nature portfolio

Peer Review File

Gut-primed neutrophils activate Kupffer cells to promote hepatic injury in mouse sepsis

Corresponding Author: Dr Atsushi Murao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this paper, Murao et al investigated the mechanisms underlying sepsis-induced liver injury, focusing on the role of gut-liver crosstalk. The authors hypothesize that gut-trained neutrophils migrating via the portal vein release neutrophil extracellular traps (NETs), which activate Kupffer cells and exacerbate hepatic damage. Their findings demonstrate increased NETs in the liver of septic mice and show that NET-mediated Kupffer cell activation leads to elevated cytokine production (IL-6 and TNFα), contributing to hepatocyte death. Additionally, gut intraepithelial lymphocytes (IELs) were found to enhance neutrophil NET production in response to LPS, further amplifying Kupffer cell activation. These effects were attenuated by inhibiting neutrophil elastase, PAR-1, or in PAD4-deficient mice.
Despite significance, I have many concerns regarding the methods, controls and also regarding the logic of the scientific track throughout the paper.

1. NET release and detection: NETs are described as being released extracellularly by neutrophils. In this paper, the authors used cit-H3 and MPO gating to measure the frequency of NET-releasing neutrophils. However, if NETs are released, how can these neutrophils still retain positivity for these markers? Could this indicate intracellular NET formation or incomplete release, and how was this distinction addressed?

2. In vivo evidence for NETs and Kupffer cell activation: The data supporting NET production and Kupffer cell (KC) activation rely entirely on in vitro studies, with no in vivo evidence that gut-derived neutrophils modulate KC function. Would it be possible to address this by isolating neutrophils from septic mice, injecting them via the portal vein or systemic circulation, and comparing their ability to modulate KC function in vivo? For example, could you assess their differential capacity to induce cytokine production or influence bacterial clearance in KCs?

3. Neutrophil and IEL interaction: The manuscript lacks a rationale for why neutrophil interaction with IELs was hypothesized. Is there prior evidence suggesting that neutrophils interact with IELs in vivo during sepsis? Are there histological or immunohistochemical data showing such interactions or their spatial proximity in the gut during sepsis? One potential experiment could involve imaging or quantifying neutrophil-IEL interactions in the septic gut. Without such data, the motivation for these experiments remains unclear.

4. CD112 specificity: Why was CD112 specifically chosen as a focus of investigation? Was this marker identified in prior screens, or is there evidence of its unique relevance in neutrophil-IEL interactions or NET production? A stronger justification for this choice, or testing other related markers, would enhance the manuscript's clarity.

5. Mechanistic validation in vivo: The proposed mechanism of sequential interactions between neutrophils, Kupffer cells, IELs, and CD112 is central to the paper but was not directly validated in vivo. The authors inferred this mechanism using sequential in vitro incubations, but in vivo experiments are essential to substantiate this model. For example, would depleting Kupffer cells in septic mice (e.g., via clodronate liposomes) abolish the observed effects?

6. NETs as mediators of Kupffer cell activation in vivo: To establish that neutrophils interact with KCs in vivo and activate them via NET production, additional experiments could be performed. For instance, does intravenous administration of DNase block KC activation and cytokine release during sepsis? This would provide direct evidence for NET-mediated effects in vivo and strengthen the study's conclusions.

Reviewer #2

(Remarks to the Author)
Dear Authors,
I have performed the revision of your manuscript “Gut Intraepithelial Lymphocyte-Trained Neutrophils Activate Kupffer Cells to Promote Hepatic Injury in Sepsis”.

This is an interesting study demonstrating new aspects of gut intraepithelial lymphocytes (IELs) and neutrophil cross-talk and the role of neutrophil derived NETs in sepsis-induced liver injury.

The main claim of this study was to elucidate the possible role of the crosstalk between neutrophils, IELs, and Kupffer cells in gut-driven hepatic injury. The novelty of this work relies not only on the demonstration of this crosstalk in vitro and in vivo but also on description of the underlying molecular mechanisms. This work may be interesting and useful for the scientists working in the complicate field of sepsis research. The proposed work is well inserted in the frame of studies published in the last years showing that cross-talk between different immune cells during the early phase of sepsis development plays an important role in sepsis progression and outcome.

The manuscript is well written and the experiments are well conducted both in vivo and in vitro and support the claims well. Notably, the use of PAD4-/- mice in the experiments is an elegant approach. The experiments mostly could be reproduced based on descriptions or references given in “Methods”. The length and quality of discussion as well as the cited literature are appropriate.

However, I have several comments/questions.

1. What C57BL/6 mice were used in the study? Just to wright “C57BL/6 (B6)” is not sufficient.
C57BL/6J (BL6J) and C57BL/6N (BL6N) inbred sub-strains are most widely used to understand the pathological roles of target molecules in a variety of diseases. In the last decade increasing amount of publications describe that C57BL/6J and C57BL/6N sub-strains differentially influence phenotype severity in different models. These differences were demonstrated for hepatic injury (Kawashita E, Ishihara K, Nomoto M, Taniguchi M, Akiba S. A comparative analysis of hepatic pathological phenotypes in C57BL/6J and C57BL/6N mouse strains in non-alcoholic steatohepatitis models. Sci Rep. 2019 Jan 18;9(1):204. doi: 10.1038/s41598-018-36862-7. PMID: 30659241; PMCID: PMC6338790). Please give the sub-strain name correctly.
2. The use of PAD4-/- mice should be briefly explained in results. Although protein-arginine deiminase type-4 (PAD4) is mentioned briefly in the introduction, it would be helpful to remind the reader that PAD4(-/-) neutrophils are unable to form NETs upon stimulation, and therefore PAD4-/- mice can be used as a “negative control” for NET-mediated experiments .

Minor comments:

1. Fig 1C: The Y-axis is unclear. What does "PMN (10^3)" mean? Does it represent the PMN concentration per ml? Fig1 H,I: it is not clear what concentration of NETs was used for experiments with different duration. Was it 1000ng/ml?
2. What was the concentration of protease inhibitor (Fig2A,B)?
3. Line 210. The sentence: “While PAR-1 inhibition yielded a greater reduction than NE inhibition, suggesting contributions from other neutrophil proteases (e.g., proteinase 3, cathepsin G), neither completely abrogated cytokine production” is unclear. Please consider rephrasing it for better clarity and flow.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
My comments were fully addressed with new experiments. Thank you.

Reviewer #2

(Remarks to the Author)

Reviewer #3

(Remarks to the Author)
Murao et al. investigated the mechanisms underlying liver injury during sepsis, with a particular focus on gut–liver interactions. They propose that neutrophils primed in the gut migrate through the portal circulation and release neutrophil extracellular traps (NETs), which subsequently activate Kupffer cells and exacerbate hepatic damage.
I have carefully reviewed the revised manuscript prepared in response to the first round of review. I have no further queries beyond those initially raised, which were thorough and fully acceptable. The authors have addressed all comments appropriately and revised the manuscript accordingly.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.
In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.
The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.
To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/

Reviewer #1 (Neutrophil, sepsis) (Remarks to the Author):

In this paper, Murao et al investigated the mechanisms underlying sepsis-induced liver injury, focusing on the role of gut-liver crosstalk. The authors hypothesize that gut-trained neutrophils migrating via the portal vein release neutrophil extracellular traps (NETs), which activate Kupffer cells and exacerbate hepatic damage. Their findings demonstrate increased NETs in the liver of septic mice and show that NET-mediated Kupffer cell activation leads to elevated cytokine production (IL-6 and TNFα), contributing to hepatocyte death. Additionally, gut intraepithelial lymphocytes (IELs) were found to enhance neutrophil NET production in response to LPS, further amplifying Kupffer cell activation. These effects were attenuated by inhibiting neutrophil elastase, PAR-1, or in PAD4-deficient mice.
Despite significance, I have many concerns regarding the methods, controls and also regarding the logic of the scientific track throughout the paper.

Response: Thank you for your excellent summary of our work and your immensely valuable comments and feedback. We have addressed each of your comments by performing additional new experiments and/or providing clear, relevant explanations in the revised manuscript. We believe our revisions have fully addressed your comments and markedly strengthened our work.

1. NET release and detection: NETs are described as being released extracellularly by neutrophils. In this paper, the authors used cit-H3 and MPO gating to measure the frequency of NET-releasing neutrophils. However, if NETs are released, how can these neutrophils still retain positivity for these markers? Could this indicate intracellular NET formation or incomplete release, and how was this distinction addressed?

Response: We apologize for the inadequate description of neutrophil extracellular trap (NET) release and detection. NETs are web-like DNA structures decorated with citrullinated histone H3 (cit-H3) and myeloperoxidase (MPO) released from neutrophils. They are initially tethered to the neutrophil, and the base of these web-like structures remains in contact with the cell during NET release. The flow cytometry-based NET assessment used in this study, first reported by Gavillet *et al*. in *American Journal of Hematology* in 2015 (PMC4715743, new ref #32), has been utilized by a large number of laboratories. This method uses non-permeabilized neutrophils, ensuring that detected cit-H3 and MPO are extracellular, indicative of NETs. The original paper validated this approach in both mouse and human neutrophils. They used NET-deficient mice as a negative control to ensure specificity and compared flow cytometry data with microscopy. Our lab has published multiple papers using this method in peer-reviewed journals, including the *Journal of Clinical Investigation* (Jin *et al*, *J Clin Invest*, 2023, PMC10348768; Murao *et al*, *FASEB J*, 2020, PMC9092350; Ode *et at*, *Sci Rep*, 2019, PMC6472421). Accordingly, we have provided a more detailed explanation of the flow cytometry-based NET assessment in the revised manuscript (page 16, line 18-22). Furthermore, acknowledging that relying on a single method for NET assessment is suboptimal, we have included microscopic images of SYTOX-stained NETs in cultured neutrophils in the original submission. In response to your valuable comment, we have now added newly generated microscopic data demonstrating increased NETs in the septic liver tissue *in vivo* (**Supplemental Fig. 1**). The *in vivo* detection of NETs in tissues using immunohistochemistry has been demonstrated by Brinkmann *et al.* (*Front Immunol,* 2016, PMC5118445), supporting the validity of our protocol for detecting NETs in tissues.

2. In vivo evidence for NETs and Kupffer cell activation: The data supporting NET production and Kupffer cell (KC) activation rely entirely on in vitro studies, with no in vivo evidence that gut-derived neutrophils modulate KC function. Would it be possible to address this by isolating

neutrophils from septic mice, injecting them via the portal vein or systemic circulation, and comparing their ability to modulate KC function in vivo? For example, could you assess their differential capacity to induce cytokine production or influence bacterial clearance in KCs?

Response: Thank you for this excellent suggestion. Accordingly, we conducted additional experiments in which neutrophils from septic mice were isolated and adoptively transferred into recipient WT mice, followed by the induction of sepsis via cecal ligation and puncture (CLP). We isolated gut-derived neutrophils from the portal vein of WT or peptidylarginine deiminase 4 $(PAD4)^{-/-}$ septic mice and intravenously injected them into WT septic mice to evaluate Kupffer cell activation. $PAD4^{-/-}$ neutrophils are defective in NET formation and therefore serve as a negative control for NET experiments. We observed increased mRNA levels of iNOS and cytokines in Kupffer cells from septic mice injected with WT septic portal vein neutrophils, whereas this Kupffer cell activation was attenuated in septic mice injected with $PAD4^{-/-}$ septic portal vein neutrophils (new **Fig. 3H–J**; page 7, lines 4–8). These new data clearly demonstrate that gut-derived neutrophils activate Kupffer cells via NETs in septic mice under *in vivo* conditions.

3. Neutrophil and IEL interaction: The manuscript lacks a rationale for why neutrophil interaction with IELs was hypothesized. Is there prior evidence suggesting that neutrophils interact with IELs in vivo during sepsis? Are there histological or immunohistochemical data showing such interactions or their spatial proximity in the gut during sepsis? One potential experiment could involve imaging or quantifying neutrophil-IEL interactions in the septic gut. Without such data, the motivation for these experiments remains unclear.

Response: We completely agree with you on those relevant points. As you suggested, we have newly generated and incorporated additional data showing the rationale for investigating neutrophil-intraepithelial lymphocyte (IEL) interaction. In the new experiments, we found a markedly increased number of neutrophils after sepsis in the gut epithelium, where IELs reside (new **Fig. 4A**; page 7, line 12-13). Furthermore, we evaluated gut tissue using immunohistochemistry and demonstrated that neutrophils were in contact with IEL in the septic gut (new **Fig. 4B**; page 7, line 14). This new data support the interaction between neutrophils and IELs *in vivo* during sepsis.

4. CD112 specificity: Why was CD112 specifically chosen as a focus of investigation? Was this marker identified in prior screens, or is there evidence of its unique relevance in neutrophil-IEL interactions or NET production? A stronger justification for this choice, or testing other related markers, would enhance the manuscript's clarity.

Response: We have previously identified CD112 expressed on neutrophils serves as an independent mediator for neutrophil-lymphocyte interaction during sepsis (Murata *et al*, *J Immunol*, 2023, PMC9852067, ref #15). As IELs are resident lymphocytes in the gut, we focused on CD112 as a mediator of the neutrophil-IEL interaction in sepsis. We found that blocking CD112 significantly attenuated IEL-induced NETs, indicating that CD112 as a critical mediator for this interaction. However, it did not result in a complete inhibition, suggesting that other factors could also mediate this interaction. We have incorporated this discussion in the revised manuscript (page 12, line 1-4). In addition, to further support the physiological relevance of neutrophil CD112, we have incorporated new *in vivo* data showing that $CD112^{+}$ neutrophils significantly increased in the septic gut epithelium (new **Fig. 4F**; page 7, line 21).

5. Mechanistic validation in vivo: The proposed mechanism of sequential interactions between neutrophils, Kupffer cells, IELs, and CD112 is central to the paper but was not directly validated

in vivo. The authors inferred this mechanism using sequential in vitro incubations, but in vivo experiments are essential to substantiate this model. For example, would depleting Kupffer cells in septic mice (e.g., via clodronate liposomes) abolish the observed effects?

Response: In response to your insightful comment and suggestion, we have conducted additional experiments involving the adoptive transfer of intraepithelial lymphocyte (IEL)-interacting neutrophils into septic mice. Compared to the control group (treated with neutrophils that did not interact with IELs), mice receiving IEL-interacting neutrophils exhibited increased iNOS and cytokine levels in Kupffer cells, as well as elevated serum liver enzymes (new **Fig. 5F-J**; page 8, lines 14-19). These effects were significantly attenuated when IEL-neutrophil interactions were blocked using an anti-CD112 antibody (new **Supplemental Fig. 4**; page 8, lines 19-21). These new data strongly suggest that neutrophils activate Kupffer cells in sepsis via CD112-mediated interaction with IELs. Kupffer cell activation was the readout of the sequential cellular interactions rather than the target of intervention in our study. We considered depleting Kupffer cells by clodronate liposomes to evaluate their impact on liver injury as per your suggestion; however, due to the potential for global monocyte/macrophage depletion and off-target effects, including its impact on neutrophils, as previously published (Mass *et al*, *J Exp Med*, 2023, PMC10067525), we opted not to use this general approach. Moreover, given the importance of Kupffer cells in combating against pathogens, a conditional knockout of the hepatotoxic content rather than complete cell depletion may be a better option for septic models. This consideration/limitation is now addressed in the revised manuscript (page 11, line 10-11)

6. NETs as mediators of Kupffer cell activation in vivo: To establish that neutrophils interact with KCs in vivo and activate them via NET production, additional experiments could be performed. For instance, does intravenous administration of DNase block KC activation and cytokine release during sepsis? This would provide direct evidence for NET-mediated effects in vivo and strengthen the study's conclusions.

Response: Thank you for this suggestion. To demonstrate *in vivo* evidence of NETs-induced Kupffer cell activation, we utilized PAD4$^{-/-}$ mice. PAD4$^{-/-}$ mice are a widely used negative control for NETs, and we found decreased proinflammatory M1 Kupffer cells (as indicated by iNOS) in PAD4$^{-/-}$ mice compared to WT mice after CLP (**Fig. 1E**). Moreover, we have additionally found increased iNOS and cytokine levels in the KCs of septic mice injected with WT septic portal vein neutrophils, whereas this Kupffer cell activation was mitigated in septic mice injected with PAD4$^{-/-}$ septic portal vein neutrophils (new **Fig. 3H-J**, page 7, line 4-8). These new data indicate that gut-derived neutrophils activate Kupffer cells via NETs in septic mice *in vivo*. Please note that our current study primarily focused on the role of proteases, rather than DNA, as NET components inducing PAR-1-mediated Kupffer cell activation. Because proteases are embedded within NETs, removing NET DNA would inevitably include proteases, making it difficult to discern the specific contribution of DNA. Therefore, to specifically investigate the role of proteases in this pathway, we have decided not to assess the effects of DNase. Nonetheless, we have acknowledged that DNA is a major NET component capable of inducing proinflammatory responses, and we have included a discussion of NET-associated DNA and the potential use of DNase in future research (page 11, line 1-4).

Reviewer #2 (Neutrophil, NET) (Remarks to the Author):

Dear Authors,
I have performed the revision of your manuscript "Gut Intraepithelial Lymphocyte-Trained Neutrophils Activate Kupffer Cells to Promote Hepatic Injury in Sepsis".

This is an interesting study demonstrating new aspects of gut intraepithelial lymphocytes (IELs) and neutrophil cross-talk and the role of neutrophil derived NETs in sepsis-induced liver injury.

The main claim of this study was to elucidate the possible role of the crosstalk between neutrophils, IELs, and Kupffer cells in gut-driven hepatic injury. The novelty of this work relies not only on the demonstration of this crosstalk in vitro and in vivo but also on description of the underlying molecular mechanisms. This work may be interesting and useful for the scientists working in the complicate field of sepsis research. The proposed work is well inserted in the frame of studies published in the last years showing that cross-talk between different immune cells during the early phase of sepsis development plays an important role in sepsis progression and outcome.

The manuscript is well written and the experiments are well conducted both in vivo and in vitro and support the claims well. Notably, the use of PAD4-/- mice in the experiments is an elegant approach. The experiments mostly could be reproduced based on descriptions or references given in "Methods". The length and quality of discussion as well as the cited literature are appropriate.

However, I have several comments/questions.

Response: Thank you for nicely summarizing our work and providing your valuable comments. Our point-to-point responses to your comments are presented below.

1. What C57BL/6 mice were used in the study? Just to wright "C57BL/6 (B6)" is not sufficient. C57BL/6J (BL6J) and C57BL/6N (BL6N) inbred sub-strains are most widely used to understand the pathological roles of target molecules in a variety of diseases. In the last decade increasing amount of publications describe that C57BL/6J and C57BL/6N sub-strains differentially influence phenotype severity in different models. These differences were demonstrated for hepatic injury (Kawashita E, Ishihara K, Nomoto M, Taniguchi M, Akiba S. A comparative analysis of hepatic pathological phenotypes in C57BL/6J and C57BL/6N mouse strains in non-alcoholic steatohepatitis models. Sci Rep. 2019 Jan 18;9(1):204. doi: 10.1038/s41598-018-36862-7. PMID: 30659241; PMCID: PMC6338790). Please give the sub-strain name correctly.

Response: Thank you for bringing up this important issue regarding mouse sub-strains and for sharing your insights on their impact on disease models. We have clarified in the revised manuscript that we used C57BL/6J mice (page 15, line 3).

2. The use of PAD4-/- mice should be briefly explained in results. Although protein-arginine deiminase type-4 (PAD4) is mentioned briefly in the introduction, it would be helpful to remind the reader that PAD4(-/-) neutrophils are unable to form NETs upon stimulation, and therefore PAD4-/- mice can be used as a "negative control" for NET-mediated experiments.

Response: Thank you for your valuable suggestion to clarify the rationale for using PAD4$^{-/-}$ mice. In the Result's section of the revised manuscript, we have incorporated the explanation of PAD4$^{-/-}$ mice as follows:

“PAD4$^{-/-}$ neutrophils are defective in forming NETs upon stimulation due to the inability of catalytic histone citrullination. Therefore, PAD4$^{-/-}$ mice can be used as a negative control for NET-mediated experiments" (page 5, line 7-9).

Minor comments:

1. Fig 1C: The Y-axis is unclear. What does "PMN (10^3)" mean? Does it represent the PMN concentration per ml? Fig1 H,I: it is not clear what concentration of NETs was used for experiments with different duration. Was it 1000ng/ml?

Response: We apologize for the ambiguous expressions in the graphs. Fig. 1C: "PMN (10^3)" means the total number of PMNs in the whole liver tissue. We have clarified in the graph that it is a number (no.) of PMNs. Fig. 1 H, I: 1000 ng/mL NETs were used for experiments with different durations, and we have clarified it in the figure legends for **Fig H, I** (page 28, line 9).