Abnormal neutrophil traps and impaired efferocytosis contribute to liver injury and sepsis severity after binge alcohol use

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Supporting Information

Supplementary Methods

Control acute sepsis model

To determine the kinetics of neutrophil infiltration following acute sepsis, mice received a single intraperitoneal (i.p.) injection of LPS (0.05 mg/kg body weight). Mice were sacrificed after 9, 12, 15 and 20 h following LPS injection.

Peritoneal neutrophil isolations from mice

Peritoneal neutrophils were isolated from wild-type mice following aged thioglycollate injections as similarly described [19]. Briefly, 1 mL aged 4% thioglycollate was injected i.p. to mice. Three hours after thioglycollate injections, mice were anesthetized with isoflurane, the abdomen cleaned with 70% ethanol, and a ventral midline incision made using

scissors. The abdominal skin was retracted to expose the intact peritoneal wall. A sterile PBS solution (5 mL) was injected into the peritoneum. Mouse abdomens were gently massaged and, using the same needle, all peritoneal fluid was slowly withdrawn and transferred to a 50 mL conical polypropylene centrifuge tube. This peritoneal wash step was repeated three times with fresh PBS solution. Pooled peritoneal neutrophils were washed three times with PBS, and then counted and either left unstained or stained with carboxyfluorescein succinimidyl ester (CFSE) as described previously [20, 21]. To induce complete neutrophil cell death (complete NETosis), harvested thioglycollate mouse peritoneal neutrophils were frozen once on ice then rapidly thawed to 37ºC. This freeze/thaw procedure led to complete neutrophil cell death.

Kinetic assay

Mice serum ALTs were assessed using a kinetic assay with reagents from Teco Diagnostics (Anaheim, CA, USA) using previously described methods [1, 2].

Histopathology of mice and human liver samples

Liver specimens from mice and humans with ALD or healthy controls were stained for immunohistology with hematoxylin/eosin (H&E), neutrophil elastase (Abcam, ab68672, Cambridge, UK), citrullinated histone (Abcam, ab5103), or MPO (Lifespan Biosciences, cat # LS-B4741, Seattle, WA, USA), or used for TUNEL assay performed by the UMMS Diabetes and Endocrinology Research Center histology core facility.

Immune cell isolation from blood and liver samples

Total immune cells from mice were obtained by lysing all red blood cells (RBCs) using the RBC lysis buffer from Sigma (St. Louis, MO, USA). Following RBC lysis, samples were centrifuged, supernatant discarded and total immune cells were recovered and washed three times with PBS. For total immune cell isolation from the liver, harvested liver samples were digested *ex vivo* as previously described [6]. Following *ex vivo* digest, samples were centrifuged at 1200 rpm for 10 min and the supernatant, which should contain mostly immune cells, was transferred to a fresh tube. Harvested total liver immune cells were washed three times with PBS. All samples were analyzed by flow cytometry to determine the relative presence of indicated immune cell populations.

Cell lines

In vitro phagocytic, clearance, and MΦ differentiation experiments using RAW 264.7 cells were maintained in low glucose Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. RAW 264.7 cells were defined as acutely exposed to alcohol after 4 h in tissue culture medium supplemented with 50 mM ethanol.

ELISA of cell culture supernatants and liver tissue lysates

Serum or tissue TNF-α, monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) concentrations were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, 100 µL of 1/10 diluted culture supernatants or tissue lysate proteins were added into flat-bottom 96-well microtiter plates pre-coated overnight with the respective capture antibody. Capture antibody-coated plates containing unknown protein samples and standards were incubated for 2 h at room temperature. Plates were washed three times, followed by the addition of 100 µL/well of respective secondary horseradish peroxidase-conjugated antibodies. Following further 1 h incubation at room temperature, plates were washed three times with wash buffer. Then 100 µL substrate solution was added, and the plates were incubated for 30 minutes in the dark. The reaction was terminated with acid stop solution (50 µL). Optical densities of plates were read at 450 nm in a microplate reader.

Western blotting of liver tissue lysates

Liver tissue samples were minced and rinsed in ice-cold PBS followed by homogenizing liver tissue in RIPA buffer supplemented with protease and phosphatase inhibitors. Samples were then centrifuged at 15000 rpm for 10 min at 4^{o}C to remove tissue debris. Supernatants containing total tissue proteins were then transferred to fresh tubes and the pellet discarded. Total tissue protein concentrations were determined using the BioRad protein assay. Total liver tissue protein lysate (50 µg) was used for western blotting as previously described [1, 2, 6]. Citrullinated histone, HMGB1, and β-Actin were analyzed.

Real-time quantitative PCR analysis for liver tissue mRNA expression

Total liver tissue RNA was extracted using the RNeasy kit (Qiagen) with on-column DNAse treatment according to the manufacturer’s specifications similar to recently described methods [1, 2, 7]. Total RNA (1 µg) was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s

specifications. Quantitative real-time PCR using SYBR Green reagent (Bio-Rad) was performed on the CFX96 Touch Real-Time PCR detection system (Bio-Rad). The ΔΔCt method compared to baseline control was used to determine differential changes in gene expression following experimental treatments. 18S ribosomal RNA was used as an internal control as previously described [1, 2].

Blood alcohol and endotoxin assay.

Blood alcohol for study participants was determined using the Analox Alcohol Analyzer similar to previous description [8]. Serum endotoxins for all study subjects were assayed using and Endotoxin kit (Cape Cod Inc., East Falmouth, MA, USA) following the manufacturer's specifications.

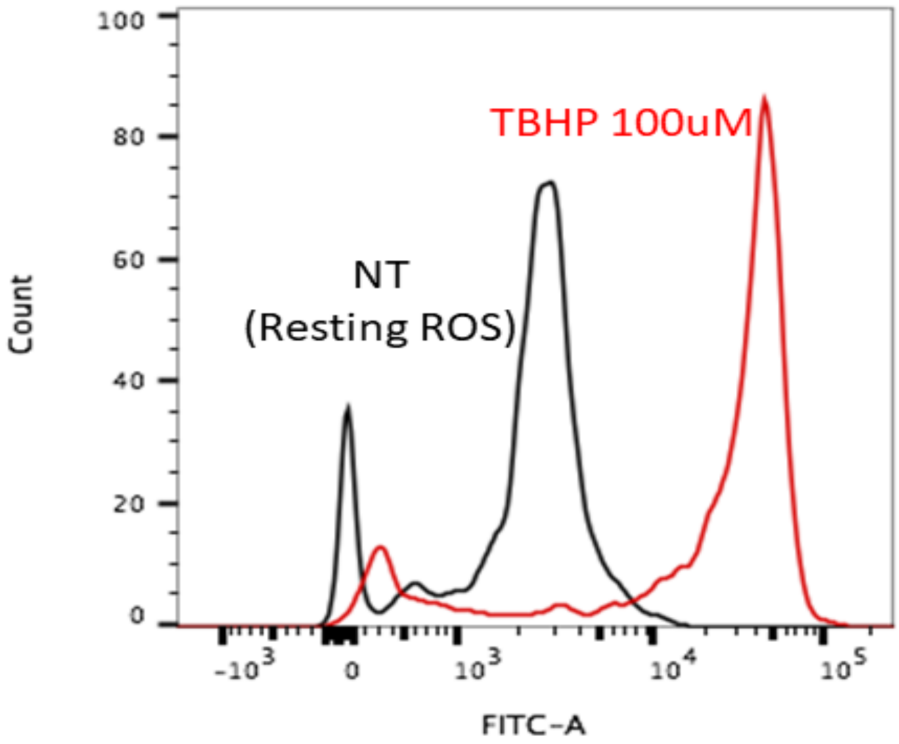
Flow Cytometry

Following experimental treatment, RAW 264.7 cells were re-suspended in approximately 10^6 cells per 50μl in FACS staining buffer containing anti-mouse BD Fc Block from BD Pharmingen (San Jose, CA, USA) to block nonspecific binding to Fcγ receptors and incubated for 15 min at 4°C. Then we added F4/80, CD11b, CD80, and CD206 antibodies (eBioscience, San Diego, CA, USA) for surface staining and incubated for 30 min in the dark at 4°C. For the negative control, the cells were stained with isotype-matched control antibody (eBioscience, San Diego, CA). Cells were washed with FACS buffer and acquired on a BD-LSR II (BD Biosciences, San Diego, CA). Data obtained were acquired at the same time and analyzed using FlowJo software.

Supplementary Figures and Legends:

Supporting Figure 1

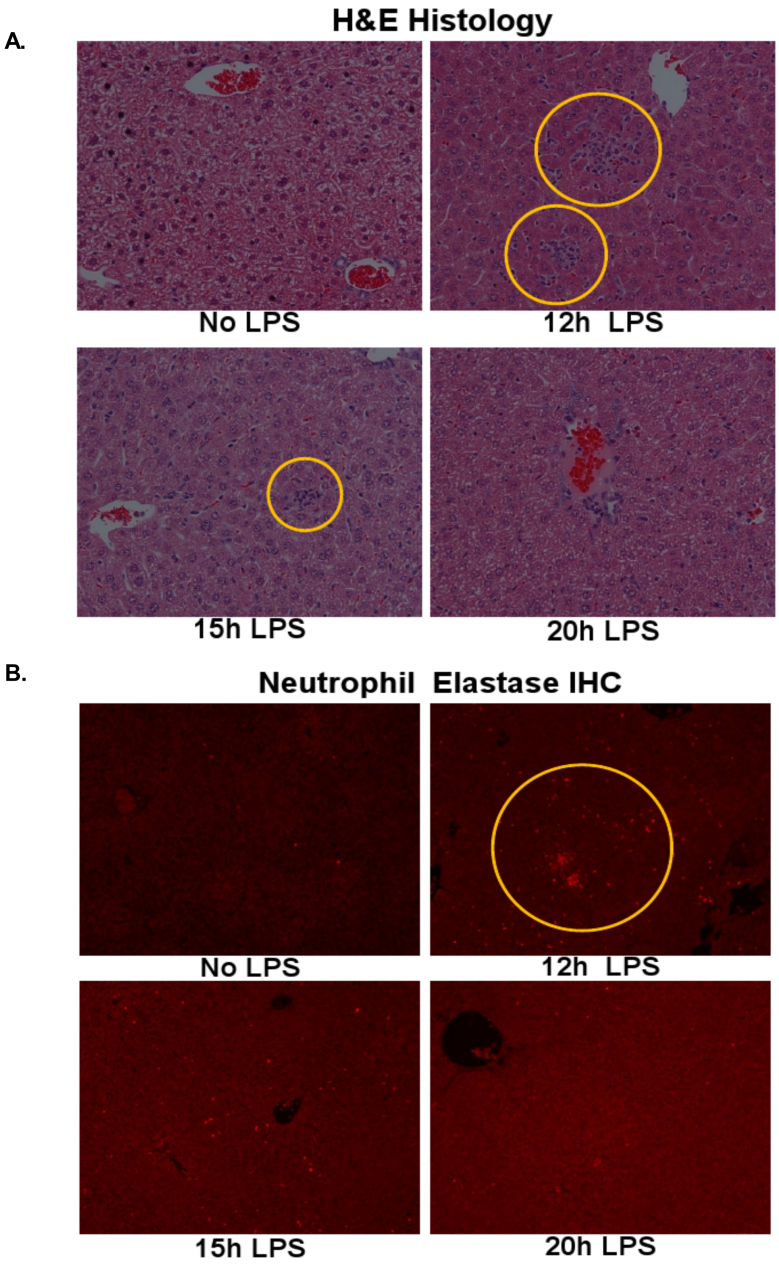
A.



Sup. Fig. 1: Isolation of blood neutrophils is not associated with cellular activation

Human blood neutrophils were isolated using modified ficoll separation methods and assessed for cellular activation by flow cytometry.

Supporting Figure 2

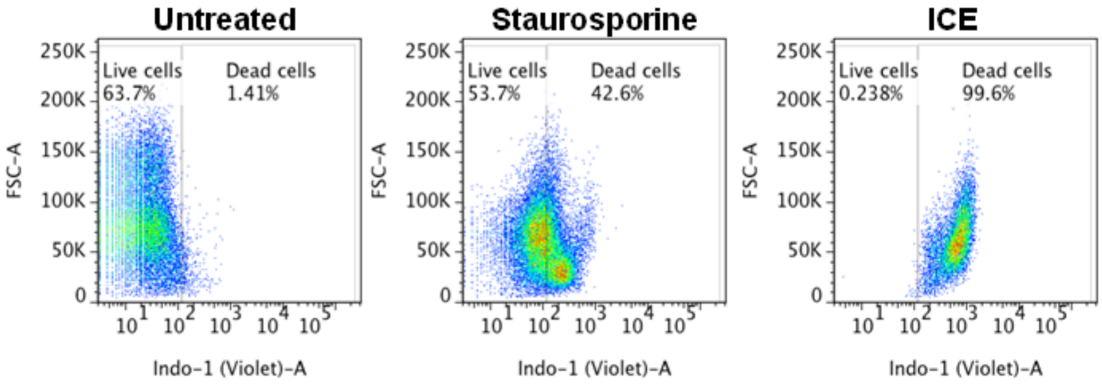


Sup. Fig. 2: Hepatic neutrophil infiltration kinetics associated with acute sepsis in mice.

C57BL/6 mice received sugar or ethanol gavage daily for three days followed by a single i.p. LPS injection (0.05mg/kg). Mice were sacrificed 12, 15, and 20 h after LPS injection and liver samples analyzed by H&E histology (A) and immunohistology for neutrophil elastase (B). Samples are representative of 5 mice per experimental group.

Supporting Figure 3

A.



Sup. Fig. 3: Neutrophil viability analysis by flow cytometry
Peritoneal mouse neutrophils were isolated from mice as detailed in the methods, then maintained at 37oC, treated with Staurosporine (20nM) or frozen on ice then rapidly brought to 37oC. Cells were then analyzed by flow cytometry for viability.

References

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