Format for ANSWERING REVIEWERS

April 14, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: *ESPS Manus 9451, Harrison-Findik 04.4.14.doc*).

Title: TLR4 signaling and the inhibition of hepcidin expression by alcohol

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Name of Journal: World Journal of Gastroenterology

ESPS Manuscript NO: 9451

We thank the reviewers for valuable comments and have performed new experiments, as requested. Our responses to the comments are below and the new data is incorporated into the revised manuscript (as indicated). The revisions have been highlighted in yellow in the new manuscript.

To reviewer 1:

1- As requested, densitometric analysis has been carried out for all the western blots and graphs have been inserted into the appropriate figures of the revised manuscript.

2a- We understand the concern with nuclear NF-κB phosphorylation. We performed new western blots. The new figure including quantitative data (***see Figure 3A-F in revised manuscript, page 23***) now clearly shows the difference in nuclear NF-κB phosphorylation between wildtype and mutant mice with better controls. Namely, the livers of wildtype, but not mutant, mice show that an ethanol-mediated decrease in cytosolic NF-κB phosphorylation is clearly accompanied by a significant increase in nuclear NF-κB phosphorylation. This strongly suggests that NF-κB is activated in wildtype, but not mutant, mice, as supported by the well-documented evidence in the literature that upon activation, phosphorylated NF-κB translocates to the nucleus.

2b- As requested by the reviewer, we have also performed western blots with IκB alpha. These findings have been incorporated into the revised manuscript (***see Figure 4A-B in revised manuscript, page 24***) and support NF-κB data in figure 3.

2c- The reviewer is asking whether TNFα receptor (TNFR1) could be involved in the regulation of hepcidin by alcohol in our TLR4 mouse model. We have previously published data (*Harrison-Findik, D. et al. (2009). Am. J. Physiol. Gastrointest. Liver Physiol. 296: G112-G118*), which clearly shows that TNFα signaling is not involved in the regulation of hepcidin by ethanol by using TNFα receptor (TNFR1

&TNFR2) knockout mice. We therefore think that it is highly unlikely that TNFR1 signaling plays a role in the regulation of hepcidin by alcohol in our TLR4 mouse model.

3a- The reviewer is inquiring whether the p65 subunit of NF-κB (like p50 subunit, as shown in the original manuscript) also binds to hepcidin promoter. We performed additional CHIP analysis using anti-p65 antibodies. Our new findings show that, unlike with p50, hepcidin promoter is not occupied by p65 in wildtype and TLR4 mutant mice. These findings have been discussed in the revised manuscript (***see bottom of page 11***).

3b- The reviewer requested the examination of a typical NF-κB -responsive gene to show the inhibition of NF-kB in ethanol-treated TLR4 mutant mice. SAA3 is a well-known target of NF-κB [Reference 37: *Reigstad CS, et al. PLoS One 2009;* ***4****(6)*] and we determined liver SAA3 gene expression by real-time PCR. Our results show that ethanol significantly up-regulates SAA3 expression in wildtype, but not in TLR4 mutant, mice (***see figure 4C in revised manuscript, page 24***), which indicates inhibition of NF-κB function in TLR4 mutant mice following chronic ethanol exposure.

4- The reviewer is asking whether there is any functional consequence to increased Stat3 phosphorylation in TLR4 mutant mice. Since the main aim of this manuscript is the regulation of hepcidin, we performed CHIP experiments to examine the binding of Stat3 to hepcidin promoter. We observed Stat3 occupancy of hepcidin promoter in alcohol-fed TLR4 mutant, but not wildtype, mice (***see figure 6C in revised manuscript, page 26***). These new findings suggest that alcohol-mediated increase in Stat3 phosphorylation in ethanol-fed TLR4 mutant mice results in Stat3 binding to hepcidin promoter and might have functional consequences.

5- The reviewer is asking about the role of different cell populations in the liver in the regulation of hepcidin by ethanol. We agree that hepcidin is expressed in hepatocytes but the major activation site for LPS and TLR4 signaling are Kupffer cells. However, we have previously published data clearly showing that Kupffer cells are not involved in the regulation of hepcidin by acute or chronic alcohol exposure in rats and mice by inactivating (GdCl3) or depleting (liposomes containing clodronate) Kupffer cells in the liver (*Harrison-Findik, D. et al. (2009). Am. J. Physiol. Gastrointest. Liver Physiol. 296: G112-G118).*

6- The reviewer is agreeing with the protein-protein interaction between SHP and NF-κB (*Figure 8 in revised manuscript*) but questioning alcohol-mediated changes in this interaction given the differences in sub-cellular SHP expression in the livers of wildtype and TLR4 mutant mice (*Figure 7 in revised manuscript*). We have incorporated quantitative analysis of nuclear and cytosolic SHP expression in the livers of

wildtype and mutant mice (***see figures Figure 7E and 7F in revised manuscript***). Alcohol-mediated significant changes in liver SHP expression were observed only in the cytosol and not in the nucleus. Furthermore, the changes in cytosolic SHP expression occurred in the livers of wildtype, but not in TLR4 mutant, mice (*see figures 7E and 7F*). We therefore believe that alcohol-mediated increase in cytosolic SHP expression (*figure 7E*) correlates with alcohol-mediated decrease in protein-protein interaction between SHP and NF-κB (*figure 8, lane 2*). As the reviewer suggested, we exercised caution with the interpretation of these results in the Discussion section (***see highlighted sections on page 13 of revised manuscript***).

Minor comments

1. We have included the method describing the isolation of cytosol and nuclear fractions in the Methods section of the revised manuscript (***see Material and Methods section in revised manuscript, page 6***).

2. We have revised the Discussion section of the manuscript, as requested and avoided the over-interpretation of results such as SHP and NF-κB interaction. The changes have been highlighted in yellow (***see Discussion section in revised manuscript, pages 12 & 13***).

To reviewer 2:

1- The reviewer is asking for the quantification of figures 2 to figure 6 (*figure 8 in revised manuscript*). We have performed densitometric analysis for all the western blots and immunoprecipitation, and graphs have been inserted into the appropriate figures of the revised manuscript.

2- The reviewer is asking about animal numbers. As requested, we have incorporated this information into Material and Methods section and into the figure legends.

3- We agree with the reviewer regarding data showing inflammation outcome in our mouse model following alcohol exposure. We performed ELISA assays to measure the level of proinflammatory cytokine, TNFα, which is known to be induced by alcohol-mediated inflammation. These new results have been incorporated into the manuscript (***see figure 2 in revised manuscript, page 22***).

Minor comments:

1- The reviewer is asking about alcohol-mediated increase in Stat3 activation in TLR4 mutant mice in figure 4 (*figure 6 in revised manuscript*). We quantified Stat3 protein expression levels (***see figure 6B in revised manuscript***) and performed CHIP assays to show the functional consequence of Stat3 activation in alcohol-fed TLR4 mutant mice (***see figure 6C in revised manuscript***). These findings have been

discussed in the revised manuscript (*see Discussion section, page 12 in revised manuscript*).

2- The reviewer pointed out the repetition of information in the first paragraph of Discussion section regarding the role of TLR4 in ALD. As requested, we have deleted this information and simplified the relevant section (*see first paragraph on page 11 in revised manuscript*).

We believe that we have addressed the issues and thereby have improved the manuscript according to the reviewers' comments. A copy of the revised manuscript with all of the changes highlighted in yellow is provided. We trust that the revised manuscript is now acceptable for publication in W. J. Gastroenterology. We appreciate your consideration.

Sincerely

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