Supplementary Material

AOAH-mediated LPS inactivation limits macrophage endotoxin tolerance and promotes inflammation and fibrosis in MASH

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Supplementary Methods

Kupffer cell isolation and stimulation

Primary Kupffer cells were isolated from 8–10-week-old male wild-type or AOAH$^{-/-}$ mice by in situ liver perfusion with collagenase type IV (C5138, Sigma-Aldrich), followed by 25/50% Percoll (17-0891-02, GE Healthcare, Madison, WI) gradient separation. Cells were plated in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% FBS and 0.6% penicillin/streptomycin. For in vitro LPS tolerance induction, Kupffer cells were isolated, stabilized for 4 hours, then pre-treated with 10 ng/mL LPS (first simulation) for 16–18 hours to induce tolerance, followed by washing and re-stimulation with 20 ng/mL LPS (second stimulation) for 6 hours. For in vivo LPS tolerance experiments, mice were injected intraperitoneally with 50 LPS and liver macrophages were isolated 3 days post-injection; these cells were re-stimulated with LPS (10 ng/mL) in vitro for 6 hours. To compare tolerance between WT and AOAH$^{-/-}$ Kupffer cells, mice were injected with 20 μg LPS, cells were isolated after 3 days, and re-stimulated with 10 ng/mL LPS. For lipoteichoic acid (LTA) stimulation experiments, Kupffer cells were conditioned as above, then stimulated with LTA (5 μg/mL; L2515, Sigma-Aldrich) for 6 hours. Culture supernatants were collected for cytokine analysis, and cells were harvested for qPCR.

Kupffer cell supernatant ELISA and cytokine array

For ELISA and cytokine membrane array analysis of cell supernatants, culture supernatants from Kupffer cells were harvested, stored at −80 °C, and centrifuged at 3000 rpm for 5 minutes before use. Commercial ELISA kits for mouse TNF-α, IL-6, and IL-12 (DY410, DY406 and DY419, R&D Systems, Minneapolis, MN, USA) were utilized according to the manufacturer's instructions. Absorbance was measured at 450 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, San Jose, CA, USA). For cytokine profiling, the Proteome Profiler Mouse Cytokine Array Kit, Panel A (ARY006, R&D Systems) was used following the provided protocol, and signal detection was performed using the Fusion Solo 6X imaging system (Vilber, Collegien, France).

Plasma assays

Blood was drawn into EDTA-treated tubes and centrifuged at 6,000 rpm for 10 minutes at 4°C. The plasma supernatant was harvested and stored at −80°C until analysis. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a Dri-chem NX500i hematology analyzer (Fujifilm, Tokyo, Japan). Plasma samples were thawed on ice and directly analyzed using the manufacturer's assay cartridge. For plasma LPS ELISA, LPS concentrations were measured using a Lipopolysaccharide ELISA Kit (OKEH02594, Aviva Systems Biology, San Diego, CA). Plasma samples were diluted with distilled water and heat-inactivated at 75°C for 7 minutes. For mouse plasma, 40 μL of plasma was mixed with 80 μL of distilled water. For human plasma, 75 μL of plasma was mixed with 75 μL of distilled water. Absorbance was read at 450 nm using a SpectraMax microplate reader (Molecular Devices). Plasma TLR4 activity was measured using HEK-Blue LPS detection kit (rep-lps2, InvivoGen, San Diego, CA). Plasma samples were heat-inactivated at 75°C for 7 minutes and then pre-incubated overnight with 1 μg/mL LPS prior to reporter assay. For reporter assays, 20 μL of the LPS-

incubated diluted plasma samples were co-cultured with HEK-Blue hTLR4 cells for 18–24 hours. SEAP activity was quantified by transferring supernatants to new plates and incubating with QUANTI-Blue™ reagent at 37°C. Absorbance was measured at 620–655 nm. Plasma cytokine bead array was conducted using LEGENDplex Mouse Inflammation Panel (740446, BioLegend, San Diego, CA) on a V-bottom plate. Plasma samples were diluted 1:2, incubated with capture beads for 2 hours, washed, incubated with detection antibodies for 1 hour, then with SA-PE for 30 minutes. Samples were analyzed on a FACS Lyric™ flow cytometer (BD Biosciences, Oxford, UK), and data were analyzed with FlowJo (BD Biosciences).

Liver histology

Liver tissues were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned (5 μm), deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E; ab245880, Abcam, Cambridge, UK) or Sirius Red (ab246832, Abcam). For immunofluorescence, antigen retrieval was performed, and sections were incubated with antibodies against type I collagen (1:50; 1310-01, Southern Biotech, Birmingham, AL), α-SMA (1:50; M085129-2, Agilent DAKO, Santa Clara, CA), and desmin (1:50; ab15200, Abcam). Secondary antibodies (Alexa Fluor 488, 594, 647; Thermo Fisher Scientific) were used at 1:200 dilution. Fluorescence images were acquired on Stellaris 5 confocal microscope (Leica Microsystems). Inflammatory foci and collagen-positive areas were quantified using ImageJ. Histology was evaluated by blinded pathologists.

Measurement of hepatic hydroxyproline

Frozen liver tissue was homogenized in distilled water using a TissueLyser II (Qiagen, Hilden, Germany), followed by acid hydrolysis in 12 N HCl at 120°C for 3 hours. Hydroxyproline content was quantified using the PicoSense Total Collagen Assay Kit (BM-COL-100, BioMax, Daejeon, Korea). Absorbance was measured at 560 nm using a SpectraMax plate reader (Molecular Devices).

Flow cytometry

Liver non-parenchymal cells were isolated by collagenase digestion and density gradient centrifugation. Briefly, liver tissues were digested in collagenase D solution (11088858001, Sigma-Aldrich) using a GentleMACS Dissociator (Miltenyi Biotec, Auburn, CA). After digestion, cell suspensions were filtered and centrifuged at 500 rpm for 3 minutes to pellet hepatocytes. The supernatant was collected and further centrifuged at 1,700 rpm for 5 minutes. The resulting cell pellet was resuspended and layered over 26% Percoll (17-0891-02, GE Healthcare), followed by centrifugation at 1,700 rpm for 5 minutes to enrich hepatic non-parenchymal cells (NPCs). For flow cytometry staining, single-cell suspensions were first incubated with anti-CD16/32 antibody (553142, BD Biosciences, San Jose, CA) at 1:200 dilution for Fc receptor blockade. Cells were washed and incubated for 30 min at 4°C with fluorochrome-conjugated antibodies at 1:300 dilution, including: BV510-anti-CD45, PE-Cy7-anti-CD11b, PerCP-Cy5.5-anti-Ly6C, FITC-anti-Ly6G, APC-anti-CD115 (103138, 101216, 128012, 127606, 135510, BioLegend), and PE-F4/80 (12-4801-82, eBioscience, San Diego, CA). Dead cells were excluded using DAPI (D9542, Sigma-Aldrich). Data acquisition was performed on a FACS Lyric™ (BD Biosciences), and data were analyzed using FlowJo software (BD Biosciences).

Quantitative real-time PCR (qPCR)

Liver tissue was homogenized using easy-BLUE™ Total RNA Extraction Kit (17061, iNtRON, Seongnam, Korea) and TissueLyser II (Qiagen). Total RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration and quality were assessed using a UV-Vis Nabi spectrophotometer (MicroDigital, Seoul, Korea). Reverse transcription was performed using 0.04–1 μg of total RNA with the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific). Quantitative PCR was conducted using Power SYBR® Green Master Mix (4367659, Applied Biosystems, Foster City, CA) and a StepOnePlus Real-Time PCR System (Applied Biosystems). The relative expression of target genes (*Tnfa, Il6, Ccl3, Cxcl10, Col1a1, Acta2, Timp1, Best1, Naip1, Gpr157, Hebp1, Dhrs1,* etc.) was normalized to 18S rRNA using the 2^-ΔCt method. Primer sequences can be provided upon adequate request. Results were expressed as fold change relative to control groups.

Immunoblot

Liver tissue lysates were prepared in RIPA buffer (R0278, Sigma-Aldrich) containing protease inhibitors, homogenized using a TissueLyser II (Qiagen), and centrifuged at 14,000 rpm for 10 minutes at 4°C. Protein concentrations were determined using the BCA assay. Equal amounts of protein (20–30 μg) were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked in TBS-T containing 5% skim milk and incubated overnight at 4°C with primary antibodies against αSMA (Agilent DAKO) and HSP60 (SC-13115, Santa Cruz Biotechnology, Dallas, TX). After washing, membranes were incubated with HRP-conjugated secondary antibodies (Bio-Rad, Hercules, CA) for 1 hour at room temperature. Bands were visualized using enhanced chemiluminescence (Thermo Fisher Scientific) and imaged with the Fusion Solo 6X system (Vilber, Collegien, France). Band intensities were quantified using ImageJ.

Kupffer cell phagocytosis assay

LPS-tolerized and control Kupffer cells were incubated with FluoSpheres™ Carboxylate-Modified Microspheres, 0.5 μm, red fluorescent (F8812, Thermo Fisher Scientific, Waltham, MA) at 3×10^8 beads/well for 1 hour. Cells were then washed extensively, fixed, and analyzed for bead uptake by flow cytometry.

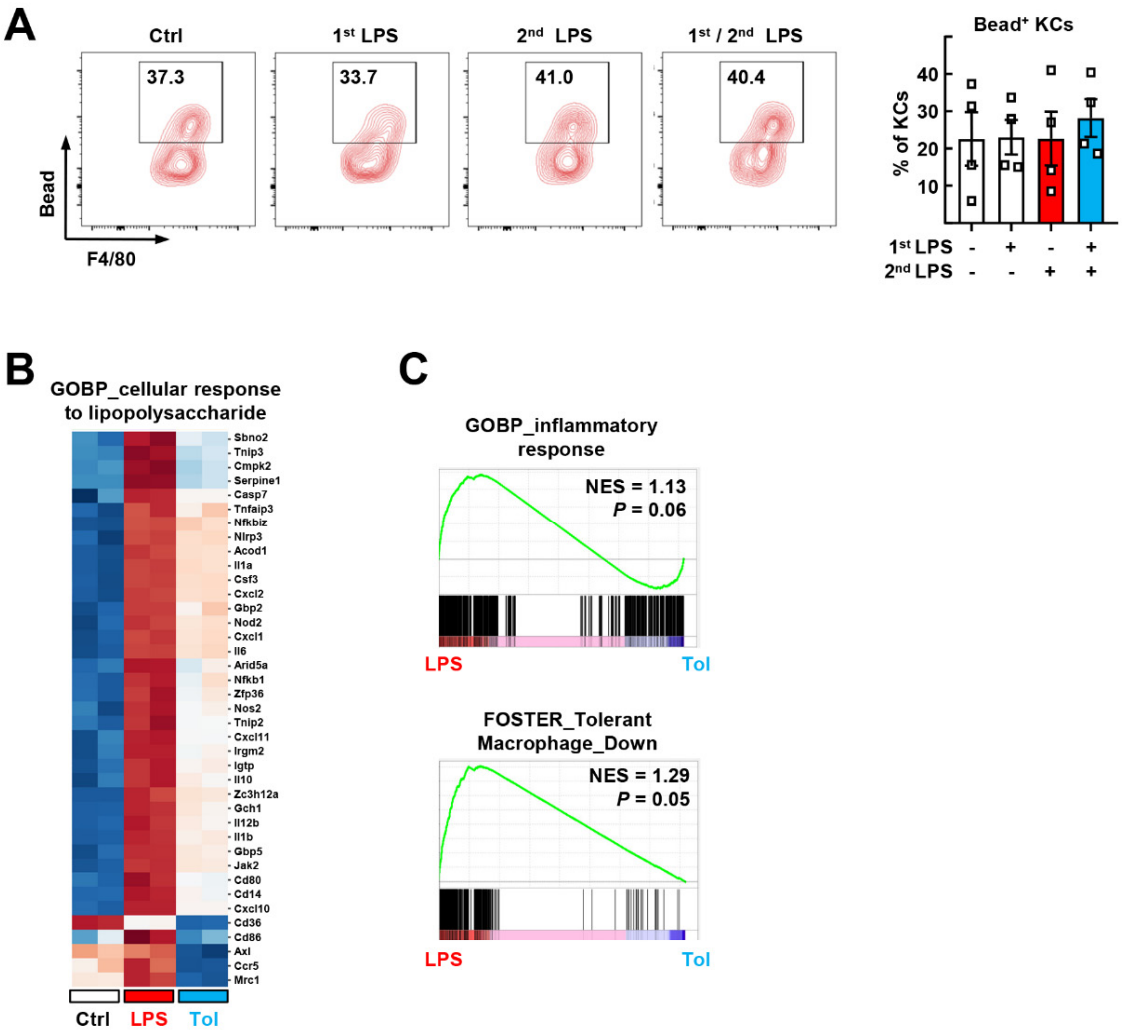
Chromogenic limulus amebocyte lysate (LAL) assay

Plasma endotoxin levels were quantified using the Pierce™ Chromogenic Endotoxin Quant Kit (A39553, Thermo Fisher Scientific) according to the manufacturer's instructions. Plasma samples were diluted 1:3 with distilled water and heat-inactivated at 75°C for 7 minutes. Then, 50 μL of each sample or endotoxin standard was added to a 96-well plate, followed by 50 μL of reconstituted amebocyte lysate reagent. The plate was incubated at 37°C. Afterward, 100 μL of pre-warmed chromogenic substrate solution was added, and the plate was further incubated for 6 minutes at 37°C. Absorbance was measured at 405 nm using a microplate reader.

Hepatic triglycerides (TGs) assay

Frozen liver tissues were homogenized in RIPA buffer (Sigma-Aldrich, St. Louis, MO) using a TissueLyser II (Qiagen, Hilden, Germany). Following centrifugation at 14,000 rpm for 10 min, the lipid-containing upper layer was collected. Hepatic triglyceride content was quantified at 570 nm absorbance using an EnzyChrom™ Triglyceride Assay Kit (BioAssay Systems, Hayward, CA) and a SpectraMax plate reader (Molecular Devices).

Supplementary Figures



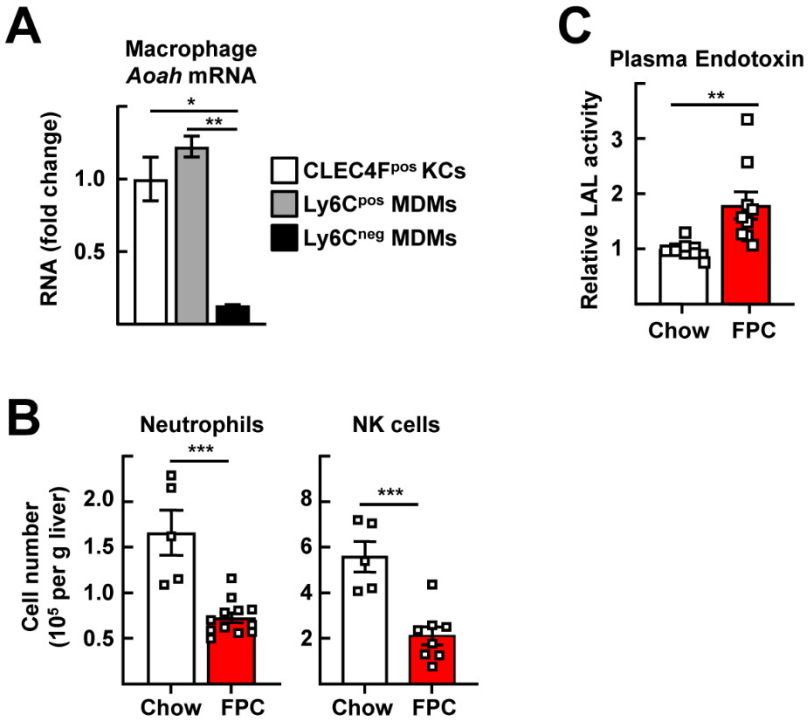
Supplementary Fig. 1. Characteristics of tolerant Kupffer cells

(**A**) Representative fluorescence plots (left) and quantification (right) of Bead$^+$ Kupffer cells 1 hour after in vitro bead treatment. (n=4)

(**B**) Heatmap of gene expression related to GO cellular response to lipopolysaccharide.

(**C**) GSEA plots showing enrichment of the "GOBP_inflammatory response" and "FOSTER_TOLERANT_MACROPHAGE_DOWN" gene sets in LPS-stimulated versus tolerant Kupffer cells.

The data represent mean ± SEM. The data were analyzed by the one-way ANOVA with Tukey's *post hoc* test for multiple groups.



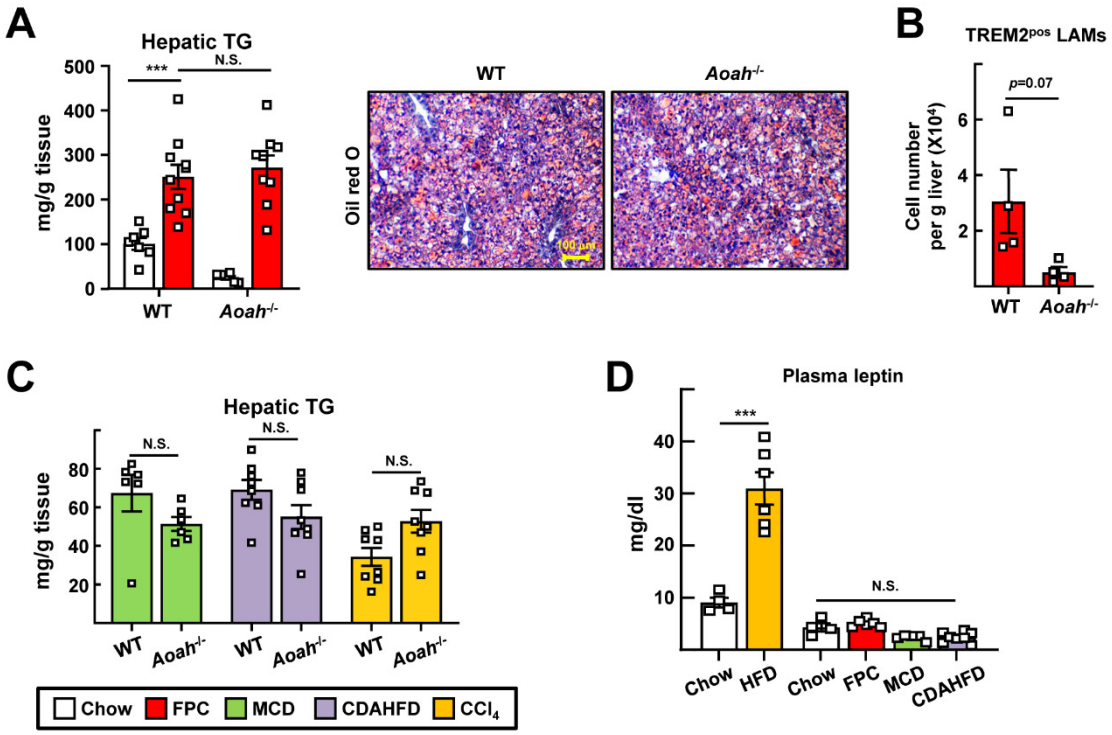
Supplementary Fig. 2. AOAH expression and LPS activity in MASH model

(**A**) Measurement of *Aoah* mRNA levels in CLEC4F^{pos} KC, Ly6C^{pos} Mo, and Ly6C^{neg} Mo sorted from FPC diet-fed liver tissue. (n=3)

(**B**) Hepatic cell numbers of neutrophils (Ly6G+ CD11b-) and NK cells (TCRb- CD19- NK1.1+) was measured after 16 weeks feeding of chow or FPC diet. (n = 5-12)

(**C**) Plasma endotoxin activity was measured by Limulus amebocyte lysate (LAL) assay in chow- and FPC-fed mice. (n=9)

$*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. The data represent mean ± SEM. The data were analyzed by the unpaired two-sided Student's t test for simple comparisons or one-way ANOVA with Tukey's *post hoc* test for multiple groups.



Supplementary Fig. 3. Hepatic lipid accumulation by knockout of AOAH

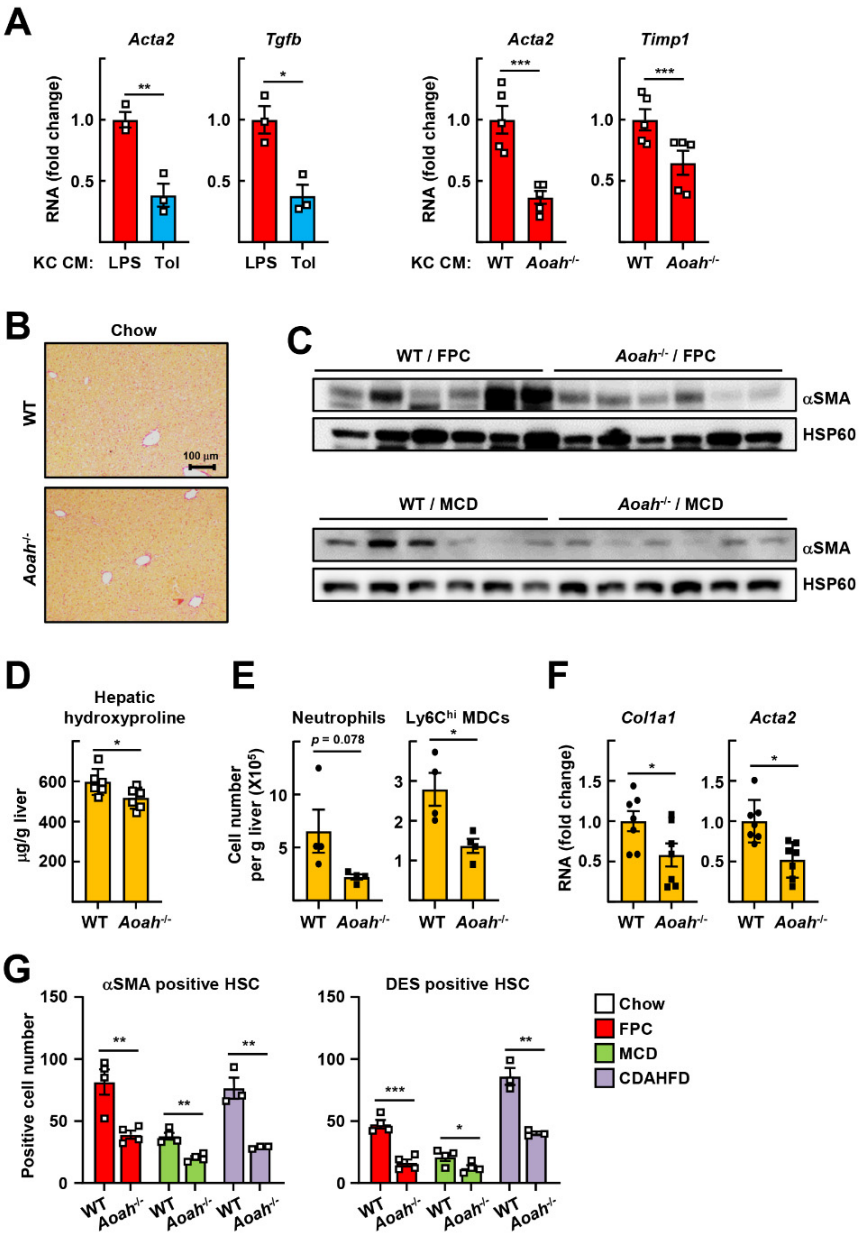
(**A**) Hepatic TG levels were measured. (n=5-10)

(**B**) The number of TREM2+ lipid-associated macrophages (LAMs) in the liver was analyzed by flow cytometry. (n=4)

(**C**) Hepatic triglyceride levels were measured. (n = 6-8)

(**D**) Plasma levels of leptin was measured. (n = 4-8).

$**p < 0.01$ and $**p < 0.001$. The data represent mean ± SEM. The data were analyzed by the unpaired two-sided Student's t test for simple comparisons or one-way ANOVA with Tukey's *post hoc* test for multiple groups.



Supplementary Fig. 4. Fibrotic changes by knockout of AOAH

8-week-old C57BL/6J WT and AOAK KO mice were fed MASH diets.

(**A**) Hepatic stellate cells (HSCs) were treated with Ctrl, LPS and Tol conditioned media of KCs. Measurement of mRNA levels of fibrotic genes was performed by qPCR. (n=3 or 5)

(**B**) Sirius Red staining of liver sections from chow-fed mice.

(**C**) Representative immunoblot analysis in liver for αSMA and HSP60 in liver tissues from FPC- and MCD diet-fed mice.

8-week-old C57BL/6J WT and AOAK KO mice were administered with CCl_4 for 6 weeks.

(**D**) Hepatic hydroxyproline content was measured in WT and AOAK KO mice. (n=6)

(**E**) The numbers of neutrophils and Ly6Chi MDCs were analyzed by flow cytometry in the liver. (n=4)

(**F**) Measurement of mRNA levels of fibrotic genes such as *Col1a1* and *Acta2*. (n=7)

(**G**) Immunofluorescence staining and quantification of αSMA and Desmin (DES) positive HSC number in sectioned liver (X100 field) of WT and AOAH KO mice fed an FPC, MCD, or CDAHFD diet (n=3–4).

$*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. The data represent mean ± SEM. The data were analyzed by the unpaired two-sided Student's t test for simple comparisons or one-way ANOVA with Tukey's *post hoc* test for multiple groups.