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Short- or long-term high fat diet feeding plus acute ethanol binge synergistically induce acute liver injury in mice: an important role for CXCL1

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Abstract

Obesity and alcohol consumption often coexist and work synergistically to promote steatohepatitis; however, the underlying mechanisms remain obscure. Here, we demonstrated that feeding mice a high-fat diet (HFD) for as little as 3 days markedly exacerbated acute ethanol binge-induced liver neutrophil infiltration and injury. Feeding mice with a HFD for 3 months plus a single binge of ethanol induced much more severe steatohepatitis. Moreover, 3-day or 3-month HFD-plus-ethanol binge (3d-HFD+ethanol or 3m-HFD+ethanol) treatment markedly upregulated the hepatic expression of several chemokines, including chemokine (C-X-C motif) ligand 1 (*Cxcl1*), which showed the highest fold (approximately 20-fold and 35-fold, respectively) induction. Serum CXCL1 protein levels were markedly elevated after the HFD+ethanol treatment. Blockade of CXCL1 with a CXCL1 neutralizing antibody or genetic deletion of the *Cxcl1* gene reduced the HFD+ethanol-induced hepatic neutrophil infiltration and injury; whereas overexpression of the *Cxcl1* exacerbated steatohepatitis in HFD-fed mice. Furthermore, the expression of *Cxcl1* mRNA was upregulated in hepatocytes, hepatic stellate cells and endothelial cells isolated from HFD+ethanol-fed mice compared to mice that were only given a HFD, with the highest fold induction observed in hepatocytes. *In vitro* stimulation of hepatocytes with palmitic acid upregulated the expression of *Cxcl1* mRNA, and this upregulation was attenuated after treatment with ERK1/2, JNK, or NF- κ B inhibitors. In addition, hepatic or serum levels of free fatty acids (FFAs) were higher in HFD+ethanol-fed mice than in the control groups. *In conclusion*, a HFD combined with acute ethanol consumption synergistically induces acute liver inflammation and injury via the elevation of hepatic or serum FFAs and subsequent upregulation of hepatic CXCL1 expression and promotion of hepatic neutrophil infiltration.

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Keywords

free fatty acid; palmitic acid; inflammation; alcoholic hepatitis

Introduction

It has been recognized for many years that being overweight or obese and consuming excess amounts of alcohol synergistically promote the development and progression of alcoholic liver injury, alcoholic hepatitis, cirrhosis, and hepatocellular carcinoma in patients.¹⁻⁹ In a recent study, multivariate regression analysis of several factors indicated that abdominal girth is positively correlated with the liver fibrosis score in alcoholics, suggesting that visceral fat accumulation, independent of body mass index and metabolic syndrome criteria, plays an important role in promoting alcoholic liver injury.³

The synergistic effects of obesity and alcohol on steatohepatitis have also been observed in several animal models. For example, providing mice *ad libitum* with a high-fat diet (HFD) and alcohol in the drinking water synergistically increased the hepatic expression of profibrogenic genes, activation of hepatic stellate cells (HSCs) and extracellular matrix deposition.¹⁰ Moreover, feeding rats a HFD for one month followed by gavage with ethanol every 12 h for the last three days increased hepatic oxidative stress but did not increase serum ALT levels,¹¹ and intragastric overfeeding of rats or mice with a HFD and co-administration of ethanol synergistically induced an elevation of serum ALT levels, hepatic steatosis, liver inflammation and fibrosis.^{12, 13} The data from mechanistic studies further indicate that alcohol and HFDs synergistically induce nitrosative, endoplasmic reticulum (ER), and mitochondrial stress and an upregulation of hepatic toll-like receptor 4 (TLR4), thereby contributing to steatohepatitis.^{10, 13} Additionally, moderate ethanol binges induce significant liver damage (hepatocyte apoptosis) in genetically obese (*ob/ob*) mice by increasing TNF- α and decreasing nuclear NF- κ B activity.¹⁴ Moreover, a recent study reported that chronic ethanol feeding of genetically obese (*ob/ob*) mice exacerbated hepatic steatosis and injury via the impairment of hepatic lipid metabolism pathways mediated by the hepatic SIRT1-AMPK signaling system.¹⁵ In the present study, we developed a simple model of mixed steatohepatitis by feeding mice a HFD followed by gavage with a single dose of ethanol. The most striking finding was that feeding mice a HFD for as little as 3 days, which is known to impair glucose tolerance and hepatic insulin sensitivity,¹⁶⁻¹⁸ markedly exacerbated the acute ethanol binge-induced neutrophilia, hepatic neutrophil infiltration, and liver injury. Long-term (3 months) HFD feeding plus gavage of a single dose of ethanol caused much more severe steatohepatitis.

In response to tissue injury or infection, neutrophils are recruited from the blood into the affected tissue; this process is orchestrated by multiple adhesion molecules, chemokines and cytokines.¹⁹⁻²¹ These factors include endothelium-expressed molecules, such as E-selectin, P-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), neutrophil-expressed proteins, such as L-selectin (CD62L), E-selectin ligand 1 (ESL-1), P-selectin glycoprotein ligand 1 (PSGL-1) and CD44, and members of the CXC chemokine family including chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2 and

CXCL8 (IL-8).^{19, 20} To examine the mechanisms underlying the synergistic effects of HFD-plus-binge ethanol feeding on neutrophil infiltration, we analyzed the expression of an array of adhesion molecules, chemokines and cytokines. Among these factors, CXCL1 showed the highest induction (up to 20-fold and 30-fold) in the liver after 3d-HFD+ethanol and 3m-HFD+ethanol feeding, respectively.

CXCL1 belongs to the CXC chemokine family and has several other names, including keratinocyte-derived chemokine (KC), cytokine-induced neutrophil chemoattractant-1 (CINC-1), growth-regulated protein oncogene-1 (Gro1), Gro- α , neutrophil-activating protein 3 (NAP-3) and melanoma growth-stimulating activity alpha (MSGA- α).²² In human, this protein is encoded by the *CXCL1* gene. CXCL2 is also referred to as macrophage inflammatory protein 2- α (MIP2- α), Gro- β and Gro-2. *CXCL1* and *CXCL2* are 90% identical in their amino acid sequences and elicit their functions by signaling through the chemokine receptor CXCR2 (also known as IL-8 binding protein), which is a G protein-coupled chemokine receptor expressed on neutrophils.^{20, 22} Hepatic and serum levels of CXCL1 are markedly elevated, whereas hepatic CXCL2 expression is downregulated in patients with alcoholic hepatitis.²³ Hepatic *CXCL1* mRNA levels correlated positively with hepatic neutrophil infiltration in these patients.²³ In addition, the *CXCL1* rs4074 A allele is associated with the elevated levels of CXCL1 protein and is a genetic risk factor for alcoholic cirrhosis.²⁴ In the current paper, we demonstrated that HFD-plus-binge ethanol synergistically upregulated hepatic and serum levels of CXCL1. Furthermore, we demonstrated that PA, oleic acid (OA) and linoleic acid (LA) upregulated the expression of *Cxcl1* mRNA in mouse hepatocytes and that HFD+ethanol feeding resulted in elevated hepatic or serum free fatty acids (FFAs). Finally, we provided evidence suggesting that CXCL1 plays an important role in inducing liver neutrophil recruitment and liver injury after HFD+ethanol treatment.

Materials and Methods

Mice—Eight- to twelve-week-old male C57BL/6 mice were used in this study. Mice were housed in a temperature-controlled room with a 12-h dark/12-h light cycle. Wild-type, *Tlr4*^{-/-} mice on a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, Maine). *Cxcl1*^{-/-} mice on a C57BL/6J background were kindly provided by Dr. Sergio Lira (Mount Sinai, NY).²⁵ All animals were cared for in accordance with the National Institutes of Health guidelines, and all animal experiments were approved by the National Institute on Alcohol Abuse and Alcoholism animal care and use committee.

HFD+ethanol binge feeding model—Mice were fed a HFD (60% kcal% fat; Cat# D12492, Research Diet, New Brunswick, NJ) for 3 days or 3 months. On the last day of HFD feeding, mice were given a single dose of ethanol (5 g/kg body weight as a 31.25% solution in water for 3d-HFD+ethanol model and 53% solution in water for 3m-HFD+ethanol model) or isocaloric dextrin-maltose by oral gavage and then euthanized at various time points following this treatment. Mice were also fed a control diet (10% kcal% fat; Cat# D12450, Research Diet) for 3 days or 3 months, followed by gavage of a single dose of ethanol or maltose.

Statistical analysis—Data are shown as the mean \pm SD of 6-20 mice per group. Data from two groups were compared using an unpaired *t*-test. Data from three or more groups were analyzed by one-way ANOVA with Tukey's multiple comparisons test. Differences were considered significant when the *P* value was less than 0.05.

Other methods are described in the supporting materials

Results

Short-term or long-term HFD-plus-binge ethanol feeding synergistically induces liver injury

To examine the effects of HFD and acute ethanol binge on liver injury, mice were fed a HFD for 3 days (3d, short-term) or 3 months (3m, long-term), followed by gavage of a single dose of maltose or ethanol. As illustrated in Fig. 1A, serum ALT and AST levels were markedly elevated in 3m-HFD+ethanol mice, and also elevated in 3d-HFD+ethanol mice but to a lesser extent. Those levels returned to the basal levels 24 h post ethanol gavage in both groups (Supporting Fig. 1A-1B). In addition, feeding with a HFD alone for 3 months but not 3 days also elevated serum ALT and AST levels. Oral administration of ethanol alone did not elevate the serum ALT or AST levels in chow-fed mice.

H&E staining (Fig. 1B) revealed that 3d-HFD feeding caused obvious hepatic steatosis, which is consistent with previous reports that short-term HFD feeding, i.e. for 3-4 days, is sufficient to induce hepatic steatosis and insulin resistance.¹⁶⁻¹⁸ Feeding mice a HFD for 3 months caused severe steatosis with obvious fat droplets in more than 90% hepatocytes. Acute ethanol binge did not further elevate steatosis in HFD-fed mice (Fig. 1B, supporting Fig. 1C)

HFD-plus-binge ethanol induces hepatic neutrophil infiltration and neutrophilia

Liver inflammation in HFD-plus-binge ethanol-fed mice was examined by immunostaining of the neutrophil marker myeloperoxidase (MPO) and real-time PCR analysis of inflammatory markers as well as flow cytometry analysis of hepatic leukocytes. As illustrated in Fig. 1C and Supporting Fig. 2A, the number of MPO⁺ neutrophils in the liver was markedly elevated in the 3d- and 3m-HFD-plus-binge ethanol groups with a higher number in the latter group than in the former group. Such neutrophil infiltration was observed as early as 3h post gavage in 3m-HFD+ethanol mice with peak effects occurring at 6h and 9h post gavage, and significant neutrophil infiltration was still observed 24h post gavage and declined 48h post gavage (Supporting Fig. 2B). Interestingly, MPO⁺ neutrophils were diffused in the parenchymal regions at 9 h post ethanol gavage in 3d-HFD- or 3m-HFD-fed mice (Fig. 1C). However, clusters of neutrophils were frequently observed around steatotic hepatocytes in 3m-HFD+ethanol group 24h post ethanol gavage (Supporting Fig. 2B).

Furthermore, as shown in Fig. 2A, gavage with a single dose of ethanol increased hepatic expression of *Ly6G* (a neutrophil marker) in chow- and HFD-fed mice, with higher levels in HFD-fed mice than in chow-fed mice. The hepatic *Ly6G* mRNA levels were much higher in 3m-HFD+ethanol-fed mice than in 3d-HFD+ethanol-fed mice (55-fold induction in 3m-

HFD+ethanol *versus* 8-fold in 3d-HFD+ethanol). Interestingly, acute ethanol gavage downregulated hepatic expression of *F4/80* (a marker of Kupffer cells and macrophages) in 3d-chow, 3d-HFD, and 3m-chow-fed mice compared to maltose gavage in these groups (Fig. 2B). Feeding mice with HFD for 3m increased hepatic expression of *F4/80* about 2 folds compared to chow-fed mice, such expression was slightly increased after acute ethanol gavage (Fig. 2B).

Flow cytometric analysis (Fig. 2C) of liver leukocytes demonstrated that acute ethanol gavage elevated the percentage and the total number of neutrophils (Gr1^{high}CD11b⁺) in the livers of chow-fed mice and 3d- or 3m-HFD-fed mice with a higher number in the 3m-HFD+ethanol group than in the 3d-HFD-ethanol group. In contrast, acute ethanol binge reduced monocytes/macrophages (Gr1^{low}CD11b⁺) in chow or 3d-HFD-fed mice, and such reduction was comparable between these two groups. Interestingly, the number of monocytes/macrophages (Gr1^{low}CD11b⁺) was higher in 3m-HFD-fed mice than in chow or 3d-HFD-fed mice, and was not further decreased after acute ethanol binge (Fig. 2C), which is in line with the results in Fig. 2B. In addition, the reduction of monocytes/macrophages was further confirmed by flow cytometric analysis using *F4/80* and *CD11b* markers (Supporting Fig. 2C); while the percentage of hepatic NK and NKT cells was not altered in 3m-HFD-fed mice after acute ethanol binge compared to maltose binge (Supporting Fig. 2D-E).

In addition to liver neutrophils, we also examined circulating neutrophils. As illustrated in Fig. 2D, the percentage and the number of circulating neutrophils were elevated after ethanol gavage in chow group, and were further increased in HFD+ethanol groups with higher levels in 3m-HFD+ethanol mice than in 3d-HFD+ethanol mice (Fig. 2D).

HFD-plus-binge ethanol synergistically upregulates hepatic and serum levels of CXCL1

To explore the mechanisms by which HFD+ethanol induces hepatic neutrophil accumulation, we examined hepatic expression of pro-inflammatory mediators and chemokines that promote hepatic neutrophil infiltration. As indicated in Supporting Fig. 3, acute ethanol gavage did not alter hepatic expression of several pro-inflammatory cytokines in 3d-HFD+ethanol mice, while hepatic expression of several cytokines was elevated in 3m-HFD-fed mice after acute ethanol gavage. Furthermore, as illustrated in Fig. 3, in the 3d-HFD group, acute ethanol binge upregulated hepatic expression of *Cxcl1* mRNA by 20-fold, while other chemokines and adhesion molecules were either upregulated to a much lesser extent or not altered. In contrast, in the 3m-HFD group, hepatic expression of many chemokines and adhesion molecules was markedly elevated after acute ethanol binge, including approximately 30-fold induction of *Cxcl1*. Hepatic expression of several chemokine receptors was not upregulated and some of them (eg. *Cxcr5* and *Cxcr6*) were even downregulated after acute ethanol binge in both 3d- and 3m-HFD-fed mice except *Ccr1*. Hepatic expression of *Ccr1* was markedly upregulated in 3m-HFD+ethanol mice compared with 3m-HFD+maltose group (Fig. 3B).

Because *Cxcl1* mRNA showed the highest fold upregulation among other chemokines in both 3d- and 3m-HFD+ethanol-fed mice, we focused on the CXCL1 in the current paper, and further measured serum CXCL1 protein levels. As illustrated in Fig. 3C, the 3d- and 3m-HFD+ethanol treatment resulted in synergistically elevated serum CXCL1 levels to

approximately 800 pg/ml and 1500 pg/ml, respectively, while 3d- or 3m-HFD+maltose feeding only slightly elevated the serum levels of CXCL1 protein.

TLR4 is not involved in the 3d- or 3m-HFD+ethanol-mediated elevation of serum CXCL1, hepatic neutrophil infiltration and injury

LPS, through binding to TLR4, has been shown to elevate serum CXCL1 levels during endotoxemic liver injury in mice.²⁶ To determine whether the LPS/TLR4 axis was involved in the 3d- or 3m-HFD+ethanol-induced CXCL1 elevation and liver injury, WT and *Tlr4*KO mice were subjected to 3d- or 3m-HFD+ethanol feeding. As illustrated in supporting Fig. 4A and 4B, serum ALT and AST level, and serum CXCL1 protein levels were comparable between 3d- or 3m-HFD+ethanol-fed WT and *Tlr4*KO mice. As expected, hepatic *Tlr4* mRNA was not detected in *Tlr4*KO mice, while hepatic expression of *Ifng*, *IL-1b*, *IL-6*, *Tnfa*, *Ly6G* was comparable between 3m-HFD+ethanol-fed WT and *Tlr4*KO mice (Supporting Figs. 4C, D). There was also no difference in the number of MPO⁺ neutrophils, hepatic triglyceride contents, hepatic and serum FFA levels between these two groups (Supporting Figs. 4D-G). Collectively, TLR4 does not contribute to 3d- or 3m-HFD +ethanol-induced elevation of CXCL1, liver neutrophil infiltration and injury.

Elevation of *Cxcl1* expression in hepatocytes, and to a lesser extent in HSCs and sinusoidal ECs, following HFD+ethanol feeding

To identify which organ is mainly responsible for the elevated serum CXCL1 levels, we measured *Cxcl1* mRNA in different organs. As illustrated in Figure 4A, acute ethanol gavage elevated *Cxcl1* mRNA by 30-fold and 5-fold in the liver and epididymal fat, respectively, in 3d-HFD-fed mice, while its expression was not elevated in the heart, spleen, kidney and subcutaneous fat.

It has been reported that hepatocytes and liver non parenchymal cells (NPCs) can produce CXCL1 after stimulation with a variety of inflammatory mediators.²⁶⁻²⁹ To further understand the mechanisms by which 3d-HFD-ethanol feeding upregulates hepatic *Cxcl1* mRNA expression, we investigated which cell types express *Cxcl1* in the liver. We first isolated hepatocytes and NPCs and examined *Cxcl1* mRNA in these cells. As illustrated in Fig. 4B, *Cxcl1* mRNA levels were upregulated in both hepatocytes and NPCs from 3d-HFD +ethanol-fed mice compared to 3d-HFD+maltose-fed mice. Next, hepatic stellate cells (HSCs) and sinusoidal endothelial cells (ECs) were isolated from the total NPCs of mice in 3d-HFD+maltose or 3d-HFD+ethanol groups, and *Cxcl1* mRNA was measured. As illustrated in Fig. 4B, the levels of *Cxcl1* mRNA were increased in HSCs and sinusoidal ECs by approximately 2-fold. Collectively, *Cxcl1* mRNA expression was increased in hepatocytes, HSCs and ECs from 3d-HFD+ethanol-fed mice compared to 3d-HFD+maltose-fed mice, with the highest induction observed in hepatocytes.

Immunostaining analysis revealed that a few steatotic hepatocytes expressed CXCL1 in 3m-HFD+maltose mice, whereas a much greater number of hepatocytes strongly expressed CXCL1 in 3m-HFD+ethanol mice (Fig. 4C).

HFD+ethanol feeding elevates hepatic or serum FFAs, which upregulate Cxcl1 expression in hepatocytes

Previous studies revealed that treatment with PA induced the production of IL-8 (CXCL8) in human hepatocytes via NF- κ B- and JNK-dependent mechanisms.²⁹ This notion led us to hypothesize that PA may contribute to the elevated serum CXCL1 levels observed after HFD+ethanol feeding. To test this hypothesis, we first measured serum FFA levels. As illustrated in Fig. 5A, serum FFA levels were significantly elevated in 3m-HFD+ethanol-fed mice but not in 3d-HFD+ethanol-fed mice compared to other groups. Next, we measured hepatic FFA levels, and the results revealed that hepatic FFA levels were markedly elevated in 3d-HFD+ethanol mice compared to HFD+maltose mice (Fig. 5B). Interestingly, hepatic FFA levels were higher in 3m-HFD-fed mice than in 3m-chow-fed mice. Acute ethanol binge did not further elevate hepatic FFA levels (Fig. 5B).

In vitro treatment with PA, OA or LA but not EPA or ELA upregulated the expression of *Cxcl1* mRNA in primary mouse hepatocytes (Fig. 5C). Fig. 5D-E shows that PA treatment upregulated *Cxcl1* mRNA expression in the mouse hepatocyte cell line AML12 or primary mouse hepatocytes. Such *Cxcl1* mRNA induction was diminished by the treatment with ERK1/2, JNK and NF- κ B specific inhibitors. TLR4 was shown to contribute to FFA-mediated effects;³⁰ but these studies were not reproduced by others.³¹ Here we demonstrated that treatment with PA induced comparable elevation of *Cxcl1* mRNA expression in wild-type and *TLR4*KO hepatocytes (Supporting Fig. 5), suggesting PA-mediated upregulation of *Cxcl1* mRNA is TLR4-independent.

Blockade of CXCL1 ameliorates HFD+ethanol-induced neutrophilia, hepatic neutrophil infiltration and liver injury

To define the functions of CXCL1 in HFD+ethanol-induced hepatic neutrophil infiltration and injury, HFD+ethanol-fed mice were treated with an anti-CXCL1 antibody or a control IgG antibody. As illustrated in Fig. 6A, compared to IgG treatment, administration of the anti-CXCL1 antibody markedly reduced the percentage and the total number of circulating neutrophils. In contrast, treatment with the anti-CXCL1 antibody did not affect the total number of circulating monocytes and lymphocytes in these HFD+ethanol-fed mice (Supporting Fig. 6). Real-time PCR and immunostaining analyses demonstrated that hepatic neutrophil infiltration, as measured by hepatic gene expression of the neutrophil marker *Ly6G* and the total number of MPO⁺ neutrophils, was markedly attenuated in anti-CXCL1 antibody-treated mice compared to IgG-treated mice (Fig. 6B, and Supporting Fig. 7). Finally, serum ALT and AST levels were significantly lower after anti-CXCL1 treatment compared to IgG treatment in 3d- or 3m-HFD-ethanol-fed mice (Figs. 6C), whereas hepatic triglyceride levels were not altered by anti-CXCL1 antibody treatment (Supporting Fig. 8).

Disruption of the Cxcl1 gene reduces HFD+ethanol-induced liver inflammation and injury, while overexpression of Cxcl1 exacerbates steatohepatitis in 3m-HFD-fed mice

The functions of CXCL1 in HFD+ethanol induced liver inflammation and injury were further determined by using *Cxcl1*KO mice. As illustrated in Fig. 7A, the total number and the percentage of neutrophils in the blood, and the number of MPO⁺ neutrophils in the liver were markedly lower in *Cxcl1*KO mice compared to those in WT mice post 3d- or 3m-HFD

+ethanol feeding. In line with this, *Cxcl1*KO mice had lower levels of serum ALT and AST compared to WT mice (Fig. 7B).

To further determine whether acute ethanol binge-induced elevation of CXCL1 contributes to liver inflammation and injury in HFD-fed mice, a gain-of-function approach was used via the administration of Ad-*Cxcl1* into HFD-fed mice to induce elevation of CXCL1. As illustrated in Fig. 7C, administration of Ad-*Cxcl1* elevated serum CXCL1 protein levels to approximately 4000 pg/ml in chow or HFD-fed mice. The number of MPO⁺ neutrophils was markedly elevated in Ad-*Cxcl1*-treated mice with much higher levels in HFD-fed mice than in chow-fed mice (Fig. 7C, supporting Fig. 9). In addition, serum levels of ALT and AST were elevated in Ad-*Cxcl1*-treated chow-fed and HFD-fed mice compared to Ad-control virus-treated mice, and those levels were much higher in HFD-fed mice than in chow-fed mice (Fig. 7D).

CXCL1 has no direct cytotoxicity on primary mouse hepatocytes in vitro

A previous paper suggests that CXCL1 may directly induce hepatocyte death without inducing neutrophil recruitment in a model of CCl₄-induced liver injury, but there was no direct evidence.³² To confirm whether CXCL1 had the direct hepatotoxicity, we examined *Cxcr2* (a receptor for CXCL1) mRNA expression and the cytotoxicity of CXCL1 in primary mouse hepatocytes. As shown in Fig. 8A, compared to the spleen, the liver expressed very low levels of *Cxcr2* mRNA. The expression of *Cxcr2* mRNA was upregulated about 2-fold in the livers from HFD+ethanol-fed mice. Furthermore, expression of *Cxcr2* mRNA in hepatocytes was 40- to 50-fold lower than that in splenocytes and neutrophils (Fig. 8B).

Incubation of primary mouse hepatocytes with CXCL1 protein even at high concentrations (up to 100 ng/ml) did not induce cell death detected by TUNEL staining (Supporting Fig. 10) and ALT or AST release into the cell culture supernatant (Fig. 8C), whereas positive controls from incubation with TNF- α plus actinomycin D demonstrated marked hepatocyte death (Fig. 8C, and Supporting Fig. 10). In addition, incubation of steatotic hepatocytes isolated from HFD-fed mice with CXCL1 protein did not enhance ALT or AST release into the cell culture supernatant (Fig. 8D).

Discussion

Alcohol and HFD consumption are known to synergistically induce nitrosative, ER, and mitochondrial stress and an upregulation of hepatic TLR4, which are important mechanisms contributing to the synergistic effect of alcohol and HFD on the induction of steatohepatitis.^{10, 13} In the present study, we have identified another important mechanism by which HFD-plus-binge ethanol synergistically exacerbates acute steatohepatitis via the induction of CXCL1 and subsequent hepatic neutrophil infiltration (Fig. 8E).

The most striking finding from this study was that feeding mice a HFD for as little as 3 days markedly exacerbated the acute ethanol-induced hepatic neutrophil infiltration and liver injury. This may not be very surprising because short-term HFD feeding, i.e. for 3-4 days, is sufficient to induce hepatic steatosis and hepatic insulin resistance.¹⁶⁻¹⁸ Although feeding mice a HFD alone for 3 days induced steatosis, hepatic neutrophil infiltration and serum

ALT levels were not elevated. Acute gavage with a single dose of ethanol resulted in slight elevation of serum ALT and AST, increased hepatic neutrophil infiltration and steatosis in chow-fed mice, and all of these effects (except steatosis) were markedly aggravated in 3d-HFD-fed mice. Furthermore, feeding mice a HFD for 3 months plus a single binge of ethanol caused much more severe steatohepatitis than 3d-HFD+binge ethanol feeding.

One of the most fascinating features in this HFD+binge ethanol feeding model is dramatic hepatic neutrophil infiltration. Neutrophil infiltration has been implicated in promoting liver damage in various types of liver diseases³³ including drug-induced liver injury³⁴⁻³⁷ and alcoholic liver injury.^{38, 39} The significant increase in hepatic neutrophil infiltration likely also participates in HFD+ethanol-induced liver damage because blockade of CXCL1 by an anti-CXCL1 neutralizing antibody or disruption of the *Cxcl1* gene prevented hepatic neutrophil infiltration and hepatocellular damage. In contrast to marked hepatic neutrophil infiltration, hepatic macrophages were reduced in 3d-HFD or slightly elevated in 3m-HFD fed mice post ethanol binge. It has been shown that short-term ethanol feeding or acute ethanol gavage induces apoptosis of Kupffer cells or macrophages *in vivo*,^{40, 41} and that *in vitro* incubation with ethanol directly induced apoptosis of mouse peritoneal macrophages or macrophage cell line J774.⁴¹ Thus, the reduction of hepatic macrophages after acute ethanol binge in 3d-HFD-fed mice was probably due to the pro-apoptotic effects of ethanol on Kupffer cells/macrophages. These apoptotic Kupffer cells/macrophages likely produce damage-associated molecular pattern molecules that subsequently contribute to neutrophil activation in this HFD-plus-binge ethanol model.

Another important finding from this study is the identification of CXCL1 as an important chemokine that was highly elevated in the liver and serum from HFD+ethanol-fed mice. Among several chemokines and adhesive molecules that we examined, hepatic *Cxcl1* mRNA was upregulated by more than 20-fold and 35-fold in the 3d- and 3m-HFD-plus-binge ethanol groups, respectively, compared to their corresponding HFD+maltose groups. Serum CXCL1 protein levels were also significantly elevated in 3d- and 3m-HFD+ethanol-fed mice, reaching approximately 800 pg/ml and 1500 pg/ml, respectively. Moreover, the fact that HFD+ethanol feeding highly upregulated *Cxcl1* mRNA in the liver but not in the other organs suggests that the liver is the major source of the increased serum CXCL1 levels in this model. The next obvious question was which cell type in the liver was mainly responsible for CXCL1 production. Previous studies have reported that hepatocytes and NPCs can produce CXCL1.^{27, 28} Here, we demonstrated that *Cxcl1* mRNA expression was upregulated in hepatocytes, HSCs and ECs after 3d-HFD+ethanol binge feeding, with the highest induction observed in hepatocytes. Given that hepatocytes constitute 80% of the liver volume and 60% of the total liver cells, we concluded that hepatocytes are largely responsible for the upregulation of *Cxcl1* in the liver after HFD-plus-binge ethanol feeding; NPCs also contribute to this CXCL1 production but to a lesser extent.

The expression of CXCL1 is upregulated by LPS²⁶ and the LPS receptor TLR4 has been shown to promote HFD-induced nonalcoholic steatohepatitis.^{42, 43} However, we demonstrated that genetic deletion of the LPS receptor TLR4 had no effect on 3d- or 3m-HFD-plus-binge ethanol-induced hepatic CXCL1 expression, neutrophilia, and liver injury, suggesting that TLR4 does not contribute to HFD+ethanol binge-induced elevation of

CXCL1, acute liver injury and inflammation. Why TLR4 contributes to HFD-induced steatohepatitis but is not involved in HFD-plus-binge ethanol binge-induced liver injury is not clear. One reason may be that 3m-HFD+ethanol feeding caused much greater elevation of serum CXCL1 than 3m-HFD alone does (1500 pg/ml circulating CXCL1 protein in 3m-HFD+ethanol vs 300 pg/ml in 3m-HFD alone), and this high elevation of CXCL1 in HFD+ethanol is independent TLR4. A recent study reported that serum CXCL1 levels were elevated in chronic-plus-binge ethanol feeding model, but those levels were much lower than in HFD+ethanol-fed mice (200 pg/ml in chronic-plus-binge ethanol model⁴⁴ versus 600-1500 pg/ml in HFD+ethanol model). Both TLR2 and TLR9 contribute to chronic ethanol-plus-binge ethanol-mediated elevation of serum CXCL1 protein.⁴⁴ However, we found that HFD+ethanol treatment induced similar levels of CXCL1 elevation and liver injury between WT and TLR9KO mice (unpublished data), suggesting that the TLR9 pathway does not contribute to HFD+ethanol-induced elevation of CXCL1. Instead, we identified a different mechanism underlying hepatic CXCL1 upregulation in the HFD+ethanol model. First, acute ethanol binge markedly elevated hepatic and serum FFA levels in 3d- and 3m-HFD-fed mice, respectively. Second, *in vitro* experiments demonstrated that several FFAs were able to directly stimulate *Cxcl1* mRNA expression in hepatocytes, which was dependent on proper ERK, JNK and NF- κ B activation. Collectively, our data suggest that acute ethanol binge induces elevation of hepatic or serum FFAs in HFD-fed mice, which directly upregulate hepatic CXCL1 production and induce acute liver injury in those mice.

Roh et al. recently reported that a selective CXCR2 blockade inhibits hepatic neutrophil infiltration and injury in chronic-plus-binge ethanol feeding model.⁴⁴ Because CXCR2 is a receptor for CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8,⁴⁵ therefore, all of those chemokines may contribute to chronic-plus-binge ethanol-induced liver inflammation and injury. In the current study, by using loss-of-function and gain-of-function approaches, we clearly demonstrated that CXCL1 plays an important role in promoting HFD+ethanol-induced acute steatohepatitis. First, HFD-plus-binge ethanol feeding markedly upregulated hepatic *Cxcl1* mRNA expression and elevated serum CXCL1 levels. Second, blockade of CXCL1 with a neutralizing antibody or genetic deletion ameliorated HFD+ethanol-induced neutrophilia, hepatic neutrophil infiltration and injury but did not affect hepatic steatosis. Previous studies have implicated CXCL1 in promoting neutrophil infiltration and liver injury induced by D-galactosamine/endotoxin (D-Gal/ET),²⁶ ischemia/reperfusion⁴⁶ and focal brain damage.⁴⁷ However, Dorman *et al.*⁴⁸ reported that treatment with anti-KC and anti-MIP-2 antibodies did not attenuate D-Gal/ET-induced liver injury in C3Heb/FeJ mice, and no differences in liver injury were observed between wild-type BALB/cJ and CXCR2^{-/-} mice. The reason behind the discrepancy between these experiments is not clear but may be related to the different backgrounds of the mice, liver injury models and the severity of liver injury used in these studies. Furthermore, overexpression of CINC (a rat homolog of CXCL1) via adenovirus-mediated gene delivery markedly induced neutrophilia and liver injury in rats.⁴⁹ In the current paper, we demonstrated that administration of adenovirus-*Cxcl1* resulted in hepatic *Cxcl1* overexpression and elevated hepatic neutrophil infiltration and injury in HFD-fed mice, providing direct evidence that CXCL1 promotes steatohepatitis. All of these data support the

notion that CXCL1 promotes hepatic neutrophil infiltration that subsequently contributes to liver injury. Surprisingly, Stefanovic et al. reported that treatment of mice with CXCL1 protein exacerbated liver damage without inducing hepatic neutrophil infiltration in a model of CCl₄-induced liver injury,³² Thus, authors concluded that CXCL1 directly induced hepatocyte death. However, Stefanovic et al. did not test whether CXCL1 had direct cytotoxicity against hepatocytes *in vitro*. In the present study, we observed that treatment with CXCL1 protein did not induce hepatocyte death *in vitro*, which is consistent with extremely low levels of CXCR2 expression on hepatocytes. Therefore, in this HFD-plus-binge ethanol model, the hepatotoxicity of CXCL1 is mediated via the induction of hepatic neutrophil infiltration and is not due to the direct cytotoxicity on hepatocytes.

Clinical implications of this study

The finding that a single oral binge exposure to ethanol can trigger acute liver injury in the presence of short-term or long-term HFD feeding highlights the risk of even episodic binge drinking in obese/overweight individuals. Although this model does not represent a model of chronic ASH, it can trigger acute liver injury. The obvious clinical implication of this finding is that obese individuals should avoid binge drinking large amounts of alcohol, as this would likely cause significant acute liver damage. These findings should also promote clinical studies on the effect of the interaction between obesity and binge drinking on neutrophilia, liver inflammation and hepatic injury. For example, the hepatic expression of CXC chemokines (*e.g.*, *CXCL1/Gro- α* , *IL-8*, *CXCL5*, and *CXCL6*) was found to be upregulated and correlated with neutrophil infiltration and the severity of disease in patients with alcoholic hepatitis.²³ Hepatic expression of *CXCL1*, *IL-8* and their receptors is also upregulated in the liver of obese patients with NASH.⁵⁰ Thus, it would be interesting to examine whether obesity, as measured by BMI, and binge alcohol drinking synergistically promote increased hepatic levels of CXC chemokines, neutrophilia, and the severity of liver disease in these patients. Another important feature of the present model is the rapidity of the development of neutrophil-mediated steatohepatitis, which would make it uniquely suitable for future mechanistic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALT	alanine aminotransferase
CINC-1	cytokine-induced neutrophil chemoattractant-1
CXCL1	(C-X-C motif) ligand 1
d	day(s)
ECs	endothelial cells
FFAs	free fatty acids
h	hour(s)
HFD-binge	high-fat diet feeding plus binge ethanol
HSC	hepatic stellate cell
KC	keratinocyte-derived chemokine
LA	linoleic acid
m	months
NPCs	non parenchymal cells
MPO	Myeloperoxidase
OA	oleic acid
PA	palmitic acid
TLR4	toll-like receptor 4

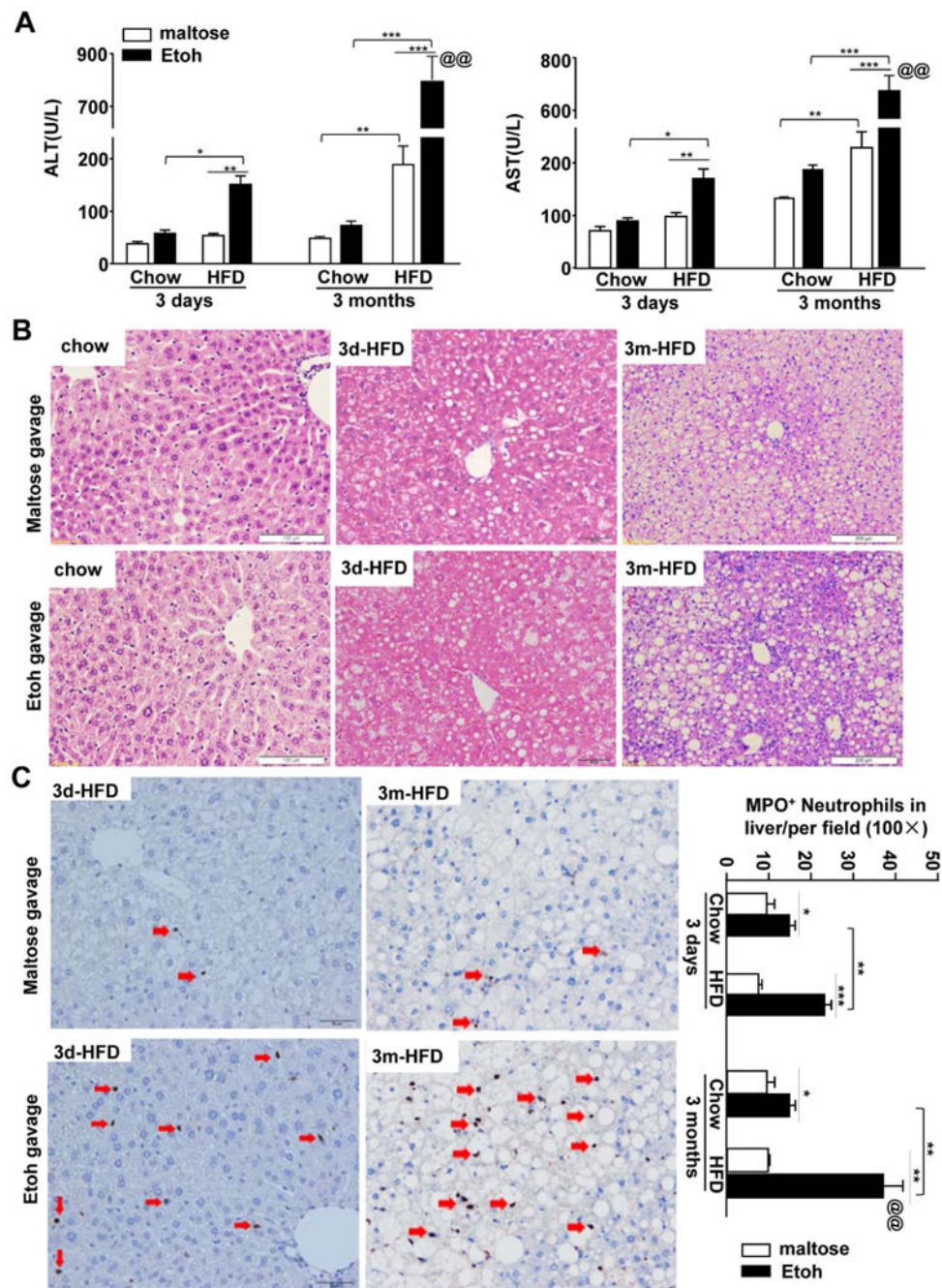


Figure 1. Short-term or long-term HFD feeding exacerbates acute ethanol-induced liver injury
 C57BL/6J mice were fed a HFD diet or chow for 3 days or 3 months, and then given maltose or ethanol by oral gavage 9 h prior to euthanization. (A) Serum ALT and AST levels. (B) Representative H&E staining of liver tissue sections. (C) Representative MPO staining is shown. The number of MPO⁺ cells/per field was determined and is shown on the right. Values represent the mean ± SD (n=8-20). ** $P < 0.01$, *** $P < 0.001$, as indicated. @ @ $P < 0.01$ in comparison with 3d-HFD+Etoh group.

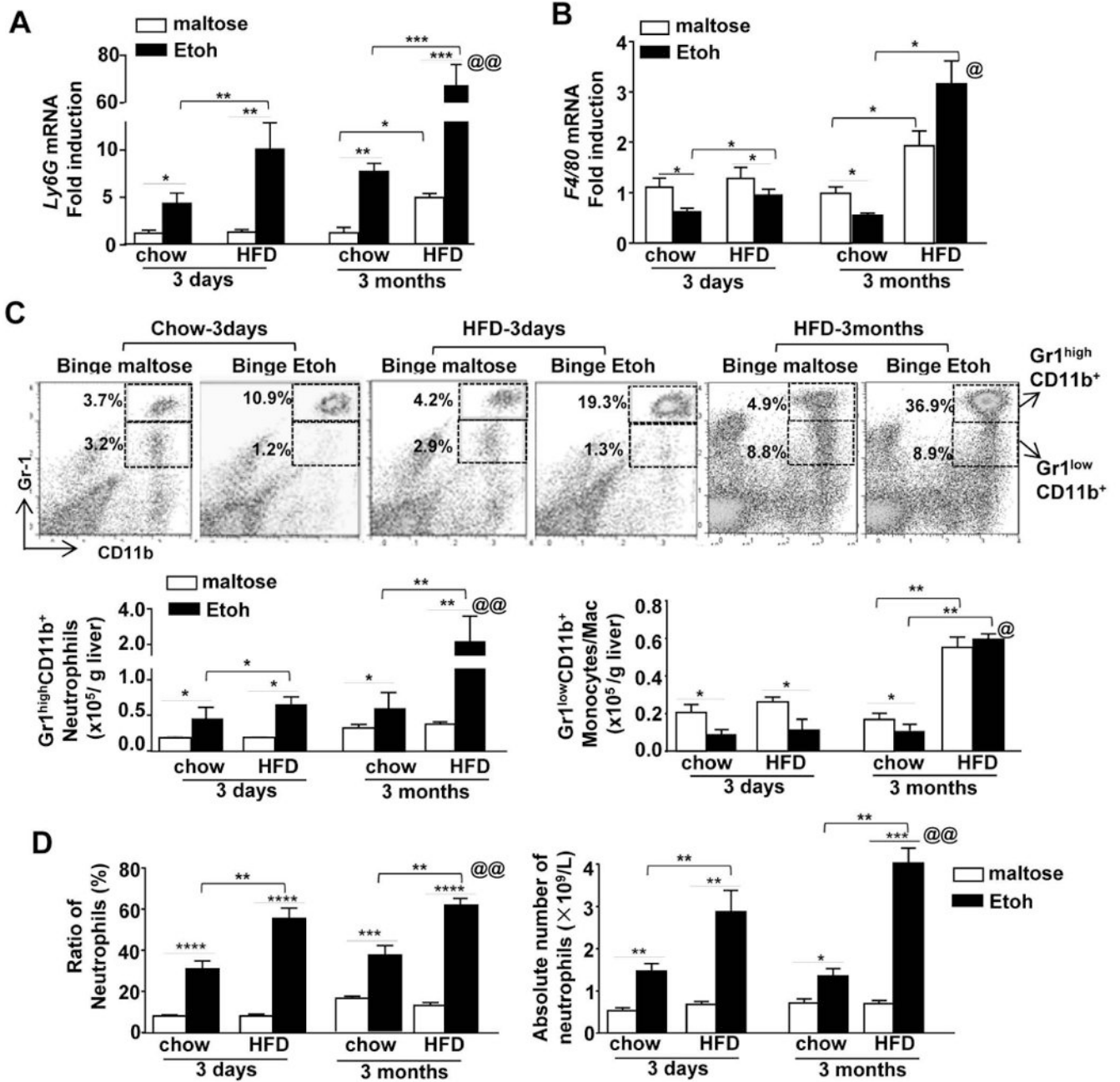


Figure 2. HFD-plus-binge ethanol feeding synergistically induces liver neutrophil infiltration and neutrophilia

Mice were fed a HFD diet or chow for 3 days or 3 months, and then given maltose or ethanol by oral gavage 9 h prior to euthanization. (A, B) Hepatic expression of *Ly6G* or *F4/80* mRNA levels was determined by real-time quantitative PCR. (C) Liver leukocytes from 3d- or 3m-HFD+ethanol-fed mice were isolated and subjected to flow cytometric analysis. The total numbers of neutrophils (Gr1^{high}CD11b⁺) and monocytes/macrophages (Gr1^{low}CD11b⁺) were determined. (D) The total number and percentage of circulating neutrophils were determined. Values represent the mean ± SD (n=5-15). *P<0.05;

** $P < 0.01$; *** $P < 0.001$, as indicated. @ $P < 0.05$; @@ $P < 0.01$ in comparison with 3d-HFD +Etoh group.

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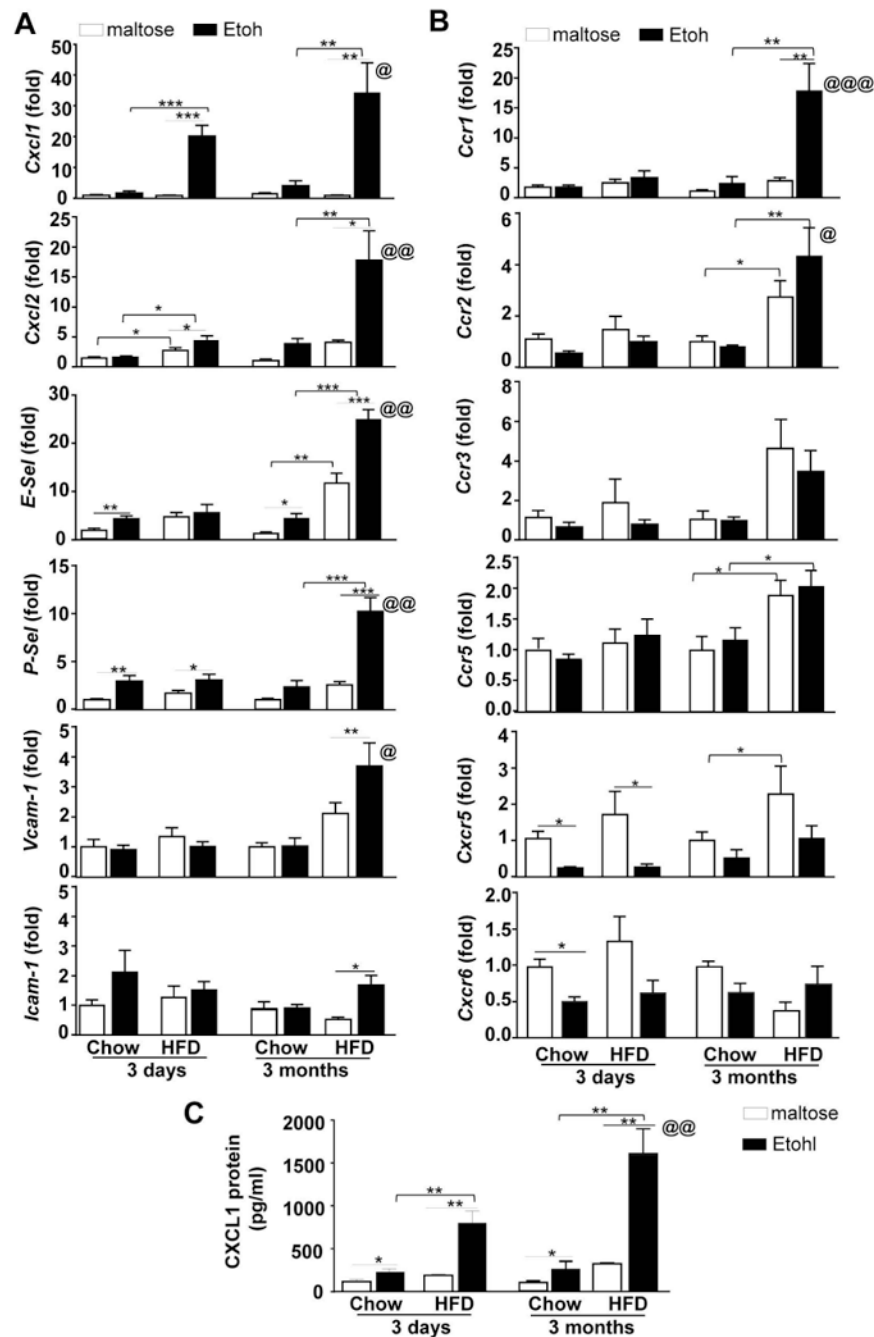


Figure 3. HFD-plus-binge ethanol synergistically increases hepatic *Cxcl1* mRNA and serum CXCL1 levels

(A, B) Mice were fed chow or a HFD for 3 days or 3 months, followed by gavage with a single dose of maltose or ethanol. Mice were euthanized 9 h post-gavage. (A, B) Liver tissues were collected, and total RNA was isolated and subjected to real-time PCR analysis. (B) Sera were collected to measure CXCL1 levels with an ELISA kit. Values represent the mean \pm SD (n=5-10). * P <0.05; ** P <0.01; *** P <0.001 as indicated. @ P <0.05, @@ P <0.01, @@@ P <0.01 in comparison with 3d-HFD+EtOH group.

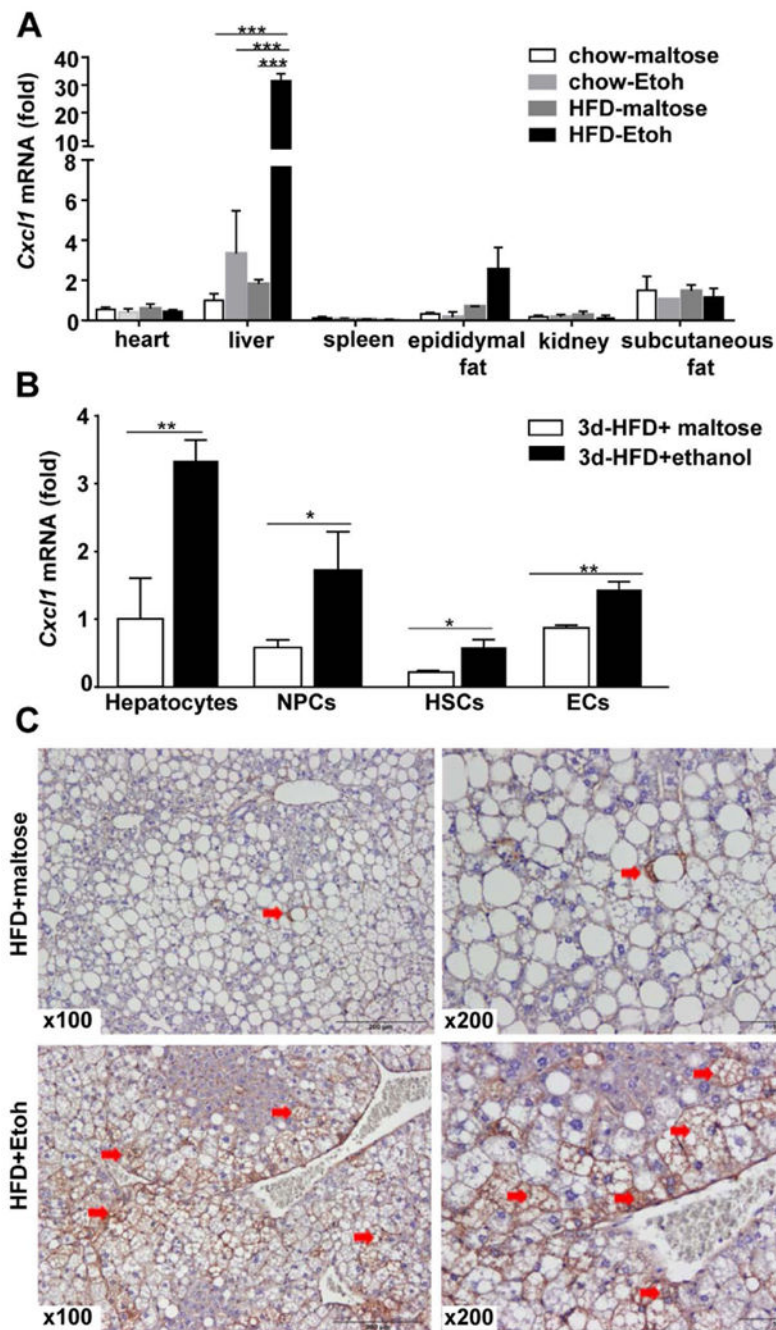


Figure 4. Upregulation of *Cxcl1* mRNA expression in the liver and liver cells from HFD-plus-binge ethanol-fed mice

(A, B), Mice were fed a HFD for 3 days followed by a single dose of ethanol or maltose; 9 h after gavage, tissues and cells were collected. Various organs were collected and subjected to real-time PCR analysis of *Cxcl1* mRNA (panel A). Hepatocytes, NPCs, HSCs and ECs were isolated and subjected to real-time PCR analysis to determine the levels of *Cxcl1* mRNA (panel B). (C) Mice were fed a HFD for 3 months, followed by a single dose of ethanol or maltose; 9 h after gavage, liver tissues were collected for immunohistochemistry

analyses of CXCL1 protein. Values represent the mean \pm SD (n=5-10). * P <0.05; ** P <0.01; *** P <0.001 as indicated. Arrows in panel C indicate positive CXCL1 staining in hepatocytes.

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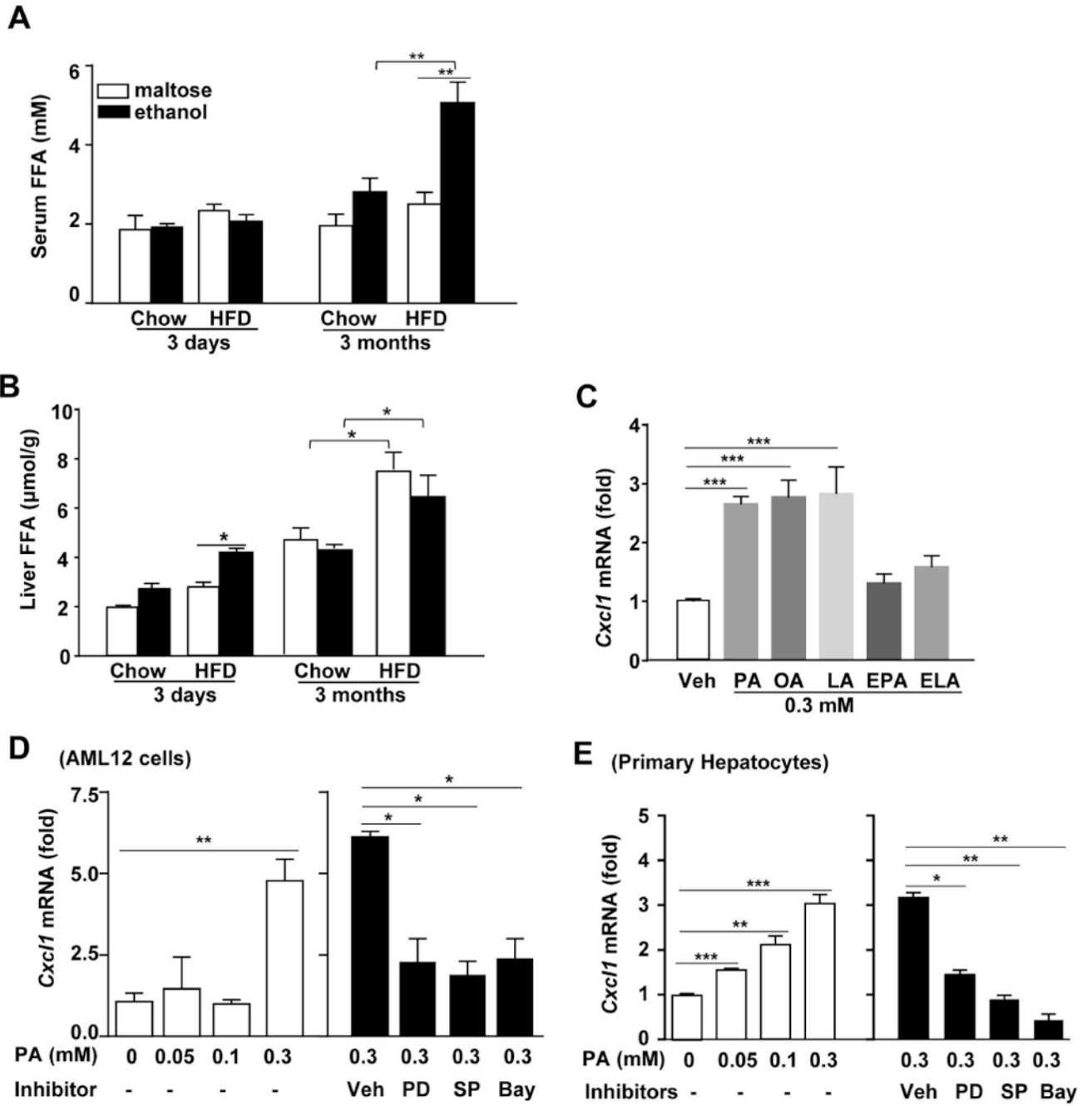


Figure 5. HFD-plus-binge ethanol feeding elevates hepatic or serum levels of FFAs, and FFAs upregulate *Cxcl1* mRNA expression in mouse hepatocytes

(A, B) Mice were fed a HFD for 3 days or 3 months, followed by gavage of a single dose of ethanol or maltose. Serum and hepatic FFAs levels were determined 9 h post-gavage. (C) Primary mouse hepatocytes were stimulated with 0.3 mM palmitic acid (PA), oleic acid (OA), linoleic acid (LA), ethyl palmitic acid (EPA) or ethyl linoleic (ELA) for 3 h, and *Cxcl1* mRNA expression was determined by real-time PCR. (D, E) AML12 cells and primary mouse hepatocytes were treated with various doses of PA with or without pre-

treatment with an ERK1/2 inhibitor (PD98059, 100 μ M), JNK inhibitor (SP600125, 50 μ M) or NF- κ B inhibitor (Bay-7085, 10 μ M). *Cxcl1* mRNA expression was determined by real-time PCR. Values represent the mean \pm SD (n=4-6). * P <0.05; ** P <0.01; *** P <0.001 as indicated.

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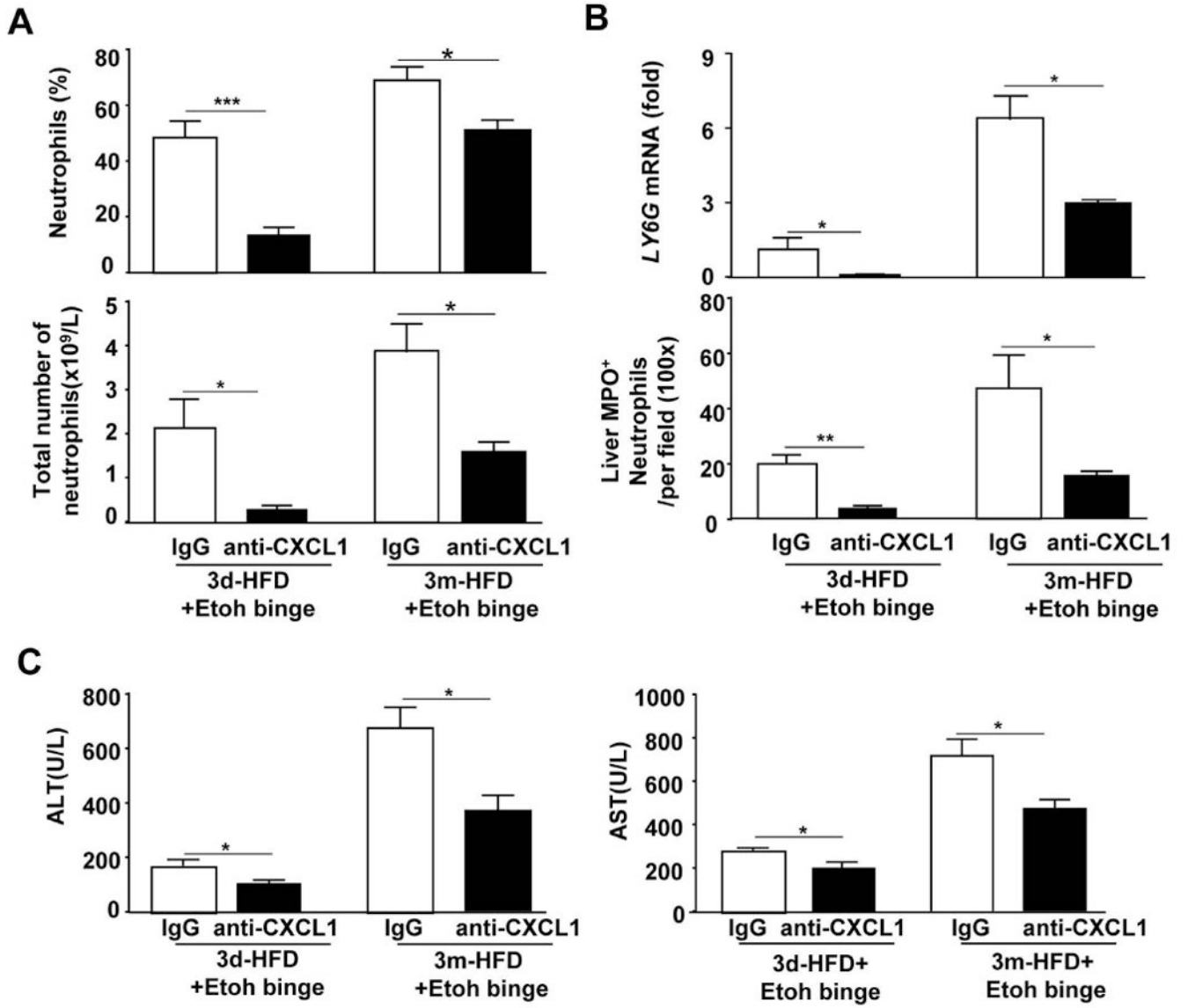


Figure 6. Blockade of CXCL1 ameliorates HFD-plus-binge ethanol-induced neutrophilia, hepatic neutrophil infiltration, and liver injury

Mice were fed a HFD for 3 days or 3 months, followed by gavage with a single dose of ethanol. An anti-CXCL1 antibody or IgG antibody was administered to the mice 15 min before gavage. Mice were euthanized 9 h post-gavage. (A) Blood leukocytes were collected, analyzed and counted. (B) Liver tissues were collected for real-time PCR analysis of *Ly6G* mRNA or subjected to immunostaining with an anti-MPO antibody, and the number of MPO⁺ cells/per field was determined. (C) Serum levels of ALT and AST were measured. Values represent the mean ± SD (n=5-8). **P*<0.05, ****P*<0.001 as indicated.

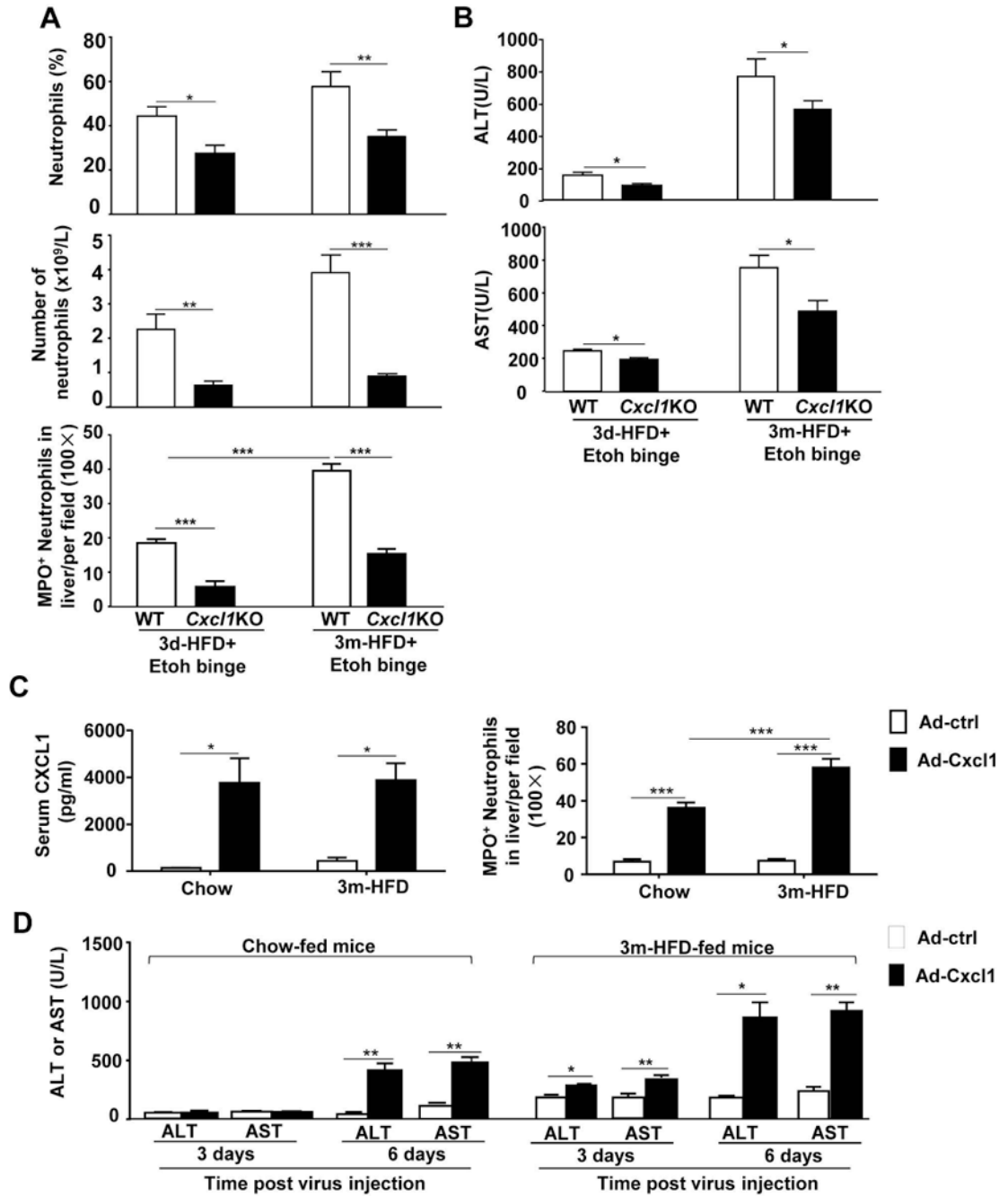


Figure 7. Genetic deletion of the *Cxcl1* gene ameliorates HFD-plus-binge ethanol-induced hepatic neutrophil infiltration and injury, while overexpression of the *Cxcl1* exacerbates liver inflammation and injury

(A, B) Mice were fed a HFD for 3 days or 3 months, followed by gavage with a single dose of ethanol. Mice were euthanized 9 h post-gavage. Circulating neutrophils and hepatic neutrophils were determined and are shown in panel A. Serum levels of ALT and AST are shown in panel B. (C, D) Chow- and 3m-HFD-fed mice were injected with Ad-control or Ad-*Cxcl1* viruses, serum CXCL1 protein levels and hepatic neutrophil infiltration were determined 6 days post virus injection (panel C); serum ALT and AST levels were

determined (panel D). Values represent the mean \pm SD (n=6-10). * P <0.05; ** P <0.01; *** P <0.001 as indicated.

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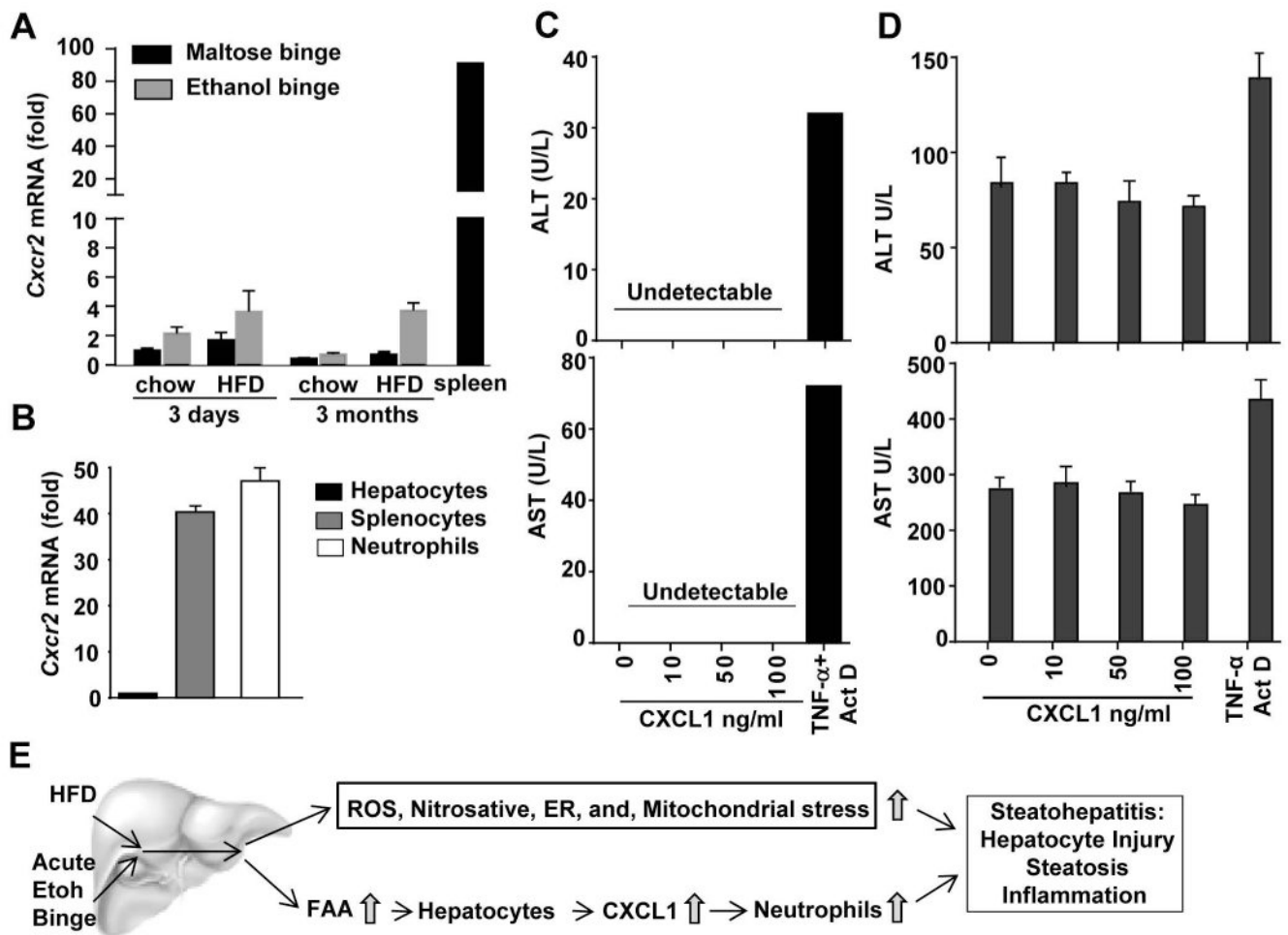


Figure 8. Hepatocytes express low levels of *Cxcr2* mRNA, and CXCL1 does not induce hepatocyte death *in vitro*

(A) Mice were fed chow or a HFD for 3 days or 3 months, followed by gavage with a single dose of maltose or ethanol. Mice were euthanized 9 h post-gavage. Liver tissues were collected for real-time PCR analysis of *Cxcr2* mRNA. Spleen tissues from normal C57BL/6 mice were used as positive controls for *Cxcr2* mRNA expression. (B) Hepatocytes, splenocytes, and blood neutrophils were isolated from C57BL/6 mice, and subjected to real-time PCR for *Cxcr2* mRNA. (C, D) Hepatocytes were isolated from chow-fed (panel C) or 3m-HFD-fed mice (panel D), and were incubated with various concentrations of CXCL1 or TNF- α +Actinomycin D. Supernatant was collected 24 h post incubation and used for measurement of ALT and AST. (E) **The mechanisms underlying the synergistic effect of HFD and acute ethanol binge on steatohepatitis.**