Author's response to reviews

Title: Moderate activation of IKK2-NF-kB in unstressed adult mouse liver induces cytoprotective genes and lipogenesis without apparent signs of inflammation or fibrosis

Authors:

Hong Lu (luh@upstate.edu)
Xiaohong Lei (leix@upstate.edu)
Qinghao Zhang (zhangqinghao1982@gmail.com)

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Author's response to reviews: see over
Dear Editor:

Enclosed is our revised manuscript entitled “Moderate activation of IKK2-NF-kB in unstressed adult mouse liver induces cytoprotective genes and lipogenesis without apparent signs of inflammation or fibrosis” (MS: 1924509767154248) by Hong Lu, Xiaohong Lei, and Qinghao Zhang. Please consider it for publication as a research article in BMC Gastroenterology.

We thank the reviewer for helping us improve the quality of our manuscript. We have read the reviewer’s comments carefully and made the corresponding modifications to our manuscript. All co-authors agreed on the content of this manuscript. We hope that the revised manuscript has addressed the major concerns of the reviewer.

Our detailed responses to the reviewer’s comments are as follows:

Major Compulsory Revisions

On the first sight, the story from Hong Lu and colleagues sounds interesting and indicate a dose-dependent effect of constitutive active IKK2 in hepatocytes, which is in contrast to previous published studies. However, the study is based only on expression profiles without the confirmation on protein levels to indicate a physiological relevance. It would be helpful to analyze 2-3 genes on protein levels for each subclass of target genes (cytokines, antioxidative genes etc.). Moreover, the result part is a listing of more than 100 genes and makes it quite difficult to read.

Answer: We have determined protein levels of two key cytokines, IL-6 and Ccl2/Mcp-1. Consistent with the lack of hepatic induction of mRNA expression of these two cytokines, blood protein levels of these two cytokines remained unchanged (Paragraph 2, Page 11). These results further confirm the lack of proinflammatory response in our model of mice with liver-specific activation of Ikk2. Additionally, we have decreased the number of genes described in the manuscript by moving 37 genes that were not differentially expressed to Additional files 2 and 3 in the revised manuscript.

The authors state, that in contrast to previous published studies, their constitutive active IKK2 mice, is moderate expressed in livers (even lower than endogenous IKK2) and therefore cannot induce the previous described pro-inflammatory and fibrogenic phenotype. This might indicate an insufficient experimental setting. Furthermore, it is well known that the Albumin-Cre mice lead to an inefficient deletion of floxed sequences (for example c-FLIP-deletion, Sci Signal. 2013 Jan15;6(258):pe2. doi: 10.1126/scisignal.2003845.) and therefore it is unclear if the IKK2ca is really overall expressed in these mice. The paper would benefit with additional experiments to prove the overactivation of NF-kB via IKK2ca, eg EMSA and nuclear p65 immunohistochemistry. Moreover, EMSA-analyses should be also performed for the non-canonical pathway because the authors indicate an important anti-inflammatory effect for this pathway.

Answer: The Alb-cre has been widely used for liver-specific knockout or knockin of the floxed transgene. The Ikk2ca protein has a Flag tag. Thus, we used real-time PCR to determine hepatic mRNA expression of the Ikk2ca transgene, using forward and reverse primers specifically targeting the Flag tag and Ikk2 cDNA, respectively. Ikk2ca mRNA was very low in
the wild-type (Ikk2ca fl/+; Alb-cre/-) livers (Mean Ct value ~30), whereas the Ikk2ca mRNA expression levels in Liv-Ikk2ca mice (mean Ct value 24.7) appeared comparable to the expression levels of endogenous Ikk2 mRNA in wild-type mice (mean Ct value 24.3), estimated by the comparable Ct values of the Ikk2ca and mouse Ikk2 in these mice. Thus, our new real-time PCR data support our claim of a moderate expression of Ikk2ca transgene in the current Liv-Ikk2ca mouse model which is likely lower than the two previous Liv-Ikk2ca mouse models in which the Ikk2ca transgene was driven by strong promoters from albumin and liver activator protein. We have added this information to the revised manuscript (Paragraph 1, Page 10). Additionally, we have conducted Western blot quantification of Ikk2 and Ikk1. Our results showed that Liv-Ikk2ca mice had an additional band in the cytosol detected by the Ikk2 antibody, which might be the transgenic Ikk2ca protein or post-translationally modified Ikk2 protein (Fig. 8). We had technical difficulty in EMSA assay of NF-kB pathways. As an alternative approach, we used ChIP-qPCR to determine DNA-binding of NF-kB p50 to promoters of genes which had altered expression in our Liv-Ikk2ca mice. Our ChIP-qPCR results (Fig. 9) showed that the global DNA-binding and locus-specific binding of p50 were markedly increased in Liv-Ikk2ca mice, which is consistent with a marked increase in the nuclear p50 in Liv-Ikk2ca mice. Thus, our new results further confirmed that the NF-kB pathway is activated by the Ikk2ca protein in our Liv-Ikk2ca mice.

Furthermore, the results shown in Figure 8b are uncompleted showing nuclear fraction with a GADPH loading control whereas it would be necessary to use a nuclear marker such as TBP. This figure needs to be updated regarding this point, and both fractions (cytoplasmic and nuclear) should be load side by side on a blot.

Answer: we have conducted Western blot quantification of Ikk1, Ikk2, and NF-kB subunits in both nuclear and cytosolic fractions (Fig. 8), and histone H3 was used as nuclear marker.

Minor Essential Revisions Finally, it would be necessary to discuss a possible NF-kB independent function of IKK2 that might suppress inflammation in the context of low expression.

Answer: After literature search, we found that Ikk2 is known to phosphorylate tuberous sclerosis 1, resulting in activation of the mTOR pathway. mTOR has multifunctional role in inflammation; inhibition of mTOR causes distinct inflammatory side effects such as fever, pneumonitis, glomerulonephritis or anemia of chronic disease. Thus, the NF-kB-independent function of IKK2, such as activation of the mTOR pathway, might suppress inflammation in the context of low expression of activated Ikk2. We have added the above discussion in the revised manuscript (Paragraph 2, Page 20).

Sincerely yours,

Hong Lu, Ph.D.
Assistant Professor
Department of Pharmacology
SUNY Upstate Medical University
6303 Weiskotten Hall Addition
750 E Adams ST
Syracuse, NY 13210