EMBO reports

# Endothelial-immune crosstalk contributes to vasculopathy in non-alcoholic fatty liver disease

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## Review Timeline:


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*Editor: Achim Breiling*


## Transaction Report:

(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Cheung,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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11) Please order the manuscript sections like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - DAS - Acknowledgements - Author contributions - Conflict of interest - References - Figure legends - Expanded View Figure legends

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Editor

EMBO Reports

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Referee #1:

Review of: Endothelial-immune crosstalk contributes to vasculopathy in 2 non-alcoholic fatty liver disease

While the role of pathological angiogenesis, including increased chemotaxis mediated by altered endothelial cell (dys)function, is known in NAFLD pathogenesis, the authors use an elegant way to enhance the field of research related to NAFLD-associated (cardio)vascular alterations. The use of BOECs to study vasculopathy related to NAFLD is novel and shows an excellent tool, that nicely reflects mechanisms that have been identified in the liver using experimental animal models. In addition, there results point to a new therapeutic target (CXCL12-CXCR4 axis) for which the authors show proof in in vitro assays. The paper is well written, and figures are clear.

General remarks:

1) Given that the authors interfered in the CXCL12-CXCR4 axis in in vitro assays, they could confirm their results by the use of AMD3100 in vivo, including characterization of endothelium and immune cell infiltration using AMD3100 in in vivo models.

2) The authors focus on endothelial-T-cell interactions while endothelial-macrophage interactions are equally important in NAFLD/NASH. For example, binding of CXCL12 to CXCR4 stimulates adipose tissue monocyte accumulation in mice fed a high-fat diet (Kim D, Kim J, Yoon JH, et al. CXCL12 secreted from adipose tissue recruits macrophages and induces insulin resistance in mice. Diabetologia 2014;57(7):1456-65.). Can the authors include this in the discussion (line 428)?

Specific comments per section:

Introduction
-Line 61: and can progress to fibrosis
-Line 77: evidence
-Line 92: , whereas
-Line 97: delete 'etc'
-Line 130: leads to

Results:
-line 108-135:
Given the reported role of pathological angiogenesis in NAFLD/NASH, it is interesting to see that the doubling time of BOECs is equal in healthy and NAFLD patients. Can the authors comment on this in the discussion?
Figure legend 1: panel (d) does not contain fibroblasts.

-Line 276-and further: the results of such a small patient cohort could be strengthen by additional (targeted) approaches such qPCR analyses on PBMCs of larger cohorts.

Discussion:
-Line 505: "We present the molecular underpinning of endothelial dysfunction in NAFLD...": as these data do not fully reflect all mechanisms involved, please tone down this sentence.

References:
-The reference 'Her et al.' is 2x present (paper of the same group).

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Referee #2:

The current study by Ng et al proposes an endothelial to immune cell cross talk specifically in patients with non alcoholic fatty liver disease (NAFLD) via endothelial expressed CXCL12. The authors identify CXCL12 via unbiased analysis from blood outgrowth endothelial cells and confirm their findings in two animal models of NAFLD. CXCL12 was already proposed to be involved in NAFLD however in a different context. Especially work from the Perlemuter-Group suggested increased affinity of T-cells towards CXCL12 through a dysfunctional CXCR4. The paper is of a very high technical standard although a biological confirmation of the proposed mode of action is missing. Currently, the paper is geared towards a gastroenterology audience, data on endothelial cell changes and immune cell interaction within the e.g. aorta would make the paper also more appealing to the cardiology community.

Specific and technical concerns:

1. The paper does not discuss the work already published from the Perlemuter-Group. Especially two papers in direct line to the research (Bigorgne et al Gastroenterology 2008 and Boujedidi et al Clin Sci 2014) need to be discussed in relation to the biological importance of the current finding. Quoting directly from the second paper: CXCL12/CXCR4 pathway contributes in both mice and patients to the enhanced recruitment of CD4+ T-cells in NASH. An increased affinity of CXCL12 to CXCR4 rather than a higher expression of the chemokine or its receptors is involved in this process. Especially with the data in Figure 5D it would be interesting if this change is already established in NAFLD patients.
2. Patient cohorts should include medication. Did patient biopsies show sign of inflammation and immune cell recruitment?
3. In Figure 1B the total cell amount obtained via the blood outgrow method looks substantially different and the total amount of cells from NAFLD patients looks reduced compared to healthy controls. Was there a difference in total cell number of obtained endothelial cells between controls and patients? In which passage were the cells used for the different experiments? I am unsure if the protocol would reflect an unwanted artificial in vitro aging if initial cell numbers were different but cells were proliferated and used regardless of the initial obtained cell number.
4. Supplementary Figure S3b showes plasma concentrations of CXCL12 in healthy control patients and NAFLD patients. There appears to be two distinct groups within the patients consisting of 4 patients with high CXCL12 values and the remaining patients with similar or even lower values of CXCL12. Can those patients be also linked back into the previous experiments, eg did the data from Fig. 2 contain outgrow cells from these four high donors? How do those patients differ from the remaining patient population?
5. The question is if the chemotactic property of SDF-1 is enough locally to have the proposed impact. Did the authors observe increased retention of leukocytes on endothelial cells when analyzing their mouse models? Was there an increased amount of immune cells within fatty streaks around the aorta within their models? Did the authors observe an increased plasma concentration of CXCL12 in the mouse models?
6. Please reevaluate the use of statistical analysis and use proper analysis for parametric and non-parametric tests. Visually, eg Fig 5D or 3D do not look normally distributed.

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Referee #3:

Major Comments:
- Are the results shown in Suppl. Fig 1 only from healthy controls? If so, please also show data from NAFLD cells, including a direct comparison with healthy controls.
- Please show all graphs as bar-graph including individual data-points. This is especially important for figures in e.g. 3A showing a very large degree of variation. Is there any correlation with features of the donor that might explain this variation (e.g. gender). Such correlations should be tested as this will provide valuable additional information.
- Regarding Figure 3C. Another very relevant stimulus and perhaps one where ECs are more responsive to are TNF-a or oxLDL. It would be highly interesting and informative to investigate these stimuli as well.
- Is there a correlation between CXCL10/CXCL12 plasma levels from the control/NAFLD patients and the chemokine levels measured from the related BOECs?
- It remains unclear how the analysis of Figure 3E and 3F were exactly performed. How many cells were quantified, were all CDH5 positive cells taken into account? There is no overlapping staining for the liver sections, how are endothelial cells identified?
- Results from Figure 4 should be further validated in independent experiment, at least on gene-levels, preferably on protein level (e.g. effects on NF-kB signaling, on HLA expression, IFNGR expression).
- Regarding Figure 5D, the variation in the CD8- fraction is enormous and clearly show 2 populations. Is the composition of these clusters different? As this effects the results dramatically. Please show Healthy and NAFLD in same graph, it looks like the CD8+ fraction is reduced in NAFLD patients? It would be very interesting to also separate CD4+ T cells, from the non-T-cell-fraction, to further elucidate whether the effects are T-cell specific.
- Figure 5E. Show NAFLD and healthy in same graph. Please also include a similar experiment with either CXCL12 blocking and/or CXCL12 supplementation.

Minor Comments:
- Figure 1D. Please increase the size of the quantification graphs (y-axis is not readable).
- Figure 1E. The merged image of Fibroblast shows a nuclear marker I assume. Please specify this in the figure/legend.
- N-numbers in figure legends do not match the amount of data-points in the graphs. For example for Fig. 1F there are 30-40 data points shown, while the legends states n=3. Please provide more information in all panels where this is the case, stating clearly how many individual replicates are used and what the data-points exactly reflect.
- Increase font size in Figure 4A, this is not readable at the moment.
- CXCR7 is now called ACKR3. Please change accordingly.

**Point-by-point response to Reviewers' comments**

Referee #1:
Review of: Endothelial-immune crosstalk contributes to vasculopathy in 2 non-alcoholic fatty liver disease.
While the role of pathological angiogenesis, including increased chemotaxis mediated by altered endothelial cell (dys)function, is known in NAFLD pathogenesis, the authors use an elegant way to enhance the field of research related to NAFLD-associated (cardio)vascular alterations. The use of BOECs to study vasculopathy related to NAFLD is novel and shows an excellent tool, that nicely reflects mechanisms that have been identified in the liver using experimental animal models. In addition, there results point to a new therapeutic target (CXCL12-CXCR4 axis) for which the authors show proof in in vitro assays. The paper is well-written, and figures are clear.

**Response: We are grateful for your feedback and support of our manuscript.**

General remarks:
1) Given that the authors interfered in the CXCL12-CXCR4 axis in in vitro assays, they could confirm their results by the use of AMD3100 in vivo, including characterization of endothelium and immune cell infiltration using AMD3100 in in vivo models.

**Response: Thank you for bringing up this important consideration. Before we could effectively validate the CXCL12-CXCR4 axis *in vivo*, we have added new data, clarifying that enhanced endothelial-derived CXCL12 may play a greater role than immune cell CXCR4 expression in mediating the NAFLD endothelial-immune crosstalk. Hence, we have tested the use of AMD3100 (CXCR4 inhibitor) and CXCL12 neutralization. Firstly, Figure 3 data substantiated the chemokine activation in NAFLD patient endothelial cells, as well as upregulated endothelial-associated CXCL12 in the aortae and liver vasculatures of diet-induced NAFLD mouse models. In our new data (Fig. 4d; results on page 8), we attempted to validate CXCR4 protein expressions on CD8+ T cells, CD8- lymphocytes and monocytes from NAFLD (*n* = 14) and healthy (*n* = 6) PBMC samples. About 20-50% of each immune subset expressed CXCR4 but there was no significant difference between NAFLD and healthy groups. Besides chemokine receptor expression, it was still unknown to us whether the chemokine binding affinity of CXCR4 receptor could be different in various immune subsets or altered under disease context as reported in other studies (PMIDs: 21725996, 25074471).**

**Secondly, we learnt that CXCR4 can be both protective and pathogenic depending on cell-specific functions and different stages of atherosclerosis development (PMIDs: 34494495, 32078474). We further explored CXCL12 inhibition since it has been shown that a CXCL12 neutralizing compound may confer greater efficacy than CXCR4 blockade with AMD3100 in decreasing macrophage infiltration in the lungs of rat models of pulmonary hypertension (PMID: 31173066). In our new data (Fig. 5e; results on page 9), we tested a CXCL12 neutralization antibody to counteract the effect of enhanced chemokine secretion from NAFLD BOECs. Indeed, the use of anti-CXCL12 IgG led to a significantly greater inhibition of NAFLD CD8+ T cell chemotaxis than in the healthy group (Fig. 5e). Anti-**

**CXCL12 IgG was similarly effective at moderating the chemotaxis of CD8- cell fraction in both NAFLD and healthy groups. The data suggest that modulating the chemokine milieu of NAFLD endothelial cell may reduce their interaction with effector immune cells.**

**Finally, we were motivated to find out if the intensified endothelial-associated CXCL12 within the aortae and liver tissues of NAFLD mice could exert chemotactic effect locally to recruit immune cells. In our previous study, T lymphocyte infiltration into liver was apparent in mice on high fat high calorie (HFHC) diet (PMID: 33013934). In our new data (Fig. 3g; results on page 7), we observed significantly increased retention of CD45+ leukocytes in the aortic walls of mice fed with NAFLD-inducing LIDPAD diet as compared to those on chow diet.**

**While intervening CXCR4-CXCL12 axis may be a viable avenue to moderate immune cell infiltration into blood vessel walls, we have discussed in the revised manuscript that it is important for future *in vivo* testing to establish the effectiveness of blocking chemokine and/or its receptor.**

2) The authors focus on endothelial-T-cell interactions while endothelial-macrophage interactions are equally important in NAFLD/NASH. For example, binding of CXCL12 to CXCR4 stimulates adipose tissue monocyte accumulation in mice fed a high-fat diet (Kim D, Kim J, Yoon JH, et al. CXCL12 secreted from adipose tissue recruits macrophages and induces insulin resistance in mice. Diabetologia 2014;57(7):1456-65.). Can the authors include this in the discussion (line 428)?

**Response: We have included this point in Discussion (page 11) "*As obesity is often associated with NAFLD, obesity-induced adipose tissue inflammation has been shown to involve CXCL12 secreted from adipose tissue, which recruits macrophages and induces insulin resistance in mice fed with high-fat diet (Kim et al., 2014).*"**

Specific comments per section:
Introduction
-Line 61: and can progress to fibrosis
-Line 77: evidence
-Line 92: , whereas
-Line 97: delete 'etc'
-Line 130: leads to

**Response: We have edited the Introduction accordingly.**

Results:
-line 108-135:
Given the reported role of pathological angiogenesis in NAFLD/NASH, it is interesting to see

that the doubling time of BOECs is equal in healthy and NAFLD patients. Can the authors comment on this in the discussion?

**Response: Indeed, based on cell doubling time (Fig. 1c) and angiogenic sprouting assay (Fig. 1f), we did not observe significant differences in the angiogenic attributes between NAFLD and healthy endothelial cells. However, we have added more data to Fig. EV1 (results on page 4). During sprouting angiogenesis, endothelial cells undergo a series of dynamic changes in their tip- and stalk-like cell phenotypes to facilitate cell migration, proliferation and stabilization, which ultimately leads to sprout formation (PMID: 23085847). At the molecular level, we found that sprouted NAFLD BOECs might be primed for angiogenesis as demonstrated by their significantly higher gene expressions for tip cell markers than sprouted healthy BOECs (Fig. EV1).**

- Figure legend 1: panel (d) does not contain fibroblasts.

**Response: We have edited the figure legend accordingly.**

-Line 276-and further: the results of such a small patient cohort could be strengthen by additional (targeted) approaches such qPCR analyses on PBMCs of larger cohorts.

**Response: We have added new data in Fig. 4d. We conducted magnetic-activated immune cell sorting of a larger sample size of PBMCs from NAFLD (*n* = 14) and healthy (*n* = 6) subjects. The protein expressions of CXCR4 and CX3CR1 in various immune subsets, namely CD8+ T cells, CD8- lymphocytes (mainly CD4+ T cells and NK cells) and monocytes were analyzed by flow cytometry.**

Discussion:
-Line 505: "We present the molecular underpinning of endothelial dysfunction in NAFLD...": as these data do not fully reflect all mechanisms involved, please tone down this sentence.

**Response: We have toned down our claim to "*We present that vasculopathy in NAFLD may involve endothelial chemokine activation and vascular-immune interactions*."**

References:
-The reference 'Her et al.' is 2x present (paper of the same group).

**Response: We have corrected the duplicated references.**
**Thank you for your time and efforts in evaluating our revised manuscript.**

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Referee #2:

The current study by Ng et al proposes an endothelial to immune cell cross talk specifically in patients with non alcoholic fatty liver disease (NAFLD) via endothelial expressed CXCL12. The authors identify CXCL12 via unbiased analysis from blood outgrowth endothelial cells and confirm their findings in two animal models of NAFLD. CXCL12 was already proposed to be involved in NAFLD however in a different context. Especially work from the Perlemuter-Group suggested increased affinity of T-cells towards CXCL12 through a dysfunctional CXCR4. The paper is of a very high technical standard although a biological confirmation of the proposed mode of action is missing. Currently, the paper is geared towards a gastroenterology audience, data on endothelial cell changes and immune cell interaction within the e.g. aorta would make the paper also more appealing to the cardiology community.

**Response: We are grateful for your constructive comments that have helped us strengthen our manuscript. Please refer to our responses below with regard to your requested new data and discussion of our work in the context of previous findings from Perlemuter-Group.**

Specific and technical concerns:
1. The paper does not discuss the work already published from the Perlemuter-Group. Especially two papers in direct line to the research (Bigorgne et al Gastroenterology 2008 and Boujedidi et al Clin Sci 2014) need to be discussed in relation to the biological importance of the current finding. Quoting directly from the second paper: CXCL12/CXCR4 pathway contributes in both mice and patients to the enhanced recruitment of CD4+ T-cells in NASH. An increased affinity of CXCL12 to CXCR4 rather than a higher expression of the chemokine or its receptors is involved in this process. Especially with the data in Figure 5D it would be interesting if this change is already established in NAFLD patients.

**Response: Thank you for highlighting this excellent point. We have provided more data clarifying that endothelial cells in NAFLD may be providing a chemokine milieu that preferentially support the interaction with immune cells, instead of differential chemokine receptor expressions between NAFLD and healthy immune cells.**

**Firstly, Figure 3 data substantiated the chemokine activation in NAFLD patient endothelial cells, as well as upregulated endothelial-associated CXCL12 in the aortae and liver vasculatures of diet-induced NAFLD mouse models. In our new data (Fig. 4d; results on page 8), we attempted to validate CXCR4 protein expressions on CD8+ T cells, CD8- lymphocytes and monocytes from NAFLD (*n* = 14) and healthy (*n* = 6) PBMC samples. About 20-50% of each immune subset expressed CXCR4 but there was no significant difference between NAFLD and healthy groups. Besides chemokine receptor expression, it was still unknown to us whether the chemokine binding affinity of CXCR4 receptor could be different in various immune subsets or altered under disease context as reported in Perlemuter-Group's studies (PMIDs: 21725996, 25074471).**

**Secondly, we learnt that CXCR4 can be both protective and pathogenic depending on cell-specific functions and different stages of atherosclerosis development (PMIDs: 34494495, 32078474). We further explored CXCL12 inhibition since it has been shown that a CXCL12 neutralizing compound may confer greater efficacy than CXCR4 blockade with AMD3100 in decreasing macrophage infiltration in the lungs of rat models of pulmonary hypertension (PMID: 31173066). In our new data (Fig. 5e; results on page 9), we tested a CXCL12 neutralization antibody to counteract the effect of enhanced chemokine secretion from NAFLD BOECs. Indeed, the use of anti-CXCL12 IgG led to a significantly greater inhibition of NAFLD CD8+ T cell chemotaxis than in the healthy group (Fig. 5e). Anti-CXCL12 IgG was similarly effective at moderating the chemotaxis of CD8- cell fraction in both NAFLD and healthy groups. The data suggest that modulating the chemokine milieu of NAFLD endothelial cell may reduce their interaction with effector immune cells.**

**We have discussed these data in relation to the published findings from Perlemuter-Group. For your ease of reading, the text has been extracted here. On page 12, "*CXCL12-CXCR4 axis has been shown to mediate intrahepatic infiltration of immune cells in mouse diet-induced models of obesity and NASH, where it has been postulated that change in membrane cholesterol may alter CXCR4 receptor conformation, hence increasing its chemokine binding affinity (Bigorgne et al., 2008a, Boujedidi et al., 2015). These studies report no change in either liver expression of CXCL12 or intrahepatic T cell expression of CXCR4. Also, it has been shown in their studies that liver CXCL12 originates mainly from bile duct epithelial source and not from endothelial cells. Here, our work demonstrated an endothelial source of CXCL12 in patient cells, as well as in the aortae and liver vasculatures of NAFLD mouse models. Since we did not notice significant differences of CXCR4 expressions in NAFLD and healthy immune cells, the upregulation of endothelial CXCL12 in NAFLD might be primarily responsible for immune cell recruitment to vasculatures. We recognize the limitation that we had not investigated the chemokine binding affinity of CXCR4 receptors on our patient immune cells*."**

2. Patient cohorts should include medication. Did patient biopsies show sign of inflammation and immune cell recruitment?

**Response: All patient biopsies have been analyzed and scored for inflammation. Our liver histopathology assessment was performed based on the NASH CRN Histologic Scoring System by HistoIndex's AI-based diagnostic technology. This information, together with medical history and medication records, have been updated in our Appendix Table S3.**

3. In Figure 1B the total cell amount obtained via the blood outgrow method looks substantially different and the total amount of cells from NAFLD patients looks reduced compared to healthy controls. Was there a difference in total cell number of obtained endothelial cells between controls and patients? In which passage were the cells used for the different experiments? I am unsure if the protocol would reflect an unwanted artificial in vitro aging if initial cell numbers were different but cells were proliferated and used regardless of the initial obtained cell number.

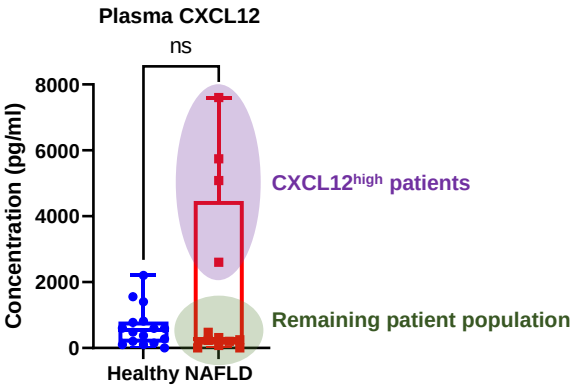
**Response: In our previous Fig. 1b, the cells from healthy control appeared visibly more than that in NAFLD group. We realized that it was our mistake as the phase contrast image of healthy blood outgrowth endothelial colony was taken before we washed off the non-adherent excess PBMCs in culture, giving the impression that there were substantially more healthy endothelial cells. Therefore, we have replaced with a more representative image of an outgrowth endothelial colony (Fig. 1b).**

**We have added the following information to Appendix Material and Method, page 10. We typically seeded 20-22 million PBMCs from each donor. Then we obtained an average of 1-2 blood outgrowth endothelial colonies per 10 million cultivated PBMCs based on a consistent seeding density of 2 million PBMCs/cm$^2$. The initial endothelial cell number obtained from both healthy subjects and NAFLD patients did not seem to differ greatly. Both healthy and NAFLD endothelial cells also had comparable cell doubling time (Fig. 1c). Hence, it was unlikely for the NAFLD endothelial cells to incur unnecessary *in vitro* aging due to the need for more proliferation/ expansion.**

**We have devoted Figure 1 and Expanded View Fig. EV1 to perform quality control characterizations (i.e. cell purity, endothelial marker expressions and functional assay) on the obtained endothelial cells. Only early passage endothelial cell lines (passages 2-6) that passed quality control validations would be utilized for experimentations such as RNA-sequencing, mechanistic validation, chemotaxis and transmigration assays.**

4. Supplementary Figure S3b shows plasma concentrations of CXCL12 in healthy control patients and NAFLD patients. There appears to be two distinct groups within the patients consisting of 4 patients with high CXCL12 values and the remaining patients with similar or even lower values of CXCL12. Can those patients be also linked back into the previous experiments, eg did the data from Fig. 2 contain outgrow cells from these four high donors? How do those patients differ from the remaining patient population?

**Response: This is an interesting observation. The patient samples used for RNA-seq in Figure 2 were <u>not</u> among the CXCL12$^{high}$ donors in Expanded View Fig. EV3b (also shown next page). Instead, we found the patients whose samples were used for RNA-seq in the remaining patient population. We analyze the demographics, and find that both the CXCL12$^{high}$ patients and the rest of patient population are Asians, age-matched, all have steatosis and about 25% of them with comorbidities (i.e. diabetes or hypertension). Gender composition is slightly different - CXCL12$^{high}$ patients (all females) versus the remaining population (75% females). In terms of their liver biopsy histopathology, 50% of the CXCL12$^{high}$ patients have lobular inflammation or fibrosis versus 25% of the remaining population have lobular inflammation. We believe that the plasma concentration data may reflect true variations among the NAFLD population, but there is also a possibility that the CXCL12$^{high}$ patients may be associated with more advanced NAFLD.**




5. The question is if the chemotactic property of SDF-1 is enough locally to have the proposed impact. Did the authors observe increased retention of leukocytes on endothelial cells when analyzing their mouse models? Was there an increased amount of immune cells within fatty streaks around the aorta within their models? Did the authors observe an increased plasma concentration of CXCL12 in the mouse models?

**Response: In both our mouse models of diet-induced NAFLD, we had not observed overt vascular lesions in the aortic and liver tissues. From our literature review, vascular pathologies in NAFLD *in vivo* models remain understudied. Diet-induced NAFLD models may at best manifest some levels of vascular inflammation, while development of atherosclerosis and macrovascular lesions still require the combination of genetic knockout models (e.g. *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$) with NAFLD-inducing diet (PMIDs: 28507343, 32883049).**

**We were motivated to find out if the intensified endothelial-associated CXCL12 within the aortae and liver tissues of NAFLD mice could exert chemotactic effect locally to recruit immune cells. In our previous study, T lymphocyte infiltration into liver was apparent in mice on high fat high calorie (HFHC) diet (PMID: 33013934). In our new data (Fig. 3g; results on page 7), we observed significantly increased retention of CD45+ leukocytes in the aortic walls of mice fed with NAFLD-inducing LIDPAD diet as compared to those on chow diet.**

**We then profiled the systemic levels of CXCL12 in serum samples of mice that had been fed on LIDPAD or chow diets for 12 and 16 weeks. In our new data (Fig. EV3c, results on page 6), we did not resolve significant differences of CXCL12 in the mouse sera between LIDPAD and control groups. This could be due to soluble CXCL12 in circulation potentially contributed by several sources of cells, including the liver sinusoidal endothelial cells, cholangiocytes and adipocytes (PMIDs: 22121892, 21725996, 24744121).**

6. Please reevaluate the use of statistical analysis and use proper analysis for parametric and non-parametric tests. Visually, eg Fig 5D or 3D do not look normally distributed.

**Response: We have used Kolmogorov-Smirnov test to check for normality. Fig. 3d is non-parametric so we have used Mann-Whitney method to test for statistical differences. It was an error that we initially stated 't-test' in the figure legend but have now corrected it to 'Mann-Whitney test'. The previous Fig. 5d is now revised Fig. 5c as we have increased the sample size. Mann-Whitney test has been used in the revised Fig. 5c as well.**

**Thank you for your time and efforts in evaluating our revised manuscript.**

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Referee #3:

Major Comments:
- Are the results shown in Suppl. Fig 1 only from healthy controls? If so, please also show data from NAFLD cells, including a direct comparison with healthy controls.

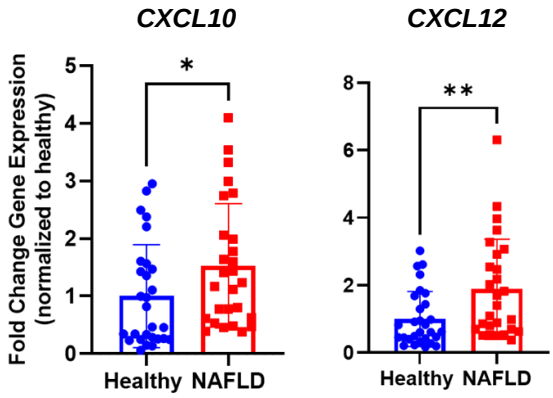
**Response: We are grateful for your constructive comments that have helped us strengthen our manuscript.**

**We have added more data to Expanded View Fig. EV1, results on page 4. We found that sprouted NAFLD BOECs might be primed for angiogenesis as demonstrated by their significantly higher gene expressions for tip cell markers than sprouted healthy BOECs (Fig. EV1). Concomitantly, the role of pathological angiogenesis in NAFLD is well known, likely driven by pro-angiogenic factors arising from chronic inflammation, tissue hypoxia and hepatocyte injury (PMIDs: 30797053, 30259536, 22658783).**

- Please show all graphs as bar-graph including individual data-points. This is especially important for figures in e.g. 3A showing a very large degree of variation. Is there any correlation with features of the donor that might explain this variation (e.g. gender). Such correlations should be tested as this will provide valuable additional information.

**Response: We have revised the bar graphs to include individual data points. In Fig. 3a, we re-examined *CXCL10* and *CXCL12* expressions (also shown below), which were significantly higher in NAFLD patients than healthy subjects, and analyzed the demographics of patients. We found that the patients who had greater CXCL10 gene expressions (> 75$^{th}$ percentile) are <u>not</u> those who had greater CXCL12 gene expressions (> 75$^{th}$ percentile), ruling out outliners who might have chemokine hyperactivation that skewed the graphs.**

**Next, to understand how data variation may be link to their biological correlates, we analyzed their demographics and liver biopsy histopathology (table next page). All patients are Asians, rather close in age and all had liver steatosis. For gender composition, the greater the proportion of females seemed to give rise to higher *CXCL10* expressions, but this trend was not observed in *CXCL12* expressions. In terms of comorbidities, higher proportion of patients with other risk factors (i.e. diabetes, hypertension or dyslipidemia) did not necessarily translate into higher chemokine expressions. Likewise, patients with more advanced features of NAFLD (i.e. lobular inflammation and liver fibrosis) were not associated with higher percentile of chemokine expressions. Therefore, we are inclined to believe that the chemokine gene expression data may reflect true variations among the NAFLD population. We recognize the limitation that human samples tend to have greater inter-individual variabilities, and that we have not noticed obvious correlation of chemokine expressions with their qualitative biological traits.**




| Genes of interest | Percentile of gene expressions | Gender (% females) | Age in years (interquartile range) | Ethnicity | 1 or more comorbidities (diabetes, hypertension and/ or dyslipidemia) | Histology - Steatosis | Histology - Lobular inflammation | Histology - Ballooning | Histology - Fibrosis |
|---|---|---|---|---|---|---|---|---|---|
| *CXCL10* | > 75th | 100% | 40 (39.5 - 40.5) | Asian | 0% | 100% | 0% | 0% | 0% |
| | 25th - 75th | 66.7% | 31 (28.8 - 34) | Asian | 33.3% | 100% | 16.7% | 0% | 33.3% |
| | < 25th | 50% | 33.5 (30.8 - 36.3) | Asian | 50% | 100% | 0% | 0% | 0% |
| | | | | | | | | | |
| *CXCL12* | > 75th | 50% | 29.5 (28.8 - 30.3) | Asian | 50% | 100% | 0% | 0% | 0% |
| | 25th - 75th | 80% | 38.4 (38 - 39) | Asian | 20% | 100% | 20% | 0% | 40% |
| | < 25th | 33.3% | 27.3 (25.5 - 29.5) | Asian | 33.3% | 100% | 0% | 0% | 0% |

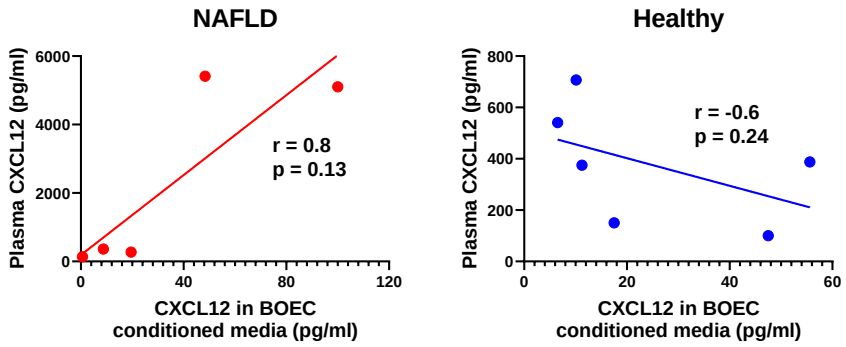
- Regarding Figure 3C. Another very relevant stimulus and perhaps one where ECs are more responsive to are TNF-a or oxLDL. It would be highly interesting and informative to investigate these stimuli as well.

**Response: Thank you for raising this point. We agree that TNFα/ oxLDL are very relevant stimuli for endothelial activation, given their well-established role in vascular dysfunction and atherogenesis (PMIDs: 19118493, 16253212, 21660303). While it is common that NAFLD patients may have shared cardiometabolic risk factors, NAFLD itself can be an independent risk factor for cardiovascular mortality (PMID: 20879883). In line with this, plasma concentrations of TNFα/ oxLDL are also elevated in NAFLD patients than controls, and plasma TNFα/ oxLDL were recognized as biomarkers to detect NAFLD progression and risk of NASH (PMIDs: 34447378, 23183522). To address your point, we have performed endothelial stimulation with autologous plasma (Expanded View Fig. EV3a; results on page 6). Patients' own plasma may be representative of disease-relevant stimuli and contain physiological levels of TNFα/ oxLDL. Upon exposure of the endothelial cells to their autologous plasma, CXC chemokine ligands, namely *CXCL10*, *CXCL11* and *CXCL12*,**

**were significantly upregulated in NAFLD endothelial cells than in healthy controls (Fig. EV3a).**

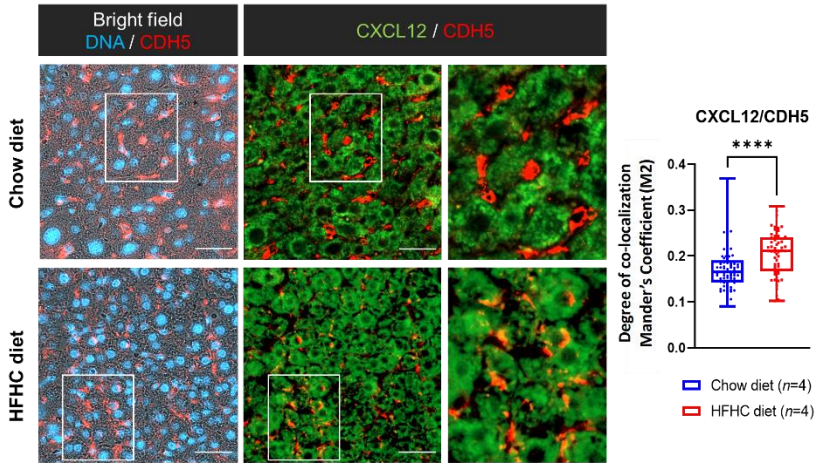
- Is there a correlation between CXCL10/CXCL12 plasma levels from the control/NAFLD patients and the chemokine levels measured from the related BOECs?

**Response: Excellent question. We have performed nonparametric Spearman correlation between plasma CXCL12 levels and the secreted chemokine levels in conditioned media of their related BOECs (data below). Interestingly, NAFLD group has a positive correlation, while healthy group has a negative correlation. Endothelial cell-derived CXCL12 may play a greater role in NAFLD setting but both groups did not achieve statistical significant correlations. This could be due to insufficient data points. Also, plasma CXCL12 could be contributed by several potential sources of cells including liver sinusoidal endothelial cells, cholangiocytes and adipocytes (PMIDs: 22121892, 21725996, 24744121). On the other hand, CXCL10 levels in BOEC conditioned media were either very low or undetectable so we did not have sufficient data points to correlate with their corresponding plasma CXCL10.**




- It remains unclear how the analysis of Figure 3E and 3F were exactly performed. How many cells were quantified, were all CDH5 positive cells taken into account? There is no overlapping staining for the liver sections, how are endothelial cells identified?

**Response: In the liver sections (Fig. 3e), there is overlapping staining of CXCL12 and CDH5. For better clarity, we have revised the figure to include zoom-in images as shown next page. Co-localization of CDH5-expressing liver endothelial cells with CXCL12 was quantified using Mander's Coefficient (M2), an established software plugin for visualizing and measuring co-localization in cells (PMID: 30361629).**



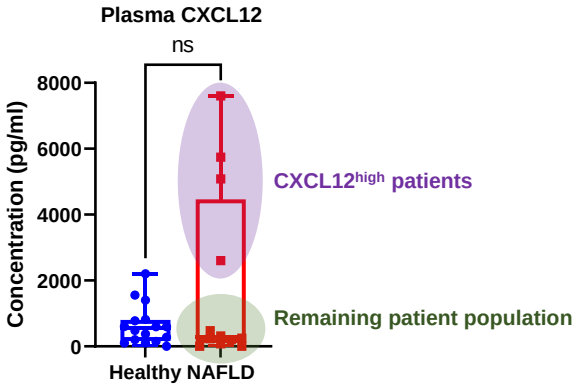

**In the aortic sections (Fig. 3f), we identified CDH5-positive endothelial cells which showed intercellular junctional staining pattern. Quantitatively, CXCL12 expressions within regions of interest strictly containing just CDH5-positive endothelial lining in the aorta were analyzed. To ensure robustness, there were 10-20 independent images/ regions of interest analyzed per mouse liver/ aorta for image quantification. All CDH5 positive cells were taken into account in all regions of interest that we studied. We have elaborated more details of image analysis in our Appendix Methods, page 12.**

- The authors show that mouse endothelial cells express higher CXCL12 levels. Can the authors please also measure CXCL12 concentrations in the mouse plasma samples? This evaluation would be very important in the interpretation of the connection between NAFLD and CVDs. Plasma concentration of CXCL12 in NAFLD patients were not altered significantly and showed clusters within the group. Here, the mouse model would help to better clarify this potential link.

**Response: We have profiled the systemic levels of CXCL12 in serum samples of mice that had been fed on the new modified Liver Disease Progression Aggravation Diet (LIDPAD; Teklad Custom Diet, TD.140897, Envigo, USA) that contains 43.3 kcal% of fat, or on the control diet (purified diet containing 14% protein, 76% carbohydrates, 10% fat) for 12 and 16 weeks. However, we did not resolve significant differences of CXCL12 in the mouse sera between LIDPAD and control groups (<u>new data in Expanded View Fig. EV3c</u>). This could be due to soluble CXCL12 in circulation potentially contributed by several sources of cells, including liver sinusoidal endothelial cells, cholangiocytes and adipocytes (PMIDs: 22121892, 21725996, 24744121).**

**To address your comment on NAFLD patient plasma CXCL12 that were not altered significantly and showed clusters within the group (Expanded View Fig. EV3b, also shown below), we have reanalyzed their demographics. Both the CXCL12$^{high}$ patients and the rest of patient population are Asians, age-matched and all have steatosis. While it is common that NAFLD patients may have shared cardiometabolic risk factors, NAFLD itself can be an independent risk factor for cardiovascular mortality (PMID: 20879883). We found that both the CXCL12$^{high}$ patients and the remaining patient population were also matched for**

**cardiovascular risk factors - about 25% of them had comorbidities (i.e. diabetes or hypertension) within each cluster. Gender composition is slightly different - CXCL12$^{high}$ patients (all females) versus the remaining population (75% females). In terms of their liver biopsy histopathology, 50% of the CXCL12$^{high}$ patients have lobular inflammation or fibrosis versus 25% of the remaining population have lobular inflammation. We believe that the plasma concentration data may reflect true variations among the NAFLD population, but there is also a possibility that the CXCL12$^{high}$ patients may be associated with more advanced NAFLD.**




- While the expressions of the candidate chemokines seem higher in NAFLD endothelial cells, expressions of their respective receptors shown in Figure 4a are clearly higher in healthy PBMCs. Could the authors please comment on this? Moreover, interestingly healthy B cells seem to be expressing more CXCR4 compared to NAFLD B cells. B cell specific CXCR4 was shown to protect against atherosclerosis (https://doi.org/10.1161/CIRCRESAHA.119.316142). Please discuss.

**Response: This is an important consideration. We have added more interpretations on page 8. For your ease of reading, we have extracted the text here - "*Even though there were increased expressions of candidate chemokines in NAFLD endothelial cells, the expressions of their counter receptors, particularly CXCR4 and CX3CR1, appeared lower in NAFLD PBMCs than healthy controls (Fig. 4a). This might be due to the diminished proportions of NK cells and monocytes in NAFLD (Fig. 4c), which were among the main chemokine receptor-expressing populations. Interestingly, B cell subset in healthy controls seemed to be expressing more CXCR4 compared to that in NAFLD (Fig. 4a). B cell-specific CXCR4 has been shown to be atheroprotective through its regulation of IgM levels (Doring et al., 2020). Whether lower CXCR4 has a casual role in NAFLD-related vasculopathy requires further interrogation as CXCR4 can be both protective and pathogenic depending on cell-specific functions and different stages of atherosclerosis development (Murad et al., 2021).*"**

- Line 340: 'As the chemokine receptor-expressing populations mainly involved CD4+ T cells, NK cells and monocytes (Fig. 4)...' here B cells were left out, especially considering that they were among the highest CXCR4 expressing cell types looking at Figure 4a.

**Response: We have added B cells to the statement "*The chemokine receptor-expressing populations were more consistently found in T lymphocytes, natural killer (NK) cells, monocytes and B cells (Fig. 4b).*"**

- Results from Figure 4 should be further validated in independent experiment, at least on gene-levels, preferably on protein level (e.g. effects on NF-kB signaling, on HLA expression, IFNGR expression).

**Response: We have added <u>new data</u> (<u>Fig. 4d</u>; results on page 8) to validate protein expressions of CXCR4 and CX3CR1 in immune cell subsets. We conducted magnetic-activated immune cell sorting to derive CD8+ T cells and CD8- cell fractions (largely include monocytes, CD4+ T cells, NK cells and B cells) from NAFLD (n = 14) and healthy (n = 6) PBMCs, followed by flow cytometry characterization. About 20-50% of each immune subset expressed CXCR4 but there was no significant difference between NAFLD and healthy groups. Both CD8+ T cells and CD8- lymphocytes subsets had relatively higher level of CX3CR1+ cells (30-80%), while CX3CR1 expression was lower in monocytes (10-25%). Overall, there were no significant differences of chemokine receptor expressions in the immune cell subsets comparing NAFLD and healthy groups.**

**We also performed flow cytometry on NAFLD and healthy endothelial cells to evaluate their human leukocyte antigens and interferon-γ receptors at the protein levels. In our <u>new data</u> (<u>Fig .4f</u>; results on page 8), almost all NAFLD and healthy BOECs expressed HLA class I antigens (HLA-A/ B/ C), but there were minimal expressions for HLA-DM class II antigens (Fig. 4f, upper panel). We did not notice major differences of IFN-γR1 expressions between NAFLD and healthy BOECs. Interestingly, epitope density of HLA class I antigens was significantly higher in NAFLD BOECs than in healthy controls (Fig. 4f, lower panel: mean fluorescence intensity). The data thus far suggest that endothelial cells in NAFLD may be providing a chemokine milieu and partially enhanced antigen presentation, which could potentially promote their interaction with immune cells.**

- Regarding Figure 5D, the variation in the CD8- fraction is enormous and clearly show 2 populations. Is the composition of these clusters different? As this effects the results dramatically. Please show Healthy and NAFLD in same graph, it looks like the CD8+ fraction is reduced in NAFLD patients? It would be very interesting to also separate CD4+ T cells, from the non-T-cell-fraction, to further elucidate whether the effects are T-cell specific.

**Response: To address inter-donor variations, we have added more donor samples to perform the endothelial-immune chemotaxis assays (n = 11 NAFLD and n = 6 healthy). In our <u>revised Fig. 5c</u> (previously Fig. 5d), while we still observe relatively huge variations, the**

**data points on the graphs now reflect a continuum with the bigger sample size, rather than the initial clusters when we had fewer samples previously.**

**Compared to healthy BOECs, NAFLD BOECs were able to recruit significantly more CD8+ T cells, while no apparent difference was observed for the chemotaxis of CD8- cell fraction (Fig. 5c). This could be in consensus with NAFLD BOECs possessing higher epitope density of HLA class I antigens (Fig. 4f) that mediate interaction with CD8+ T cells. However, it appears that the numbers of recruited CD8+ T cells were generally lower than that of CD8- cell fraction. This was despite no major difference of CXCR4 expressions on the various immune cell subsets of NAFLD and healthy subjects which we described in Fig. 4d. Besides chemokine receptor expression, it was still unknown to us whether the chemokine binding affinity of CXCR4 receptor could be different in various immune subsets or altered under disease context as reported in other studies (PMIDs: 21725996, 25074471).**

**We agreed that it would be interesting to separate CD4+ T cells from the CD8- lymphocytes fraction in order to elucidate T-cell specific effect. Unfortunately, we have depleted most of our PBMC samples by now. We have only collected 5-9 ml of peripheral blood from each donor and a large proportion of the isolated PBMCs (20-22 million cells/ donor) were cultivated for better successes in deriving outgrowth endothelial cells. Therefore, we have prioritized the use of PBMCs for endothelial cell generation and other critical revision experiments such as the endothelial-immune chemotaxis assays. Thank you for your understanding.**

- Figure 5E. Show NAFLD and healthy in same graph. Please also include a similar experiment with either CXCL12 blocking and/or CXCL12 supplementation.

**Response: We have revised our Fig. 5d (previously Fig. 5e), as well as added <u>new data</u> (<u>Fig. 5e</u>; results on page 9) on CXCL12 blocking. We tested a CXCL12 neutralization antibody to counteract the effect of enhanced chemokine secretion from NAFLD BOECs. Indeed, the use of anti-CXCL12 IgG led to a significantly greater inhibition of NAFLD CD8+ T cell chemotaxis than in the healthy group (Fig. 5e). Anti-CXCL12 IgG was similarly effective at moderating the chemotaxis of CD8- cell fraction in both NAFLD and healthy groups. The data suggest that modulating the chemokine milieu of NAFLD endothelial cell may reduce their interaction with effector immune cells.**

Minor Comments:
- Figure 1D. Please increase the size of the quantification graphs (y-axis is not readable).

**Response: We have increased the size of graphs and font size of axis labels.**

- Figure 1E. The merged image of Fibroblast shows a nuclear marker I assume. Please specify this in the figure/legend.

**Response: We have edited the figure label to include 'nuclei' marker.**

- N-numbers in figure legends do not match the amount of data-points in the graphs. For example for Fig. 1F there are 30-40 data points shown, while the legends states n=3. Please provide more information in all panels where this is the case, stating clearly how many individual replicates are used and what the data-points exactly reflect.

**Response: We have further clarified in Fig. 1f figure legend that the quantifications of sprout lengths and numbers of sprout per bead were obtained from *n* = 3 healthy subjects, *n* = 3 NAFLD patients, with 4-5 beads analyzed per donor. There were typically 5-11 sprouts per bead, accounting for the many data points on the 'sprout length' graph.**

- Increase font size in Figure 4A, this is not readable at the moment.

**Response: We have enlarged the font size accordingly.**

- CXCR7 is now called ACKR3. Please change accordingly.

**Response: We have corrected CXCR7 to ACKR3.**
**Thank you for your time and efforts in evaluating our revised manuscript.**
---

Dear Dr. Cheung,

Thank you for the submission of your revised manuscript to our editorial offices. I have received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support publication of your work in EMBO reports.

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- Please provide the abstract written in present tense throughout and with not more than 175 words.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main and EV figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

- Please add scale bars of similar style and thickness to the microscopic images using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, for some panels the sale bars are rather faint.

- Please move all methods information and references from the Appendix into the main manuscript text.

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The authors adequately addressed my comments and modified the manuscript leading to further improvement.

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Referee #2:

The authors have done a very thorough revision and answered my questions.

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Referee #3:

Strong revision. The manuscript is now acceptable for publication.

The authors have addressed all minor editorial requests.

Dr. Christine Cheung
Nanyang Technological University
Lee Kong Chian School of Medicine
Singapore

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**Abridged guidelines for figures**

**1. Data**

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

**2. Captions**

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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|---|---|---|
| New materials and reagents need to be available; do any restrictions apply? | Not Applicable | |

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|---|---|---|
| For **antibodies** provide the following information:<br>- Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number<br>- Non-commercial: RRID or citation | Yes | Appendix Table S5 - Antibodies |

| DNA and RNA sequences | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| **Short novel DNA or RNA including primers, probes**: provide the sequences. | Yes | Appendix Table S5 - Primer sequences for qPCR |

| Cell materials | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| **Cell lines:** Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID. | Not Applicable | |
| **Primary cultures:** Provide species, strain, sex of origin, genetic modification status. | Yes | Appendix Tables S1 and S3 |
| Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination. | Not Applicable | |

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|---|---|---|
| **Laboratory animals or Model organisms:** Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID. | Yes | Materials and Methods - Diet-induced NAFLD murine model; Humanized NAFLD murine model |
| **Animal observed in or captured from the field:** Provide species, sex, and age where possible. | Not Applicable | |
| Please detail **housing and husbandry conditions**. | Yes | Materials and Methods - Diet-induced NAFLD murine model; Humanized NAFLD murine model |

| Plants and microbes | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| **Plants:** provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens). | Not Applicable | |
| **Microbes:** provide species and strain, unique accession number if available, and source. | Not Applicable | |

| Human research participants | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants. | Yes | Appendix Tables S1, S3 and S4 |

| Core facilities | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| If your work benefited from core facilities, was their service mentioned in the acknowledgments section? | Yes | Acknowledgements (NTU Optical Bio-Imaging Centre, NOBIC; Spatial and Single Cell Genomics Platform at Genome Institute of Singapore) |

**Design**

| Study protocol | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI. | Not Applicable | |
| Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable. | Not Applicable | |

| Laboratory protocol | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Provide DOI OR other citation details if **external detailed step-by-step protocols** are available. | Not Applicable | |

| Experimental study design and statistics | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Include a statement about **sample size** estimate even if no statistical methods were used. | Not Applicable | Statistical methods have been used. |
| Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described? | Yes | Materials and Methods - Humanized NAFLD murine model; Immunohistochemical staining and image analysis |
| Include a statement about **blinding** even if no blinding was done. | Yes | Materials and Methods - Immunohistochemical staining and image analysis |
| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Not Applicable | |
| For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared? | Yes | Materials and Methods - Statistical analysis |

| Sample definition and in-laboratory replication | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| In the figure legends: state number of times the experiment was **replicated** in laboratory. | Yes | Figure legends, Materials and Methods |
| In the figure legends: define whether data describe **technical or biological replicates**. | Yes | All figure legends |

## Ethics

| Ethics | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. | Yes | Materials and Methods - Study approvals, patient enrolment and sample collection |
| Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | Yes | Materials and Methods - Study approvals, patient enrolment and sample collection |
| Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained. | Not Applicable | |
| Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations. | Yes | Materials and Methods - Animal studies approval |
| Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why. | Not Applicable | |

| Dual Use Research of Concern (DURC) | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm | Not Applicable | |
| If you used a select agent, is the security level of the lab appropriate and reported in the manuscript? | Not Applicable | |
| If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript? | Not Applicable | |

## Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

| Adherence to community standards | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided. | Not Applicable | |
| For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | Not Applicable | |
| For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | Not Applicable | |

## Data Availability

| Data availability | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section? | Yes | Availability of Data and Materials |
| Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement? | Yes | Availability of Data and Materials |
| Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided? | Not Applicable | |
| If publicly available data were reused, provide the respective **data citations in the reference list**. | Not Applicable | |