JCI The Journal of Clinical Investigation

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J Clin Invest. 2024. https://doi.org/10.1172/JCI178616.

Research Letter In-Press Preview Hepatology

IL-8^{+} neutrophils drive inexorable inflammation in severe alcohol-associated hepatitis

Yukun Guan[1*], Brandon Peiffer[2*], Dechun Feng[1*], Maria A. Parra[2], Yang Wang[1], Yaojie Fu[1], Vijay H. Shah[3], Andrew M. Cameron[2], Zhaoli Sun[2], Bin Gao[1]

[1]Laboratory of Liver Diseases, National Institute on Alcohol Abuse and Alcoholism, NIH, Bethesda, MD, USA; [2]Department of Surgery, Johns Hopkins University, Baltimore, MD, USA; [3]Department of Medicine, Mayo Clinic, Rochester, MN, USA

*****YG, BP, and DF contributed equally to this work.

Addresses corresponding to: Bin Gao, NIAAA, NIH, 5625 Fishers Lane, Bethesda, MD. Email: bgao@mail.nih.gov; or Zhaoli Sun, 700 Rutland Ave, Johns Hopkins, Baltimore, MD. Email: zsun2@jh.edu

Conflict of interest: The authors have declared that no conflict of interest exists.

To the Editor: Alcohol-associated liver disease (ALD), one of the major chronic liver diseases worldwide, includes a spectrum of liver disorders from simple steatosis to steatohepatitis and cirrhosis. Patients with chronic ALD may develop alcohol-associated hepatitis (AH) with major clinical signs such as jaundice (1). Severe AH (sAH), especially those with alcohol-associated cirrhosis (AC), have high short-term mortality and lack effective pharmacological therapies (1). To explore the mechanisms underlying the transition from AC to sAH, we performed single cell RNA (scRNA) sequencing analysis of livers and peripheral white blood cells (WBC) from sAH and AC patients. We also included the published scRNA data of 5 healthy donor livers and 2 AC patient livers from the GEO database (2) in our analysis. We identified 15 clusters of cell populations using 30 principal components under the resolution of 0.1, with most of the cells being immune cells (**Fig. 1A, Supplemental Fig. 1A-D**). Intriguingly, we found that the major difference between sAH and AC was that sAH livers had a markedly higher number of neutrophils (Cluster 0) than AC livers, while the differences in other subsets of immune cells between AC and sAH were less evident (**Fig. 1A)**, suggesting that hepatic neutrophil infiltration is an important factor that promotes AC to sAH.

Next, we further focused on the neutrophil cluster. We found two distinct liver sAH-specific neutrophil clusters 2 and 4 and one circulating sAH-specific neutrophil cluster 3 (**Fig. 1B, Supplemental Fig. 1C**). The sAH-specific liver neutrophil clusters 2 and 4 exhibit unique gene profiles notably characterized by heightened expression of *CXCL8*, a gene encoding IL-8, defined as IL-8$^+$ neutrophils (**Fig. 1C, Supplemental Fig. 1C**). The gene expression pattern including transcription factor-related genes in the cluster 2 and 4 is different from that in other clusters (**Supplemental Fig. 2A**). Pathway analysis revealed that top three differentially expressed hallmark pathways in liver IL-8$^+$ neutrophils are related to TNF-α, IFN-γ, and inflammatory response compared to those in circulating neutrophils with upregulation of TNF-α and inflammatory genes but downregulation of IFN-related genes (**Supplemental Fig. 2B-C**). We then conducted immunostaining to validate sAH-specific liver IL-8$^+$ neutrophils identified by scRNA seq. **Fig. 1D** shows that sAH livers exhibited a much greater number of neutrophils, with most of these neutrophils displaying higher levels of IL-8 expression than those in AC livers. Further staining of IL-8 in isolated liver nonparenchymal cells (NPCs) and WBCs showed that sAH WBCs had ~20% IL-8$^+$ cells

but IL-8$^+$ cells were barely detected in AC WBCs, and sAH liver NPCs had ~70% IL-8$^+$ cells, while only ~5% IL-8$^+$ cells were detected in AC NPCs (**Supplemental Fig. 3A**). As IL-8 is a key chemokine to activate neutrophils (3), accumulation of IL-8$^+$ neutrophils in sAH likely contributes to self-sustained neutrophil activation and liver inflammation in these patients.

To understand the mechanisms underlying upregulation of IL-8 in infiltrating neutrophils in sAH, we analyzed the genes (such as IL-1β, TNF-α) and transcriptional factors that are known to upregulate IL-8 gene (3), in neutrophils and liver tissues. Our scRNA sequencing data revealed that neutrophil populations expressed high levels of *IL-1R* and *TNFR* genes than other cell populations (**Supplemental Fig. 3B**), specifically IL-8$^+$ neutrophil cluster 2 and 4 highly express *TNFRSF1A/B* and *IL1R1/2,* respectively (**Fig. 1E**). The transcriptional factors (REL and FOS) that are known to upregulate IL-8 gene expression (3) were detected at higher levels in IL-8$^+$ clusters 2 and 4 than other clusters (**Fig. 1E**). Moreover, our bulk RNA sequencing data in **Supplemental Fig. 3C** revealed marked upregulation of genes related to the IL-1 and TNF families, and their associated receptors, in sAH when compared to healthy donor and AC livers. Finally, activated/phosphorylated p38MAPK, an important signaling pathway that activates IL-8 (3), was detected in sAH liver neutrophils (**Fig. 1F and Supplemental Fig. 3D**).

To understand the mechanism underlying neutrophil recruitment to the sAH but not AC livers, we measured the mRNA and protein levels of neutrophil chemokines in the liver. We found that most of these chemokines were higher in sAH livers compared to those in healthy and AC livers with the highest fold elevation of IL-8 in sAH patients (**Supplemental Fig. 4, A and B**). We then explored the cell types that express these chemokines by analyzing our scRNA sequencing data. We observed *CXCL8* in neutrophils, *CXCL6* in hepatocytes, and *CXCL5* in hepatic stellate cells, while *CXCL1* was detected in both hepatocytes and neutrophils (**Fig. 1G**). Such expression patterns were further confirmed by multiplex staining (**Supplemental Fig. 4C**).

In conclusion, although the elevation of IL-8 and neutrophils in AH has been known for many years (1), their roles in AH pathogenesis have yet to receive enough attention and targeting neutrophils has not been tested clinically in sAH. Our current study has

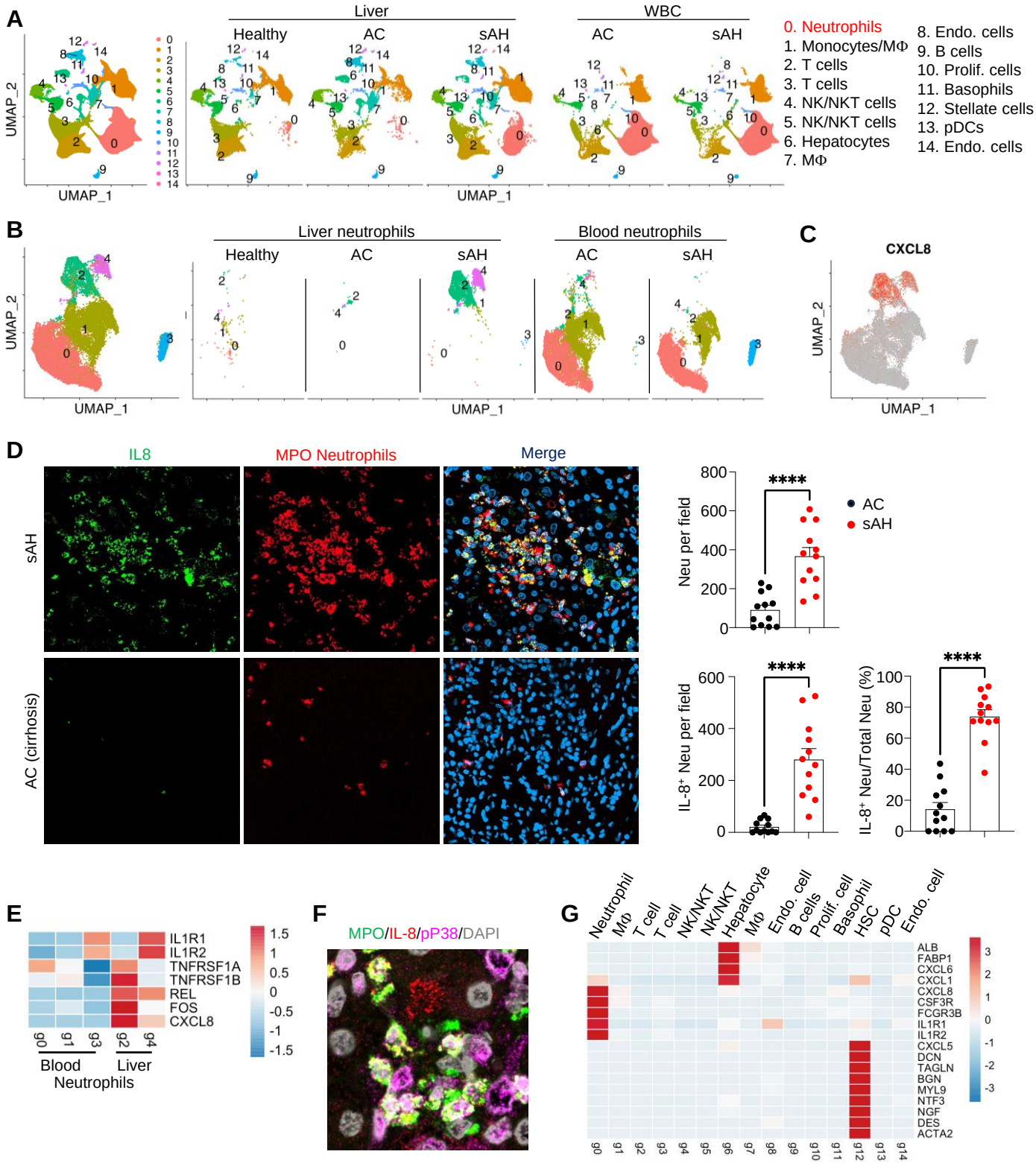
demonstrated that sAH had self-sustaining IL-8^{+} neutrophil accumulation that likely drives inexorable liver inflammation and failure in sAH. Targeting IL-8^{+} neutrophils could be a promising therapeutic strategy for sAH by directly blocking IL-8 signaling (via anti-IL-8 antibodies or CXCR1/2 antagonists) or indirectly blocking the inflammatory signals that induce IL-8 (3).

References:

1. Bataller R, et al. Alcohol-Associated Hepatitis. *N Engl J Med.* 2022;387(26):2436-48.
2. Ramachandran P, et al. Resolving the fibrotic niche of human liver cirrhosis at single-cell level. *Nature.* 2019;575(7783):512-8.
3. Cambier S, et al. The chemokines CXCL8 and CXCL12: molecular and functional properties, role in disease and efforts towards pharmacological intervention. *Cell Mol Immunol.* 2023;20(3):217-51.

Fig. 1. sAH is associated with accumulation of self-sustaining IL-8$^+$ neutrophils

(A) scRNA sequencing data of liver cells from 5 healthy donors, 3 AC and 5 sAH; and peripheral white blood cells (WBC) from 3 AC and 4 sAH. In total, 87,105 cells were analyzed, integrated and clustered by Seurat. UMAP plots of each group are shown. **(B)** Neutrophils (CD16$^+$CD114$^+$, Cluster 0 in Fig. 1A) from all groups were re-clustered. UMAP plots of all the neutrophils from each group are shown. **(C)** Feature plots for the gene expression of *CXCL8* (a gene encoding IL-8 protein) among all groups of neutrophils. **(D)** sAH and AC liver tissues were stained with IL-8 and neutrophil marker MPO. Representative images are shown. The number and percentage of IL-8$^+$ neutrophils are presented as means ± SEM. ****P < 0.0001. **(E)** Heatmap of genes that related to IL-8 upregulation in neutrophils from scRNA seq data. **(F)** sAH liver tissues were stained with IL-8, phospho-p38 MAPK and neutrophil marker MPO. A representative merged image is shown, and the image with each individual antibody staining is shown in Supplemental Figure 3D. **(G)** Heatmap of neutrophil chemokine and cell maker genes from scRNA seq data.


A
Liver
Healthy
AC
sAH
WBC
AC
sAH
UMAP_1
UMAP_2
0. Neutrophils
1. Monocytes/MΦ
2. T cells
3. T cells
4. NK/NKT cells
5. NK/NKT cells
6. Hepatocytes
7. MΦ
8. Endo. cells
9. B cells
10. Prolif. cells
11. Basophils
12. Stellate cells
13. pDCs
14. Endo. cells
B
Liver neutrophils
Healthy
AC
sAH
Blood neutrophils
AC
sAH
C
CXCL8
D
IL8
MPO Neutrophils
Merge
sAH
AC (cirrhosis)
Neu per field
AC
sAH
****
IL-8⁺ Neu per field
IL-8⁺ Neu/Total Neu (%)
E
IL1R1
IL1R2
TNFRSF1A
TNFRSF1B
REL
FOS
CXCL8
Blood Neutrophils
Liver
F
MPO/IL-8/pP38/DAPI
G
Neutrophil
Mφ
T cell
T cell
NK/NKT
NK/NKT
Hepatocyte
Mφ
Endo. cell
B cells
Prolif. cell
Basophil
HSC
pDC
Endo. cell
ALB
FABP1
CXCL6
CXCL1
CXCL8
CSF3R
FCGR3B
IL1R1
IL1R2
CXCL5
DCN
TAGLN
BGN
MYL9
NTF3
NGF
DES
ACTA2