Author’s response to reviews

Title: MORG1+/- mice are protected from histological renal damage and inflammation in a murine model of endotoxemia

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Dear Professor Sharpe,

We thank you and the reviewer for the interest in our manuscript. According to the issues raises, we have performed many additional experiment and have thoroughly modified our contribution. We would like to answers to the reviewers point by point in detail:

First of all we would like to thank to the reviewers for the critical reading of our manuscript and for the fruitful comments and recommendation in order to improve the quality of the manuscript.

Answers to the Reviewer 1.

1. “However, I am not convinced by the data shown in Figures 3 and 4 suggesting that MORG mutants have lower levels of PHD3 protein and higher levels of HIF 2 alpha protein after LPS treatment as these are based on "quantitative" analysis of IHC. Two issues here: PHD3 staining is not convincing in Fig 3A, and while HIF2A staining in Fig 4A is nice, quantification of staining intensity in this way is not a valid method to quantify protein expression. You could either: a) quantify positive cell numbers; and/or b) use western blots to quantify protein.”

• In order to answer your suggestions and to improve the quality of submitted manuscript we performed a new staining for PHD3 detection in kidney sections and to be better visualize the positive immunoreactivity we used as an HRP-substrate the DAB chromogen, not AEC chromogen as in the originally submitted version of the Fig. 3a. We believe that this improved the figure quality and in addition performed the evaluation of the immunoreactivity as you have suggested by counting the number of positively stained cells per treatment and expressed this
Graphically in addition to the representative images which are shown in a similar manner as in the originally submitted Fig. 3. The new data are presented in the current Fig. 3a, b.

- Similarly we performed a new quantification of the images of the HIF2 alpha staining and presented the quantification as well in number of positive cells per treatment. The new data are presented in Fig. 4b.

2. “Abstract: the statements that "although accumulation of renal HIF-2 was significantly higher in the kidneys of MORG1+/− mice than in wild-type mice, we detected diminished phosphorylation of IB-and IKK," needs to be better explained”.

- We modified the formulation of the sentence in the Abstract in order not to further increase the size of the Abstract through additional explanation, which need be done if the sentence is present into the Abstract.

3. “Background: is rather disorganized and difficult to follow and should be rewritten in a more linear fashion. In addition, remove "hypoxic preconditioning" from the last sentence as you are not studying this.”

- The Background is rewritten and we removed the "hypoxic preconditioning" from the sentence. You are absolutely right, we did not study hypoxic preconditioning but our studies on the MORG1 heterozygous animals demonstrate that the reduced expression of MORG1 is associated with an elevated basal levels of HIFs in the renal tissue, which in a way “mimic” the effect of induction of hypoxia preconditioning, where is aimed the induction of HIFs activation. But as we do not study in fact this in the present work we agreed to delete the statement from the sentence in the background.

4. “Methods: a) state whether analyses were blinded or not; b) state time tissues, blood and urine were analyzed after LPS treatment; and c) IHC quantification, see previous comments”

- To a): We checked again the Method part and it was already written that the scoring system we used in the analyses of the protein expression based on the immunohistology studies was performed from a person unaware of the experimental protocol (thus “blinded”). Therefore the scoring was performed blinded only we did not use exactly this word because it is difficult to judge microscopic slides, if you are indeed blind. Nevertheless we have written at the present version of the manuscript that the analyses were performed blind, from a person person unaware…

- To b): It was already explained that the treatments of the experimental protocol were performed for 24 h but we added as well additional explanation of the time issue in every other part of the Methods.

- To c): We performed according to your suggestion a new evaluation of the IHC quantification by counting the cell number of the positively stained cells per field. In addition we introduced that the analyses are were “blinded” as it was done in fact.
5. “Results: a) including timing of analyses in figure legends for clarity; b) format of Fig 6C is different from all of the others: suggest this is changed”

- To a): we included the timing of the analysis in the figure legends.
- To b): We changed the format of the Fig. 6C.

6. “Discussion: an alternative interpretation of the data is that MORG mutant are less sensitive to the effects of LPS because they have lower levels of TLR receptors and/or LPS is metabolized differently. This cannot easily be addressed in vivo but should be discussed”

- You are right that an alternative interpretation could be the lower sensitivity of the MORG1 mutant to LPS, which we did not analyze in the current contribution, but is an interesting issue to look for in future studies. We address as well this possibility in the discussion which was in general re-written as also suggested form the Reviewer 2.

- We would like to point that due to the re-writing of the Background and Discussions parts of the manuscript as well as shown results of new analyses of KIM1 protein expression as an additional marker of injury, there was a large change in the reference’s numbering and order as some references were removed and new references were included into the present revised version of the manuscript.
- All changes are highlighted in yellow in the revised version of the manuscript.

Answers to the Reviewer 2:

1. “Abstract: Too long”

- We shortened the abstract.

2. “Introduction: Also too long. Typo "gramm" negative”

- We also shortened the Background part of the manuscript and corrected the typo “gramm” negative to “gram negative”. We would like to add that the Background part was re-written as suggested from the Review 1.

3. “Methods: Were +/- controls age, sex and weight matched? Were they littermate or purchased controls?”

- The “+/- controls” were littermates in all experiments and were not purchased controls. Therefore we labeled them as MORG1+/- wild-type controls and not only a wild-type control. In addition we explained at the revised version of the manuscript that the MORG1 heterozygous mice were generated previously in our laboratory for better clarity. “The MORG1+/- mice were generated as described in (Hammertschmidt et al., Am J Physiol-Renal 2009, 297(5):F1273-F1287) and were backcrossed for more than 12 generation with C57BL/6J genetic background.”

All experiments were performed with male mice as it is written in the Methods part. The age and
weight of the “+/+ control” mice matched between the other experimental mice used in this experimental protocol.

4. “Does the dose of LPS cause hypotension in the mice?”

- We did not measure the blood pressures of mice used in this experimental procedure. We can only add that looking carefully the PAS staining of all the mice the tubular vasodilation was obvious in the wild-type endotoxemic mice it was also detected in endotoxemic MORG1 heterozygous mice tubular dilatation but to a lesser extent. As one of the characteristics of the hypotension is the tubular dilatation we assume, but indirectly, that in our experiment the i.p. injection of 5 mg/ml LPS probably induced slight hypotension, although Knotek et al., Kidney Int 59, 2243, 2001 did not measure a significant reduction of the MAP after application of the same dose of LPS in mice. The differences in our results could be also related to the fact that we did not infused intravenously the mice during the entire procedure with 6% albumin, as in Knotec et al., KI 2001, and did not kept the mice in a sedative state throughout the experimental protocol. In addition in the Supplementary Fig.1 we assessed the iNOS expression by real time PCR and have found a higher increase in the mRNA of iNOS in the wild-type endotoxemic mice, what is also known in LPS treated mice to be associated to the induced hypotension as inhibitors of iNOS reverse the LPS dependent hypotension at least in rat model of endotoxemia (Tunctan, B. et al., Nitric Oxide-Biol Ch 2013, 33:18-41.)

5. “Results: Fig 1 Tubular injury is less in +/- mice. There should be arrows in the pictures demonstrating the different features of tubular injury “

- We added the asterisk to label the tubular damage on the images.

6. “Results: Fig 2: Plasma NGAL is not really a marker of KIDNEY injury but rather a systemic response. Comment on this

- NGAL was suggested as a novel marker to study early AKI and specially related to septic conditions and nephrotoxicity (Bolignano, D. et al., Am J Kidney Dis. 2008 Sep;52(3):595-605). NGAL levels in plasma are a more related to the systemic response as it is released not only from activated neutrophils in response to bacterial infection but also from many other cells types as kidney tubuli, and also from other injured organs as liver. Thus the plasma levels of NGAL are more related to the systemic response to inflammation as to specifically renal damage. Nevertheless a lot of researchers investigate NGAL as a marker for early renal injury as it rapidly rises in response to injury much earlier than e.g. serum creatinine levels. We also believe that NGAL is more representative marker for the system response in inflammation related injury therefore we think that the urinary NGAL levels and/or renal NGAL levels are more representative for the renal damage, compared with the plasma NGAL levels. This was actually supported as well as from our results presented in Fig. 2 a, b and c.

7. “Results: Was BUN, serum creatinine or inulin GFR measured. Inulin GFR, but not BUN, SCr, has been found to be decreased with LPS (Knotek at el. Kidney Int 59, 2243, 2001)"
• We presented in the revised version of the manuscript the data of the plasma levels of Creatinine and BUN concentrations. We found an elevation in both during the treatment with 5 mg/kg BW LPS as it is presented in the new Fig. 2e and f. We did not measure in our study the inulin GFR.

8. “Results: Fig 3: The decrease in PHD3 protein expression in +/- is very small (does not seem to merit P<0.05) and there is no decrease in mRNA. Major conclusions are drawn about PHD3 in Abstract and Discussion based on this weak data. Conclusions about PHD3 should be toned down, limitations of data discussed OR more experiments performed to look at PHD3.”

• The decrease of the PHD3 protein expression in renal section as detected by scoring between MORG1 +/- and MORG1 +/- mice was P<0.05 since we have analysed a large number of PHD3 staining images per experimental group but the scores were close to each other. In the revised version we repeated the PHD3 stain in renal sections as suggested from Review 1 and quantified the PHD3 staining and protein expression based on the PHD3 positive cells per analysed field. The new data are presented in the Fig. 3a (representative images) and Fig. 3b (PHD3 protein expression – graphic presentation). The reduced PHD3 expression and as well PHD3 prolylhydroxylation activity is associated with the increase basal HIF2-

9. “Results: Fig 4 HIF 2 alpha protein expression is increased in +/- Fig 5 IL-6 and INF (//typo for IFN) data is not new Fig 6 TNF protein not increased in +/- Fig 7 NF-KB not increased in +/- Fig 8 CD4 T cells and Caspase-3 not increased in +/- Should say "Caspase-3" and not extrapolate to apoptosis without showing changes in apoptosis on histology (Fig 1) Fig 9 Is there a way of determining whether the data is statistically significant discussion too long.

A lot of previous studies and background data is discussed rather than commenting on each of the multiple findings.”

• In the revised version of the manuscript we have re-written the Discussion and try to address if not all at least most of our findings. Unfortunately this does not made the discussion part shorter than in the originally submitted version. We also followed your suggestions and remove the apoptosis from the section with analysis of Caspase-3 activation, as well as corrected the typo for IFN HIF2-

• Survival analyses presented in Fig. 9 are based on the Kaplan-Maier survival analyses method. Regardless, that we detected that all endotoxemic heterozygous mice survived the 72 h period, while two of the wild-type endotoxemic mice (20%) died due to endotoxemic shock (even at this dose of LPS 5 mg/kg BW). The results are not significant according to this method which is a standard method to analyze the survival ability in animal models. If one would like to analyze the mice by pairwise comparison using Mann-Whitney method the results between the
endotoxemic groups will be then significant but this is unfortunately not a correct presentation of the results for survival studies. Therefore the data are not yet statistically significant.

- All changes are highlighted in yellow in the revised version of the manuscript.

In summary, we have performed several additional experiments, present new data, and have rewritten our manuscript. We think that we have comprehensively addressed the critiquae raised in the review process and hope that our manuscript is now acceptable for publication in BMC-Nephrology.

Prof. Dr. Gunter Wolf, MD, MHBA on behalf of all authors