Supporting Materials

Protective and Detrimental Roles of p38α MAPK in Different Stages of Nonalcoholic Fatty Liver Disease

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Supporting Materials and Methods

Isolation of human peripheral neutrophils

Human blood neutrophils were isolated from the blood donated by healthy individuals that was obtained from the Clinical Center, National Institutes of Health (Bethesda, MD) according to a standard density gradient separation method as described before.[1,2]

Isolation of mouse bone marrow-derived neutrophils

Mouse bone marrow was collected from femur and tibia and passed through a 70 μm cell strainer in PBS, and the cell suspension was centrifuged at 300×g for 5 min. The resulting leukocyte pellet was resuspended in ACK lysing buffer (BioWhittaker, Walkersville, MD). After incubation for 2 min on ice, the cells were washed in PBS. The leukocytes were subjected to neutrophil isolation by using Neutrophil Isolation Kit (Miltenyi Biotec, San Diego, CA) according to the manufacturer's instructions.

Isolation and culture of primary human hepatocytes

Freshly isolated primary human hepatocytes were obtained from the University of Pittsburgh Medical Center (Pittsburgh, PA) and cultured in DMEM containing 10% fetal bovine serum, penicillin-streptomycin, insulin (100 nM), and dexamethasone (100 nM).

Isolation and culture of primary mouse hepatocytes

Mice were anesthetized with 30 mg/kg pentobarbital sodium intraperitoneally. Portal vein was cannulated and perfused with ethylene glycol tetraacetic acid and the liver tissue was digested with 0.075% collagenase (Worthington, Lakewood, NJ).[3] Primary hepatocytes were collected after centrifugation at 50×g for 5 min and cultured in DMEM containing 10% fetal bovine serum, penicillin-streptomycin, insulin (100 nM), and dexamethasone (100 nM). After 4 hr of culture, culture media were replaced, and hepatocytes were treated with vehicle or PA (100 μM) for 24 hr. PA (Sigma, St. Louis, MO) was prepared by conjugation to FA-free bovine serum albumin (Sigma, St. Louis, MO) as described before.[4] In other experiments, hepatocytes were treated with vehicle or H_2O_2 (500 μM, 15 min or 5 hr) with or without pretreatment with MG132 (20 μM, 3 hr). After the treatment period, ALT measurement, Western blot analyses, RT-qPCR analyses, and oil red O staining were performed as described in the separate sections below.

Culture of AML12 cells

Mouse hepatocyte cell line AML12 cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium with 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium, 40 ng/mL dexamethasone, 10% fetal bovine serum, and penicillin-streptomycin. When cells reached 80% confluence, cells were treated with LY-2228820 (1 or 3 μM), PH-797804 (1 or 3 μM), or vehicle for 1 hr followed by a treatment with PA (100 μM, 24 hr), H_2O_2 (500 μM, 15 min or 5 hr), or vehicle. In some experiments, cells were pretreated with MG132 (20 μM) for 3 hr to block proteasomal activity. After the treatment period, ALT measurement, Western blot analyses, and oil red O staining were conducted as described in the separate sections below.

Co-culture of neutrophils with or without primary hepatocytes and chemotaxis assay

Primary human or mouse hepatocytes, or absence of hepatocytes as control were cultured in the bottom chamber of the transwell system and treated with TNF-α, IL-1β, or IL-6 of the corresponding species (Peprotech, Rocky Hill, NJ) for 9 hr, followed by an addition of neutrophils

of the corresponding species to the insert (1×10^6 cells, mouse; 1×10^6 or 4×10^6 cells, human). After 3-hr incubation, migrating neutrophils were collected from the bottom chamber media and subjected to hemocytometer-assisted cell counting after low-speed centrifugation to remove primary hepatocytes. The levels of CXCL1 and IL-8 in the culture media were measured after 12-hr incubation with TNF-α, IL-1β, or IL-6 using Mouse CXCL1/KC Quantikine ELISA kit, Human CXCL1/KC Quantikine ELISA kit, and Human IL-8/CXCL8 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Migrating neutrophils were collected from the bottom chamber media and subjected to hemocytometer-assisted cell counting after low-speed centrifugation to remove primary hepatocytes

In vitro ALT measurement

Culture medium was collected and centrifuged at 1000×g for 10 min. ALT levels were determined from the resulting supernatant using ALT (SGPT) Kinetic Kit (Teco Diagnostics, Anaheim, CA) according to the manufacturer's instructions.

Oil red O staining

Cells were fixed for 15 min with 4% paraformaldehyde, followed by 3 times of washing with PBS. After 45 min of staining with 0.6% oil red O solution in 60% isopropyl alcohol, cells were washed with 60% isopropyl alcohol and distilled water (3 times each), and counterstained with hematoxylin. Positive areas in 5 randomly selected high-power fields were analyzed.

Serum analysis

Serum samples were prepared from the mouse blood drawn from the retro-orbital plexus. Serum ALT levels were measured by ALT (SGPT) Kinetic Kit (Teco Diagnostics, Anaheim, CA) according to the manufacturer's instructions. Serum levels of mouse CXCL1 and human IL-8 were measured by Mouse CXCL1/KC Quantikine ELISA kit and Human IL-8/CXCL8 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Measurement of hepatic triglyceride content

Approximately 50 mg of the frozen liver tissues were used for determination of triglyceride content with Triglyceride Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI), according to the assay protocol.

Histological and immunohistochemical analysis

Formalin-fixed liver samples were processed, and 4-μm-thick paraffin sections were stained with Sirius Red dyes (Sigma, St. Louis, MO). TUNEL staining was performed with an ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Burlington, MA). For immunohistochemistry, after heat-induced epitope retrieval, paraffin-embedded sections were incubated in 3% H_2O_2 and blocked in 3% normal serum buffer. Sections were incubated with primary antibodies overnight at 4°C. Vectastain Elite ABC Staining Kit and DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA) were used to visualize the staining according to the manufacturer's instructions. Primary antibodies used include those specific to MPO (Biocare Medical, Concord, CA), Ly6G (BioXCell, West Lebanon, NH), MDA (Genox, Baltimore, MD), 4-HNE (Genox, Baltimore, MD), F4/80 (Cell Signaling Technology, Danvers, MA), and α-SMA (Agilent DAKO, Santa Clara, CA). Positive cells and positive areas in 10 randomly selected high-power fields were analyzed.

Western blot analysis

Liver tissues and cells were homogenized or lysed in RIPA buffer containing a cocktail of protease inhibitors (Santa Cruz Biotechnology, Dallas, TX) according to the manufacturer's instructions. Protein extracts were loaded onto 4-12% Bis-Tris protein gels (Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes (Thermo Fisher, Waltham, MA). Protein bands were visualized with Pierce ECL Western Blotting Substrate (Thermo Fisher, Waltham, MA). The antibodies against cleaved CASP3, CASP3, p-p38 (Thr180/Tyr182), p38, BCL2, CHOP, BIP, p-eIF2α (Ser51), eIF2α, p-PERK (Thr980), PERK, p38α, and p-MK2 (Thr334) were purchased from Cell Signaling Technology (Danvers, MA). The antibodies against p-PPARα (Ser12) and PPARα were purchased from Thermo Fisher (Waltham, MA). The antibodies against β-actin, p-BCL2 (Ser87), and MK2 were purchased from Abcam (Cambridge, MA).

Total RNA isolation and RT-qPCR

Total RNA was purified from liver tissues or cell cultures using TRIzol reagents (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA). The expression levels of mRNA were measured by RT-qPCR with ABI7500 RT-PCR system (Applied Biosystems, Foster City, CA). *Gapdh* or *Apob* was used as the invariant control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the level of mRNA. The primer sequences used for PCR reactions are listed in Supporting Table 1.

References

1) Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. Nat Med 2016;22:146-153.

2) Li H, Feng D, Cai Y, Liu Y, Xu M, Xiang X, et al. Hepatocytes and neutrophils cooperatively suppress bacterial infection by differentially regulating lipocalin-2 and neutrophil extracellular traps. Hepatology 2018;68:1604-1620.

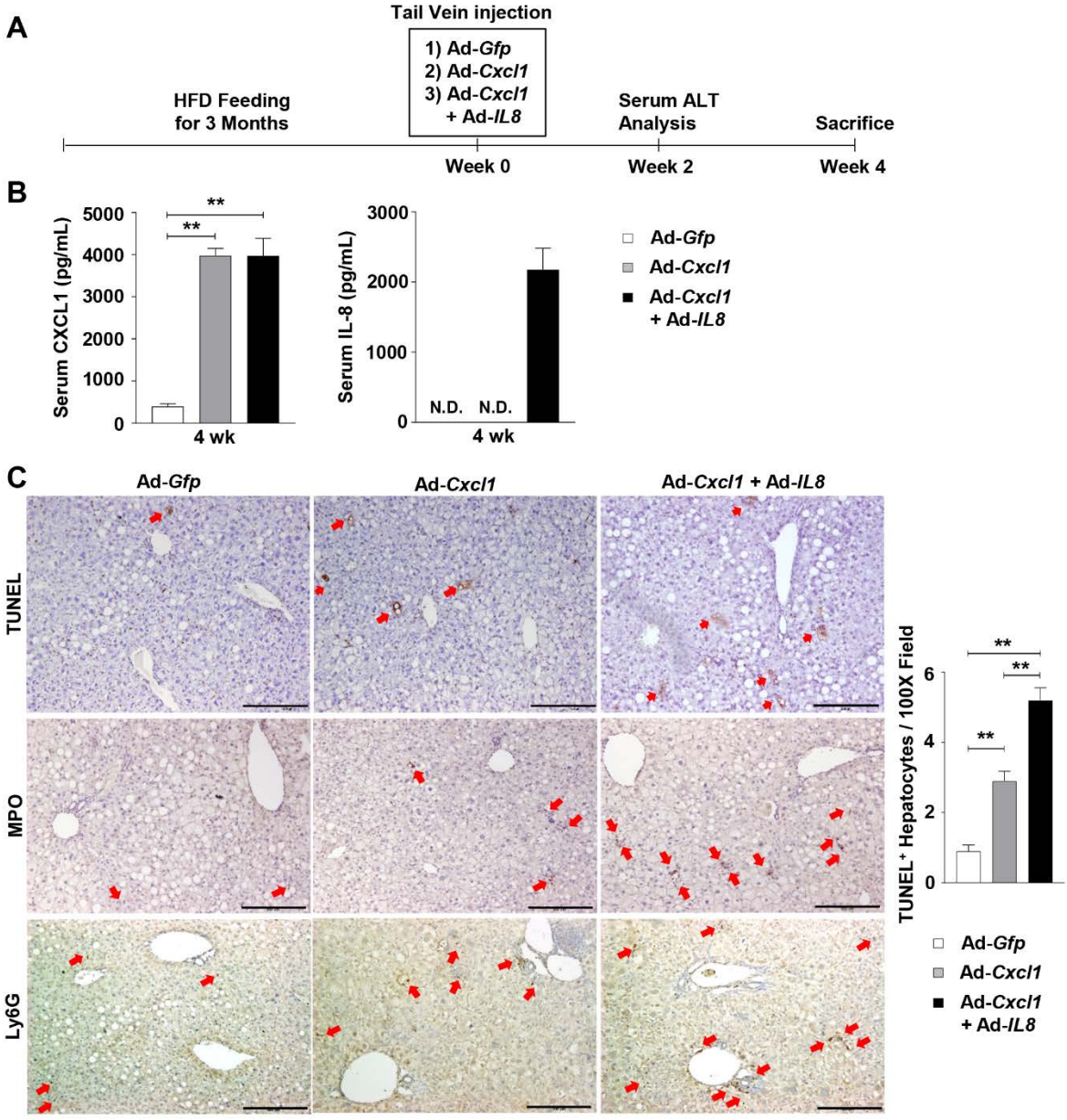
3) He Y, Hwang S, Cai Y, Kim SJ, Xu M, Yang D, et al. MicroRNA-223 Ameliorates Nonalcoholic Steatohepatitis and Cancer by Targeting Multiple Inflammatory and Oncogenic Genes in Hepatocytes. Hepatology 2019;70:1150-1167.

4) **Chang B, Xu MJ,** Zhou Z, Cai Y, Li M, Wang W, et al. Short- or long-term high-fat diet feeding plus acute ethanol binge synergistically induce acute liver injury in mice: an important role for CXCL1. Hepatology 2015;62:1070-1085.

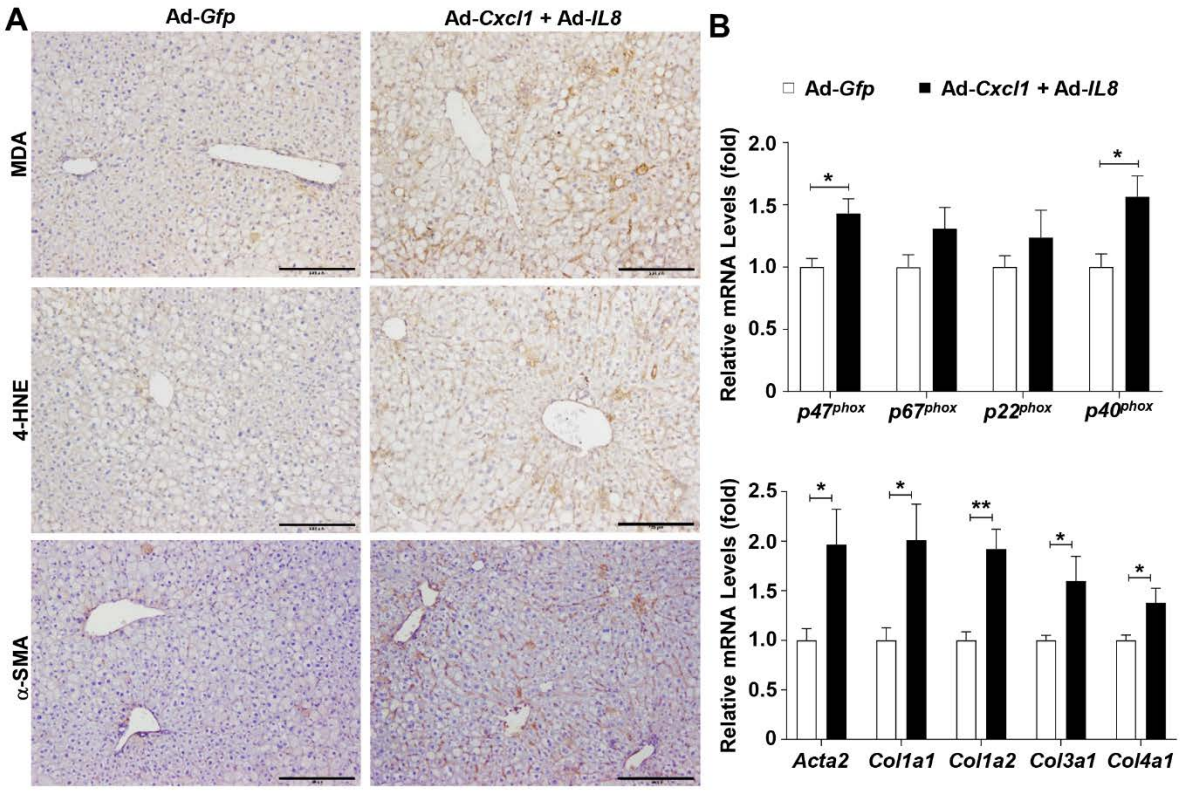
Author names in bold designate shared co-first authorship.

Supporting Table 1. Primer sequences for RT-qPCR

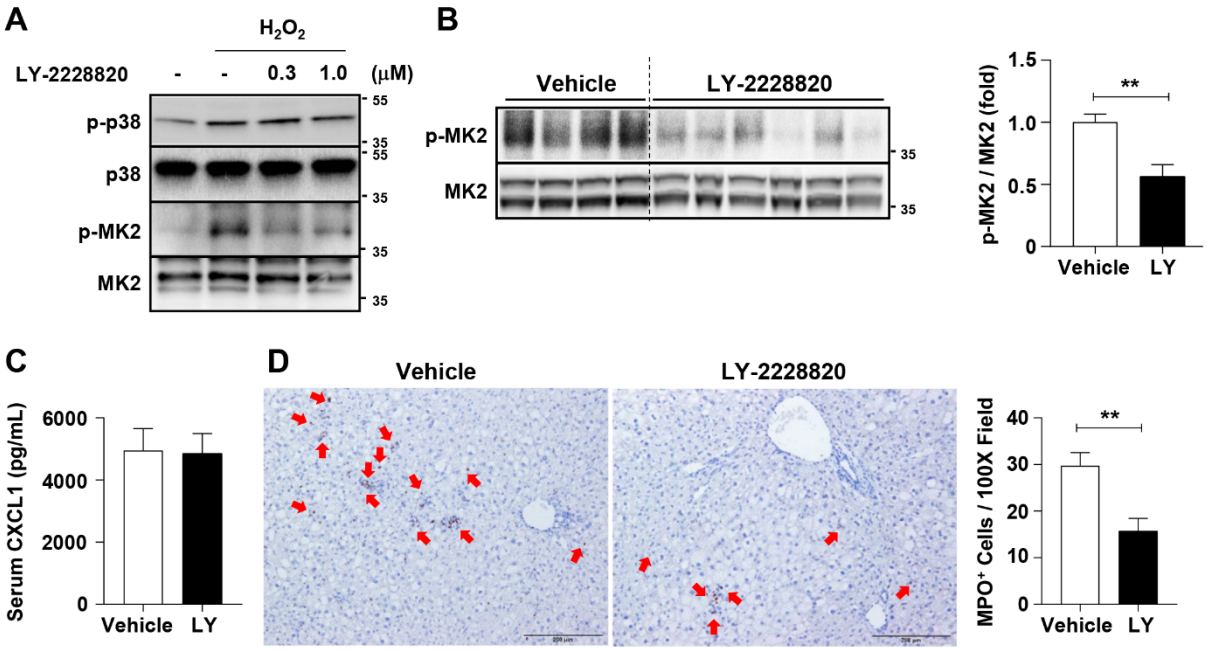
Gene Name	Forward (5'-3')	Reverse (5'-3')
Acc1	TGGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA
Acox1	AGATTGGTAGAAATTGCTGCAAAA	ACGCCACTTCCTTGCTCTTC
Acta2	TCCTGACGCTGAAGTATCCGATA	GGTGCCAGATCTTTTCCATGTC
Apob	CGTGGGCTCCAGCATTCTA	TCACCAGTCATTTCTGCCTTTG
Ccl2	TCTGGACCCATTCCTTCTTGG	TCAGCCAGATGCAGTTAACGC
Cd36	CCTGCAAATGTCAGAGGAAA	GCGACATGATTAATGGCACA
Col1a1	TAGGCCATTGTGTATGCAGC	ACATGTTCAGCTTTGTGGACC
Col1a2	GGTGAGCCTGGTCAAACGG	ACTGTGTCCTTTCACGCCTTT
Col3a1	TAGGACTGACCAAGGTGGCT	GGAACCTGGTTTCTTCTCACC
Col4a1	CACATTTTCCACAGCCAGAG	GTCTGGCTTCTGCTGCTCTT
Col4a2	GACCGAGTGCGGTTCAAAG	CGCAGGGCACATCCAACTT
Cpt1	CACCAACGGGCTCATCTTCTA	CAAAATGACCTAGCCTTCTATCGAA
Cpt2	AGCCTACCTGGTCAATGCATATC	GGGTTTGGGTATACGAGTTGAATT
Dgat1	TCCGTCCAGGGTGGTAGTG	TGAACAAAGAATCTTGCAGACGA
Dgat2	GCGCTACTTCCGAGACTACTT	GGGCCTTATGCCAGGAAACT
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Fasn	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
Fatp1	CCGTATCCTCACGCATGTGT	CTCCATCGTGTCCTCATTGAC
Fatp2	TCCTCCAAGATGTGCGGTACT	TAGGTGAGCGTCTCGTCTCG
Fatp5	GTTCTCCCGTCCAAGACCATT	GCTCCGTACAGAGTGTAGCAAG
Fgf21	CCTCTAGGTTTCTTTGCCAACAG	AAGCTGCAGGCCTCAGGAT
Gapdh	AGCAGCCGCATCTTCTTGTGCAGTG	GGCCTTGACTGTGCCGTTGAATTT
gp91phox (Nox2)	GACCATTGCAAGTGAACACCC	AAATGAAGTGGACTCCACGCG
Gpat1	ACAGTTGGCACAATAGACGTTT	CCTTCCATTTCAGTGTTGCAGA
Il1b	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
Il6	ACAAGTCGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC
Lcas	GGCTACTTGAAAGACCCAGCAA	AATGTCCCCCGTGTGTAACC
Ly6g	TGCGTTGCTCTGGAGATAGA	CAGAGTAGTGGGGCAGATGG
p22phox	ATGGAGCGATGTGGACAGAAG	TAGATCACACTGGCAATGGCC
p40phox (Ncf4)	ATCGTCTGGAAGCTGCTCAA	CCCATCCATCTGCTTTTCTG
p47phox (Ncf1)	TCCTCTTCAACAGCAGCGTA	CTATCTGGAGCCCCTTGACA
p67phox (Ncf2)	TCTATCAGCTGGTTCCCACG	TGGCCTACTTCCAGAGAGGA
Ppara	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
Pparg	CACAATGCCATCAGGTTTGG	GCTGGTCGATATCACTGGAGATC
Scd1	CCGGAGACCCCTTAGATCGA	TAGCCTGTAAAAGATTTCTGCAAACC
Srebp1c	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT
Timp1	GCATCTCTGGCATCTGGCATC	GCGGTTCTGGGACTTGTGGGC
Timp3	CTTCTGCAACTCCGACATCGT	GGGGCATCTTACTGAAGCCTC
Tgfb1	CAACCCAGGTCCTTCCTAAA	GGAGAGCCCTGGATACCAAC
Tnfa	AGGCTGCCCCGACTACGT	GACTTTCTCCTGGTATGAGATAGCAAA



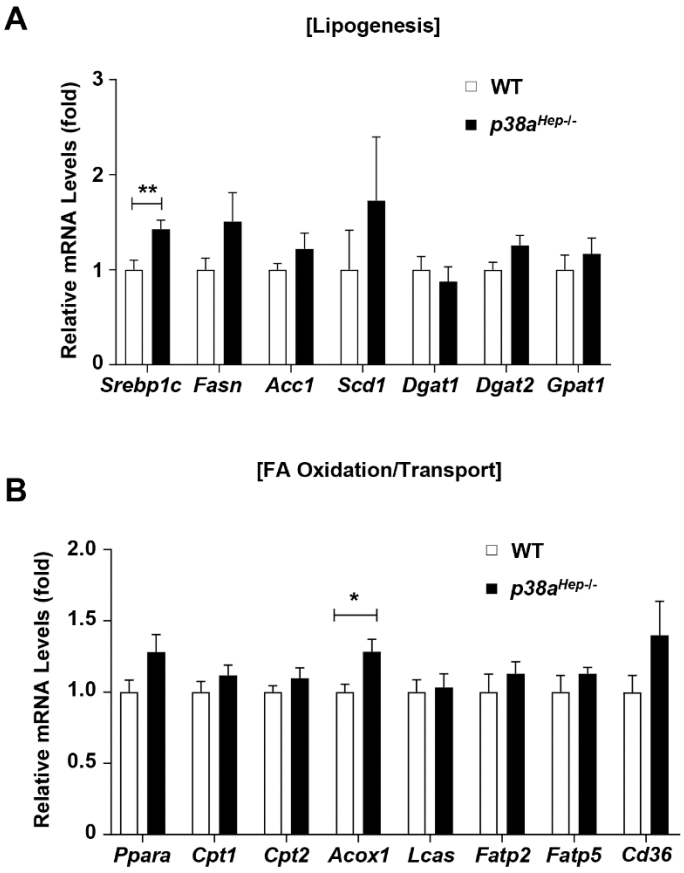
Supporting Fig. S1. Hepatic overexpression of *Cxcl1* and *IL8* in HFD-fed mice elevates serum levels of CXCL1 and IL-8 and stimulates hepatocyte death. (A-C) C57BL/6J mice fed an HFD for 3 months were infected with Ad-*Gfp*, Ad-*Cxcl1,* or Ad-*Cxcl1*+Ad-*IL8* and were subjected to serum ALT analysis at 2 weeks and sacrificed at 4 weeks after infection (n=4-6/group). A schematic illustration of the experimental design (panel A). Serum levels of CXCL1 and IL-8 (panel B). Paraffin-embedded liver sections were subjected to TUNEL, MPO, and Ly6G staining (panel C). The number of TUNEL$^+$ hepatocytes per 100X field were counted and shown in the right. Red arrows indicate positive cells. Scale bars indicate 200 μm. Values represent mean ± SEM. Statistical evaluation was performed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons (**p<0.01). N.D., not detected.



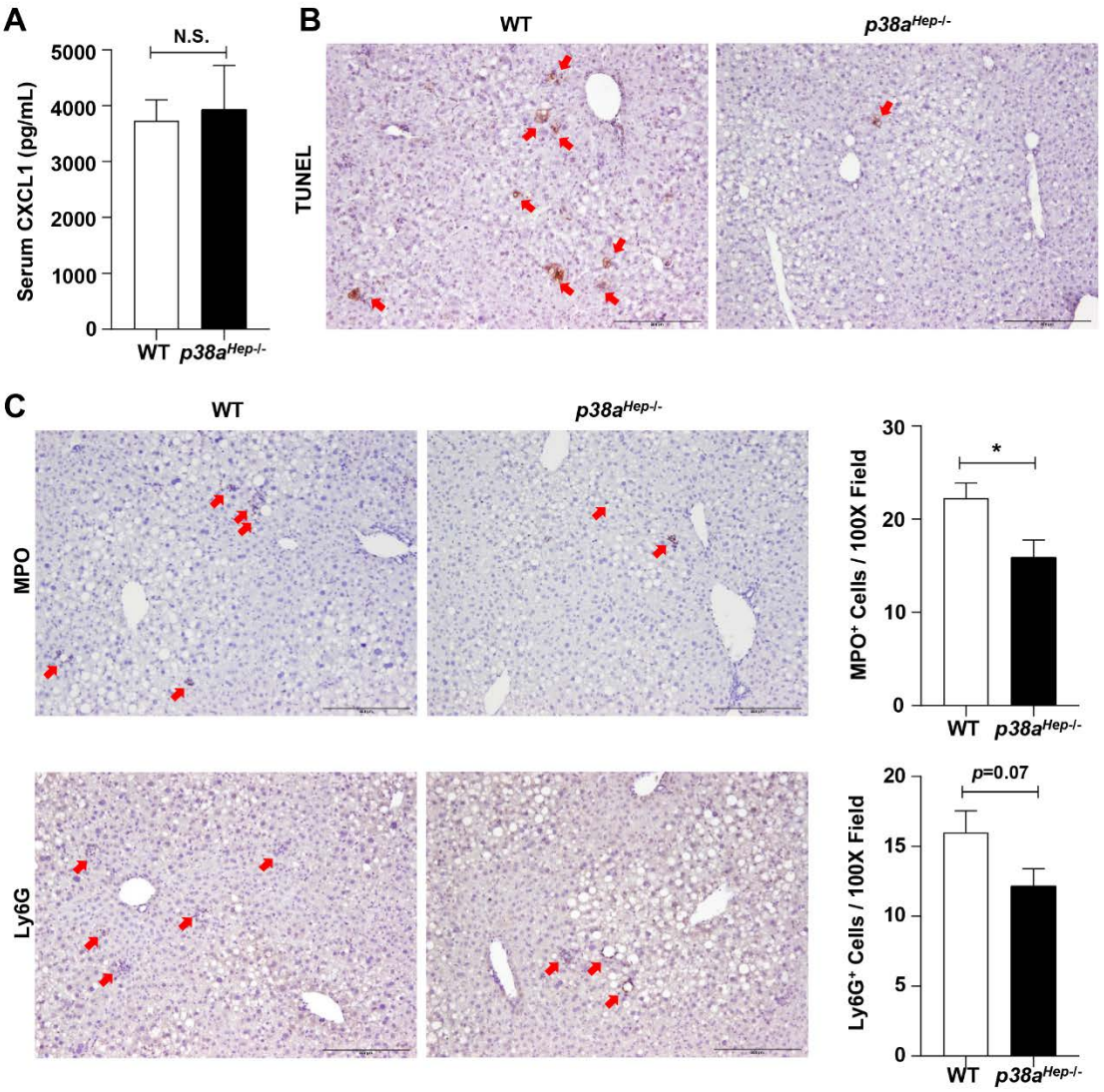
Supporting Fig. S2. Hepatic overexpression of *Cxcl1* and *IL8* in HFD-fed mice induces oxidative stress, inflammation, and fibrosis in the liver. (A-B) C57BL/6J mice fed an HFD for 3 months were infected with Ad-*Gfp* or Ad-*Cxcl1*+Ad-*IL8* and sacrificed at 4 weeks after infection (n=4-6/group). Paraffin-embedded liver sections were subjected to various types of staining (panel A). Scale bars indicate 200 μm. Liver tissues were subjected to RT-qPCR analyses of the genes involved in neutrophil oxidative burst (panel B, top) and fibrosis (panel B, bottom). Values represent mean ± SEM. Statistical evaluation was performed by Student's t-test (*p<0.05; **p<0.01).



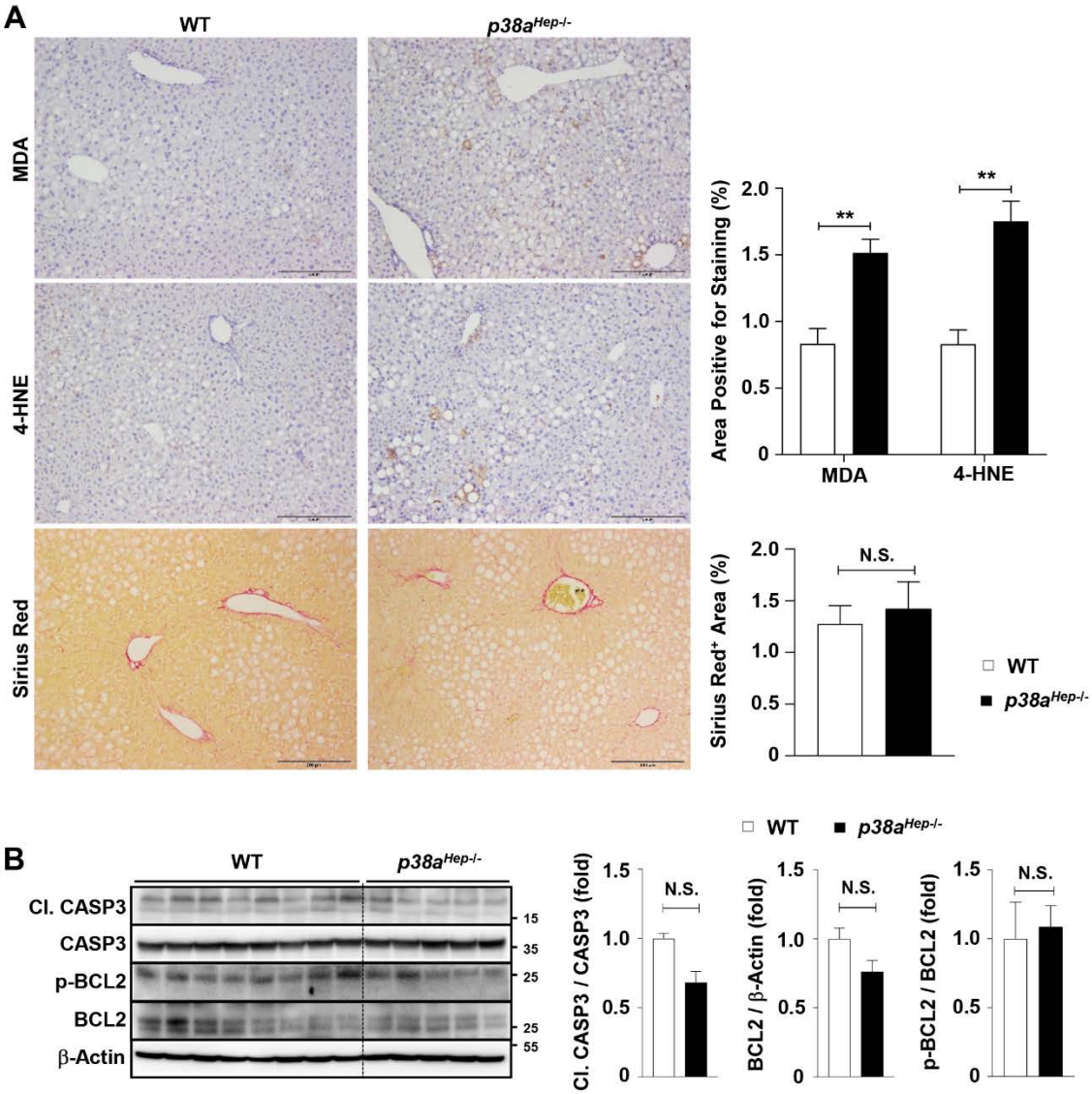
Supporting Fig. S3. Treatment of LY-2228820 inhibits p38 activity in primary mouse hepatocytes and in the liver of HFD-fed mice and reduces hepatic neutrophil infiltration. (A) Primary mouse hepatocytes from C57BL/6J mice were treated with H_2O_2 (1 mM) or vehicle for 4 hr following 1-hr pretreatment with LY-2228820 or vehicle. Western blot analyses of total cell lysates revealed that LY-2228820 reduced H_2O_2-induced phosphorylation of MK2, which is the downstream target of p38, indicating that LY-2228820 suppressed the activity of p38. (B) C57BL/6J mice were subjected to the experiment as described in Fig. 2B and the livers of mice were subjected to western blot analyses. LY-2228820 reduced the phosphorylation of MK2 similarly to the result in panel A (panel B, top). Quantification of the ratio of p-MK2 to MK2 (panel B, bottom). (C) Serum of mice was subjected to ELISA analysis of CXCL1 (n=5-6/group). (D) Paraffin-embedded liver tissue sections were subjected to MPO staining. Representative images are shown (left), and the number of MPO^+ cells were counted (right). Red arrows indicate MPO^+ cells, and scale bars indicate 200 μm. Values represent mean ± SEM. Statistical evaluation was performed by Student's t-test (**p<0.01).



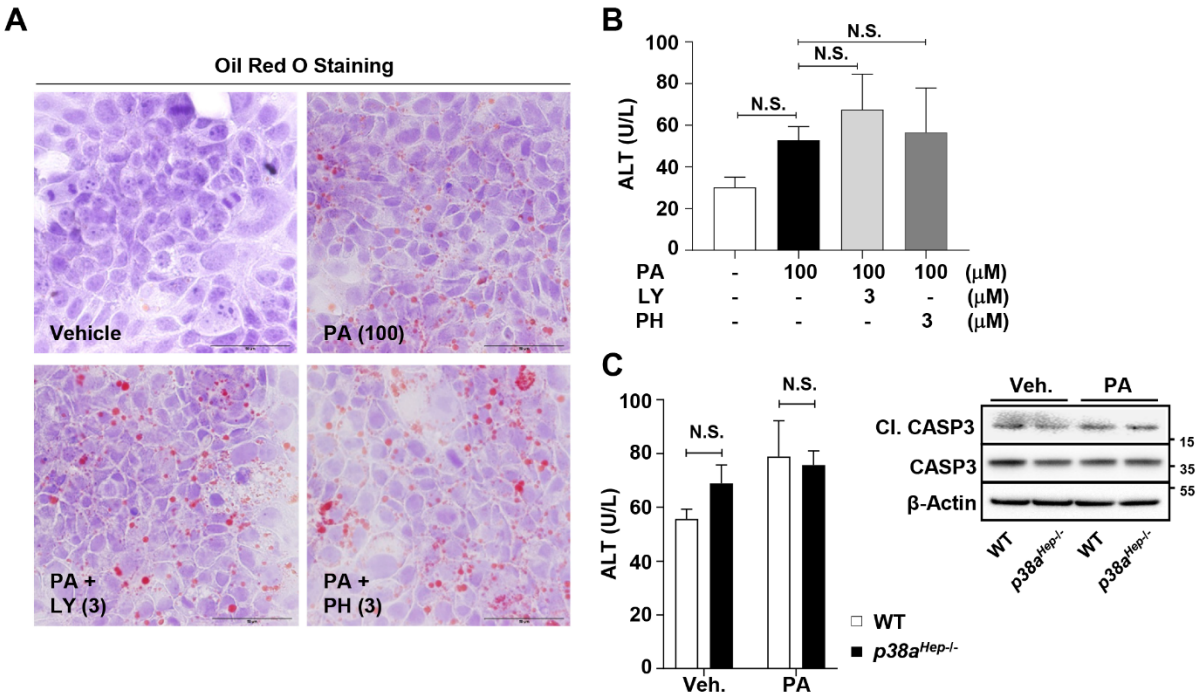
Supporting Fig. S4. Hepatocyte-specific deletion of *p38a* does not significantly affect the expression of the genes involved in lipogenesis or FA oxidation/transport in mice with HFD^{+Cxcl1}-induced NASH. (A-B) WT and *p38a*$^{Hep-/-}$ mice fed an HFD for 3 months were infected with Ad-*Cxcl1* for 2 weeks, and livers were subjected to RT-qPCR analyses of the genes involved in lipogenesis (panel A) and FA oxidation/transport (panel B) (n=8/group). Values represent mean ± SEM. Statistical evaluation was performed by Student's t-test (*p<0.05; **p<0.01).



Supporting Fig. S5. Hepatocyte-specific deletion of *p38a* does not affect serum CXCL1 levels but reduces HFD^{+Cxcl1}-induced hepatocyte death and neutrophil infiltration in the liver. (A-C) Three-month HFD-fed WT and *p38a*$^{Hep-/-}$ mice were infected with Ad-*Cxcl1* for 2 weeks (n=8/group). Serum CXCL1 levels (panel A). Livers of mice were subjected to TUNEL staining (panel B) and MPO and Ly6G staining (panel C, left). The number of MPO$^+$ cells and Ly6G$^+$ cells were counted (panel C, right). Red arrows indicate TUNEL$^+$ hepatocytes, MPO$^+$ cells, or Ly6G$^+$ cells depending on the type of staining. Scale bars indicate 200 μm. Values represent mean ± SEM. Statistical evaluation was performed by Student's t-test (*p<0.05). N.S., not significant.

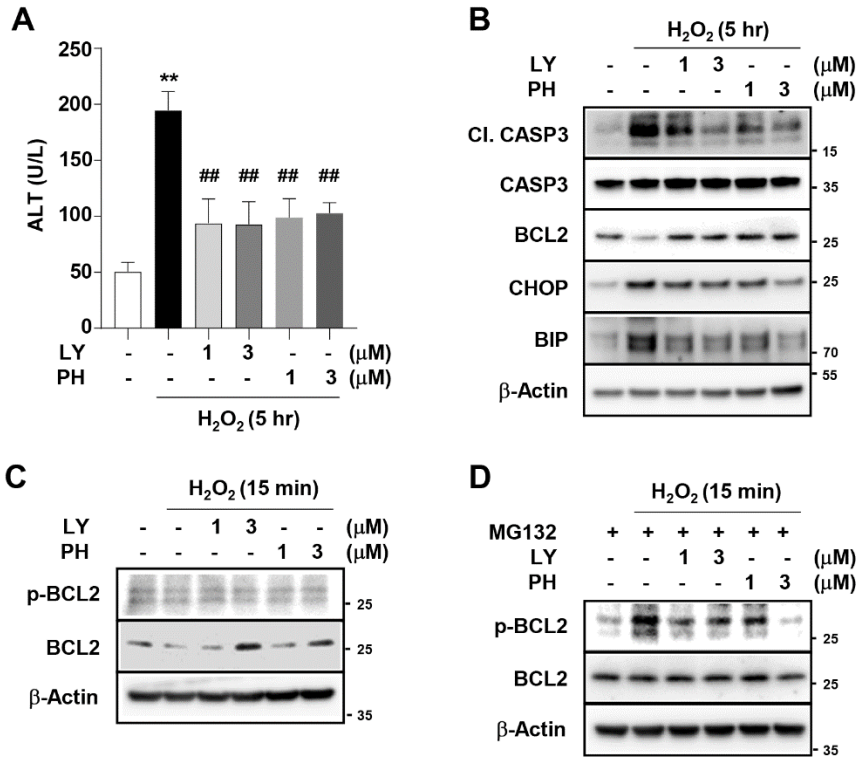


Supporting Fig. S6. Hepatocyte-specific deletion of *p38a* causes mild oxidative stress without a significant effect on fibrogenesis or apoptotic signaling in the liver of 3-month HFD-fed mice. WT and *p38a*$^{Hep-/-}$ mice were fed an HFD for 3 months (n=5-8/group). (A) Liver tissues were subjected to MDA, 4-HNE, and Sirius red staining. Scale bars indicate 200 μm. Quantification data are shown in the right. (B) Liver tissues were subjected to western blot analyses of factors involved in apoptosis (left) and the blots were quantified (right). Values represent mean ± SEM. Statistical evaluation was performed by Student's t-test (**p<0.01). Cl. CASP3, cleaved form of CASP3; N.S., not significant.



Supporting Fig. S7. (A) Pharmacological inhibition of p38 enhances PA-induced fat accumulation in AML12 cells. AML12 cells were treated with PA (100 μM) for 24 hr following 1-hr pretreatment with LY-2228820 (3 μM) or PH-797804 (3 μM). Representative images of oil red O staining are shown. Scale bars indicate 50 μm.

(B-C) A low concentration of PA does not induce cell death in AML12 cells or primary mouse hepatocytes. (B) AML12 cells were treated with PA (100 μM) for 24 hr following 1-hr pretreatment with LY-2228820 (3 μM) or PH-797804 (3 μM). The supernatant of AML12 culture media was subjected to ALT analysis to examine the death of AML12 cells. (C) Primary mouse hepatocytes from WT and *p38a*$^{Hep-/-}$ mice were treated with vehicle or PA (100 μM) for 24 hr. ALT levels were measured from the supernatant of the culture media (left), and total lysates of hepatocytes were subjected to western blot analysis (right). Values represent mean ± SEM. Statistical evaluation was performed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. N.S., not significant; Veh., Vehicle; Cl. CASP3, cleaved form of CASP3.



Supporting Fig. S8. Pharmacological inhibition of p38 attenuates H_2O_2-induced death of AML12 cells. (A-B) AML12 cells were treated with vehicle or H_2O_2 (500 μM) for 5 hr following 1-hr pretreatment with LY-2228820 (1 or 3 μM) or PH-797804 (1 or 3 μM). The supernatant of AML12 culture media was subjected to ALT analysis to examine the death of AML12 cells (panel A). Total cell lysates were subjected to western blot analyses of factors involved in apoptosis and ER stress (panel B). (C) AML12 cells were treated with vehicle or H_2O_2 (500 μM) for 15 min following 1-hr pretreatment with LY-2228820 (1 or 3 μM) or PH-797804 (1 or 3 μM). Total cell lysates were subjected to western blot analyses. (D) After 3-hr treatment with MG132 (20 μM), AML12 cells were treated with LY-2228820 (1 or 3 μM) or PH-797804 (1 or 3 μM) for 1 hr followed by 15-min treatment with H_2O_2 (500 μM). Total cell lysates were subjected to western blot analyses. Values represent mean ± SEM. Statistical evaluation was performed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons (**p<0.01 vs. vehicle only; $^{\#\#}p$<0.01 vs. H_2O_2 only). Cl. CASP3, cleaved form of CASP3.