Supplementary data to:

C-X-C Motif Chemokine 10 Impairs Autophagy and Autolysosome Formation in Non-alcoholic Steatohepatitis

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Supplementary Methods

Immunofluorescence

Frozen liver tissues were embedded in Optimal Cutting Temperature (O.C.T.) and snap frozen in liquid nitrogen. The frozen tissue was cut into 4-μm sections and fixed in 3% (v/v) paraformaldehyde (PFA). Cells grown on coverslips were fixed with 3% PFA at 4°C. To block the unspecific binding of antibodies, liver sections and cells

were incubated with 3% bovine serum albumin (BSA) for 30min at room temperature. After that, the tissue sections and cells on coverslips were incubated with primary antibody against LC3B (1:100, Novus Biologicals), CXCL10 (2μg/mL, R&D systems), LAMP2 (1:1000, Novus Biologicals), LAMP1 (1:50, sc-19992, Santa Cruz) or p62/SQSTM1 (1:50, Novus Biologicals) at 4°C overnight. Cells were then probed with Alexa Fluor 488 goat anti-rabbit secondary antibody, Alexa Fluor 594 donkey anti-mouse secondary antibody or Alexa Fluor 594 goat anti-rat secondary antibody (1:500, Thermo Fisher Scientific, Waltham, MA) at room temperature for another 2h. The liver sections and cells were then mounted with ProLong Gold Antifade Mountant with DAPI (P-36931, Thermo Fisher Scientific) and observed under LSM 510 Zeiss confocal microscope (ZEISS, Göttingen, Germany).

Isolation of primary hepatocytes

Primary hepatocytes were isolated from C57BL/6 WT mice and $CXCL10^{-/-}$ mice fed with HFHC diet. *In situ* liver perfusion was performed with ethylene glycol tetraacetic acid buffer followed by collagenase IV (Thermo Fisher Scientific) digestion. Hepatocytes were collected after centrifugation at 40x g for 5 min. Cell viability was confirmed by trypan blue staining.

Lysosomal pH measurement

Lysosomal pH was determined by Lysosensor Yellow/Blue dextran (Invitrogen) in hepatocytes. LO2 human hepatocytes with or without anti-CXCL10 mAb treatment were cultured in MCD medium for 24h. Cells were then harvested (1 X 10^6 cells/mL) and stained with 1mg/mL Lysosensor Yellow/Blue dextran for 1h at 37°C with 5% CO_2 in the incubator. pH calibration was performed in control cells treated with 10μM

monensin and 10μM nigericin in MES buffer (5mM NaCl, 115mM KCl, 1.3mM $MgSO_4$, 25mM Mes) with the pH adjusted from 3 to 7. Cells were then aliquoted at 100μL into a black 96-well microplate and read in a Fluo Star Optima Fluorometer with excitation at 355nm. The 535/440nm fluorescence ratio was calculated for each sample and pH values were determined from the linear standard curve.

Detection of mitochondrial reactive oxygen species (ROS)

Primary hepatocytes and AML-12 hepatocyte cell lines were grown in a dish cultured with medium. The medium was then removed and pre-warmed staining solution containing cell-permeant MitoTracker Red CMXRos probes was added (200nM, Thermo Fisher Scientific). Cells were then placed in the incubator for 30min with humidified air at 37°C. Afterwards, the stained cells were washed and collected by trypsinization. Fluorescence emission of MitoTracker was measured through a 590nm long pass barrier filter by Accuri C6 cytometer (BD Biosciences, Franklin Lakes, NJ).

Quantification of hepatic mRNA expression levels

Total RNA was isolated from cells with TRIzol (Invitrogen), and 2μg total RNA was reverse transcribed to cDNA with First-Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). Real-time PCR was performed using Maxima SYBR Green Master Mix (Roche) and a Light Cycler 480 real-time PCR system (Roche). mRNA expression of genes was analyzed with the following primer: CXCL10 (F: TGTTGAGATCATGCACGAT; R: CTCTCTGCTGTCCATCCAT).

Oil Red O staining and biochemical assay

Cells grown on coverslips were fixed with 3% PFA at 4°C. Cells were stained with Oil Red O solution for 10min and rinsed with 60% isopropanol. Images were acquired under microscopy for analysis. The Oil Red O dye was eluted by 100% isopropanol and OD was measured at 500nm for quantification. Cellular lipid peroxidation was quantified by measuring malondialdehyde using thiobarbituric acid-reactive substances (TBARS) assay.

Serum CXCL10, p62 concentration and tissue NADPH oxidase

Serum CXCL10 and p62 levels from human subjects were measured by ELISA assay (R&D systems). NADPH/NADP ratio in mice tissues from MCD-fed mice treated with control or anti-CXCL10 mAb was detected by ELISA according to the manufacturer's instructions (Abcam, Cambridge, MA).

Transwell migration assay

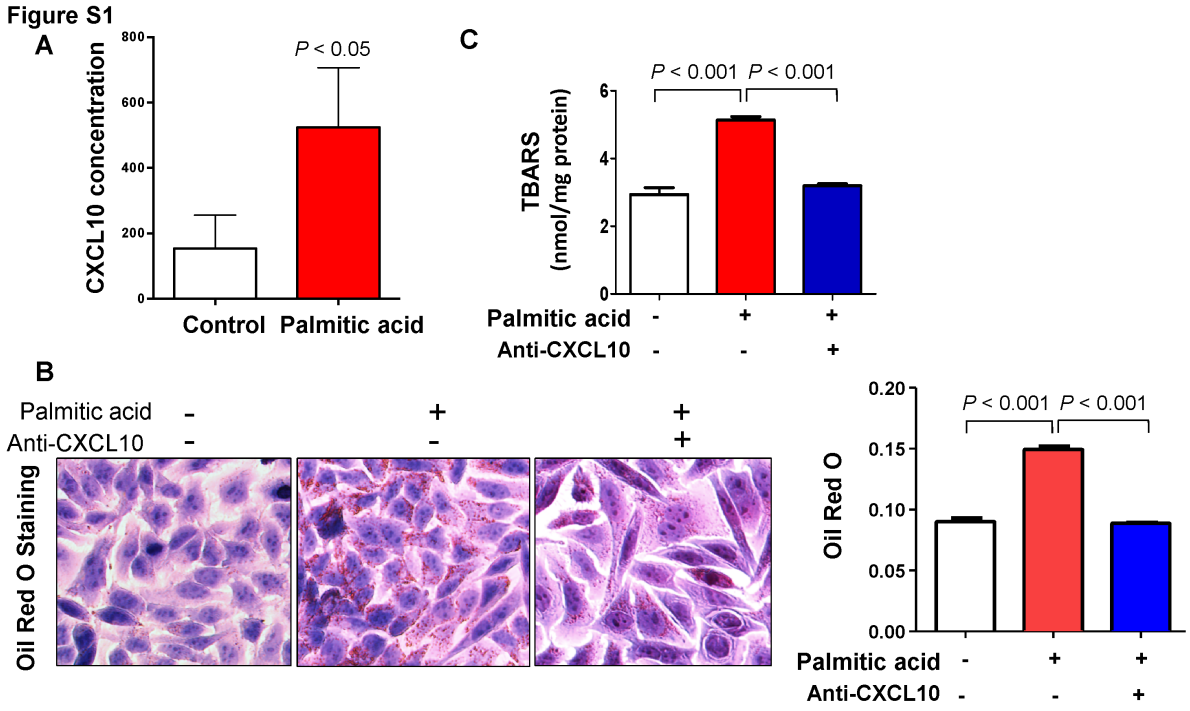
RAW 264.7 macrophages were seeded in the upper chamber of a Transwell insert (8 mM pore size, Corning Costar, Chorges, France) in complete culture medium. AML-12 hepatocytes were seeded in the lower chamber in control or MCD medium supplemented with control IgG2A or anti-CXCL10 mAb. Assays were incubated at 37℃ for up to 3 days. To stop migration, cells were fixed in methanol and stained in crystal violet. Cells on the upper side of the membrane were wiped off with a cotton swap and cells that had migrated through the pores to the underside of the membrane were quantified.

Table S1 Clinical characteristics of the human subjects

	Control	NAFLD
All	66	129
Male	34 (51.5)	72 (55.8)
Age	48 (9.87)	48 (9.61)
Metabolic syndrome*	8 (12.1)	100 (77.5)
ALT (IU/L)*	24.8 (11.3)	73 (44.6)
Fasting glucose (mmol/L)*	5.0 (0.4)	6.5 (2.5)
Triglyceride (mmol/L)*	1.3 (1.21)	2.1 (1.09)
Steatosis grade 1/2/3		46/46/37
Lobular inflammation 0/1/2		42/83/4
Ballooning 0/1/2		45/76/8
Fibrosis 0/1/2/3/4		50/46/13/9/11

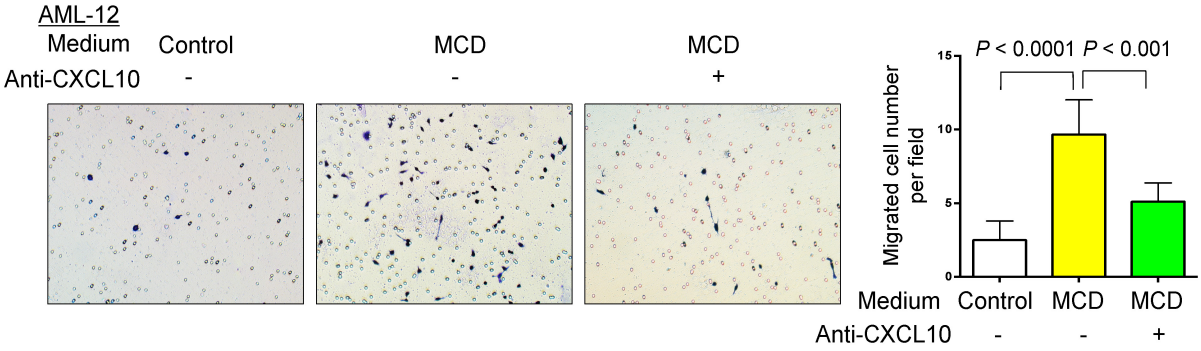
Numbers in parentheses indicates the percentage for categorical data or standard deviation for numerical data. *$P < 0.01$ between control subjects and NAFLD patients.

Figure S1-7



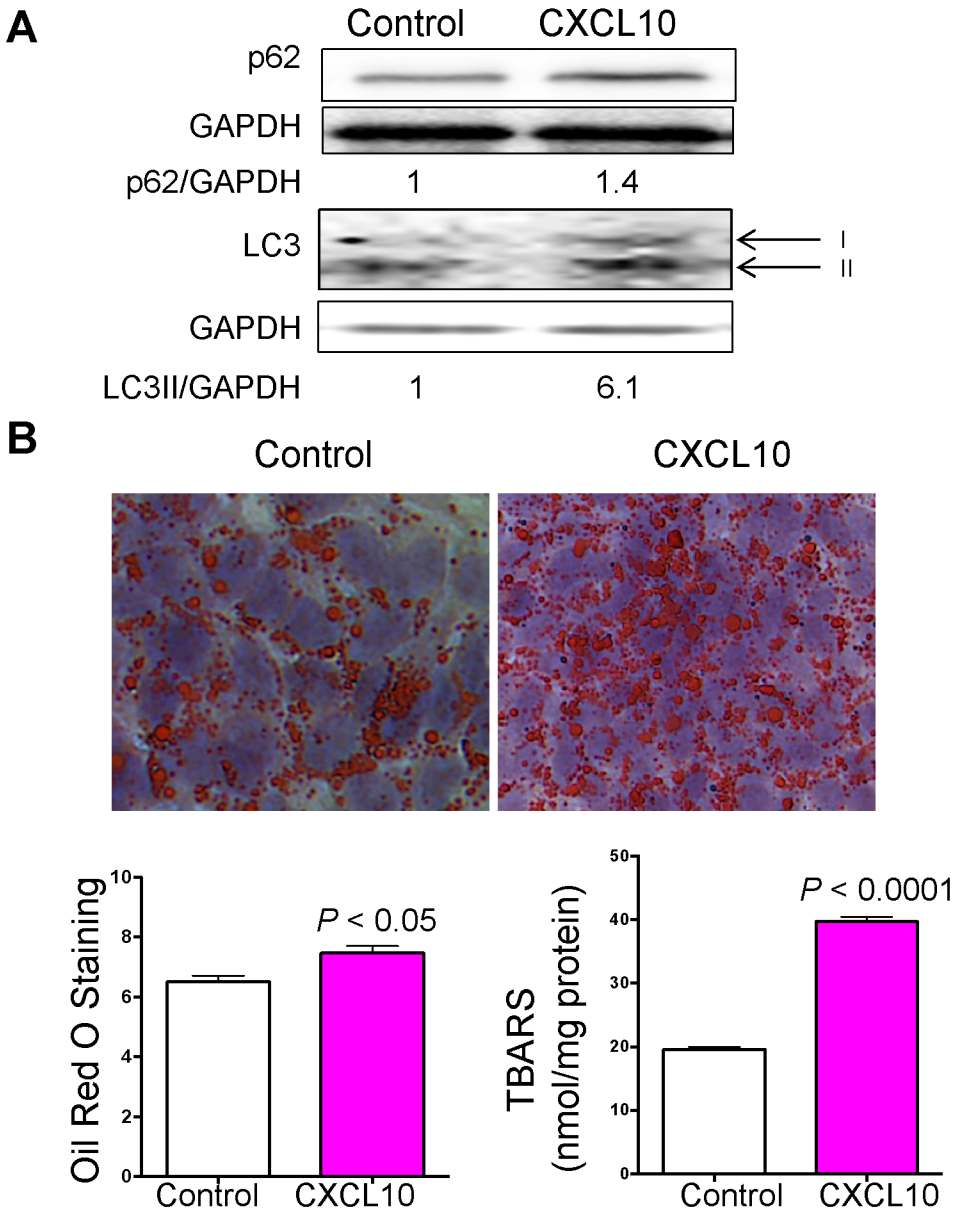
Effects of CXCL10 neutralization on steatohepatitis changes in HepG2 hepatocytes. (A) CXCL10 expression was up-regulated in the culture medium by palmitic acid treatment in HepG2 cells; **(B)** Representative Oil Red O staining pictures and quantitative analysis of Oil Red O staining in palmitic acid-treated HepG2 cells administrated with 1μg/ml of anti-CXCL10 mAb for 24 hours; **(C)** cellular lipid hydroperoxide levels in palmitic acid-treated HepG2 cells administrated with 1μg/ml of anti-CXCL10 mAb for 24 hours. Control IgG2A mAb was used as control.

Figure S2



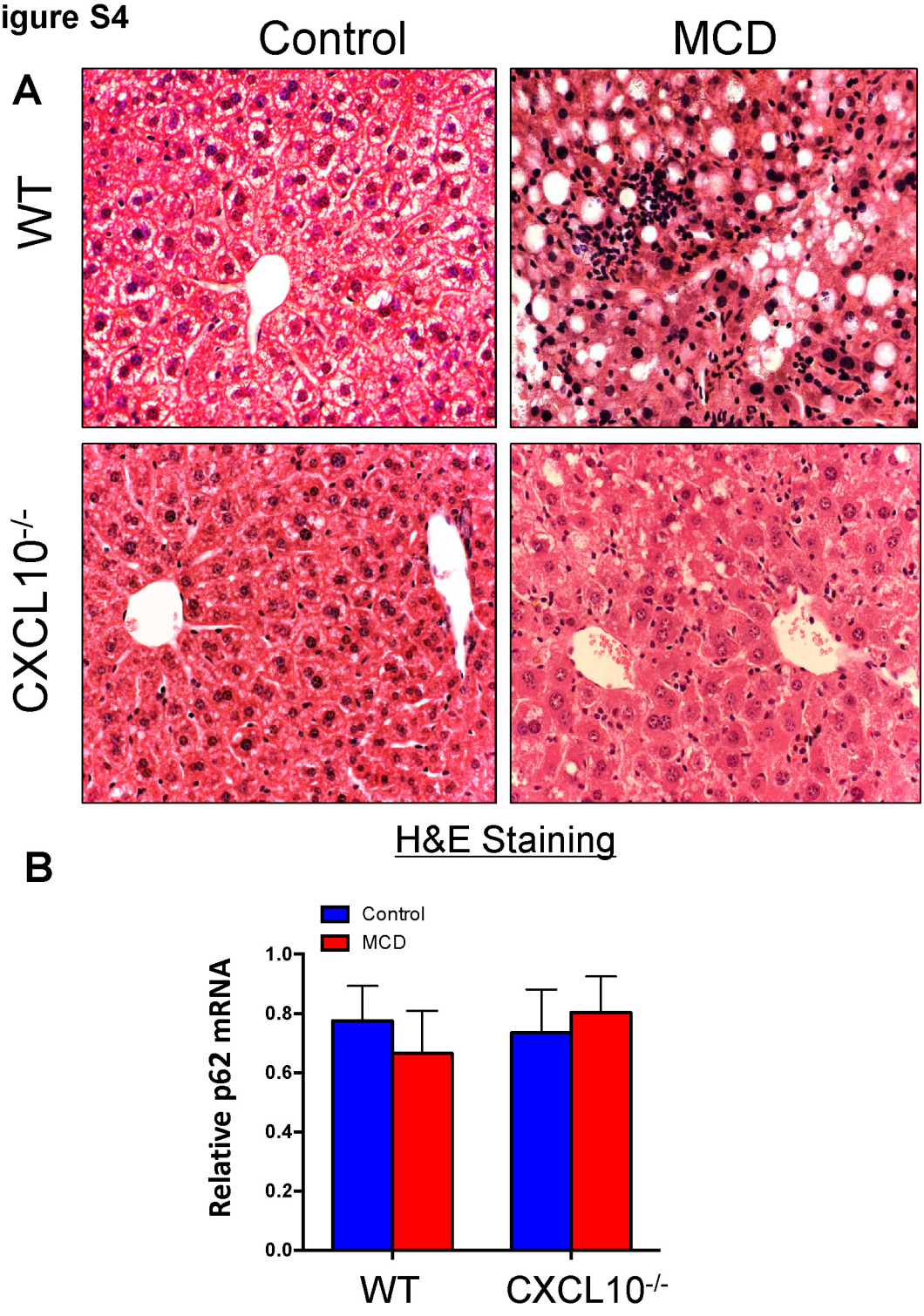
Anti-CXCL10 mAb inhibits macrophage chemotaxis using transwell assay. AML-12 hepatocytes were cultured in control or MCD medium in the presence of anti-CXCL10 mAb or control IgG2A antibody in the lower transwell chembers. RAW 264.7 macrophages were seeded in the upper transwell chambers. Medium was renewed every day. Migration was stopped at indicated time points and cells that had migrated through the Transwell inserts were counted in 8 microscopic fields.

Figure S3

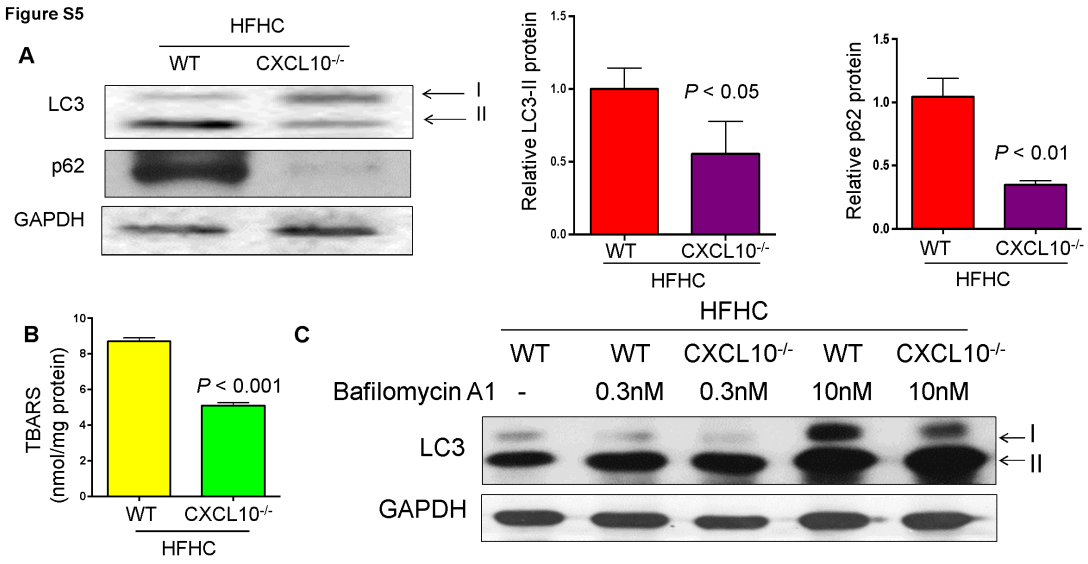


CXCL10 impairs autophagy in AML-12 hepatocytes. **(A)** LC3 and p62 protein levels; **(B)** Oil Red O staining and cellular lipid hydroperoxide levels in MCD medium cultured AML-12 cells administrated with 10ng/mL CXCL10 recombinant protein.

Figure S4

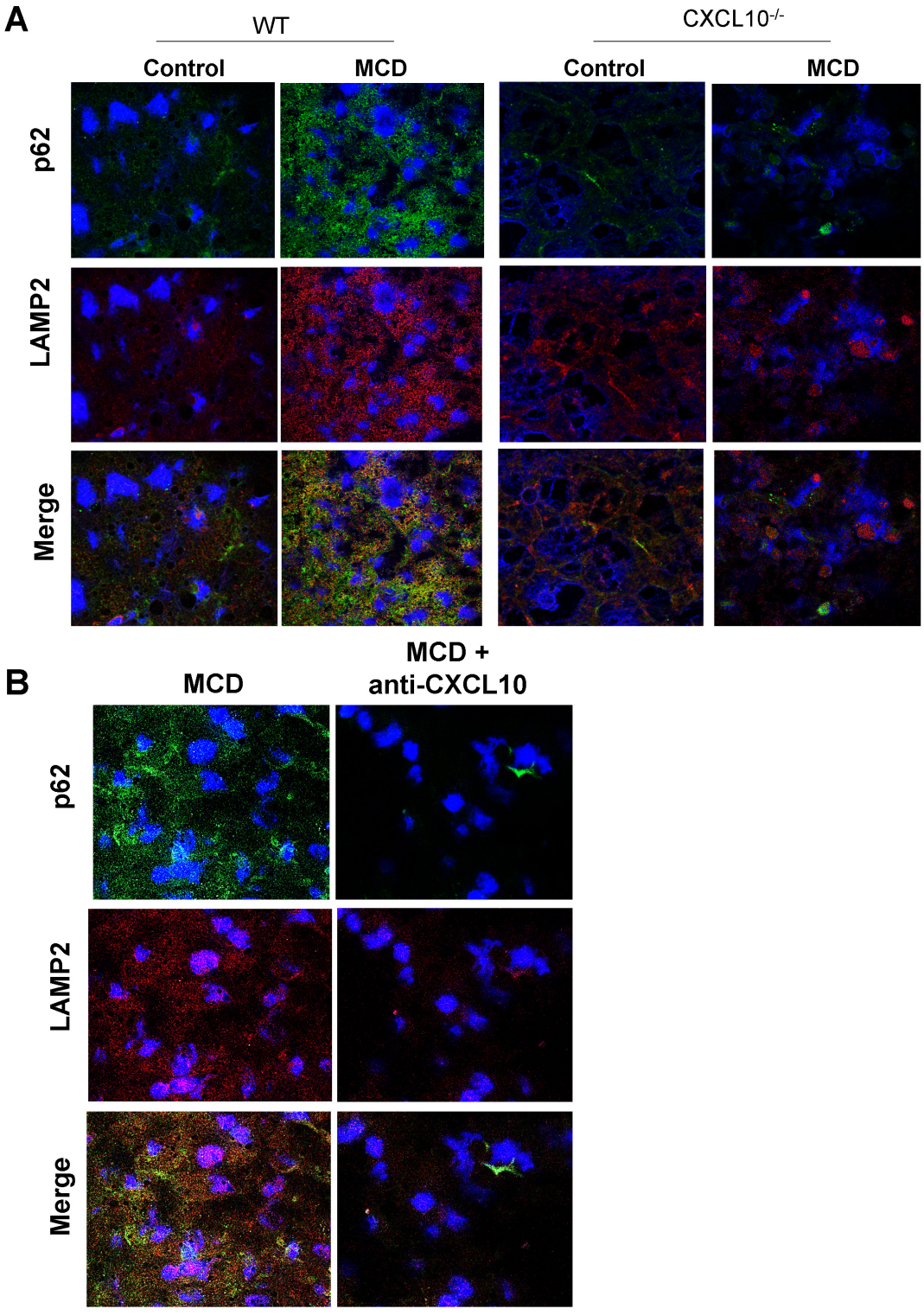


(A) Representative H&E staining from liver sections of WT and CXCL10$^{-/-}$ mice fed with control or MCD diet for 4 weeks; **(B)** p62 mRNA levles in WT and CXCL10$^{-/-}$ mice fed with control or MCD diet for 4 weeks. MCD, methionine and choline deficient.



(A) LC3 and p62/SQSTM1 protein levels in primary hepatocytes isolated from HFHC-fed CXCL10$^{-/-}$ and WT mice; **(B)** Cellular lipid hydroperoxide levels in primary hepatocytes isolated from HFHC-fed WT and CXCL10$^{-/-}$ mice; **(C)** CXCL10 knockout could not decrease LC3-II levels in the presence of bafilomycin A1 at low (0.3nM) and high doses (10nM) in primary hepatocytes. HFHC, high-fat-high-cholesterol.

Figure S6



(A) Co-localization of p62/SQSTM1 and LAMP2 was diminished in MCD-fed WT mice administrated with CXCL10 mAb; **(B)** Co-localization of p62/SQSTM1 and LAMP2 was decreased in mice liver tissues from MCD-fed CXCL10$^{-/-}$ mice compared to those from MCD-fed WT mice.

Figure S7

Rubicon protein levels in AML-12 cells administrated with 10ng/mL CXCL10 recombinant protein.