Supporting Materials

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

Binxia Chang,[1,3]Ming-Jiang Xu,[1]Zhou Zhou,[1]Cai Yan,[1]Man Li,[1]Wei Wang,[1] Dechun Feng,[1]Adeline Bertola,[1]Hua Wang,[1]George Kunos,[2]Bin Gao[1]

[1]Laboratory of Liver Diseases, [2]Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD 20892, USA; [3]Diagnosis and Treatment Center for Non-Infectious Liver Diseases, Institute of Alcoholic Liver Disease, Beijing 302 Hospital, Beijing.

Materials and Methods:

Histopathological analysis. Liver tissue was collected at various time points post-gavage and samples were fixed in 10% formalin and paraffin-embedded following standard procedure. Embedded liver tissue was cut to 4μm thickness and subjected to staining with hematoxylin and eosin (H&E) or subjected to immunohistochemical staining for myeloperoxidase (MPO) by using a prediluted rabbit anti-MPO polyclonal antibody (Biocare Medical, LLC, Concord, CA) and a rabbit ABC staining kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instruction. MPO positive cells were quantified randomly from 10 fields at 100x magnification per mouse.

Biochemical assays. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using a Catalyst Dx Chemistry Analyzer (IDEXX Laboratories, Inc, Westbrook, ME). Liver triglycerides were extracted with a 2:1 chloroform:methanol mixture and measured using the EnzyChrom Triglyceride Assay Kit (BioAssays Systems, Hayward, CA).

Measurement of serum and liver free fat acids (FFAs). Serum and liver FFAs were measured with a commercial kit from BioVision (Catalog #K612-100; Milpitas, CA). Samples were prepared and measured according to the manufacturer's instruction.

Isolation of hepatic leukocytes and flow cytometry analysis. Liver tissues were passed through a 70 μm cell strainer in phosphate buffered saline (PBS) and the cell suspension was centrifuged at 30*g* for 5 minutes to pellet the hepatocytes. The supernatant enriched liver leukocytes was centrifuged at 300*g* for 10 minutes. The pellet was resuspended in 15 ml of 35% Percoll (GE Healthcare, Pittsburgh, PA) and centrifuged at 500*g* for 15 minutes. The resulting pellet containing leukocytes was resuspended in 2 ml of ACK lysing buffer (BioWhittaker, Walkersville, MD). After incubation for 5 minutes on ice, cells were washed in PBS containing 2% fetal bovine serum. Cells were preincubated with Mouse BD Fc Block™ (purified rat anti-mouse CD16/CD32, clone 2.4G2, BD Biosciences, San Diego, CA) at 4°C for 10 minutes, and then stained with antibodies of interest for 30 minutes at 4°C. The following antibodies were used: anti-F4/80 (clone BM8, eBioscience, San Diego, CA), anti-Gr-1 (clone RB6-8C5, eBioscience), and anti-CD11b (clone M1/70, BD Biosciences). Flow cytometry analysis was performed using a FACSCalibur (BD Biosciences).

Isolation of hepatocytes, hepatic stellate cells (HSCs), sinusoidal endothelial cells (ECs) for real-time PCR analyses. For isolation of mouse hepatocytes, mice weighing 18‑22 g were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally), and the portal vein was cannulated under aseptic conditions. The liver was perfused first with a solution containing 5.4 mmol/L KCl, 0.44 mmol/L KH2PO4, 140 mmol/L NaCl, 0.34 mmol/L Na2HPO4, 0.5 mmol/L EGTA, and 25 mmol/L Tricine, pH 7.2 (EGTA solution) and then with DMEM containing 0.075% type I collagenase (Sigma), followed by an additional digestion step (0.009% collagenase at 37°C with agitation for 15 min), and centrifugation as described previously.[1] The isolated mouse hepatocytes were immediately subjected to total RNA extraction using TRIzolreagent (Invitrogen) and real-time PCR analyses.

Mouse HSCs were isolated by *in situ* collagenase perfusion and differential centrifugation on OptiPrep (Sigma) density gradients. Mouse livers were perfused in situ first with EGTA solution as described above, then with perfusion buffer (0.075% collagenase type I in GBSS buffer with 0.02% DNase I), and digested in digestion buffer (0.009% collagenase type I in GBSS buffer with 0.02% DNase I) at 37°Cfor 20-30 min. The homogenate was filtered, centrifuged at 25g for 5 min at roomtemperature to remove the hepatocytes. The supernatant was transferred to a new

tube andcentrifuged at 400g for 10 min at 4°C. The cell pellet was then resuspended in 5 mL of 15%OptiPrep, and loaded carefully with 5 mL of 11.5% OptiPrep, and centrifuged at 1400g for17 min at 4°C. The cell fraction in the GBSS and 11.5% OptiPrep interphase was gentlyaspirated, mixed with GBSS, and centrifuged at 1400g for 10 min at 4°C. After anotherwash, the final cell pellet wasimmediately subjected to total RNA extraction using TRIzolreagent (Invitrogen) and real-time PCR analysis.

Mouse liver sinusoidal ECs were isolated as described previously.[2] The isolated ECs from several mice were pooled and subjected to total RNA extraction using TRIzol reagent and real-time PCR analysis.

Primary mouse hepatocyte culture and treatment with FFAs and CXCL1 in vitro. Primary hepatocytes were isolated from C57BL/6J mice and cultured *in vitro* as described previously.[3] Various doses of FFAs were added to the culture. Palmitic acid (PA; Catalog #10006627) and oleic acid (OA; Catalog #90260) were from Cayman Chemical Company (Ann Arbor, MI); linoleic acid (LA; Catalog #CDS003308), ethyl palmitic acid (EPA; Catalog #P9009) and ethyl linoleic (ELA; Catalog #L1751) were from Sigma-Aldrich. All FFAs were conjugated to bovine serum albumin (BSA, Sigma-Aldrich Catalog #A7030) by adding FFAs to 10% BSA-Tris buffer (pH 8.0) and shaking over-night at 37ºC. The final FFA concentration was 8 mM with the ratio of 5:1 for FFAs to BSA.

In some experiments, several kinase inhibitors were added 30 min before addition of FFAs. Three hours later after incubation with FFAs, hepatocytes were collected for real-time PCR analyses for CXCL1 mRNA.

Various doses of CXCL1 were added to the culture. ALT and AST levels in the supernatant were measured 24 h post culture. TNFa+actinomycin D were used as positive controls to induce hepatocyte death.

Real-time quantitative polymerase chain reaction (Real-time PCR). Total RNA was extracted using TRIzol Reagent (Invitrogen) following the manufacturer's protocols. cDNA was

synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed in duplicate for each sample using the ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). The reaction mixture contained 10 µl of SYBR Green Master Mix (Applied Biosystems), 0.5 µM of forward and reverse primers (Invitrogen) and 5µl of cDNA (corresponding to 50 ng of RNA) in a total volume of 20 µl. The PCR conditions were: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Amplification of specific transcripts was confirmed by melting curve profiles generated at the end of the PCR program. Expression levels of target genes were normalized to the expression of 18S and calculated based on the comparative cycle threshold Ct method ($2^{-\Delta\Delta Ct}$). Primer sequences are shown in SupplementaryTable 1.

Supplemental Table I: Primer sequences for real-time PCR.

Target gene	Forward primer (5'→3')	Reverse primer (5'→3')
TNF-a	AGGCTGCCCCGACTACGT	GACTTTCTCCTGGTATGAGATAGCAAA
IL-1β	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
IL-6	ACAAGTCGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC
IL-4	TTGAACGAGGTCACAGGAGA	AAATATGCGAAGCACCTTGG
IFN-g	TAGCCAAGACTGTGATTGCGG	AGACATCTCCTCCCATCAGCAG
CXCL1	TCTCCGTTACTTGGGGAC	CCACACTCAAGAATGGTCGC
CXCL2	TCCAGGTCAGTTAGCCTTGC	CGGTCAAAAAGTTTGCCTTG
CXCR2	ATGCCCTCTATTCTGCCAGAT	GTGCTCCGGTTGTATAAGATGAC

The primer sequences for SELP, SELE, ICAM, VCAM and other chemokines (CCR1, CCR2 etc) are described previously.[4, 5]

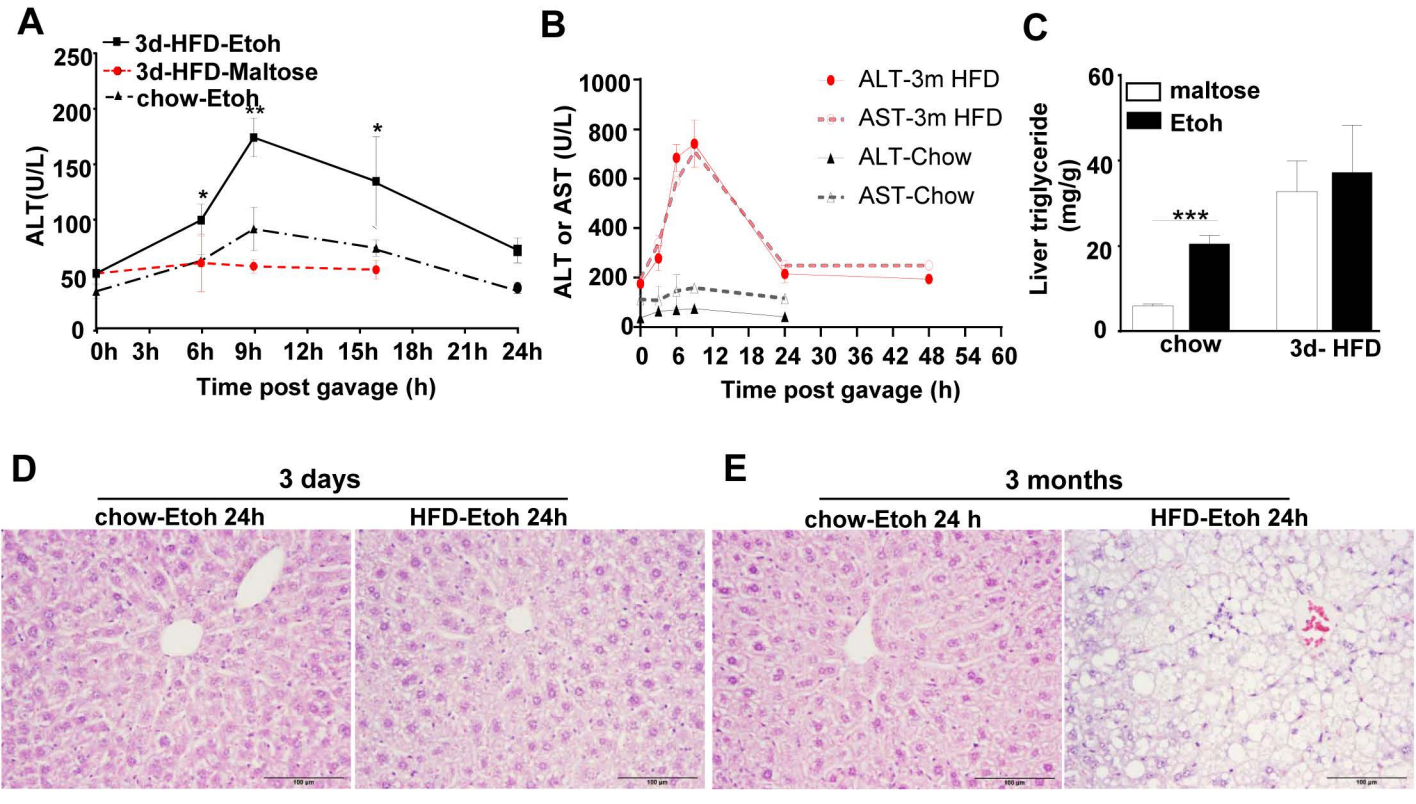
CXCL1 blockade. CXCL1 was blocked by intravenous injection of a mouse CXCL1/KC neutralizing antibody (150 mg/mouse) (MAB453, R&D systems, Minneapolis, MN) 15 min before gavage. Control animals were injected with a rat IgG2a isotype control antibody (2A3, BioXCell, West Lebanon, NH).

Treatment of mice with adenovirus-Cxcl1 and control virus. Adenovirus expression mouse *Cxcl1* was made and purchased from Applied Biological Materials Inc.(Richmond, BC, Canada). Mice were fed chow diet or a HFD for 3 months, followed by intravenous injection of control (Ad-control) or mouse *Cxcl1* (Ad-*Cxcl1*) adenovirus ($2.5x10^8$ *pfu*) for 6 days. Serum and liver tissues were collected for experiments.

Reference:

1. Sun R, Park O, Horiguchi N, et al. STAT1 contributes to dsRNA inhibition of liver regeneration after partial hepatectomy in mice. Hepatology 2006;44:955-66.
2. Vrochides D, Papanikolaou V, Pertoft H, et al. Biosynthesis and degradation of hyaluronan by nonparenchymal liver cells during liver regeneration. Hepatology 1996;23:1650-5.
3. Sun R, Tian Z, Kulkarni S, et al. IL-6 prevents T cell-mediated hepatitis via inhibition of NKT cells in CD4+ T cell- and STAT3-dependent manners. J Immunol 2004;172:5648-55.
4. 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;58:1814-23.
5. Jaruga B, Hong F, Kim WH, et al. IFN-gamma/STAT1 acts as a proinflammatory signal in T cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: a critical role of IRF-1. Am J Physiol Gastrointest Liver Physiol 2004;287:G1044-52.

Supporting Fig. 1

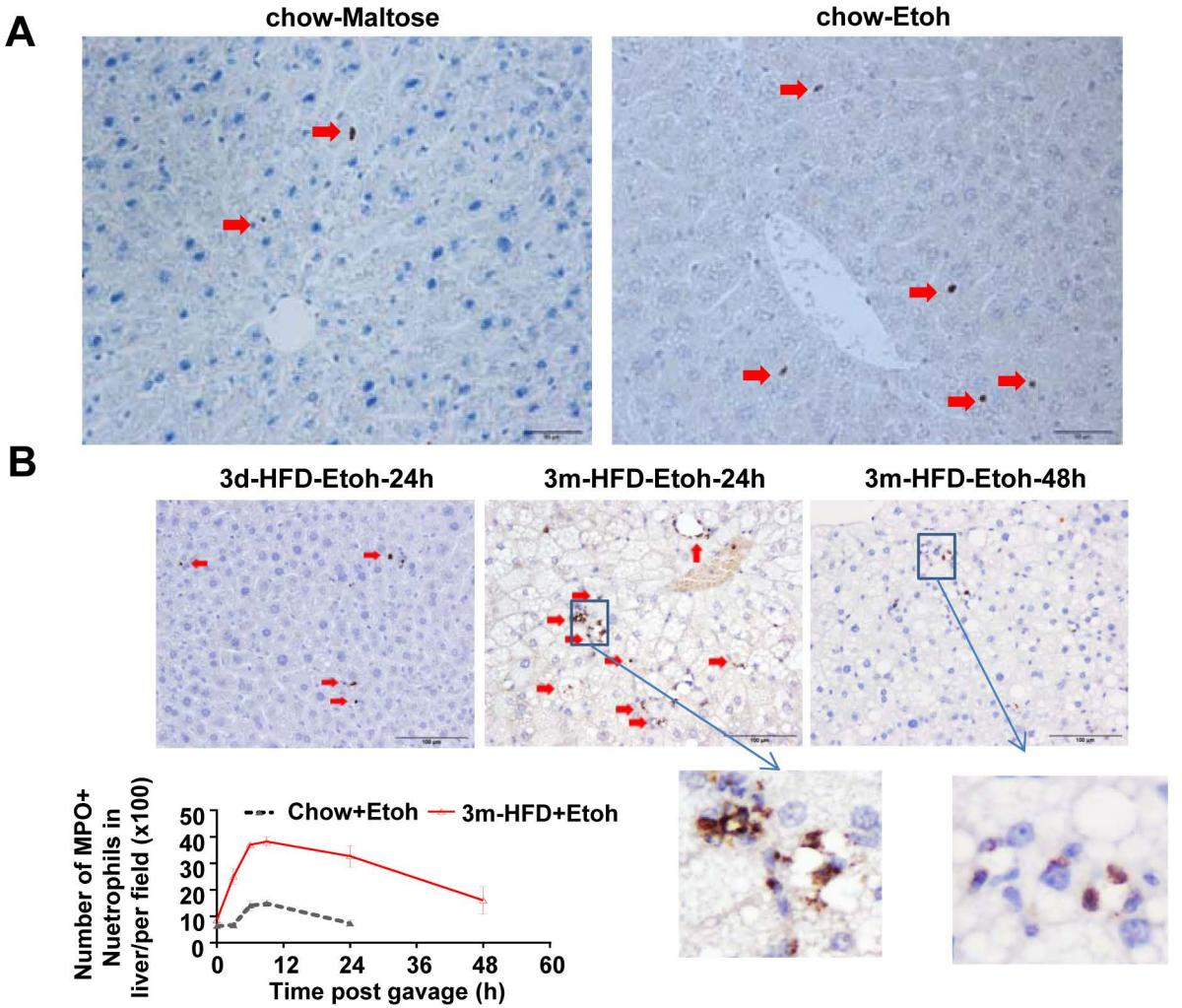


Supporting Fig. 1A and 1B. Serum levels of ALT at various time points post gavage of a single dose of ethanol or maltose in 3d- or 3m-HFD or chow-fed mice. C57BL/6J mice were fed a HFD diet or chow for 3 days or 3 months, and then given maltose or ethanol by oral gavage, mice were then euthanized at various time points. Serum ALT levels were measured. Values represent the mean ± SD (n=3-10). **P*<0.05, ***P*<0.01, ****P*<0.01 in comparison with corresponding chow-Etoh groups.

Supporting Fig. 1C. Hepatic triglyceride levels in 3d-HFD+ethanol binge fed mice. C57BL/6J mice were fed a HFD diet or chow for 3 days, and then given maltose or ethanol by oral gavage 9 h prior to euthanization. Liver triglyceride levels were measured. Values represent the mean ± SD (n=3-10). ****P*<0.001 in comparison with corresponding maltose groups. **Note:** As expected, hepatic triglyceride levels were increased in 3d-HFD-fed mice A single ethanol binge increased hepatic triglyceride levels in chow-fed mice but there was no difference between the 3d-HFD+ethanol and 3d-HFD+maltose groups.

Supporting Fig. 1D and 1E. H&E staining of liver tissues from various groups. C57BL/6J mice were fed a HFD diet or chow for 3 days (panel D) or 3 months (panel E), and then given ethanol or maltose by oral gavage, mice were then euthanized 24 h post gavage. Liver tissues were collected for H&E staining. **Note**: In panel D, hepatic steatosis was reduced 24 h post ethanol gavage in 3d-HFD+ethanol group compared to 3d-HFD+maltose or 3d-HFD+ethanol 9 h groups (as shown in Fig. 1B). In panel E: after gavage of a single dose of ethanol in these 3m-HFD-fed mice, steatosis was not further increased 9 h (as shown in Fig. 1B) and 24 h post ethanol gavage

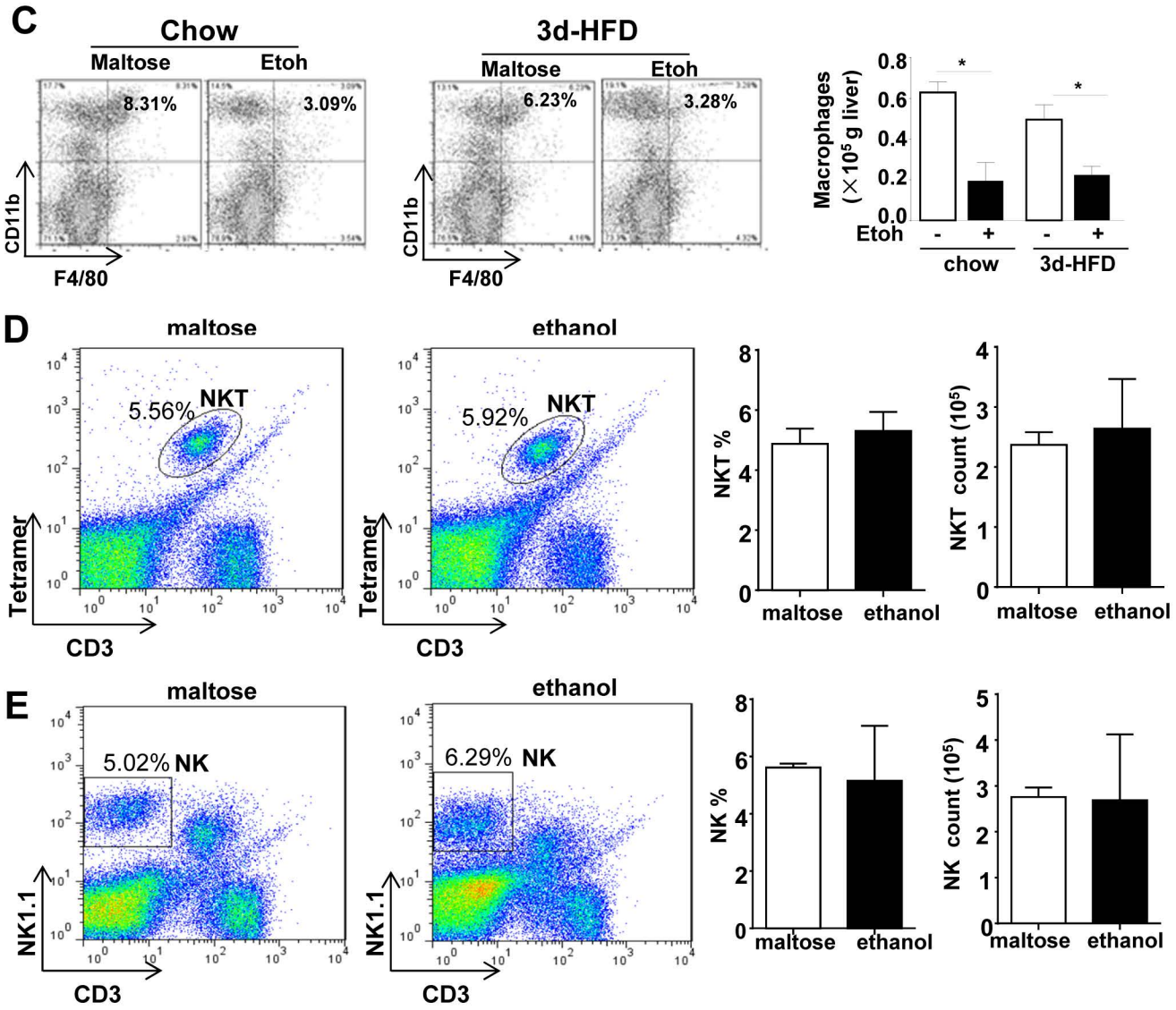
Supporting Fig. 2A, 2B, 2C



Supporting Fig. 2A. Acute ethanol gavage induces liver neutrophil infiltration. Mice were gavaged with a single dose of maltose or ethanol. Mice were euthanatized 9 h post-gavage. Liver tissues were subjected to immunostaining with an anti-MPO antibody. Representative immunostaining pictures are shown. Red arrows indicate MPO^+ neutrophils.

Supporting Fig. 2B. HFD feeding plus acute ethanol binge induce hepatic neutrophil infiltration. C57BL/6J mice were fed a HFD diet for 3 days or 3 months, and then given ethanol by oral gavage 24 h or 48 h prior to euthanization. Liver tissues were collected for MPO staining. Representative immunostaining pictures are shown. Red arrows indicate MPO^+ neutrophils. The number of MPO+ neutrophils in the liver was accounted and is shown.

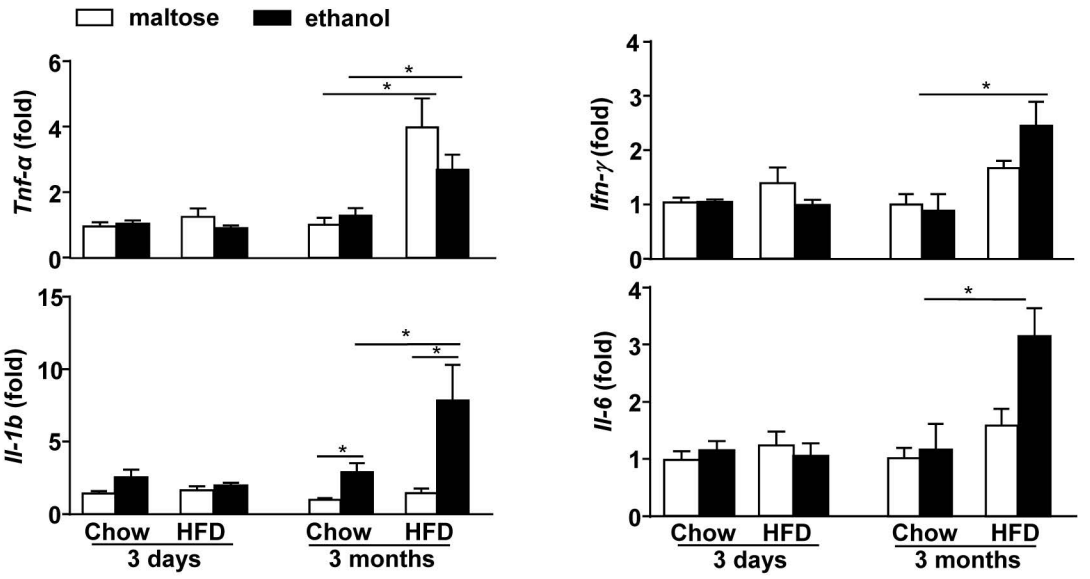
Supporting Fig. 2C, 2D, 2E



Supporting Fig. 2C. Acute ethanol gavage reduces liver macrophages. Mice were fed a HFD diet or chow for 3 days, and then given maltose or ethanol by oral gavage 9 h prior to euthanization. Liver leukocytes were isolated and subjected to flow cytometric analysis. The total numbers of macrophages ($F4/80^+CD11b^+$) were determined. *P<0.05

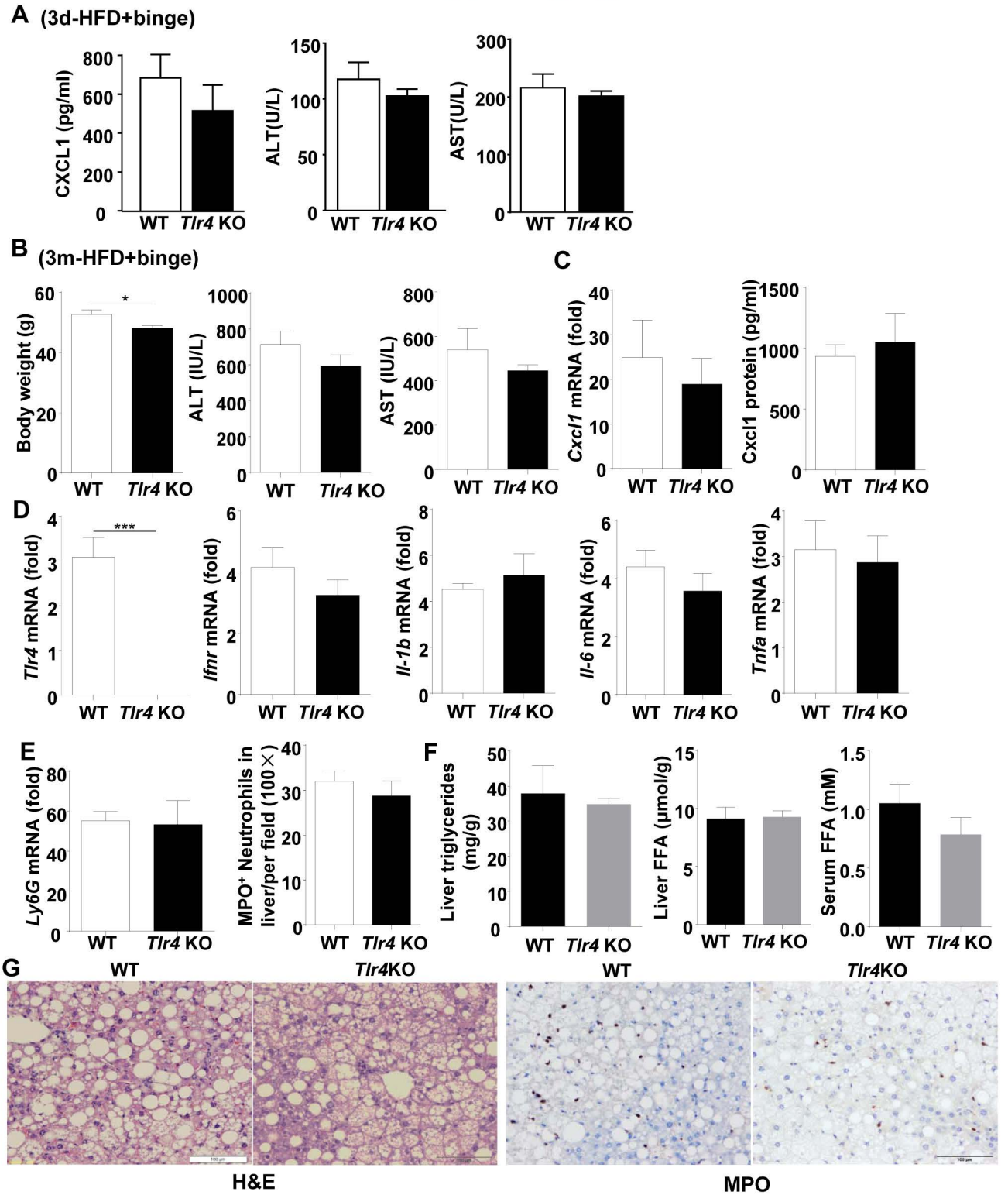
Supporting Fig. 2D, 2E. Acute ethanol gavage had no effect on liver NKT and NK cells in HFD-fed mice. Mice were fed a HFD diet for 3 months, and then given maltose or ethanol by oral gavage 9 h prior to euthanization. Liver leukocytes were isolated and subjected to flow cytometric analysis. The percentage and total numbers of NKT and NK cells were determined.

Supporting Fig. 3



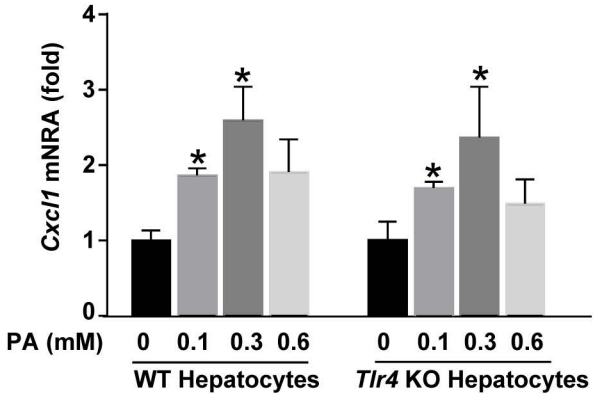
Supporting Fig. 3. Effects of acute ethanol and 3d-HFD feeding or 3m-HFD feeding on hepatic cytokine mRNA expression. Mice were fed chow or a HFD for 3 days or 3 months, followed by gavage with a single dose of maltose or ethanol. Mice were euthanatized 9 h post-gavage. Liver tissues were collected for total RNA isolation and real-time PCR analysis. Values represent the mean ± SD (n=5-8). *P<0.05

Supporting Fig. 4



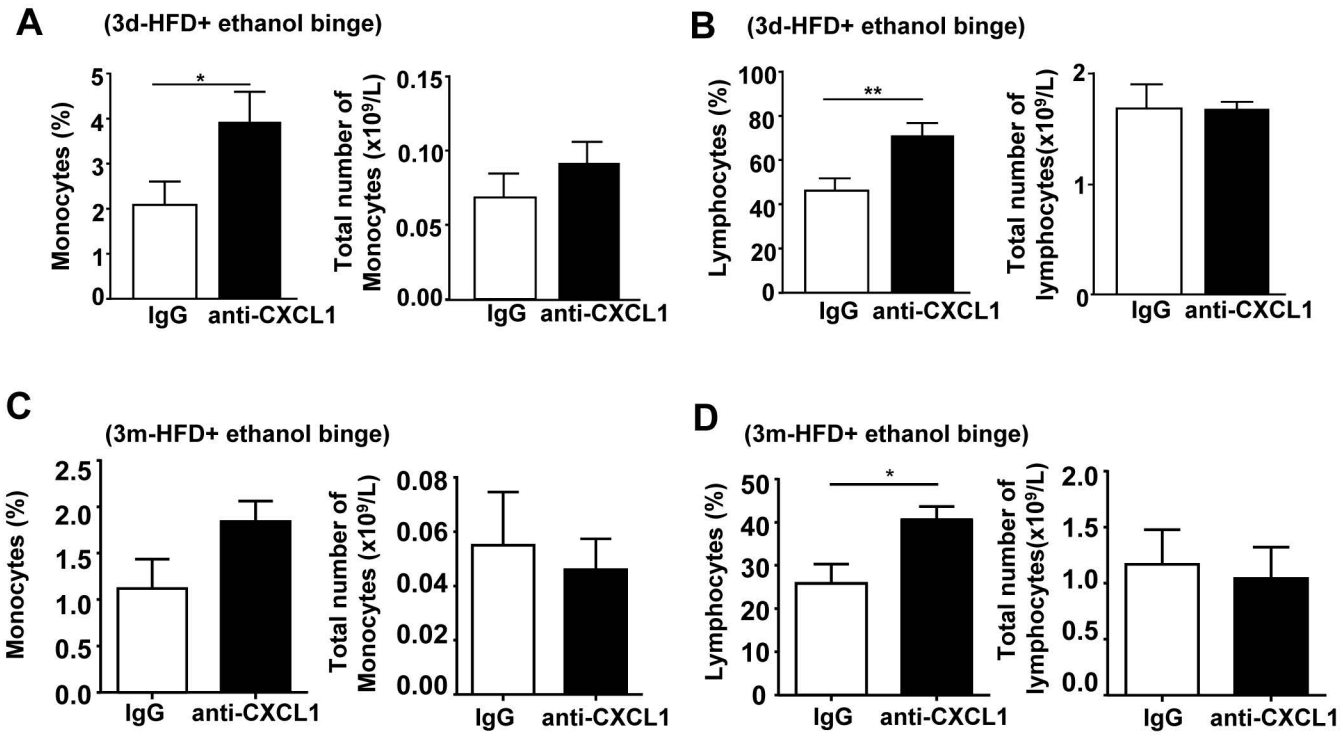
Supporting Fig. 4. Effects of 3d-HFD+ or 3m-HFD+ethanol binge feeding on hepatic neutrophil infiltration and injury in *Tlr4* KO and WT mice. (A) WT and *Tlr4* KO mice were fed a HFD for 3 days, followed by gavage with a single dose of ethanol. Mice were euthanatized 9h post-gavage. The serum levels of CXCL1, ALT and AST were measured. (B-G) WT and *Tlr4* KO mice were fed a HFD for 3 months, followed by gavage with a single dose of ethanol. Mice were euthanatized 9h post-gavage. (B) The body weight, serum levels of ALT and AST were measured, (C) liver CXCL1 mRNA and serum CXCL1 level were measured, (D) real-time PCR analyses of the liver, (E) liver neutrophils were assessed by *Ly6G* gene expression and MPO staining, (F) liver TG, FFA, and serum FFA were determined, (F) representative H&E staining and MPO staining are shown.

Supporting Fig. 5



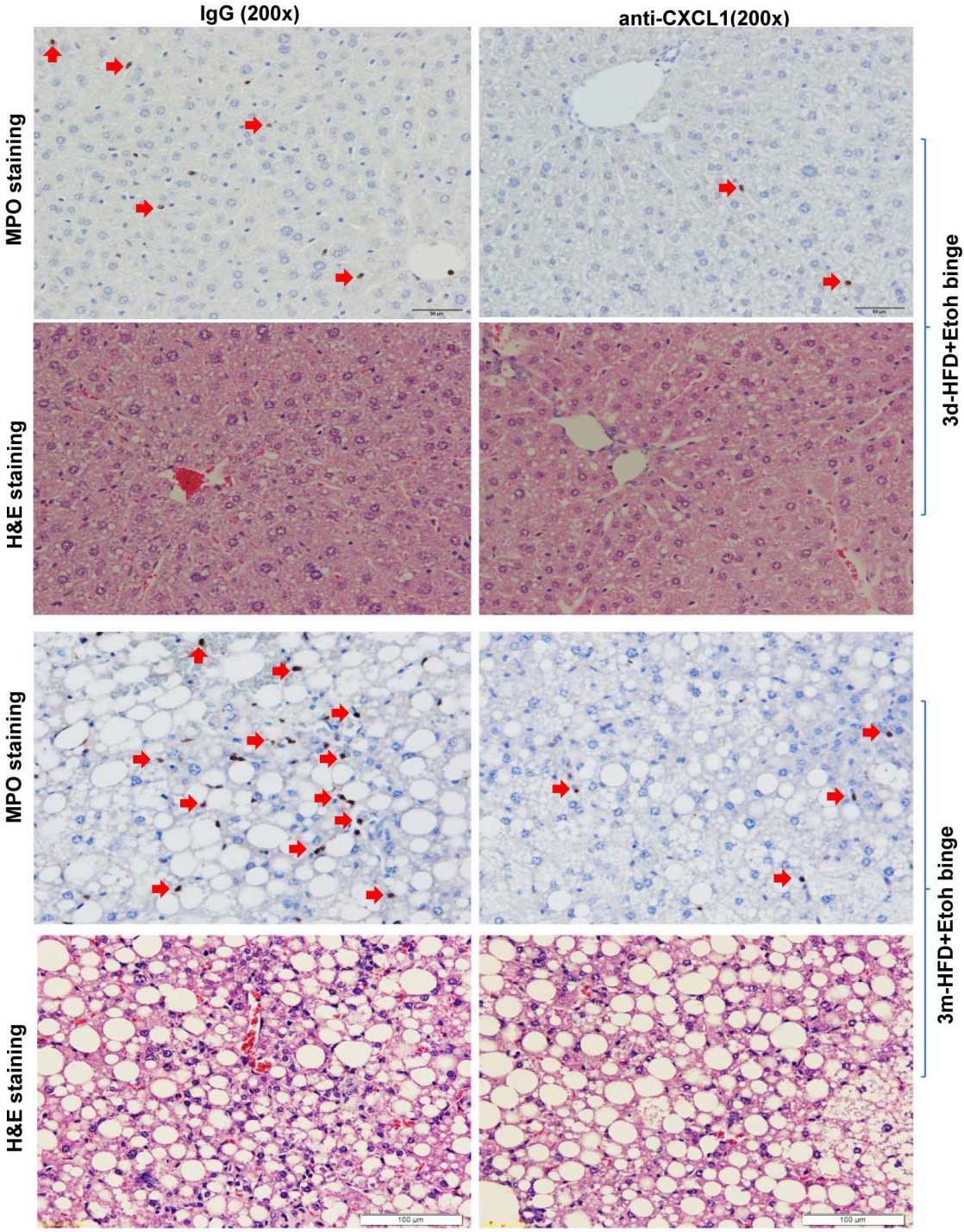
Supporting Fig. 5. PA upregulates *Cxcl1* mRNA expression in a TLR4-independent manner. Primary mouse hepatocytes from WT and *Tlr4* KO mice were stimulated with 0.3 mM PA for 3h, and *Cxcl1* mRNA expression was determined by real-time PCR. Values represent the mean ± SD (n=3). **P*<0.05 in comparison with corresponding 0 group.

Supporting Fig. 6



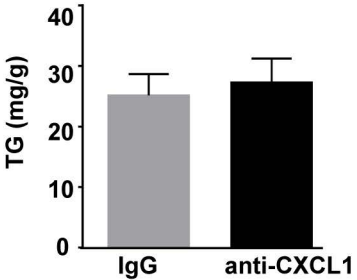
Supporting Fig. 6. Blockage of CXCL1 increases the percentage of circulating monocytes and lymphocytes without affecting their total numbers in 3d- or 3m-HFD+ethanol binge-fed mice. Mice were fed a HFD for 3 days (**A, B**) or 3 months (**C, D**), followed by gavage of a single dose of ethanol. Anti-CXCL1 antibody or IgG antibody was administrated into the mice 15 min before gavage. Mice were euthanatized 9 h post gavage. Blood leukocytes were collected, analyzed, and counted. Values represent means ± SD (n=4-6). **P*<0.05, ***P*<0.01.

Supporting Fig. 7



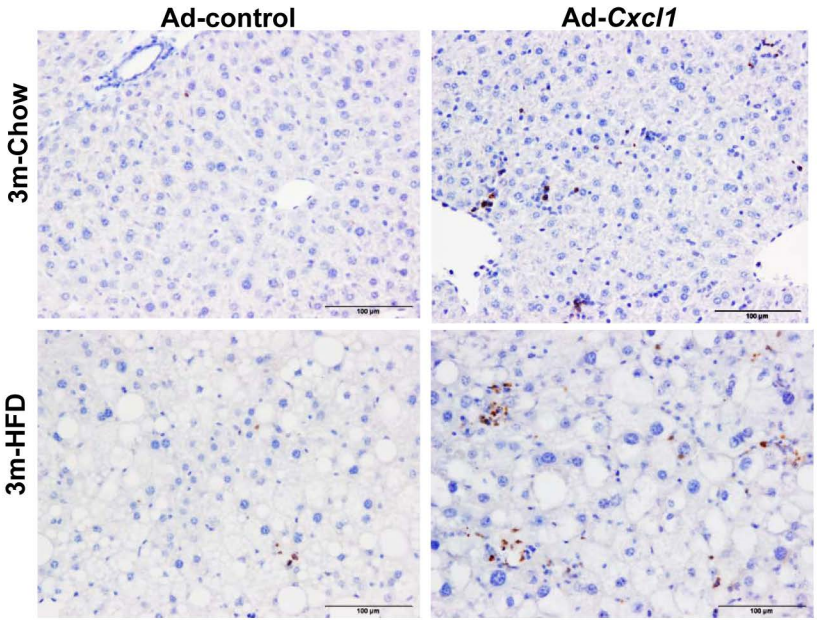
Supporting Fig. 7. Blockade of CXCL1 ameliorates 3d or 3m-HFD-ethanol binge-induced hepatic neutrophil infiltration. Mice were fed a HFD for 3 days or 3 months, followed by a single dose of ethanol. An anti-CXCL1 antibody or IgG control antibody was administered to the mice 15 min before gavage. Mice were euthanatized 9 h post-gavage. Liver tissues were subjected to immunostaining with an anti-MPO antibody or H&E staining. Representative images are shown.

Supporting Fig. 8



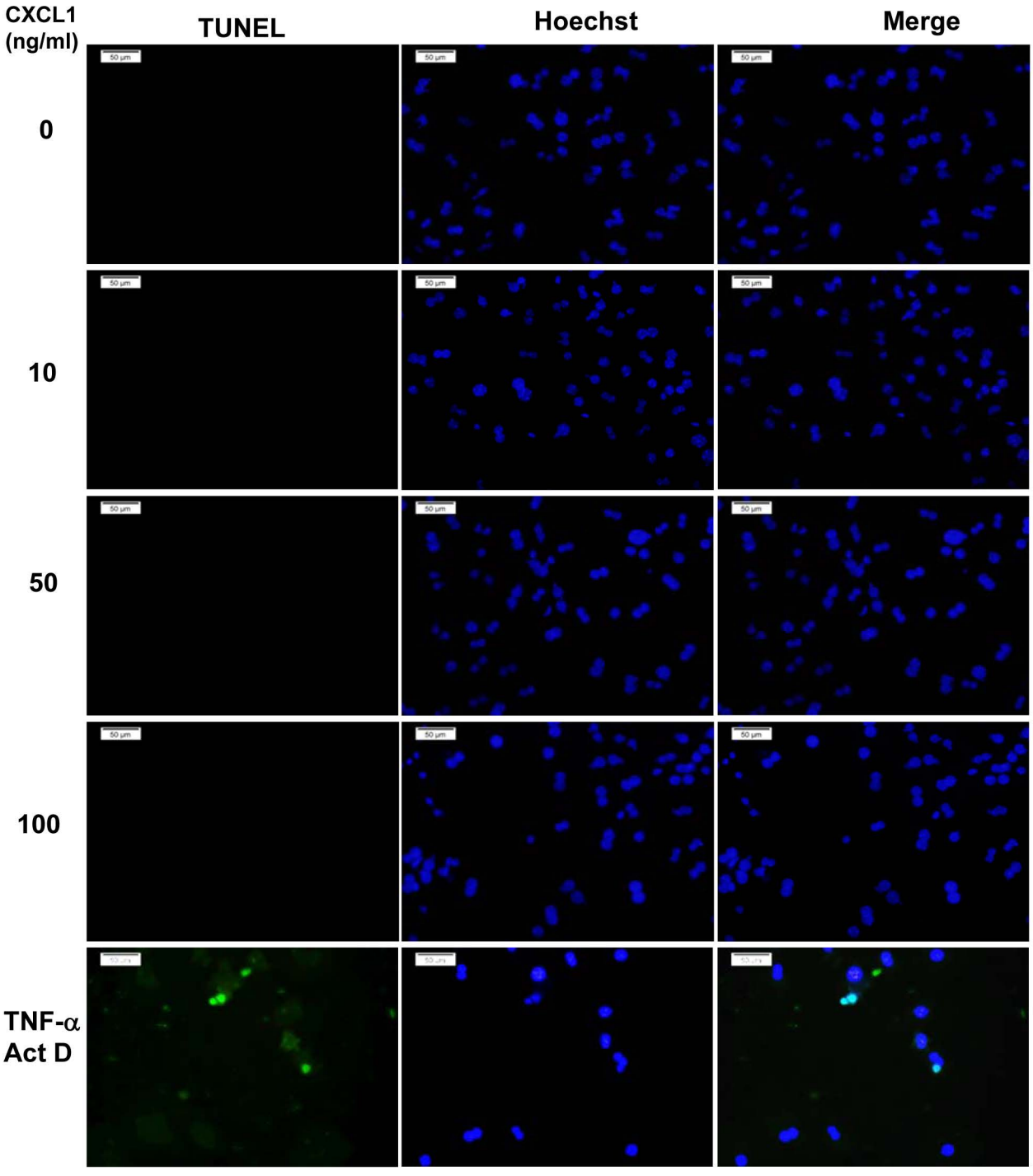
Supporting Fig. 8. Blockade of CXCL1 does not affect 3d-HFD-ethanol binge-induced hepatic steatosis. Mice were fed a HFD for 3 days, followed by a single dose of ethanol. An anti-CXCL1 antibody or IgG control antibody was administered to the mice 15 min before gavage. Mice were euthanatized 9 h post-gavage. Liver tissues were collected and hepatic triglycerid (TG) levels were measured. Values represent means $\pm$ SD.

Supporting Fig. 9



Supporting Fig. 9. Adenovirus-mediated *Cxcl1* overexpression increased neutrophils infiltration into the liver. Mice were fed chow diet or a HFD for 3 months, followed by intravenous injection of control (Ad-control) or mouse *Cxcl1* (Ad-*Cxcl1*) adenovirus (2.5×10^8 *pfu*) for 6 days. Representative MPO staining in the liver tissue is shown.

Supporting Fig. 10



Supporting Fig. 10: Primary mouse hepatocytes were treated with 0, 10, 50 and 100 ng/ml CXCL1 for 24h. Apoptosis of the cells were analyzed with an Alexa Fluor 488 labeled TUNEL staining kit. 50 ng/ml TNF-α plus actinomycin D treatment was used as a positive control for the apoptotic analysis. The images shown represent one of two independent experiments. Note: TNF-α+Act D treatment induced massive hepatocyte death, whereas CXCL1 treatment did not induce hepatocyte death.