Journal of Clinical and Translational Research
Peer review process file 201503004

IL-23 and IL-17 are not involved in hepatic ischemia reperfusion injury in mouse and man

Pim B. Olthof[1†], Rowan F. van Golen[1†], Megan J. Reiniers[1], Milan Kos[1], Lindy K. Alles[1], Martinus A. Maas[1], Joanne Verheij[2], Thomas M. van Gulik[1], Michal Heger[1*]

Corresponding author:
Michal Heger
Department of Experimental Surgery, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

Handling editor:
Yao Liu
Department of Membrane Biochemistry and Biophysics, Utrecht University, Utrecht, the Netherlands
Review timeline:

Received: 10 November, 2015
Editorial decision: 22 November, 2015
Revision received: 1 December, 2015
Editorial decision: 5 December, 2015
Published online ahead of print: 17 December, 2015

1st editorial decision:

Date: 22-Nov-2015

Ref.: Ms. No. JCTRes-D-15-00013
IL-23 and IL-17 are not involved in hepatic ischemia reperfusion injury in mouse and man
Journal of Clinical and Translational Research

Dear Dr. Heger,

Reviewers have submitted their critical appraisal of your paper. The reviewers' comments are appended below. Based on their comments and evaluation by the editorial board, your work was FOUND SUITABLE FOR PUBLICATION AFTER MINOR REVISION.

If you decide to revise the work, please itemize the reviewers' comments and provide a point-by-point response to every comment. An exemplary rebuttal letter can be found on at http://www.jctres.com/en/author-guidelines/ under "Manuscript preparation." Also, please use the track changes function in the original document so that the reviewers can easily verify your responses.

Your revision is due by Dec 22, 2015.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Yao Liu
Editorial Board Member
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: This paper has pursued a thorough exploration of the role and relevance of IL-23/IL-17 in liver I/R spanning several model systems: in vitro, in vivo in mice and humans. It is carefully written with high quality depiction of data in figures and tables.

Minor issue is the use of AML12 cell line in some of the experiments rather than primary hepatocytes; suggest rationale for this be included in either Methods and/or Discussion.

Comment regarding results 3.3 Fig 4: large variability likely due to patient variability (co-morbidities, medications) and operative procedure - this may be worthwhile mentioning in Discussion.

A suggestion for authors to assist with data analyses: Are the authors able to perform a sub-analysis of patients (shown in Table 1) that have undergone intermittent (i) occlusion vs continuous (c) vs control - intermittent occlusion group may have experienced preconditioning by virtue of technique of intermittent clamping, and could confound your findings for Fig 4 A, B.

Reviewer #2: Title and introduction:

1. It would be more appropriate if the title includes "in clinical settings" or "human subjects" instead of "man";
2. Introduction needs more background information about the about the role of Damage-associated molecular pattern molecules (DAMPs) and their intracellular signalling in the sterile inflammation response.

Material and Methods:
1. The authors did not provide evidence about the extent of cell injury/necrotic death e.g. LDH leakage after rendering the cell necrotic;
2. How the authors can prove the presence of DAMPs in the supernatant of necrotic cells. Did the authors quantify, for example, HMGB-1 or any other DAMP?
3. Throughout the paper the authors report DAMPs concentrations as (50:1, 25:1, 50C:1 and 25C:1; Figure 1 and 2), which presumably the DAMPs concentration. This need to be clarified and what this concentration represents needs to be explained. It is known that DAMPs contain

different sort of protein and non-protein members, but it is import to quantify at least one of them e.g. estimation of HMGB-1 concentration via ELISA;
4. Quantification of murine plasma cytokines title should include liver tissue;
5. Reference # 14 is a review and does not provide technical information about IRI or molecular biology procedures.

Results:

1. Again the authors describe the effects of DAMPs treatment on macrophages activation which is an interesting approach. However, no information is provided about the nature of these DAMPs. Are they HMGB-1, urates, leaked DNA, ATP etc.?
2. The human results are very interested and complement both experimental findings. The authors did not report transaminases levels after patients' surgery;
3. Figure 2 (A, B and C) has 4 bars for each time point (1 h and 6 h), but the meaning of each bar is unclear;
4. The activation of NF-kB is essential to explain the present results. The characterization of NF-kB activation should be more investigated. For example, it would be more convincing and robust if the authors combine the results of luciferase reporter assay with western blot analysis of p65 subunit nuclear translocation and/or phosphorylation.
5. Reperfusion time in figure 3 (I, L and K) should be standardized. Reperfusion time after 3 and 12 h of reperfusion does not provide any information.

Authors' rebuttal:

Manuscript ID: JCTRes-D-15-00013.
Title: IL-23 and IL-17 are not involved in hepatic ischemia reperfusion injury in mouse and man

Dear editor,

We thank the reviewers for their critical evaluation of our work, which we feel has considerably raised the level of the manuscript. Please find below a point-by-point response to the raised concerns.

Reviewer #1

This paper has pursued a thorough exploration of the role and relevance of IL-23/IL-17 in liver I/R spanning several model systems: in vitro, in vivo in mice and humans. It is carefully written with high quality depiction of data in figures and tables.

1. Minor issue is the use of AML12 cell line in some of the experiments rather than primary hepatocytes; suggest rationale for this be included in either Methods and/or Discussion.

We acknowledge that primary hepatocytes most closely resemble the in vivo situation, but deliberately used AML12 for these experiments for the following reasons. First, AML12 hepatocytes were used because these non-transformed cells better approach primary mouse hepatocytes than the more frequently used hepatoma-derived cell lines. Considering that DAMPs with a proven involvement in liver I/R injury such as HMGB1 or histones are highly conserved between species and present in cell lines, we anticipated that AML12-derived DAMPs would be equally successful in activating murine macrophages as DAMPs from primary hepatocytes. The data shown in Figure 2 corroborate this hypothesis. Second, we used an extensive number of hepatocytes (i.e., ±18 million hepatocytes per 24 wells plate) to perform the DAMP transfer experiments (including countless pilots). With a projected yield of ±6 million hepatocytes per mouse liver isolation (Liu et al., 2011), the infrastructural and financial downsides of using primary hepatocytes would not outweigh the benefits, especially since the DAMP profiles are not expected to differ significantly between primary cells and cell lines (see first point). Third, recently introduced European but also Dutch legislation on animal experiments has raised extensive barriers (botch financial and practical) to performing animal experiments when alternatives such as cell lines are available. Finally, the reviewer should note that our line of reasoning does by no means take away from the validity of the data.

2. Comment regarding results 3.3 Fig 4: large variability likely due to patient variability (co-morbidities, medications) and operative procedure - this may be worthwhile mentioning in Discussion.

We thank the reviewer for this suggestion. A statement on data variability was added to the discussion on page 22.

3. A suggestion for authors to assist with data analyses: Are the authors able to perform a sub-analysis of patients (shown in Table 1) that have undergone intermittent (i) occlusion vs continuous (c) vs control - intermittent occlusion group may have experienced preconditioning by virtue of technique of intermittent clamping, and could confound your findings for Fig 4 A, B.

We thank the reviewer for this very interesting suggestion. The effect of ischemic preconditioning has been extensively studied in many experimental models and clinical translation of these findings is essential. In our center, patients are routinely operated using intermittent vascular inflow occlusion (20 min cycles, repeated until parenchymal transection is completed). Four out of eight patients that were subjected to continuous occlusion in fact only needed the first cycle of what would have been intermittent occlusion. Therefore the ischemic

durations of continuous occlusion is median (range) 27.5 (20-52) min compared to 56 (26-120) min in the intermittent occlusion group, which is a considerable difference. In addition, we only included patients exposed to ≥20 min of liver ischemia in our study, which is twice the duration used to precondition livers to prolonged ischemia (Clavien et al., 2003). Due to these differences, we feel the intermittent and continuous occlusion groups do not qualify for subgroup analysis.

Reviewer 2

Title and introduction:

1. It would be more appropriate if the title includes "in clinical settings" or "human subjects" instead of "man"

The title was deliberately chosen because of its literary and symbolic significance. In the first place, the title pertains to the poem of Robert Burns, "To A Mouse, On Turning Up Her Nest." In this poem, the home that was carefully constructed by the mouse (in analogy, the existing work that has led to our paper) was destroyed by the cruel winter winds and bitter conditions (i.e., our study). The morale of the story is that sometimes “the best-laid schemes o' mice an 'men gang aft agley” (often go awry). That is what happened to the IL-23/IL-17 signaling axis in our study. Moreover, in Steinbeck’s novel “Of Mice and Men,” Lennie's accidental murder (i.e., our study) of Curley's wife (i.e., the existing work that led to our study) completely disrupts their lives and dreams of an easy existence (i.e., the IL-23/IL-17 signaling axis). Like the mouse in Burns’ story, neither George nor Lenny could have foreseen the drastic turn that their lives would take, despite their plans for the future. We had great expectations for this signaling axis in liver I/R, but our prospects of further elucidating the role of this signaling axis in liver I/R were abolished by the outcomes of this study. In light of this realistic symbolism, we hope that the reviewer is emphatic and accepts the title as is. Moreover, this title is technically not incorrect and readers still understand what we mean by it.

2. Introduction needs more background information about the about the role of Damage-associated molecular pattern molecules (DAMPs) and their intracellular signalling in the sterile inflammation response.

An introduction on DAMPs was added to the introduction on page 5. 'DAMPs are intracellular…-…I/R injury.'

Material and Methods:
1. The authors did not provide evidence about the extent of cell injury/necrotic death e.g. LDH leakage after rendering the cell necrotic;

The DAMP stimulation assay was modified from Bamboat ZM et al, in which necrosis was routinely >98%, as measured by flow cytometry. We performed the heat incubation according to this report and confirmed the large extent of necrosis induced by this protocol in pilot experiments using light microscopy (the cell monolayer was entirely gone) and trypan blue exclusion on cells in the supernatant (routinely >95% necrosis).

2. How the authors can prove the presence of DAMPs in the supernatant of necrotic cells. Did the authors quantify, for example, HMGB-1 or any other DAMP?

We did not quantify DAMPs in the culture supernatant. The aim of the paper was to examine whether murine macrophages produce IL-23 in response to immunogenic stimuli derived from necrotic hepatocytes. Any intracellular constituent with immunogenic properties can be considered a DAMP and only a fraction of DAMPs are most likely currently known. Furthermore, it is not known which DAMP(s) is/are most important in IL-23 production. Therefore we did not quantify specific DAMPs, as we feel measuring a randomly selected DAMP would not help to answer the main research question of our manuscript (i.e., whether the IL-1β/IL-23/IL-17A axis is activated following liver I/R).

3. Throughout the paper the authors report DAMPs concentrations as (50:1, 25:1, 50C:1 and 25C:1; Figure 1 and 2), which presumably the DAMPs concentration. This need to be clarified and what this concentration represents needs to be explained. It is known that DAMPs contain different sort of protein and non-protein members, but it is import to quantify at least one of them e.g. estimation of HMGB-1 concentration via ELISA;

The concentrations indeed indicate the amount of (necrotic) AML12 cells incubated per RAW264.7 macrophage. These concentrations were used to increase clinical translation (e.g., if

concentrations of 5.000.000:1 were needed to achieve macrophage activation, the results would not be clinically relevant). The concentrations are better addressed in the methods section 2.1.

As mentioned in our response to comment 2, we did not quantify DAMPs in culture supernatant. The pathways of IL-23 production by macrophages are currently elusive and we were solely interested in whether necrotic hepatocytes activate macrophages and whether or not this results in IL-23 production. We feel the selection of one DAMP for quantification would not add knowledge to the current manuscript. First, the cytokine and ROS measurements unequivocally show macrophage activation, attesting to the pro-inflammatory effects of necrotic hepatocytes. As we do not know which specific DAMPs are responsible for IL-23 production, neither the presence nor the absence of a randomly selected DAMP would impact the conclusion drawn from these experiments (i.e., necrotic hepatocytes activate RAW264.7 macrophages but do not elicit IL-23 production).

4. Quantification of murine plasma cytokines title should include liver tissue;

The title was changed to “Quantification of murine plasma cytokines in murine plasma and liver tissue”

5. Reference # 14 is a review and does not provide technical information about IRI or molecular biology procedures.

Reference 14 does not only include a review of literature, but also includes a large data set derived from the mouse liver I/R model and the clinical cohort presented here. The manuscript and its supplementary information do contain the information we refer to in the current manuscript.

Results:
1. Again the authors describe the effects of DAMPs treatment on macrophages activation which is an interesting approach. However, no information is provided about the nature of these DAMPs. Are they HMGB-1, urates, leaked DNA, ATP etc.?

We would like to refer the reviewer to the rationale for not quantifying specific DAMPs detailed above (response to comments 2 and 3, materials and methods section).

2. The human results are very interested and complement both experimental findings. The authors did not report transaminases levels after patients' surgery;

We did not report the ALT levels after surgery due to a recent report in Annals of Surgery that examined the clinical (ir)relevance of postoperative transaminase levels. The report by Boleslawski E et al. concluded that the postoperative transaminase levels do no correlate to the used duration of ischemia or to postoperative outcomes. Therefore the transaminase levels most likely do not reflect the extent of liver injury by IR in patients.

3. Figure 2 (A, B and C) has 4 bars for each time point (1 h and 6 h), but the meaning of each bar is unclear;

The meaning of the bars was indeed not adequately reported in the figure, which has been modified accordingly.

4. The activation of NF-kB is essential to explain the present results. The characterization of NF-kB activation should be more investigated. For example, it would be more convincing and robust if the authors combine the results of luciferase reporter assay with western blot analysis of p65 subunit nuclear translocation and/or phosphorylation.

We agree with the suggestions of the reviewer. However, we would like to emphasize that it was not our intention to provide a mechanistic rationale for our negative findings. We carried out these experiments to consolidate the data regarding the pro-inflammatory signaling that occurs following liver I/R as well as in DAMP-exposed RAW cells (Figure 2). So what we essentially demonstrated is that pro-inflammatory signaling, which is mostly under NF-kB transcriptional control, does indeed occur following DAMP priming, but that IL-23 is either not under control of the NF-kB regulatory unit or is not produced by (RAW) macrophages. This was sufficient complementary information for the purposes of this study, but should indeed be investigated further in focused IL-23 studies.

5. Reperfusion time in figure 3 (I, L and K) should be standardized. Reperfusion time after 3 and 12 h of reperfusion does not provide any info

The analysis at 3 an 12 h was performed to examine the trend of IL-17A production over time and prevent false negative conclusions. However, we agree the data does not add info to the manuscript and t=3 and t=12 were omitted from the figure.

References

Bamboat ZM et al. Toll-like receptor 9 inhibition confers protection from liver ischemia-reperfusion injury. Hepatology. 2010 Feb;51(2):621-32. [19902481]

Boleslawski E et al. Relevance of postoperative peak transaminase after elective hepatectomy. Ann Surg. 2014 Nov;260(5):815-20. [25074418]

Clavien PA et al. A prospective randomized study in 100 consecutive patients undergoing major liver resection with versus without ischemic preconditioning. Ann Surg 2003;238(6):843-50; discussion 851-2. [14631221]

Liu W et al. Sample preparation method for isolation of single-cell types from mouse liver for proteomic studies. Proteomics 2011;11(17):3556-64. [21751380]

2nd editorial decision:

Date: 5-Dec-2015

Ref.: Ms. No. JCTRes-D-15-00013R1
IL-23 and IL-17 are not involved in hepatic ischemia reperfusion injury in mouse and man
Journal of Clinical and Translational Research

Dear Dr. Heger,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

Comments from the editor and reviewers can be found below. You can see that Reviewer #2 has provided further comments upon acceptance of your paper. I kindly ask you to address these comments and send a final version of your manuscript to JCTR.

Thank you for submitting your work to JCTR.

Kindest regards,

Yao Liu
Editorial Board Member
Journal of Clinical and Translational Research

Comments from the editors and reviewers:

Reviewer #1: None.

Reviewer #2: 1. The authors did not provide evidence about the extent of cell injury/necrotic death e.g. LDH leakage after rendering the cell necrotic;
The DAMP stimulation assay was modified from Bamboat ZM et al, in which necrosis was routinely >98%, as measured by flow cytometry. We performed the heat incubation according to this report and confirmed the large extent of necrosis induced by this protocol in pilot experiments using light microscopy (the cell monolayer was entirely gone) and trypan blue exclusion on cells in the supernatant (routinely >95% necrosis).
This information should be included in the body of the manuscript as well as the cited paper to allow other researchers to recreate the conditions and the results of the experiments.
2. Reference # 14 is a review and does not provide technical information about IRI or molecular biology procedures.
Reference 14 does not only include a review of literature, but also includes a large data set derived from the mouse liver I/R model and the clinical cohort presented here. The manuscript and its supplementary information do contain the information we refer to in the current manuscript.
Please check thoroughly the cited paper: Van Golen RF, et al: The mechanisms and physiological relevance of glycocalyx degradation in hepatic ischemia/reperfusion injury. Antioxid Redox Signal 2014;21:1098-1118. The only information about IRI procedure is contained in Fig 8 "HS is released into the circulation following hepatic I/R in mice. Male C57Bl/6J mice (8-12 weeks old) were subjected to 60 min of partial liver ischemia or a sham operation (N= 5-6/group). Ethylenediaminetetraaceticacid anticoagulated blood samples were collected after 1 or 6 h of reperfusion or 6 h after sham operation". There is no information whatsoever, as stated in the current paper, about tissue sampling, molecular analysis (PCR) and histological analysis (hematoxylin and eosin staining of formalin-fixed, paraffinized tissue specimens).