Point-to-point Responses to the Reviewers' Comments

Reviewer 3

The study by Puengel et al. describes a two-pronged approach for alleviating NASH and aspects of acute liver injury by targeting CCR2/5 and FGF21. The data includes human patient data with NAFLD/NASH at different fibrotic stages, multiple mouse models using the CDAHFD diet, and an acute injury CCl4 model. Overall, the study is well designed in describing how combined therapy may be useful for targeting metabolic (FGF21) and inflammatory (CCR2/5) components of inflammatory fatty liver disease. The addition of characterization of blood and liver immune cells adds a mechanistic approach which is a strength of this study. That being said, there are areas the authors need to address to improve their study:

Response: We highly appreciate the positive evaluation of our manuscript by the reviewer.

1) My biggest concern with the data as presented has to do with the CDAHFD model and the conclusions drawn from it. Did all of the CDAHFD groups have similar consumption of the diet? All of the treatments had a weight drop after the treatment started in week 6. In particular the combined group went from ~24g to ~19g from weeks 6-8. This supports that they likely could have had reduced food intake following treatment. Without reporting this data, the conclusion could be made that all effects in the CDAHFD model were due to changes in food consumption, which reduced their exposure to the CDAHFD diet, and therefore showed anti-inflammatory and reduced hepatic lipid deposition effects compared to the vehicle group. Food consumption must be reported to alleviate this potential alternative hypothesis.

   Response: We would like to thank the reviewer for his/her detailed observation of our experimental NASH model. As mentioned in the discussion the "ideal" NASH model reflecting all aspects of the human disease does not exist but CDAHFD fed mice develop severe fibrosis and steatohepatitis (1). Nonetheless, systemic features of the metabolic dysregulation (such as obesity or insulin resistance) are less well reflected by the CDAHFD model. The CDAHFD is a commonly used diet in the NAFLD research field to induce experimental NASH in which an initial weight loss of the mice can usually be

observed followed by stabilisation of the bodyweight (2). We particularly opted for this model, because it allows to study inflammatory as well as fibrogenic pathways simultaneously, which we considered mandatory to assess the combined effects of FGF-21 analogues and CCR2/5 inhibitors in a translationally relevant study.

All experiments were performed under conditions approved by the appropriate institutional and governmental authorities according to German legal requirements with regular weight controls and no individual mouse lost ≥20% bodyweight compared to the initial weight from the start of the experiment.

Regarding the differential effects of the various compounds on body weight, we did not exactly measure the individual food intake of the mice. However, body weight loss is a well-known mechanism of action of FGF-21 agonists, which is also reflected in our data. Previous studies in other mouse models are in line with our work and show that these effects are not related to food intake. In particular, Fisher et al. showed that FGF-21 KO aggravated NAFLD in the MCD diet model, which is associated with even more weight loss compared to our CDAHFD model. FGF-21 KO increased body weight and hepatic lipid accumulation through suppression of mitochondrial beta-oxidation, while FGF-21 administration reduced lipid accumulation and inflammation (3).

In contrast, the weight loss in the CCR2/5 inhibitor-only group was not significantly different from the vehicle-treated group, in keeping with the mechanism of action, again suggesting that food intake did not play a major role in the observed outcome. To act on the reviewer´s suggestion, we extended the description in reference to the bodyweight development of the mice in the manuscript (line 170 – 175 and in the discussion line 374 to 377)

2) For human data, it would have strengthened this report to have BMI/sex-matched control patients and report their CCL2, FGF21 and CK18 values.

Response: We thank the reviewer for his/her expert comment and would like to refer to the first comment of reviewer 1. As both reviewers rightfully mentioned, we included gender-specific analyses as new Supplementary Figure 1 to the manuscript (Section 2.1, line 124 to 134 and in the discussion line 355 to 370). Matched healthy control patients were not included in our cohort, in part because liver biopsies from obese patients without signs of

NAFLD were not easily available given the lack of indication to perform the biopsy. Nonetheless, we hope that these new analyses fully answer the posed questions.

3) Validation of CCL2/5 inhibition should be shown by showing a reduction of downstream signaling such as MAPK. The other possibility would be to show that CCL2 and CCL5 mRNA were unchanged in liver following treatment with inhibitors and then subsequently adding this data to supplemental figure 1E.

   Response: We thank the reviewer for his comment and would like to point to Supplementary Figure 1E demonstrating serum concentrations of CCL2 and CCL5 as well as *Ccl2* gene expression levels from total liver tissue, labelled as *Mcp1* (monocyte chemoattractant protein 1 = CCL2). We confirmed effective dosing of the CCR2/5 inhibitor by measuring CCL2 serum concentrations, which are significantly increased upon pharmacological blockade of CCR2 as also previously reported from our group as well as from clinical trials in NASH patients (4).

   In reference to our answer of the second comment of reviewer 1, CCR5 orchestrates migration, activation, and proliferation of hepatic stellate cells (HSCs) as well as recruitment of lymphocyte populations (natural killer cells, T cells) to the liver, that is mainly related to viral or (auto-)immune hepatitis. In this study, we focus on monocytes and macrophages, since the CCR2/5 inhibition only affected these populations, which is mainly mediated by the CCR2/CCL2 axis.

4) Was there any mortality in the CCR2/5i + FGF21 groups? Their weights at 12 weeks appear to be dropping? Was this weight drop significant compared to eh CDAHFD Vhc group? Was there any change in appearance or overall well being of the mice? This needs to be reported in the text.

   Response: We thank the reviewer for his/her comment and would like to refer to our answer of the first comment. We did not observe any negative side effects due to the compound treatment, neither in the single compound nor in the combinational treatment group. In particular, no mortality was observed, as this is now stated in the manuscript (line 169 – 170). As correctly described by the reviewer, we observed a conspicuous weight loss in the FGF21 similar treated mice as well as in the combinational treatment - but less prominent in the CCR2/5 inhibitor treated mice - leading us to the conclusion that this effect

is mainly directed by the FGF21 agonism. The potential of FGF21 to protect mice from diet induced obesity is extensively reported in the literature (5, 6).

We would like to emphasize that all experiments were performed under conditions approved by the appropriate institutional and governmental authorities according to German legal requirements with regular weight controls and no individual mouse lost ≥20% bodyweight compared to baseline.

5) With the protective effects of FGF21v for some measures in the CCl4 model, is it possible that FGF21v treatment changes metabolism of CCl4 by changing cytochrome p450 activity (such as CYP2E1)? This needs to be checked for both CCR2/5i and FGF21v groups to ensure conclusions of these groups are appropriate.

Response: We thank the reviewer for his/her expert comment. The acute CCl_4 liver injury model is accompanied by hepatic inflammatory processes in which necrotic hepatocytes release danger-associated-molecular patterns (DAMPs) that activate macrophages, Kupffer cells and neutrophils. Activated immune cells release cytokines (e.g. TNF-α, IL-1β) and chemokines (e.g. CCL2 via CCR2) that result in the recruitment of bone-marrow derived monocytes and neutrophils. As we observed a comparable reduction of hepatic MoMF in experimental CDAHFD-induced NASH, we employed the acute CCl_4 liver injury model to elucidate effects of both therapies on accumulation of hepatic monocytes and MoMF. As previously shown by our group we confirmed that CCR2/5 antagonism blocks monocyte infiltration into the liver. On the contrary, we could exclude direct effects of the FGF21 similar treatment on immune cell recruitment. As FGF21 application did not influence monocyte infiltration or recruitment of other immune cells we speculate that reduced numbers of hepatic MoMFs in the experimental NASH model were rather indirect effects due to beneficial metabolic modification. To date, the mode of action of FGF21 and its downstream targets are not fully understood and future studies are definitely needed to answer open questions. Given the lack of FGF-21 effect on monocyte infiltration and hepatocyte necrosis following CCl_4 administration, it is unlikely that metabolization of CCl_4 was a major factor in its lowering of ALT levels, as otherwise these readouts would also have been affected.

Reference:

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