS-induced endotoxic shock (Zhao Q JEM 2006). All in all, with these obvious precedence, we were not convinced that the current submission does still provide the conceptual and significant advance according to the aim and scope of our more general journal and highly competitive journal.

A major conclusion of our study is that MKP5 plays a non-redundant role in protecting host from oxidant-mediated vascular injury. This conclusion is based on our original finding that deletion of Mkp5 leads to pronounced vascular response to LPS, even though other MKPs such as MKP1 is still present. We have now obtained additional experimental data to show that Mkp1 deficiency does not lead to the exaggerated vascular response to LPS (see new Figure 1). Our results also demonstrate that the Mkp1 KO neutrophils are similar to wild type neutrophils in superoxide production (new

Figure 6). It should be pointed out that the LPS-induced endotoxic shock model used by Zhao et al is different from the local Shwartzman reaction model that we used in the current study. The former results primarily from overproduction of inflammatory cytokines, whereas the latter employs an acute function (superoxide production) of neutrophils. Therefore, our study demonstrates for the first time that MKPs with similar substrate specificity can have very different functions in vivo, which is a conceptual and significant advancement in the field of MKP research.

Research on MAP kinase phosphatases is far lagging behind MAPK research. Likewise, little is known about the mechanisms that negatively regulate NADPH oxidase activation. In consideration of our additional data that further demonstrate the novelty of this work, we respectively request your consideration of our manuscript for possible publication in The EMBO Journal.

Additional Correspondence by Editor 02 April 2009

Thank you very much for your letter and the modified manuscript. Given your valuable arguments we have uploaded the paper to our system and I will send it out for peer-review.

I will come back to you as soon as I will have received comments from our referees.

2nd Editorial Decision 20 April 2009

Thank you very much for the submission of your research manuscript for consideration to The EMBO Journal editorial office. As it becomes obvious from the reports enclosed, at least two referees indicate some interest in your study that demonstrates a role of MAPK phosphatatase 5 in the regulation of neutrophile activation and LPS-induced inflammatory responses. Nevertheless, careful reading of the reports from ref#1 and #2 reveals that additional insight into the link between p38 and p47phox would be necessary to also fulfill the rather molecular demands of our journal and strengthen the so far essentially genetic results reported. In particular ref#1 is in the absence of such molecular and mechanistic insight not willing to provide the essential support for your study which is rather explicit from the remarks sent directly to our editorial office. Both referees (#1 and #2) however do also suggest various experiments that might help to solve this currently problematic issue and I would like to seriously attend to this problem.
A number of other issues were raised by the referees that I do not have to repeat here, but that would certainly also need modification/correction. Overall, we would kindly offer you the chance to carefully and thoroughly adjust the paper. I do have to remind you though that it is EMBO_J policy to allow a single round of major amendments only, i.e. if essential critiques remain unsatisfied by your responses, we might still have to reject the manuscript!

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

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors observe a heightened inflammatory response in an LPS-induced skin vascular damage model (local Schwartzmann reaction) upon disruption of the MAPK phosphatase MKP5. The phenotype of this knockout has been described previously to show elevated inflammatory responses. Accordingly, this finding per se is not that novel. The novelty is the documentation that the inflammation can be traced to elevated p38 MAPK and consequent elevated production of superoxide through a p47phox-dependent mechanism. The authors need to address to a greater degree the relationship between p38 and p47-phox-dependent superoxide production. Thus,

1) Does coadministration of the p38 inhibitor or, preferably, crossing the MKP5 knockouts with mice for which elements of the p38 pathway have been disrupted block the exacerbated LSR?

2) How does p38 regulate p47 in this system? Do you actually see elevated p47 phosphorylation at

Ser345 in the knockout mice? Is this a direct reaction or is it mediated by p38-activated kinases?

Referee #2 (Remarks to the Author):

General Comments:
The manuscript entitled ´A non-redundant role for MKP5 in limiting ROS production and preventing LPS-induced vascular injury" by Qian et al, describes the results of studies designed to investigate the role of MAPK phosphatatase 5 in the regulation of neutrophil activation and LPS-induced inflammation.
The authors show that: 1)-A single dermal injection of LPS in MPK5-/- mice induced robust and accelerated vascular inflammatory responses ; 2)-Depletion of MKP5-/- neutrophils significantly reduced the vascular injury, whereas adoptive transfer of the MKP5-/- neutrophils was sufficient to reproduce skin lesions in recipient wild type mice ; 3)-The MKP5-/- neutrophils exhibited enhanced p38 MAPK activation and increased superoxide production upon C5a and TNF- stimulation, which was abolished by pharmacological inhibition of p38 MAPK but not ERK and JNK ; 4)-Deletion of the p47phox gene diminished the LPS-induced vascular injury in MKP5-/- mice.
The authors concluded that MPK5 is implicated in regulating neutrophil oxidant production and the protection against LPS-induced vascular injury. This manuscript reports new original data in the field of ROS production and inflammation, the experiments are well described and the presentation of the data is clear, however the authors should address some concerns in order to validate the conclusion.

Major Comments:

1. To my knowledge, since this is the first study to analyze the role of MPK5 in neutrophil activation and in skin inflammation, authors should show:
a)-Western blot data showing the expression of MPK5 in WT neutrophils compared to MPK5 KO neutrophils
b)-Since MPK5 is known to localize in the nucleus and p47phox is a cytosolic protein and activated NADPH oxidase is localized at the membranes, authors should determine subcellular localization of MPK5 in resting and stimulated neutrophils
c)-If possible, authors should check MPK5 activity in resting and C5a- fMLF- and TNF -stimulated netrophils.

2. The major concern in this sudy is the lack of direct link between p38MAPK and p47phox in mice. Indeed the human p47phox has potential MAPK phosphorylation sites (Ser345 and Ser348) which are phosphorylated in vitro and in intact cells. However in the mouse-p47phox sequence these sites are missing. Authors should check:
a)-if mouse recombinant p47phox is phosphorylated in vitro by p38MAPK
b)-if phosphorylation of p47phox in mice neutrophils is inhibited by the p38 inhibitor SB203580.

3. Depletion of MKP5-/- neutrophils and reconstitution by injection: Does the anti-Gr-1 antibody deplete only neutrophils, what about other granulocytes and monocytes ? Authors should show monocyte counts in Figures 3. Also how pure are the injected neutrophils, in the Methods section authors mention that neutrophils are only 78 %. For the injection experiment authors should use more purified neutrophils.

4. The p47phox-KO experiment : As shown by the data presented in this manuscript, p47phox is necessary for the modified local Shwartzman reaction. What is the effect of p47phox deletion on classical local Schatzman reaction induced by consecutive injections of LPS and then TNF ? Authors should also add these data.

5. For migration and degranulation experiments, authors should test the effect of MPK5-KO on fMLF responses. For superoxide production experiments, authors should test the effect of MPK5-KO on fMLF and PMA responses.

Referee #3 (Remarks to the Author):

The manuscript by Qian and colleagues described interesting findings regarding the function of MKP5 in the regulation of neutrophil ROS production, which is a critical aspect of the innate immune response. The studies were nicely designed. The findings are novel and the phenotype is robust. These findings represent a major advance in our understanding of the physiological function of this group of novel protein phosphatases. The conclusions are convincing and the manuscript is, for the most part, well written. I only have some minor comments on data presentation and discussion.

1. Page 4, line 6: The sentence "Dephosphorylation of active....by a dual specificity MKPs...that involves the active site Cys and Asp..." is awkward. MKP here should be singular. The sentence is difficult to read and should be rephrased.
2. The introduction is too long, and should be condensed a little, ideally to within two to two and half pages. Perhaps it is excessive to give too much detail on the major findings in the Introduction section.
3. The first paragraph of the Results section (2^{nd} - 4^{th} sentences) should be changed. Since results from experiments are described, past tense should be used.
4. Page 7, top: "The number of neutrophils at...was determined based..." should be changed to "neutrophil infiltration was quantitated ...", because MPO assay does not tell the number of neutrophils.
5. Page 12, Discussion section, lines 1-3: The authors stated that MKPs exhibit very little activity in the absence of their physiological substrates. Such a statement is not entirely accurate, since only a subset of MKPs can be catalytically activated by interaction with their cognate kinase substrate. For example, MAPKs have little effect on the catalytic activity of MKP5 (J Biol Chem. 1999, 274(28):19949-56). Furthermore, DUSP5 also does not undergo catalytic activation by its substrate, ERK (Mol Cell Biol. 2005, 25(5):1830-45).
6. Page 13, Discussion section: The sentence "In fact, the substrate...(e.g., MKP3 and PAC1),...which are highly selective for ERK" is problematic, and needs to be rephrased. In vitro PAC1 prefers ERK and p38 as substrate (J Biol Chem. 1996, 271(11):6497-501). However, the studies by Jeffery et al. demonstrated that its physiological substrate is actually JNK (Nat Immunol. 2006, 7(3):274-83).
7. Page 13, the sentence "To better understanding the difference..." should be rephrased. The sentence sounds like that experiments have been performed in their studies to compare the structures, catalytic mechanisms, and cellular localizations of the two phosphatases. The authors can change this sentence to something like "Several differences in the biochemical properties between the two phosphatases may contribute to their functional differences described here". In addition to the differences in biochemical properties including subcellular localization, it is also plausible that differences in expression pattern could contribute to the functional differences between MKP5 and MKP1. In this regard, it would be informative to know whether MKP1 is expressed in neutrophils.
8. The Discussion section is too long. The authors should try to shorten it a little.

1st Revision - Authors' Response 20 July 2009

We would like to thank the reviewers for their insightful and constructive comments. In response to these critiques and comments, we have conducted additional experiments and addressed these concerns as detailed below.

General changes to the manuscript:

1) Approximately 60% more data have been generated during the revision process, which necessitates the creation of a Supplementary Information section for negative controls and other results not essential to the major conclusions but overall important to the paper. 2) As a result of these changes, the figures are renumbered.
3) We have agreed to add Dr. Ni Cheng as a coauthor for his significant contribution in conducting the mouse p47phox phosphorylation assays.

Our point-by-point responses to reviewers' comments are on separate pages below.

Referee #1:

The novelty is the documentation that the inflammation can be traced to elevated p38 MAPK and consequent elevated production of superoxide through a p47phox-dependent mechanism. The authors need to address to a greater degree the relationship between p38 and p47-phox-dependent superoxide production. Thus,

1) Does coadministration of the p38 inhibitor or, preferably, crossing the MKP5 knockouts with mice for which elements of the p38 pathway have been disrupted block the exacerbated LSR?

We appreciate the reviewer's comments and have determined the *in vivo* effect of SB203580 coadministration, an approach that is feasible given the 90-day revision period, which precludes additional crossing of mice for this experiment. Coadministration of SB203580 with LPS resulted in a significant reduction in skin lesions compared to administration of LPS alone (new Figure 6A). A significantly reduced level of ATF-2 phosphorylation was also observed in skin tissues receiving LPS together with SB203580, compared to skin tissues receiving LPS alone (new Figure 6B). These results support our conclusion that excessive activation of p38 MAPK contributes substantially to the LPS-induced vascular inflammation in the Mkp5-/- mice.

2) How does p38 regulate p47 in this system? Do you actually see elevated p47 phosphorylation at Ser345 in the knockout mice? Is this a direct reaction or is it mediated by p38-activated kinases?

In human neutrophils, p38 regulates p47phox through phosphorylation of Ser345. As reported by Dang et al and cited in this paper, phosphorylation at Ser345 primes NADPH oxidase in TNF-α stimulated neutrophils (*J Clin Invest*, 116, 2033-2043, 2006). Whether the mouse p47phox is directly phosphorylated by p38 MAPK was not determined prior to this study. We noticed sequence difference between human p47phox and mouse p47phox surrounding the potential phosphorylation site (new Figure 7A). To determine whether mouse p47phox is directly phosphorylated by p38 and which site is phosphorylated, we prepared GST fusion proteins of mouse p47phox that carry alanine substitution at Ser346, Ser355 or Thr356, the three potential phosphorylation sites for p38 based on sequence analysis. The recombinant GST-mp47phox proteins were used as substrates for p38 in our in vitro kinase assays. We observed equal phosphorylation of the full-length mp47phox and its S346A and S355A mutants, but the level of phosphorylation was markedly reduced with the T356A mutation (new Figure 7B, 7D). These new results demonstrate direct phosphorylation of mouse p47phox by p38.

We have tried to detect mouse p47phox phosphorylation using the antibody developed by Dang and colleagues, which recognizes phospho-Ser345 in human p47phox. Unfortunately, this antibody does not detect mouse p47phox, probably due to the sequence difference (Figure 7A) and the apparent lack of p38-mediated phosphorylation at Ser346 in mouse p47phox. In the absence of a suitable anti-phospho-antibody, we used a phosphoprotein purification column (Qiagen) to enrich phosphoproteins in C5a-stimulated neutrophils from WT and Mkp5-/- mice. As shown in the new Figure 8, C5a induced a marked increase in p47phox phosphorylation that was significantly inhibited by SB203580. The level of phosphorylation is higher in Mkp5-/- neutrophils than in WT neutrophils.

The C5a-induced phosphorylation of mouse p47phox was partially (40-50%) inhibited by the p38 inhibitor SB203580 (new Figure 8). On one hand, this result is consistent with the fact that C5a activates multiple kinases and some of them, including PKCs, are known to phosphorylate p47phox but are not sensitive to SB203580. On the other hand, the extent of inhibition is quite significant given that only Thr356 has been identified as the site for p38 phosphorylation. At present, we cannot exclude the possibility that mouse p47phox has other site(s) for p38 phosphorylation. Moreover, kinases downstream of p38 may also contribute to the phosphorylation of p47phox. Finally, p47phox phosphorylation by p38 may facilitate its phosphorylation by other kinases, which is in line with the concept of NADPH oxidase priming that potentiates subsequent activation in response to additional agonists. These possibilities are now discussed in the revised paper (p.15, second paragraph) and will be investigated in future studies.

Referee #2:

This manuscript reports new original data in the field of ROS production and inflammation, the experiments are well described and the presentation of the data is clear, however the authors should address some concerns in order to validate the conclusion.

Major Comments:

1. To my knowledge, since this is the first study to analyze the role of MPK5 in neutrophil activation and in skin inflammation, authors should show:
a)-Western blot data showing the expression of MPK5 in WT neutrophils compared to MPK5 KO neutrophils.

We agree with the reviewer on this point, and have added to Supplementary Figure S6 Western blotting results showing MKP5 expression in WT mouse neutrophils compared to MKP5 KO neutrophils.

b)-Since MPK5 is known to localize in the nucleus and p47phox is a cytosolic protein and activated NADPH oxidase is localized at the membranes, authors should determine subcellular localization of MPK5 in resting and stimulated neutrophils.

In the original manuscript, we mentioned that cytosolic localization of MKP5 might be crucial to its function in limiting p47phox phosphorylation by p38 MAPK. Since p47phox is a cytosolic component of NADPH oxidase in resting cells, we determined the relative distribution of mouse MKP5 in the cytosolic and nuclear fractions. Using Western blotting, we detected the majority of MKP5 in neutrophil cytosolic fraction, although it is also present in the nuclear fraction. C5a stimulation does not cause a detectable shift between the cytosolic and nuclear localization of MKP5 (Supplementary Figure 6S). A more detailed subcellular localization study will be carried out in the future to determine MKP5 distribution in other cellular compartments when neutrophils and macrophages are exposed to various soluble and particulate stimuli.

c)-If possible, authors should check MPK5 activity in resting and C5a- fMLF- and TNFα - stimulated netrophils.

We agree that this is an important question in MKP research, but it involves a complex regulatory process that remains controversial, and is outside the scope of the current study. We have a plan to collaborate with other investigators for a future study of the activities of MKP5 (and those of other MKPs) in phagocytes.

2. The major concern in this study is the lack of direct link between p38MAPK and p47phox in mice. Indeed the human p47phox has potential MAPK phosphorylation sites (Ser345 and Ser348) which are phosphorylated in vitro and in intact cells. However in the mouse-p47phox sequence these sites are missing. Authors should check: a)-if mouse recombinant p47phox is phosphorylated in vitro by p38MAPK. b)-if phosphorylation of p47phox in mice neutrophils is inhibited by the p38 inhibitor SB203580.

We appreciate these comments and have conducted experiments to address the issues. a) To establish a direct link between p38 MAPK and p47phox in mice, we performed in vitro kinase assay as described above in our response to Reviewer #1, and detailed in the legend to the new Figure 7. As the reviewer correctly pointed out, the sequence surrounding Ser346 in mouse p47phox is different from the sequence surrounding Ser345 in human p47phox. A phosphorylation site prediction analysis (using the PSPP software) identified Ser346, Ser355 and Thr356 as potential sites for p38 MAPK phosphorylation. Using alanine substitution, we generated mutant p47phox as substrates for in vitro kinase assays. We found that p38 MAPK-mediated phosphorylation of mouse p47phox was reduced with the Thr356 to Ala mutation (new Figure 7). These results demonstrate that mouse p47phox can be directly phosphorylated by p38 MAPK *in vitro*, and Thr356 is a site for p38 MAPK phosphorylation. At present, the possibility that p38 MAPK phosphorylates mouse p47phox at more than one site cannot be ruled out, and will need to be investigated in a future study.

b) We observed that C5a stimulated p47phox phosphorylation in mouse neutrophils, and the induced phosphorylation was inhibited by SB203580 in both WT and MKP5 KO neutrophils (new Figure 8). The inhibitory effect of SB203580 matched to its suppression of LPS-induced skin lesions when SB203580 was coadministered with LPS, an experiment suggested by the first reviewer (new Figure 6). We provided a discussion on the several possibilities for the 40-50% inhibition of phosphorylation by SB203580, based on our own results as well as previously published data on neutrophil NADPH oxidase priming (p.15, second paragraph; see also our response to the first reviewer).

3. Depletion of MKP5-/- neutrophils and reconstitution by injection: Does the anti-Gr-1 antibody deplete only neutrophils, what about other granulocytes and monocytes ? Authors should show monocyte counts in Figures 3. Also how pure are the injected neutrophils, in the Methods section authors mention that neutrophils are only 78 %. For the injection experiment authors should use more purified neutrophils.

To address this concern, we have determined that the anti-Gr-1 antibody used in this experiment (clone RB6-8C5 recognizing Ly-6G and Ly-6C) caused a small reduction in peripheral blood monocytes count, although the change is not statistically significant (Supplementary Figure S1A). To determine whether neutrophils are responsible for the LSR in the recipient mice, we prepared highly purified neutrophils based on the side scatter property of these cells (Figure S1B). The sorted cells were examined under microscope after cytospin, and no monocytes were identified out of ~250 cells examined (data not shown). Eosin positive cells are less than 1%. The sorted cells were > 99% positive when stained with an antibody (clone 1A8) that specifically recognizes Ly-6G (1A8). These purified neutrophils were then injected in recipient mice and LPS-induced LSR was determined. As shown in supplementary Figure S2, the purified neutrophils mediated a robust LSR in recipient mice after LPS injection. Together, these results confirm that Mkp5-/- neutrophils are the cell population primarily responsible for the augmented LSR in recipient mice.

4. The p47phox-KO experiment: As shown by the data presented in this manuscript, p47phox is necessary for the modified local Shwartzman reaction. What is the effect of p47phox deletion on classical local Shwartzman reaction induced by consecutive injections of LPS and then TNFα? Authors should also add these data.

To answer this question, we conducted the suggested experiment using a classic LSR model (LPS + TNFα). The results showed a slightly reduced LSR in the absence of p47phox (supplementary Figure S5). The change is not statistically significant. This result suggests that skin lesions seen in the classic LSR and in the one-injection LSR are mediated through different mechanisms. In a paper which we cited, Hirahashi and coworkers reported that neutrophil elastase is a primary cause for the vascular inflammation seen in the classic LSR, and genetic deletion of gp91phox produced a very small effect in the skin lesions. Our result using the p47phox KO mice is consistent with their findings.

5. For migration and degranulation experiments, authors should test the effect of MPK5-KO on fMLF responses. For superoxide production experiments, authors should test the effect of MPK5-KO on fMLF and PMA responses.

We have conducted the suggested experiments and found that fMLF produced effects in neutrophil migration and degranulation that are similar to those produced by C5a in WT and MKP5 KO mice (Supplementary Figure S4A and S4B). As with C5a, both fMLF and PMA induced augmented superoxide production in Mkp5-/- neutrophils, compared to WT neutrophils (Figure S4C and S4D).

Referee #3:

The manuscript by Qian and colleagues described interesting findings regarding the function of MKP5 in the regulation of neutrophil ROS production, which is a critical aspect of the innate immune response. The studies were nicely designed. The findings are novel and the phenotype is robust. These findings represent a major advance in our understanding of the physiological function of this group of novel protein phosphatases. The conclusions are convincing and the

manuscript is, for the most part, well written. I only have some minor comments on data presentation and discussion.

1. Page 4, line 6: The sentence "Dephosphorylation of active....by a dual specificity MKPs...that involves the active site Cys and Asp..." is awkward. MKP here should be singular. The sentence is difficult to read and should be rephrased.

We thank the review for these suggestions, and have rephrased the sentence and corrected the typographic error.

2. The introduction is too long, and should be condensed a little, ideally to within two to two and half pages. Perhaps it is excessive to give too much detail on the major findings in the Introduction section.

We have reduced the length of the introduction by a half page.

3. The first paragraph of the Results section (2nd-4th sentences) should be changed. Since results from experiments are described, past tense should be used.

Again, we appreciate the constructive comment and made the suggested changes.

4. Page 7, top: "The number of neutrophils at...was determined based..." should be changed to "neutrophil infiltration was quantitated ...", because MPO assay does not tell the number of neutrophils.

The reviewer is correct. We have made the change as suggested.

5. Page 12, Discussion section, lines 1-3: The authors stated that MKPs exhibit very little activity in the absence of their physiological substrates. Such a statement is not entirely accurate, since only a subset of MKPs can be catalytically activated by interaction with their cognate kinase substrate. For example, MAPKs have little effect on the catalytic activity of MKP5 (J Biol Chem. 1999, 274(28):19949-56). Furthermore, DUSP5 also does not undergo catalytic activation by its substrate, ERK (Mol Cell Biol. 2005, 25(5):1830-45).

We thank the reviewer for pointing out this error, and have responded to the comments by revising this paragraph in the Discussion section.

6. Page 13, Discussion section: The sentence "In fact, the substrate...(e.g., MKP3 and PAC1),...which are highly selective for ERK" is problematic, and needs to be rephrased. In vitro PAC1 prefers ERK and p38 as substrate (J Biol Chem. 1996, 271(11):6497-501). However, the studies by Jeffery et al. demonstrated that its physiological substrate is actually JNK (Nat Immunol. 2006, 7(3):274-83).

We agree with the reviewer on this point. As a result of reducing the Discussion section, the mentioning of the discrepancy in substrate selectivity *in vitro* and *in vivo* has been removed.

7. Page 13, the sentence "To better understanding the difference..." should be rephrased. The sentence sounds like that experiments have been performed in their studies to compare the structures, catalytic mechanisms, and cellular localizations of the two phosphatases. The authors can change this sentence to something like "Several differences in the biochemical properties between the two phosphatases may contribute to their functional differences described here". In addition to the differences in biochemical properties including subcellular localization, it is also plausible that differences in expression pattern could contribute to the functional differences between MKP5 and MKP1. In this regard, it would be informative to know whether MKP1 is expressed in neutrophils.

The reviewer is correct. The cited findings were from published reports and not our own experimental data, except that we carried out an additional experiment to demonstrate the presence of MKP5 in neutrophil cytosolic fraction (Supplementary Figure S6). Changes have been made to this paragraph to distinguish between published findings and our own results.

8. The Discussion section is too long. The authors should try to shorten it a little.

In response to this comment, we have reduced the size of the Discussion section by 19%.

