Hepatology Publish Ahead of Print
DOI:10.1097/HEP.0000000000001232

OPEN

CD44 IN MYELOID CELLS IS A MAJOR DRIVER OF LIVER INFLAMMATION AND INJURY IN ALCOHOL-RELATED LIVER DISEASE

Déborah ROUSSEAU[1]; Stéphanie BONNAFOUS[2]; Frédéric SOYSOUVANH[1]; Dorian SARRAIL[1]; Manon BOURINET[1], Axelle STRAZZULLA[1], Stéphanie PATOURAUX[2]; Arnaud BORDERIE[3]; Bastien DOLFI[4], Alexandre GALLERAND[4], Marwin A FARRUGIA[2]; Véronique ORIAN-ROUSSEAU[5]; Yingzheng XU[6], Jesse W. WILLIAMS[6]; Stoyan IVANOV[4]; Albert TRAN[2]; Rodolphe ANTY[2]; Carmelo LUCI[1]; Philippe GUAL[1$]

[1] Université Côte d'Azur, INSERM, U1065, C3M, Nice, France

[2] Université Côte d'Azur, CHU, INSERM, U1065, C3M, Nice, France

[3] Université Côte d'Azur, CHU, Department of Pathology, Nice, France

[4] Université Côte d'Azur, CNRS, LP2M, Nice, France

[5] Karlsruhe Institute of Technology (KIT), Institute of Biological and Chemical Systems – Functional Molecular Systems (IBCS-FMS), Karlsruhe, Germany

[6] Center for Immunology, Department of Integrative Biology & Physiology, University of Minnesota, USA

Keywords: Human; mouse; ALD; alcohol

Running title: CD44 on alcohol-related steatohepatitis.

$ Contact info: Philippe Gual, Ph.D., Inserm UMR1065/C3M, Bâtiment Universitaire ARCHIMED, Team "Chronic liver diseases associated with obesity and alcohol", 151 route Saint-Antoine de Ginestière, BP 2 3194, 06204 Nice Cedex 03, France, philippe.gual@inserm.fr.

Word count: 5992

Graphical Abstract

GA1

ABSTRACT

Background and Aims: Alcohol-related liver disease (ALD) is one of the leading causes of severe liver disease with limited pharmacological treatments for alcohol-related steatohepatitis (ASH). CD44, a glycoprotein mainly expressed in immune cells, has been implicated in multiple inflammatory diseases but has never been studied in the ALD context. We therefore studied its contribution to ASH development in mice and its expression in ALD patients

Approach and Results: Here, we report that liver CD44 expression is associated with liver injury and inflammation and its deficiency (*Cd44*$^{-/-}$) partially protected mice upon chronic plus binge ethanol feeding (CPB-EtOH). CD44 deletion in myeloid cells (*Cd44*$^{myel\text{-}KO}$) recapitulated the same protective effects associated with reduced inflammatory monocyte infiltration and neutrophil activation in the liver and diminished blood neutrophil-lymphocyte ratio (NLR). CD44-deficient neutrophils displayed reduced PMA-induced inflammatory mediator expression and increased phagocytosis of live bacteria. *Cd44*$^{myel\text{-}KO}$ mice were also protected against hepatic steatosis mediated by CPB-EtOH or chronic ethanol feeding, due in part to increased SIRT1 mediated fatty acid beta-oxidation. CD44 neutralization with antibodies strongly decreased liver injury and

inflammation (hepatic neutrophil frequency) and blood NLR upon CPB-EtOH. In samples from ALD patients, hepatic CD44 expression increased with ALD severity, correlated with hepatic TNFα and CD11b expression, and CD44-expressing neutrophils were enriched in alcohol-associated hepatitis. **Conclusions:** Human and experimental evidence supports CD44 as a marker of hepatic inflammation in ALD. In addition, CD44 modulates neutrophil mobilization and functions and its targeting partially prevents liver inflammation and injury in the context of acute-on-chronic alcohol drinking.

Impact and implications:

Hepatic inflammation is a key pathogenic factor to the progression of alcohol-related liver diseases, yet the molecular mechanisms on inflammation onset are not fully understood. Herein, we identified CD44, a cellular protein mainly expressed in immune cells, as a marker (mouse and human) and a key player in liver inflammation and injury in ALD by regulating neutrophil mobilization and functions. Its global targeting (deficiency or neutralization) and specific deficiency on myeloid cells improve liver inflammation and injury. Our findings not only provide mechanistic insights into ALD but also provide potential therapeutic targets.

Highlights

- CD44, a cellular protein mainly expressed in immune cells, is upregulated in human and mouse livers with ALD.
- Hepatic myeloid cells are the main contributors of CD44 expression in ALD liver.
- CD44 contributes to liver injury and inflammation associated with ASH by regulating neutrophil activity.
- Targeting of CD44 in myeloid cells prevents liver steatosis, inflammation and injury in preclinical models of ALD.
- Global CD44 neutralization corrects alcohol-related liver inflammation and injury in a preclinical study.

INTRODUCTION

Alcohol consumption is prevalent in US, with over 50% of adults over 18 years old being regular drinkers[1]. In Europe, alcohol consumption also remains high, with 10 litres of pure alcohol consumed per adult each year[2]. In addition, drinking habits have changed, particularly in terms of binge drinking and heavy drinking. A recent report (2019) by the WHO reveals that 30.4% of

people report having consumed more than 60 g of pure alcohol on a single occasion in the last 30 days[3]. Among the several complications associated with acute and chronic alcohol consumption, alcohol-related liver disease (ALD) is one of the most common chronic liver diseases worldwide and the most prevalent cause of advanced liver disease in Europe. ALD also constitutes the leading cause of death among adults with excessive alcohol consumption[4-5].

ALD encompasses a broad spectrum of disorders ranging from alcohol-associated fatty liver to steatohepatitis (ASH), characterized by steatosis, hepatocellular damage and lobular inflammation. ASH represents a progressive disease form that can evolve into fibrosis and cirrhosis. In addition, patients with underlying ASH or cirrhosis and recent excessive alcohol consumption can also develop a form of acute-on-chronic liver failure called alcohol-associated hepatitis (AH)[4-6]. Despite the constantly increasing burden of ALD, no targeted therapies are yet available (current therapies are abstinence, nutritional support, and corticosteroids for severe AH)[7].

ASH development is the consequence of aberrant activation of hepatic immune and parenchymal cells in response to inflammatory mediators from the liver, adipose tissue and gut (including PAMPs, DAMPs, metabolites). Hepatocytes and Kupffer cells concomitantly contribute to the massive recruitment and accumulation of bone marrow-derived monocytes and neutrophils into the liver, a hallmark of ASH. The expression of chemokines (CCL2…) and adhesion molecule (E-selectin…), known to promote monocyte and neutrophil tissue infiltration, is markedly upregulated and correlates with disease severity[5-6, 8]. An imbalance between hepatic anti- and pro-inflammatory resident or recruited macrophages is a key characteristic in ALD development[9]. Importantly, binge alcohol feeding markedly elevates hepatic and circulating neutrophils in chronically ethanol-fed mice and in heavy alcohol drinkers and this facilitates hepatic neutrophil infiltration and ALD progression[5, 10-11]. While chronic/excessive alcohol consumption disrupts bone remodeling, injures the hematopoietic tissue and increases the susceptibility to infections[12-13], ethanol could also modify the bone marrow myelopoiesis and subsequently the blood immune cell levels, even at the early stages of the disease.

While signaling through CD44 impacts inflammation, injury and fibrogenesis, its potential role in ALD pathogenesis has not yet been investigated. CD44 is a cell-surface glycoprotein involved in cell–cell interactions, cell-adhesion and migration and it is expressed on various cell types (mainly leukocytes). Hyaluronan (HA), osteopontin and E-selectin, which can all bind to CD44, are involved in ALD progression (inflammation, injury and fibrosis)[14-21]. In addition, we have previously reported that CD44 targeting (genetic and pharmacological) in a mouse model of metabolic steatohepatitis, strongly alleviates liver injury, inflammation and fibrosis by downregulating the recruitment of macrophages and neutrophils into the liver[22]. We also demonstrated that CD44 regulates proinflammatory macrophage polarization mediated by LPS and hepatic DAMPs, pathogenic factors share with ALD[22]. This led us to speculate that CD44 might also play a role in promoting the development of ALD. We report here that CD44 expression is upregulated in human and mouse livers during ALD and its systemic or myeloid cell-specific deletion prevented inflammation and liver damage in murine model of chronic plus binge ethanol feeding. These beneficial effects are associated with regulating the frequency of circulating immune cells and neutrophil function. In addition, CD44 blockage with a neutralizing antibody was associated with the amelioration of neutrophil mobilization (in blood and liver) and liver complications in acute-on-chronic alcohol-related liver injury

METHODS

Mice and study design. CD44-deficient mice (*Cd44*$^{-/-}$)(B6.Cg-Cd44$^{tm1Hbg/J}$) and LysM^{Cre} mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Cd44*$^{flox/flox}$ mice were kindly provided by Pr. V. Orian-Rousseau (Karlsruhe Institute, Germany)[23]. C57BL/6J/Rj wild-type, *Cd44*$^{-/-}$, *LysM*$^{cre/+}$ *Cd44*$^{flox/flox}$ and littermate control *LysM*$^{+/+}$ *Cd44*$^{flox/flox}$ mice were acclimated to our animal facilities under a 12/12h light/dark cycle at a temperature of 21±2 °C. After acclimation to a liquid diet, mice were subjected to one of two different ethanol feeding protocols (chronic and NIAAA models)[24]. **Acclimation protocol:** Female mice (24-30 weeks old for *Cd44*$^{-/-}$ and Wt mice and 12-17 weeks old for *LysM*$^{cre/+}$ *Cd44*$^{flox/flox}$ and *LysM*$^{+/+}$ *Cd44*$^{flox/flox}$ mice) were

initially fed the control Lieber-DeCarli diet (SSNIFF, Soest, Germany) ad libitum for 5 days to acclimatize them to a semi-liquid diet. **Chronic plus one binge ethanol feeding.** Then, mice were allowed free access to the ethanol Lieber-DeCarli diet (SSNIFF) containing 5-6% (vol/vol) ethanol for 10 days, and control-fed groups were pair-fed with an isocaloric control diet. On day 11, mice received a single dose of ethanol by gavage (5 g/kg body weight) or isocaloric dextrin-maltose in the early morning and sacrificed 9 hours later. **Chronic ethanol feeding.** After acclimation to a semi-liquid diet, mice were allowed free access to the ethanol Lieber-DeCarli diet (SSNIFF) containing 5% (vol/vol) ethanol for 4 weeks, and control-fed groups were pair-fed with an isocaloric control diet. **Anti-CD44 treatment**. After acclimation to a semi-liquid diet, WT C57BL/6 female mice (20 weeks of age) were allowed free access to the ethanol Lieber-DeCarli diet containing 5% (vol/vol) ethanol for 10 days. In the evening on day 10 and in the early morning on day 11 mice received one intra-peritoneal injection of either purified rat anti-mouse CD44 mAb (n=7; IM7, BD Pharmingen, catalog #553131; BD Biosciences) or purified rat IgG2b, isotype control (n=7; A95-1, BD Pharmingen, catalog #553986; BD Biosciences)(100 µg/mouse). Mice were then immediately gavaged with a single dose of ethanol (5 g/kg body weight) and 9 hours later the blood was collected and mice were immediately sacrificed, after which the liver was removed. One part of the liver was immediately frozen in liquid nitrogen and stored at -80°C until analysis. A second part was fixed in buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin-eosin. The remainder liver was used for a flow cytometry analysis. The guidelines of laboratory animal care were followed, and the local ethical committee approved the animal experiments (APAFIS#5100-2015121110477413v6; APAFIS#8495-2017010514042986v5).
For further details, please refer to the Supplementary material, Supplemental Digital Content 3, http://links.lww.com/HEP/J684 .

RESULTS

CD44 deficiency attenuated liver injury and inflammation induced by chronic plus binge ethanol feeding. We first evaluated the hepatic expression levels of *Cd44* in a dietary mouse model of acute-on-chronic liver injury, the NIAAA model[24]. This chronic plus one binge alcohol feeding mouse model specifically triggers high levels of alcohol in blood, liver injury, fatty liver and inflammation, which mimics acute-on-chronic alcoholic liver injury in patients. Upon chronic plus binge ethanol feeding, the mice displayed elevated hepatic *Cd44* expression **(Fig1A)** associated with liver injury (as evaluated by ALT activity)**(Fig1B)**, liver steatosis **(Figs1C-D)**, elevated hepatic triglyceride content **(Fig1E)** and inflammation (as evaluated by inflammatory marker expression)**(Fig1F)**. The role of CD44 in liver complications induced by the NIAAA model was then investigated using mice deficient for *Cd44*. The *Cd44*-/- mice displayed less liver damage **(Fig1B)** and hepatic inflammation **(Fig1F)** with a similar grade of hepatic steatosis **(Figs1C-D)** and hepatic triglyceride content **(Fig1E)** when compared with the livers of chronic plus acute ethanol-consuming Wt mice **(Figs1C-F)**. To gain insight into the impact of CD44 deficiency on liver complications, 3 livers of EtOH Wt and EtOH $Cd44^{-/-}$ mice were randomly selected for RNA sequencing. Following dimension reduction, the samples clustered according to mouse genotype **(FigS1, Supplemental Digital Content 1 http://links.lww.com/HEP/J682)**. From gene expression analysis, the *Cd44* deficiency had a strong influence on the expression of 244 transcripts with 80 up-regulated and 164 down-regulated compared to Wt liver **(Fig1G)**. Focusing on down regulated genes, the gene ontology analysis highlighted that the absence of CD44 mainly regulated responses related to neutrophil and innate immune cell functions including cytokine production and phagocytosis **(Fig1H-S1, Supplemental Digital Content 1 http://links.lww.com/HEP/J682).**

Myeloid cell specific CD44 deficiency strongly attenuated liver injury and steatosis induced by ethanol feeding. Since CD44 deficiency preferentially impacted innate immune and neutrophil responses, we then focused on the role of CD44 in myeloid cells. The $Cd44^{\text{myel-KO}}$ mice ($Cd44^{\text{flox/flox}}LysM^{\text{cre/+}}$ mice) displayed decreased CD44 expression in hepatic inflammatory

monocytes ($Ly6G^{-}Ly6C^{high}$ cells), macrophages ($F4/80^{+}CD11b+$ cells and $Ly6C^{-}$ $F4/80^{+}CD64^{+}Clec2^{+}CD11b^{+}Clec4F^{+}TIM4^{+}$ cells corresponding to Kupffer cells) and neutrophils ($Ly6G^{+}Ly6C^{int}$ cells) without disruption of CD44 expression in T cells ($CD3^{+}NK1.1^{-}$ cells) as evaluated by flow cytometry analysis **(FigS2, Supplemental Digital Content 1 http://links.lww.com/HEP/J682).** In addition, the CD44 deficiency in myeloid cells strongly decreases the total CD44 hepatic expression in the steady state and in response to EtOH **(Figs2A-B)**, suggesting that myeloid cells are the main hepatic populations expressing CD44. This marked reduction in liver CD44 expression was associated with the prevention of hepatic steatosis **(Figs2C-D),** hepatic triglyceride content **(Fig2E)** and liver injury **(Fig2F)**.

This protective effect of CD44 deficiency in myeloid cells on liver steatosis was also associated with the prevention of increased p53/miR34a pathway that negatively regulated hepatic beta-oxidation via the SIRT1/AMPK-dependent regulation of PPAR-alpha and CPT1 **(Fig2G)**[25-30]. With the development of hepatic steatosis, the expression of p53 (assessed by *p21* expression) and miR-34a increased **(Figs2H-I)**, while miR-34a-dependent repression of the expression of *Naprt*, *Nampt* and *Sirt1* **(Fig2J)**, as well as the master regulators of beta-oxidation *Ppar-alpha* and *Cpt1* **(Fig2K)**, decreased. CD44 myeloid cell deficiency prevented this upregulation of the p53/miR-34a pathway **(Figs2H-I)**, resulting in a partial recovery of *Naprt*, *Nampt*, *Sirt1* **(Fig2J)**, as well as *Ppar-alpha* and *Cpt1* **(Fig2K)** expression.

To mimic the chronic drinking patterns in humans, $Cd44^{myel\text{-}KO}$ and $Cd44^{F/F}$ mice were also challenged with semi-liquid diet supplemented with ethanol for 4 weeks. CD44 deletion in myeloid cells mediated the same protective effects on liver steatosis, hepatic triglyceride content and injury and expression of *Sirt1* and beta-oxidation regulators *Ppar-alpha* and *Cpt1* **(FigS3, Supplemental Digital Content 1, http://links.lww.com/HEP/J682)**.

Myeloid cell specific CD44 deficiency strongly prevented liver inflammation induced by chronic plus binge ethanol feeding. The Gao-binge model also produces hepatic inflammation,

which is characterized by a higher frequency of inflammatory monocytes and neutrophils and elevated expression of inflammatory markers. We here reported that this challenge was also associated with increased CD44 expression in liver macrophages ($F4/80^{+}CD11b^{+}$) and neutrophils ($Ly6G^{+}Ly6C^{int}$)**(Fig3B)**. Whereas these responses are clearly marked in *Cd44*$^{F/F}$ mice upon chronic plus binge EtOH feeding, the *Cd44*$^{myel\text{-}KO}$ mice displayed a substantially lower hepatic frequency of inflammatory monocytes ($Ly6C^{high}Ly6G^{-}$), activated neutrophils ($Ly6G^{+}Ly6C^{int}CD62L^{low}$)**(Fig3A)**, and CD44+ and MPO+ cells **(Fig3C)**, as well as lower hepatic expression of tumor necrosis factor (*Tnf*), C-X-C motif chemokine ligand 2 (Cxcl2) and E-selectin (*Sele*)**(Fig3D)** and lower hepatic and blood level of chemokine ligand 2 (*Ccl2*)**(Figs3D-E)**. In addition to these markers, bulk RNA-seq analysis from 3 livers of EtOH *Cd44*$^{myel\text{-}KO}$ mice versus 3 livers of EtOH *Cd44*$^{F/F}$ mice, randomly selected and well clustered according to mouse genotype **(Fig3F)**, revealed that *Cd44* deficiency in myeloid cells positively regulated the expression of 645 genes and down-regulated 800 genes compared to EtOH *Cd44*$^{F/F}$ liver **(Fig3G)**. Focusing on the preventive effect, the pathway analysis revealed that the absence of CD44 in myeloid cells mainly decreased EtOH stimulated inflammatory pathways such as those related to TNF, inflammatory responses and interleukin **(Fig3H).**

Myeloid cell specific CD44 deficiency strongly prevented the elevated neutrophil/lymphocyte ratio in blood induced by the chronic plus binge ethanol feeding. Since increased neutrophil levels in peripheral blood and liver tissue is consistently reported in ALD patients[10], we then investigated the impact of acute-on-chronic EtOH feeding and myeloid cell CD44 deficiency on blood immune cell levels in mice. In response to chronic plus binge EtOH feeding, the number of white blood cells (WBC) decreased in both genotypes **(Fig4A).** Neutrophil counts were increased in control EtOH mice and this augmentation was only partial in *Cd44*$^{myel\text{-}KO}$ mice **(Figs4B-C)**. WBC decreases likely resulted from lower blood monocyte and lymphocyte numbers **(Figs4B-C)**. The CD44 deficiency in myeloid cells partially prevented these changes in blood immune cell frequency

without altering the WBC number **(Figs4A-C).** In line with this, the blood neutrophil-lymphocyte ratio (NLR) elevation in response to EtOH feeding is reduced in *Cd44*$^{myel\text{-}KO}$ mice **(Fig4D).** It is also of interest to highlight that CD44 expression in blood neutrophils is not increased in response to ethanol **(Fig4E)**, unlike in liver neutrophils **(Fig3B)**.

CD44 deficiency in neutrophils reduced their capacity to produce inflammatory mediators, while enhancing their phagocytosis potential. It has been clearly established that the infiltrating neutrophils are the main contributor to alcohol-induced liver damage. In ALD, neutrophils are strongly activated and are important sources of reactive oxygen species (ROS) and cytokines. Although ROS production is a powerful bactericidal process, excessive ROS formation causes adverse tissue damage. We then investigated the potential role of CD44 in the neutrophil production of ROS and inflammatory factors upon phorbol 12-myristate 13-acetate (PMA) activation. While CD44 deficiency in bone-marrow neutrophils did not alter the frequency of ROS+ neutrophils **(FigS4A)(FigsS5A-C, Supplemental Digital Content 1, http://links.lww.com/HEP/J682)** or ROS production per cell **(FigS5D, Supplemental Digital Content 1, http://links.lww.com/HEP/J682)**, it strongly decreased the PMA-mediated *Tnf* and *Cxcl1* expression **(Figs5A-B)**. The activation of neutrophils by PMA was also associated with the up-regulation of CD44 **(Fig5B)**. The antimicrobial activities of neutrophils also depend on their ability to phagocytose cellular debris and/or bacteria. Incubation of BM neutrophils with labelled live E-coli bacteria was associated with an increased frequency of CD11b^{high}Ly6G^{high} neutrophils and bacteria-loaded neutrophils after 1h at 37°C **(Figs5C-F)(FigS4B, Supplemental Digital Content 1, http://links.lww.com/HEP/J682)**. The CD44 silencing amplified these responses with a higher frequency of CD11b^{high}Ly6G^{high} neutrophils, bacteria-containing neutrophils and phagocytosis intensity **(Figs5C-G).** These results indicate that CD44 deficiency modified the properties of neutrophils with higher susceptibility to remove bacteria and lower pro-inflammatory phenotypes upon activation.

CD44 neutralization corrected liver inflammation and injury induced by the chronic plus binge EtOH feeding. Our data revealed that CD44 is a central player in liver EtOH-driven injury and inflammation. To explore this novel therapeutic avenue, we then investigated if CD44 neutralization by a specific antibody could correct the liver complications induced by chronic plus binge ethanol feeding. After 10 days of chronic EtOH feeding, mice received two intraperitoneal injections (one the evening on day 10 and one early the next morning prior to gavage) of either purified rat anti-CD44 mAb or isotype control before gavage with a single dose of EtOH. The treatment with anti-mouse CD44 mAb strongly decreased WBC **(Fig6A)**, normalized blood NLR **(Fig6B)** and strongly reduced liver injury as evaluated by the ALT activity **(Fig6C).** No improvement in hepatic steatosis **(Fig6D)**, liver triglyceride levels **(Fig6E)** and hepatic expression of *p21*, *Naprt*, *Nampt*, *Sirt1, Pparα* and *Cpt1* **(FigS7, Supplemental Digital Content 1, http://links.lww.com/HEP/J682)** was observed, likely due to our experimental protocol (mAb injections just before ethanol gavage). Anti-CD44 mAb treatment was also associated with a marked reduction in liver inflammation with less hepatic enrichment in neutrophils ($Ly6G^{+}Ly6C^{int}$) and a marked reduction in hepatic levels of inflammatory markers (*Tnf*, *Ccl2* and *Cxcl2*)**(Figs6F-G).** Therefore, anti-CD44 mAb treatment efficiently lower EtOH induced liver injury and inflammation.

Liver CD44 expression increased with local inflammation and injury in heavy alcohol drinkers. We then examined the relationship between hepatic CD44 expression and human ALD progression. In 19 consecutive heavy alcohol drinkers (16 men, 3 women, mean age: 44±7 years) admitted to our Liver unit, patients were classified into 2 groups: with mild ALD and with moderate ALD on the basis of the severity of hepatic steatosis and injury as assessed by histological analysis and serum ALT activity, respectively (**Figs7A-C, FigS8, Supplemental Digital Content 1, http://links.lww.com/HEP/J682 , TableS1, Supplemental Digital Content 2.**

http://links.lww.com/HEP/J683). Liver mRNA levels of *CD44* increased with ALD severity **(Fig7D)** and correlated with liver injury **(Fig7E)**. We also found that *CD44* expression strongly correlated with liver mRNA levels of *TNF* and myeloid cell maker *ITGAM (CD11b)*, markers of hepatic inflammation **(Fig7E)**. The CD44 expression at the protein level mainly occurred in inflammatory foci and neutrophils **(Fig7A)**. With the aggravation of ALD, WBC counts decreased **(Fig7F)** and negatively correlated with liver expression of *CD44* **(Fig7G)**. Consistent with our animal studies, the liver expression of *CD44* could be a local marker of hepatic inflammation in ALD patients.

Alcohol-associated hepatitis has been associated with CD44 upregulation in liver neutrophils. Finally, we examined the relationship between hepatic CD44 expression and human AH. *CD44* mRNA levels were first evaluated in the dataset of patients with AH (n=15) versus subjects without liver complications (n=5)(GEO database GSE28619). A shown in **Fig8A**, the hepatic expression of *CD44* was increased with AH. To then explore CD44 expression in immune cells in AH, we re-analyzed single cell RNA-Sequencing (scRNA-Seq) data of livers from 5 healthy donors, 5 alcohol-associated cirrhosis (AC) and 6 severe AH (GEO database GSE136103 and GSE255772). As previously reported[31], the main difference between AH and AC was that AH livers had significantly higher numbers of neutrophils (Cluster 6) than AC livers, while differences in other myeloid cells between AC and AH were less obvious **(Figs8B-C)**. While myeloid cells (DCs, monocytes, macrophages and neutrophils) express CD44, AH is associated with a marked increase in liver neutrophils with increased CD44 expression (**Figs8D-E**). These neutrophils, including those with high CD44 expression, also showed high expression of IL8 (CXCL8) and CXCR2 (**Fig8E**), as recently described[31]. In addition, although there was no obvious difference in the number of blood neutrophils between AH and AC patients, the number of blood neutrophils expressing CD44 was higher in AH patients than blood neutrophils in AC patients **(FigS9, Supplemental Digital**

Content, http://links.lww.com/HEP/J682). CD44 upregulation in liver neutrophils is associated with severe AH.

DISCUSSION

The elevated expression of CD44 in liver upon chronic plus binge ethanol feeding is closely related to the frequency and activation level of the myeloid cells in the liver. Liver infiltration of inflammatory monocytes and activation of liver neutrophils are reduced in mice with CD44 deficiency in myeloid cells. In addition, CD44 expression is augmented in liver macrophages and neutrophils. Deletion of CD44 in these cells greatly reduced its hepatic expression level in baseline conditions and blunted its upregulation following chronic plus binge EtOH consumption.

We then assessed the consequences of this poor hepatic expression of CD44 on the development of hepatic steatosis, which is almost universal at early stages of ALD[32]. We report here that the CD44 deficiency in myeloid cells reduces liver steatosis upon chronic alcohol drinking **(FigS3, Supplemental Digital Content 1, http://links.lww.com/HEP/J682)** and chronic plus binge EtOH feeding. This preventive response has been associated with the decreased expression of inflammatory mediators known to modify lipid metabolism in hepatocytes, such as TNF and CCL2[6] and decreased p53/miR34a pathway with the recovery of SIRT1 regulator, SIRT1, PPARα and CPT1 expression, the key regulators of hepatocyte metabolism including fatty acid uptake, beta-oxidation, and triglyceride turnover[33]. The pivotal role of p53 in ALD pathogenesis has been well established [34] as well as its ability to regulate miR34a transcription[28-29]. Inhibition of miR-34a also suppressed liver lipid accumulation and ameliorated hepatic steatosis in preclinical models through restoration of SIRT1 and PPAR-α expression, resulting in increased AMPK activation and PPAR-α target gene expression, respectively[25-27]. p53 inhibition also attenuates steatosis and liver injury in a mouse model of MASLD which were dependent on regulation of p53/miR34a/SIRT1/AMPK pathway[30]. Unexpectedly, systemic CD44 deficiency did not prevent hepatic steatosis or the regulation of *p21*, *naprt*, *nampt*, *sirt1*, *pparα* and *cpt1* **(FigS7, Supplemental Digital Content 1,**

http://links.lww.com/HEP/J682). This may be due to the regulation of additional liver cells by systemic CD44 deficiency counteracting this partial prevention of hepatic steatosis observed after myeloid cell specific CD44 deficiency. Although hypothetical, and as NK cells show a high level of CD44 expression (personal data), targeting CD44 in these cells could impair the protective role against alcohol-associated liver steatosis by hepatic NK cells[35].

In addition to regulating liver monocyte infiltration, we also report that CD44 deficiency alters the frequency of liver-activated neutrophils. Key neutrophil properties were altered as we observed reduced susceptibility to produce inflammatory mediators and higher anti-inflammatory phenotypes by phagocytosis of bacteria. We note that these responses in CD44-deficient neutrophils are strongly similar to the effects mediated by neutrophils during the resolution phase of liver disease. Emerging evidence shows the importance of neutrophils in the resolution of inflammation and injury. For example, neutrophils may phagocytose pathogens and cellular debris that would otherwise prolong inflammation, drive the conversion of pro-inflammatory macrophages to reparative macrophages in acute liver injury[10, 36] and provide matrix metalloproteinases to the liver which suppress fibrosis progression in a carbon tetrachloride–induced fibrosis mouse model[10, 37]. In addition, specialized pro-resolving lipid mediators, such as lipoxins, protectins, resolvins, and maresins, promote tissue repair by inhibiting neutrophil chemotaxis, suppressing cytokine production, and enhancing phagocytosis[10, 38]. Although it is difficult to assess the relative contribution of CD44-deficent neutrophils in preventing pro-inflammatory pathways or increasing those that resolve inflammation, we report here that CD44 is a novel regulator of neutrophil function and its deficiency dampens liver inflammation and injury.

CD44 regulation of resident and monocyte-derived macrophages may also contribute to ASH development. We here reported that chronic plus binge ethanol feeding was associated with increased CD44 expression in liver macrophages (F4/80+CD11b+), CD44 deficiency in myeloid cells decreased its expression in Kupffer cells **(FigS2, Supplemental Digital Content 1, http://links.lww.com/HEP/J682)** and reduced hepatic inflammatory monocyte frequency, liver

inflammation and injury in response to chronic plus binge ethanol feeding. The role of CD44 in liver recruitment of macrophages/monocytes has previously been reported in the context of MASH[22], sterile liver injury (mature F4/80^{hi} GATA6^{+} macrophages from the peritoneal cavity)[39] and hepatic fibrosis (recruited monocytes adhere to large intrahepatic vessels via CD44 and then form superclusters (KC-type syncytia) to eliminate blood-borne pathogens)[40]. Furthermore, we have previously reported that CD44 also regulates TLR4 ligand-mediated macrophage activation, including hepatic DAMPs, LPS and saturated fatty acids[22].

The chronic plus binge EtOH feeding is also associated with leukopenia resulting from decreased levels of lymphocytes and monocytes concomitant with the elevation of neutrophils. Leukopenia is common in heavy alcohol drinkers. In addition, chronic drinkers with recent excessive drinking could have higher levels of circulating neutrophils compared to healthy controls or chronic drinkers without recent excessive drinking[41]. Although the deficiency of CD44 in myeloid cells did not completely prevent the WBC number reduction, a partial correction in the frequency of these immune cells was observed. Similarly, the neutrophil-lymphocyte ratio (NLR) was lower in mice deficient for CD44 in the myeloid cells when they were exposed to EtOH. This may relevant because elevated NLR has been associated with higher susceptibility to infection and poor clinical outcome after steroid treatment in patients with alcohol-associated hepatitis[42]. The same partial correction of the NLR in CD44$^{myel\text{-}KO}$ mice is also achieved by the preventive injection of anti-CD44 antibodies prior to binge ethanol exposure. The molecular mechanisms explaining these changes in blood immune cell frequency require future study, but this beneficial effect on the NLR in the absence of CD44 is not the result of a different mobilization of neutrophils from the bone marrow into the bloodstream (data not shown). However, we note that maturation of bone marrow CD44-deficient neutrophils was already modified in the steady state, as assessed by Ly6G and CXCR2 expression levels (**FigS6, Supplemental Digital Content 1, http://links.lww.com/HEP/J682**)[43-44].

Consistent with our animal studies, the hepatic expression of CD44 was upregulated with AH and ALD in humans. In ALD, liver CD44 (mainly expressed in immune cells and neutrophils) also correlated with liver injury and hepatic expression of inflammatory markers. Interestingly, it has been reported that the CD44 elevation in ALD patients decreased after abstinence[45]. Among myeloid cells, AH is mainly associated with a significant enrichment of hepatic neutrophils and more particularly IL-8+ neutrophils as recently reported[31]. We here reported that AH-associated liver neutrophils also showed increased CD44 expression. While CD44 deficiency in myeloid cells decreased IL8-related markers in mice in response to chronic plus binge EtOH feeding **(FigS10, Supplemental Digital Content 1, http://links.lww.com/HEP/J682)**, the frequency of CD44+ neutrophils was about three times lower than those expressing IL-8 in human AH livers. Further studies are thus required to establish a direct link between CD44 and IL-8 in neutrophils in the context of liver pathologies.

Inflammation thus seems to be an important regulator of liver CD44 expression. Among CD44 ligands, HA is highly expressed in the liver sinusoids and binds CD44 on neutrophils, providing a potential mechanism of neutrophil recruitment. McDonald *et al.* demonstrated that the blocking of the HA-CD44 interaction by anti-CD44 antibodies ameliorated liver injury and neutrophil infiltration in a mouse model of LPS-induced liver injury[46]. While circulating levels of HA correlated with the severity of hepatic fibrosis in heavy alcohol drinkers[20] and its elevated expression in AH was rapidly reduced after cessation of alcohol consumption[47], the contribution of CD44-HA complex to the liver infiltration of neutrophils remains elusive in the context of ALD.

The hepatic inflammation development associated with alcohol consumption shares pathophysiological features with MASH. We here highlighted that CD44 is a key driver of liver inflammation and injury in an acute-on-chronic drinking pattern by regulating myeloid cell mobilization and/or function (monocytes and neutrophils). We have also previously reported that CD44 regulates the recruitment of monocytes and neutrophils into the liver in a context of MASH. In addition, CD44 regulates the macrophage activation mediated by TLR4 ligands including

DAMPs, LPS and saturated fatty acids [22]. However, in the context of binge drinking, CD44-dependent neutrophil responses appear to be particularly crucial in liver injury. The beneficial effects on liver complications obtained by neutralizing CD44 using a specific antibody prevented liver inflammation and injury associated with decreased neutrophil recruitment into the liver without affecting the liver monocyte/macrophage enrichment. In a similar vein, neutrophil depletion by injection of anti-Ly6G antibody reduced liver injury in response to chronic-plus binge ethanol feeding[8]. In line with common pathogenic factors, the hepatic expression levels of E-selectin, which is expressed by activated endothelial cells and interacts with CD44, were upregulated in human and mouse livers with MASH and mouse livers upon chronic plus binge ethanol feeding[8, 48-49]. The E-selectin deletion in mice reduced liver complications in both aetiologies by regulating neutrophil recruitments (preferentially into the liver in response to alcohol and more into adipose tissue with obesity)[8, 49]. Expression of E-selectin and inflammatory markers is reduced in ALD livers when CD44 is manipulated (systemic deletion or deletion in myeloid cells). Since obesity is an emerging factor that accelerates ALD progression[2], pathogenic factors associated with both aetiologies, such as CD44, could be very promising therapeutic targets.

Some limitations in our study should be noted. While the *in vitro* studies have enhanced our understanding of the role of CD44 in neutrophils, the neutrophil functions regulated by CD44 could mediate additional responses in the liver by directly acting with the other liver cells (endothelial cells, hepatocytes, NK cells and T cells for example). In addition, it remains difficult to investigate liver fibrosis associated with alcohol drinking in such preclinical models.

In summary, the harmful functions of CD44 in ALD occur at different stages of the disease (from steatosis to inflammation and liver injury) and in different liver cells (neutrophils and macrophages). With regard to the development of hepatic steatosis, CD44 in myeloid cells could reinforce the low-grade inflammation that impairs the beta-oxidation of fatty acids. Regarding liver inflammation associated with an acute-on-chronic drinking pattern, CD44 enhances the neutrophil mobilization in blood, liver monocyte recruitment and the pro-inflammatory responses of both

neutrophils and macrophages. This elevated local inflammation subsequently contributes to liver injury. The targeting of CD44 specifically in myeloid cells could represent a new therapeutic strategy to dampen liver inflammation and injury in chronic liver diseases (ALD and MASLD).

ACKNOWLEDGMENTS: Our thanks to all INSERM-U1065 facilities (for details, please refer to the Supplementary material, Supplemental Digital Content 3, http://links.lww.com/HEP/J684) and Catherine Buchanan from the Office of International Scientific Visibility for comments on the English version of the manuscript.

ABBREVIATIONS: alcohol-associated hepatitis (AH); alcohol associated steatohepatitis (ASH); alcohol-related liver disease (ALD); carnitine palmitoyltransferase 1 (CPT1); damage-associated molecular patterns (DAMPs); ethanol (EtOH); hyaluronan (HA); neutrophil-lymphocyte ratio (NLR); pathogen-associated molecular patterns (PAMPs); phorbol 12-myristate 13-acetate (PMA); peroxisome proliferator activated receptor alpha (PPARα), reactive oxygen species (ROS), white blood cells (WBC).

Funding: This work was supported by grants from INSERM (France), charities (AFEF to PG and CL; SNFGE-FARE to RA), the National Research Agency (#ANR-19-CE14-0044-01, #ANR-21-CE14-0015-03, #ANR-22-CE14-0027-01, #ANR-22-CE14-0027-02, #ANR-23-CE14-0048-03), the "Investments for the Future" LABEX SIGNALIFE (#ANR-11-LABX-0028-01), the UCA^{JEDI} Investments in the Future project (#ANR-15-IDEX-0001) and the Deutsche Forschungsgemeinschaft DFG OR124/21-1 to VOR. FS and AS were supported by the ANR (#ANR-19-CE14-0044-01 and #ANR-22-CE14-0027-01, respectively).

Disclosures: Véronique Orian-Rousseau consults for and owns stock in Amcure. The remaining authors have not conflicts to report.

Author contributions: PG, DR and CL designed the research and wrote the paper. VOR and SI provided tools as well as their expertise. DR, SB, FS, DS, MB, AS, SP, BD, AB and AG performed the experiments. AT, MAF and RA contributed to human sample and data collection. UX

and JWW contributed to analyze of sc-RNA-Seq from human data. All authors edited and approved the final submitted draft.

REFERENCES

Author names in bold designate shared co-first authorship

[1] White A, Castle IJ, Chen CM, Shirley M, Roach D, Hingson R. Converging Patterns of Alcohol Use and Related Outcomes Among Females and Males in the United States, 2002 to 2012. Alcohol Clin Exp Res 2015;39:1712-1726.

[2] Ntandja Wandji LC, Gnemmi V, Mathurin P, Louvet A. Combined alcoholic and non-alcoholic steatohepatitis. JHEP Rep 2020;2:100101.

[3] Pruckner N, Hinterbuchinger B, Fellinger M, Konig D, Waldhoer T, Lesch OM, et al. Alcohol-Related Mortality in the WHO European Region: Sex-Specific Trends and Predictions. Alcohol Alcohol 2019;54:593-598.

[4] Mathurin P, Bataller R. Trends in the management and burden of alcoholic liver disease. J Hepatol 2015;62:S38-46.

[5] Mackowiak B, Fu Y, Maccioni L, Gao B. Alcohol-associated liver disease. J Clin Invest 2024;134.

[6] Gao B, Ahmad MF, Nagy LE, Tsukamoto H. Inflammatory pathways in alcoholic steatohepatitis. J Hepatol 2019;70:249-259.

[7] Singal AK, Shah VH. Current trials and novel therapeutic targets for alcoholic hepatitis. J Hepatol 2019;70:305-313.

[8] Bertola A, Park O, Gao B. Chronic plus binge ethanol feeding synergistically induces neutrophil infiltration and liver injury in mice: a critical role for E-selectin. Hepatology 2013;58:1814-1823.

[9] Wan J, Benkdane M, Teixeira-Clerc F, Bonnafous S, Louvet A, Lafdil F, et al. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. Hepatology 2014;59:130-142.

[10] Cho Y, Szabo G. Two Faces of Neutrophils in Liver Disease Development and Progression. Hepatology 2021;74:503-512.

[11] Khan RS, Lalor PF, Thursz M, Newsome PN. The role of neutrophils in alcohol-related hepatitis. J Hepatol 2023;79:1037-1048.

[12] Chakkalakal DA. Alcohol-induced bone loss and deficient bone repair. Alcohol Clin Exp Res 2005;29:2077-2090.

[13] Shi X, DeLucia AL, Bao J, Zhang P. Alcohol abuse and disorder of granulopoiesis. Pharmacol Ther 2019;198:206-219.

[14] Kim MJ, Sim MO, Lee HI, Ham JR, Seo KI, Lee MK. Dietary umbelliferone attenuates alcohol-induced fatty liver via regulation of PPARalpha and SREBP-1c in rats. Alcohol 2014;48:707-715.

[15] Yang YM, Noureddin M, Liu C, Ohashi K, Kim SY, Ramnath D, et al. Hyaluronan synthase 2-mediated hyaluronan production mediates Notch1 activation and liver fibrosis. Sci Transl Med 2019;11:eaat9284.

[16] Morales-Ibanez O, Dominguez M, Ki SH, Marcos M, Chaves JF, Nguyen-Khac E, et al. Human and experimental evidence supporting a role for osteopontin in alcoholic hepatitis. Hepatology 2013;58:1742-1756.

[17] Nagoshi S. Osteopontin: Versatile modulator of liver diseases. Hepatol Res 2014;44:22-30.

[18] Rostami S, Parsian H. Hyaluronic Acid: from biochemical characteristics to its clinical translation in assessment of liver fibrosis. Hepat Mon 2013;13:e13787.

[19] Katayama Y, Hidalgo A, Chang J, Peired A, Frenette PS. CD44 is a physiological E-selectin ligand on neutrophils. J Exp Med 2005;201:1183-1189.

[20] **Lavallard VJ, Bonnafous S,** Patouraux S, Saint-Paul MC, Rousseau D, Anty R, et al. Serum markers of hepatocyte death and apoptosis are non invasive biomarkers of severe fibrosis in patients with alcoholic liver disease. PLoS One 2011;6:e17599.

[21] **Patouraux S, Bonnafous S,** Voican CS, Anty R, Saint-Paul MC, Rosenthal-Allieri MA, et al. The osteopontin level in liver, adipose tissue and serum is correlated with fibrosis in patients with alcoholic liver disease. PLoS One 2012;7:e35612.
[22] **Patouraux S, Rousseau D,** Bonnafous S, Lebeaupin C, Luci C, Canivet CM, et al. CD44 is a key player in non-alcoholic steatohepatitis. J Hepatol 2017;67:328-338.
[23] Shatirishvili M, Burk AS, Franz CM, Pace G, Kastilan T, Breuhahn K, et al. Epidermal-specific deletion of CD44 reveals a function in keratinocytes in response to mechanical stress. Cell Death Dis 2016;7:e2461.
[24] Bertola A, Mathews S, Ki SH, Wang H, Gao B. Mouse model of chronic and binge ethanol feeding (the NIAAA model). Nat Protoc 2013;8:627-637.
[25] Ding J, Li M, Wan X, Jin X, Chen S, Yu C, et al. Effect of miR-34a in regulating steatosis by targeting PPARalpha expression in nonalcoholic fatty liver disease. Sci Rep 2015;5:13729.
[26] Torres JL, Novo-Veleiro I, Manzanedo L, Alvela-Suarez L, Macias R, Laso FJ, et al. Role of microRNAs in alcohol-induced liver disorders and non-alcoholic fatty liver disease. World J Gastroenterol 2018;24:4104-4118.
[27] Wang X, He Y, Mackowiak B, Gao B. MicroRNAs as regulators, biomarkers and therapeutic targets in liver diseases. Gut 2021;70:784-795.
[28] Lee J, Padhye A, Sharma A, Song G, Miao J, Mo YY, et al. A pathway involving farnesoid X receptor and small heterodimer partner positively regulates hepatic sirtuin 1 levels via microRNA-34a inhibition. J Biol Chem 2010;285:12604-12611.
[29] Hermeking H. p53 enters the microRNA world. Cancer Cell 2007;12:414-418.
[30] Derdak Z, Villegas KA, Harb R, Wu AM, Sousa A, Wands JR. Inhibition of p53 attenuates steatosis and liver injury in a mouse model of non-alcoholic fatty liver disease. J Hepatol 2013;58:785-791.
[31] Guan Y, Peiffer B, Feng D, Parra MA, Wang Y, Fu Y, et al. IL-8+ neutrophils drive inexorable inflammation in severe alcohol-associated hepatitis. J Clin Invest 2024;134.
[32] Diaz LA, Arab JP, Louvet A, Bataller R, Arrese M. The intersection between alcohol-related liver disease and nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 2023;20:764-783.
[33] Luci C, Bourinet M, Leclere PS, Anty R, Gual P. Chronic Inflammation in Non-Alcoholic Steatohepatitis: Molecular Mechanisms and Therapeutic Strategies. Front Endocrinol (Lausanne) 2020;11:597648.
[34] Derdak Z, Lang CH, Villegas KA, Tong M, Mark NM, de la Monte SM, et al. Activation of p53 enhances apoptosis and insulin resistance in a rat model of alcoholic liver disease. J Hepatol 2011;54:164-172.
[35] Cheng C, Zhang Q, Li Y, Jiang J, Xie L, Shen H, et al. Interplay Between Liver Type 1 Innate Lymphoid Cells and NK Cells Drives the Development of Alcoholic Steatohepatitis. Cell Mol Gastroenterol Hepatol 2023;15:261-274.
[36] Yang W, Tao Y, Wu Y, Zhao X, Ye W, Zhao D, et al. Neutrophils promote the development of reparative macrophages mediated by ROS to orchestrate liver repair. Nat Commun 2019;10:1076.
[37] Saijou E, Enomoto Y, Matsuda M, Yuet-Yin Kok C, Akira S, Tanaka M, et al. Neutrophils alleviate fibrosis in the CCl(4)-induced mouse chronic liver injury model. Hepatol Commun 2018;2:703-717.
[38] Serhan CN, Takano T, Chiang N, Gronert K, Clish CB. Formation of endogenous "antiinflammatory" lipid mediators by transcellular biosynthesis. Lipoxins and aspirin-triggered lipoxins inhibit neutrophil recruitment and vascular permeability. Am J Respir Crit Care Med 2000;161:S95-S101.
[39] Wang J, Kubes P. A Reservoir of Mature Cavity Macrophages that Can Rapidly Invade Visceral Organs to Affect Tissue Repair. Cell 2016;165:668-678.
[40] Peiseler M, Araujo David B, Zindel J, Surewaard BGJ, Lee WY, Heymann F, et al. Kupffer cell-like syncytia replenish resident macrophage function in the fibrotic liver. Science 2023;381:eabq5202.

[41] Li M, He Y, Zhou Z, Ramirez T, Gao Y, Gao Y, et al. MicroRNA-223 ameliorates alcoholic liver injury by inhibiting the IL-6-p47(phox)-oxidative stress pathway in neutrophils. Gut 2017;66:705-715.
[42] Rachakonda V, Bataller R, Duarte-Rojo A. Recent advances in alcoholic hepatitis. F1000Res 2020;9.
[43] Deniset JF, Surewaard BG, Lee WY, Kubes P. Splenic Ly6G(high) mature and Ly6G(int) immature neutrophils contribute to eradication of S. pneumoniae. J Exp Med 2017;214:1333-1350.
[44] Lazennec G, Rajarathnam K, Richmond A. CXCR2 chemokine receptor - a master regulator in cancer and physiology. Trends Mol Med 2024;30:37-55.
[45] Urashima S, Tsutsumi M, Ozaki K, Tsuchishima M, Shimanaka K, Ueshima Y, et al. Immunohistochemical study of hyaluronate receptor (CD44) in alcoholic liver disease. Alcohol Clin Exp Res 2000;24:34S-38S.
[46] McDonald B, McAvoy EF, Lam F, Gill V, de la Motte C, Savani RC, et al. Interaction of CD44 and hyaluronan is the dominant mechanism for neutrophil sequestration in inflamed liver sinusoids. J Exp Med 2008;205:915-927.
[47] Hill DB, Deaciuc IV, McClain CJ. Hyperhyaluronanemia in alcoholic hepatitis is associated with increased levels of circulating soluble intercellular adhesion molecule-1. Alcohol Clin Exp Res 1998;22:1324-1327.
[48] Bertola A, Bonnafous S, Anty R, Patouraux S, Saint-Paul MC, Iannelli A, et al. Hepatic expression patterns of inflammatory and immune response genes associated with obesity and NASH in morbidly obese patients. PLoS One 2010;5:e13577.
[49] Rodrigues RM, He Y, Hwang S, Bertola A, Mackowiak B, Ahmed YA, et al. E-Selectin-Dependent Inflammation and Lipolysis in Adipose Tissue Exacerbate Steatosis-to-NASH Progression via S100A8/9. Cell Mol Gastroenterol Hepatol 2022;13:151-171.

Figure 1: CD44 deficiency strongly prevented liver injury and inflammation induced by chronic plus binge ethanol feeding. After acclimation to a semi-liquid diet, Wild-type (Wt) and *Cd44*$^{-/-}$ mice were allowed free access to the ethanol Lieber-DeCarli diet containing 5% (vol/vol) ethanol or were pair-fed with an isocaloric control diet for 10 days. On day 11, mice received a single dose of ethanol (5 g/kg body weight [b.w.])(EtOH) or isocaloric dextrin-maltose (Malto) by gavage and sacrificed 9 hours later (5-17 mice/group). **(A)** Hepatic *Cd44* expression was evaluated at the mRNA level. **(B)** The serum levels of ALT were evaluated. **(C-D)** H&E staining of liver tissue section samples from Wt and *Cd44*$^{-/-}$ after chronic plus binge (CPB) EtOH feeding as indicated. Representative images **(C)** and quantification of hepatic steatosis **(D)** are shown. **(E)** Liver triglyceride content. **(A, B, D, E)** Results are expressed as means ± SEM and statistically analyzed using a two-way ANOVA test. **(F)** Hepatic mRNA expression levels of *Cd44* and markers of inflammation were analyzed by real-time quantitative PCR (4-17 mice/group). Data are presented as relative mRNA levels normalized to *Rplp0* mRNA levels. Data are expressed as means in Heat Map and statistically analyzed by a two-way ANOVA test. *P<0.05, compared with Wt Malto mice, §P <0.05, compared with Wt EtOH mice. **(G-H)** 3 livers of EtOH Wt and EtOH *Cd44*$^{-/-}$ mice were randomly selected for RNA sequencing. **(G)** Gene expression analysis. **(H)** Gene ontology analysis on down regulated genes.

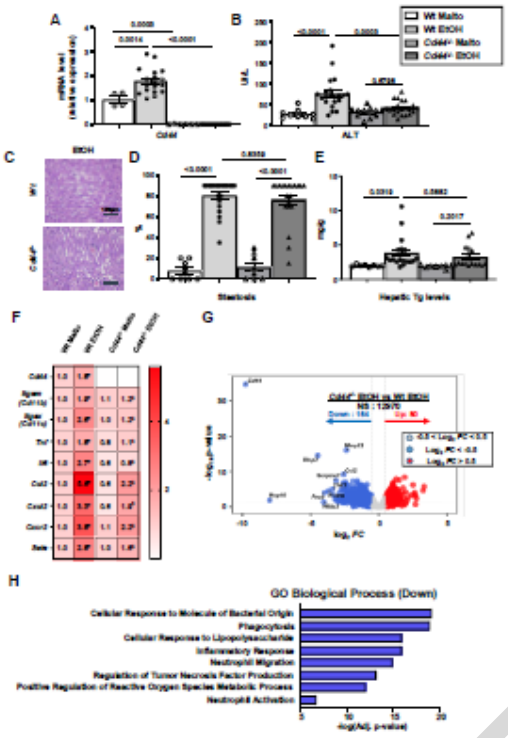

Figure 2: The CD44 deficiency in myeloid cells prevented hepatic expression of CD44, liver injury and hepatic steatosis induced by chronic plus binge ethanol feeding. (A-K) After acclimation to a semi-liquid diet, *Cd44*$^{myel\text{-}KO}$ and littermate control *Cd44*$^{F/F}$ mice were allowed free access to the ethanol Lieber-DeCarli diet containing 6% (vol/vol) ethanol or were pair-fed with an isocaloric control diet (Malto) for 10 days. On day 11, mice received a single dose of ethanol (5 g/kg body weight [b.w.])(EtOH) or isocaloric dextrin-maltose (Malto) by gavage and sacrificed 9 hours later (4-16 mice/group). **(A-B)** Hepatic *Cd44* expression was evaluated at the mRNA (9-16 mice/group)**(A)** and protein level (3-4 mice/group)**(B)**. **(C-D)** H&E staining of liver tissue section samples from *Cd44*$^{myel\text{-}KO}$ and *Cd44*$^{F/F}$ mice after CPB. Representative images **(C)** and quantification of hepatic steatosis **(D)** are shown. Liver triglyceride content (6-9 mice/group)**(E)** and serum levels of ALT **(F)** were evaluated. **(G)** Schematic diagram of the miR34a-SIRT1-AMPK pathway. **(H, J-K)** Hepatic mRNA expression levels of *p21*, miR-34a-dependent targets (*Naprt*, *Nampt* and *Sirt1)*, *Ppara and Cpt1* were analyzed by real-time quantitative PCR. Data are presented as relative mRNA levels normalized to *Rplp0* mRNA levels (4-7 mice/group). **(I)** Hepatic expression levels of miR-34a-5p were analyzed by real-time quantitative PCR. Data are presented as relative levels normalized to miR-30E-5p expression levels (4-7 mice/group). **(A, D-F, H-K)** Results are expressed as means ± SEM and statistically analyzed using a two-way ANOVA test.

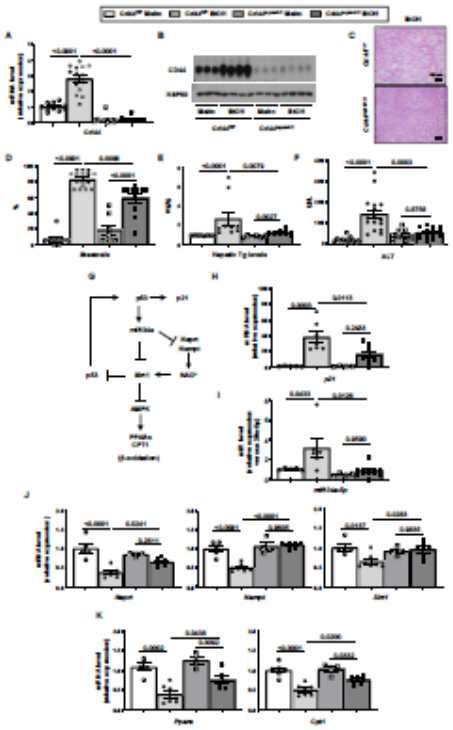

Figure 3: The CD44 deficiency in myeloid cells strongly decreased hepatic frequency of inflammatory monocytes and activated neutrophils as well as liver inflammation induced by chronic plus binge ethanol feeding. After acclimation to a semi-liquid diet, *Cd44*$^{myel\text{-}KO}$ and littermate control *Cd44*$^{F/F}$ mice were subjected to CPB EtOH or Malto feeding (11-18 mice/group). **(A-B)** Hepatic non parenchymal cells were stained for CD45, Ly6G, Ly6C, CD62L and CD44 and analyzed by flow cytometry (5-6 mice/group). **(A)** Frequency of inflammatory monocytes (Ly6C^{high} Ly6G^{-} cells), neutrophils (Ly6G^{+} Ly6C^{int} cells) and neutrophil activation levels (Ly6G^{+} Ly6C^{int} CD62L^{Low} cells) and **(B)** MFI CD44 levels in macrophages (F4/80^{+} CD11b^{+}) and neutrophils (Ly6G^{+} Ly6C^{int} cells) were assessed by flow cytometric analysis. Results are expressed as means ± SEM and statistically analyzed using a two-way ANOVA test. **(C)** αCD44 and αMPO staining of liver samples from *Cd44*$^{F/F}$ and *Cd44*$^{myel\text{-}KO}$ mice subjected to CPB EtOH. Representative fluorescent images were acquired using Nikon Confocal A1R at 40X magnification. Quantification of CD44/MPO fluorescence area (two fields per mouse and three different mice per condition) are expressed as mean ± SEM and statistically analyzed using Mann-Whitney U test. **(D)** Hepatic mRNA expression levels of *Cd44* and markers of inflammation were analyzed by real-time quantitative PCR (4-15 mice/group). Data are presented as relative mRNA levels normalized to *Rplp0* mRNA levels. Data in Heat Map are expressed as means and statistically analyzed by a two-way ANOVA test. *P<0.05, compared with *Cd44*$^{F/F}$ Malto mice, §P <0.05, compared with *Cd44*$^{F/F}$ EtOH mice. **(E)** The serum levels of CCL2 were evaluated (7-14 mice/group). Results are expressed as means ± SEM and statistically analyzed using a two-way ANOVA test. **(F-H)** 3 livers of EtOH *Cd44*$^{myel\text{-}KO}$ and EtOH *Cd44*$^{F/F}$ mice were randomly selected for RNA sequencing. **(F)** Dimension reduction method (principal component analysis). **(G)** Gene expression analysis. **(H)** Pathway analysis on down regulated genes.

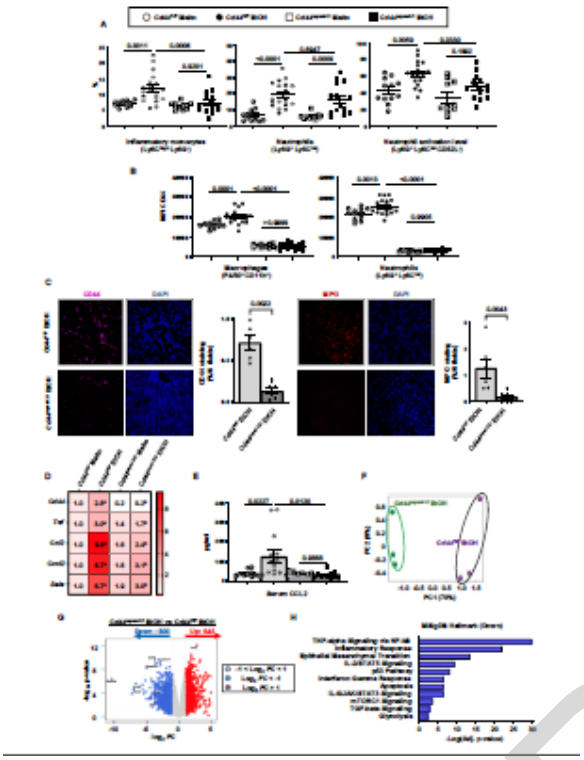

Figure 4: CD44 deficiency in myeloid cells altered the proportions of circulating immune cells and partially corrected the elevation of the neutrophil/lymphocyte ratio induced by chronic plus binge ethanol feeding. After acclimation to a semi-liquid diet, *Cd44*$^{myel\text{-}KO}$ and littermate control *Cd44*$^{F/F}$ mice were subjected to CPB EtOH or Malto feeding (9-15 mice/group). The number of white blood cells (WBC)**(A)**, neutrophils, monocytes and lymphocytes **(B)** were evaluated by blood haematology analyzer. The results were also expressed as % of blood cells **(C)** and neutrophil to lymphocyte ratio (NLR)**(D). (E)** Blood cells were stained for CD45, Ly6G, Ly6C, and CD44 and analyzed by flow cytometry (9-15 mice/group). MFI CD44 levels in neutrophils (Ly6G^{+} Ly6C^{int} cells) were evaluated. **(A-E)** Results are expressed as means ± SEM and statistically analyzed using a two-way ANOVA test.

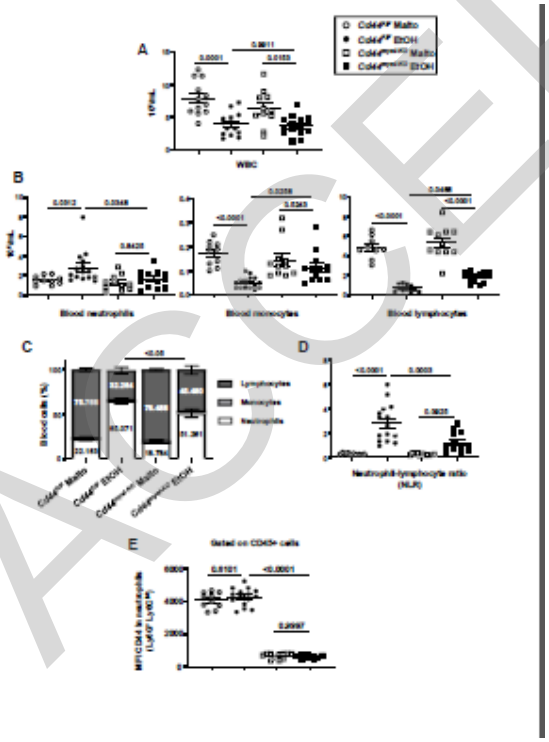

Figure 5: CD44 deficiency in neutrophils reduced their capacity to produce inflammatory mediators, while enhancing their phagocytosis potential. (A-B) Purified bone marrow neutrophils from Wt and *Cd44*^{KO} mice were stimulated with PMA (10nM) for 4h at 37°C before analyzing *Cd44* and inflammatory mediators by RT-qPCR. **(B)** Data are presented as relative mRNA levels normalized to *Rplp0* mRNA levels. Data from three independent experiments are expressed as means ± SEM and statistically analyzed using a Student's t-test. **(C-G)** Bone marrow purified neutrophils (500,000 cells/ml) were incubated or not with 100µg/ml of inactivated fluorescent E. coli bacteria for 1 hour either at 4°C or 37°C. After the quenching of the fluorescence of the bacteria on the cell surface and blocking FcgRII/III, cells were stained for Ly6G and CD11b and analyzed by flow cytometry upon excitation at 488nm (three independent experiments). **(D-E)** Frequency of CD11b^{high} Ly6G^{high} and CD11b^{meduim} Ly6G^{meduim} cells: **(D)** one out of three independent experiments is shown, **(E)** % of three independent experiments. **(F)** Frequency of neutrophils with bacteria (% versus Wt neutrophils at 37°C). **(G)** Phagocytosis intensity per cell (% Wt neutrophils at 37°C). **(E-G)** Results are expressed as means ± SEM and statistically analyzed using a Student's t-test.

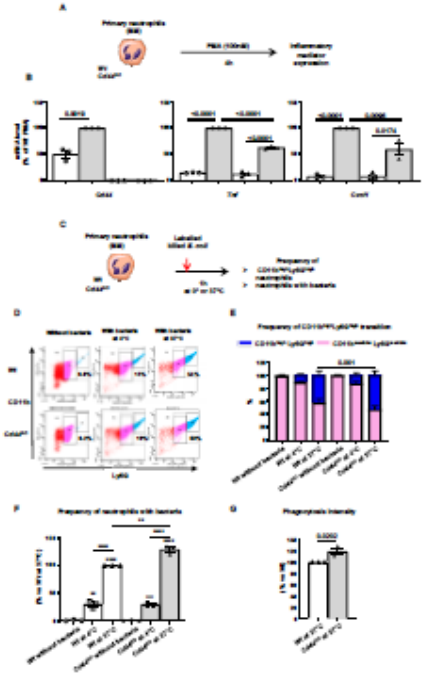

Figure 6: Neutralization of CD44 corrected the liver inflammation and injury induced by the chronic plus binge EtOH feeding. After acclimation to a semi-liquid diet, WT C57BL/6 female mice (20 weeks of age) were allowed free access to the ethanol Lieber-DeCarli diet containing 5% (vol/vol) ethanol for 10 days. In the evening on day 10 and in the early morning on day 11 mice received one intra-peritoneal injection of either purified rat anti-mouse CD44 mAb (n = 7; IM7) or purified rat IgG2b, isotype control (n = 7; A95-1)(100 µg/mouse). Mice were then immediately gavage-fed with a single dose of ethanol (5 g/kg body weight [b.w.]) and 9 hours later the blood was collected and mice were sacrificed. In blood, white blood cell (WBC) number **(A)**, neutrophil to lymphocyte ratio (NLR)**(B)** and serum ALT activity **(C)** were evaluated. In liver, H&E staining of sections samples, quantification of hepatic steatosis **(D)** and triglyceride levels **(E)**, number of liver neutrophils ($Ly6G^{+}$ $Ly6C^{int}$), inflammatory monocytes ($Ly6C^{high}$ $Ly6G^{-}$) and T cells ($CD3^{+}$ $NK1.1^{-}$)**(F)** and liver expression of CD44 and inflammatory markers at the mRNA level **(G)** were assessed. **(A-G)** Results are expressed as means ± SEM and statistically analyzed using a Mann Whitney test.

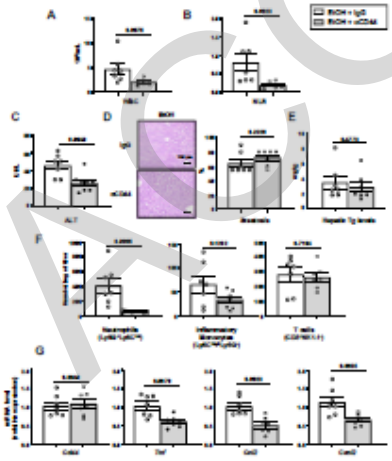

Figure 7: Liver CD44 expression increased with liver inflammation in heavy alcohol drinkers. In 19 consecutive heavy alcohol drinkers, patients were classified into 2 groups: with mild or moderate ALD on the basis of severity of liver steatosis and injury as assessed by histological analysis **(A, B)**, and by serum ALT activity **(C)**, respectively. **(A, B)** Representative images of H&E staining, αCD44 (DAB)/αCD15 (magenta) co-staining of liver tissue section samples (**A)** and quantification of hepatic steatosis **(B)** are shown. **(D)** Hepatic *CD44* mRNA expression levels were analyzed by real-time quantitative PCR. Gene expression was normalized to the mRNA levels of RPLP0 and expressed relative to the mild ALD group. Results are expressed as means ± SEM **(B, D)** or medians with 25-95 percentiles **(C)** and statistically analyzed using a Mann Whitney test. **(E, G)** Correlations between liver CD44 expression (fold change) and ALT level, WBC number and liver expression of *TNF* and *ITGAM* (CD11b) were analyzed using the Spearman correlation test. **(F)** In blood, white blood cell (WBC) number were evaluated, expressed as means ± SEM and statistically analyzed using the Mann Whitney test.

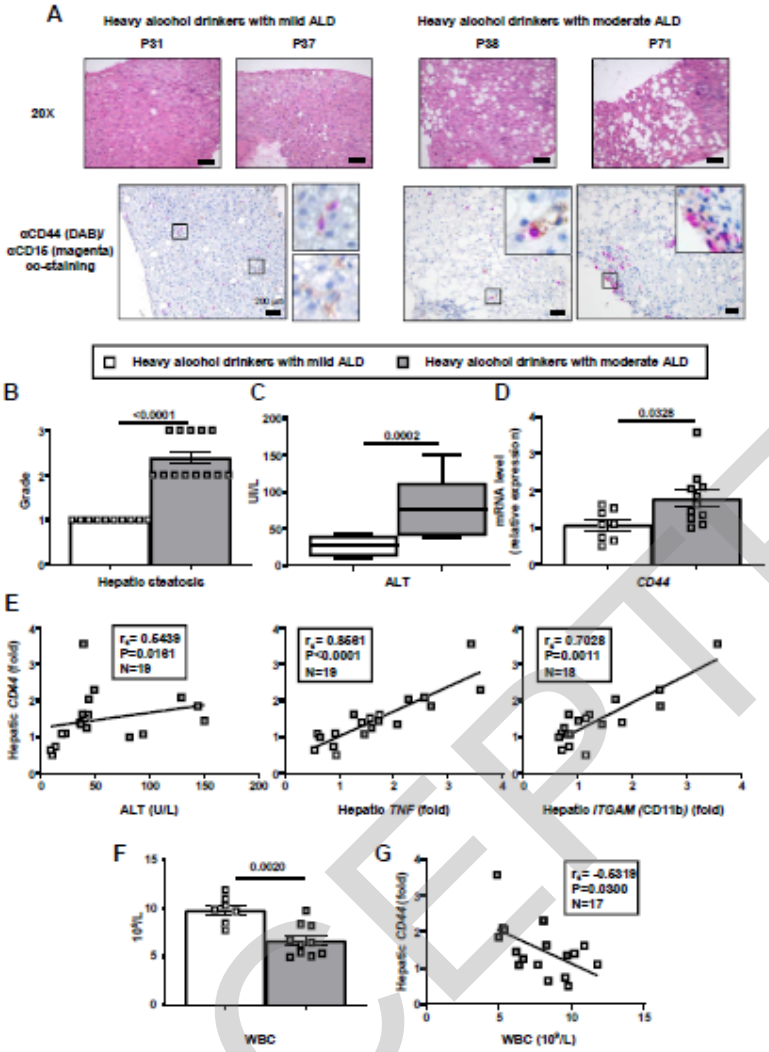

Figure 8: CD44 expression strongly increased in liver neutrophils in patients with severe alcohol associated hepatitis. **(A)** *CD44* mRNA levels was first evaluated in the dataset of patients with AH (n=15) versus subject without liver complications (n=5)(**GSE28619**). **(B-E)** scRNA-seq data deposited into GEO database GSE136103 and GSE255772 have been re-evaluated. **(B)** UMAP projection of 71382 cells, consisting of samples from 5 healthy donors, 5 alcohol-associated cirrhosis (AC) and 6 severe alcohol-associated hepatitis (AH). **(C)** Bar plot showing the composition of conditions for each group. **(D)** Histogram showing quantification of CD44-expressing myeloid cells whose CD44 expression is either above average (0.89)(red "TRUE") or below (blue "FALSE"). **(E)** Violin plot showing *CD44*, *CXCL8*, and *CXCR2* expression in neutrophils from AH or healthy condition.