Supporting Materials

Adipocyte death preferentially induces liver injury and inflammation via the activation of CCR2^{+} macrophages and lipolysis

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Material and Methods:

Biochemical assays. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), Lipase and Amylase levels were determined using a Catalyst Dx Chemistry Analyzer (IDEXX Laboratories, Westbrook, ME). Biochemical analysis of multiple parameters in Tables were performed by using automated biochemical analyzers at the Department of Laboratory Medicine at the National Institutes of Health https://clinicalcenter.nih.gov/cp/index.html.

Complete blood count (CBC) test. The anticoagulated blood was collected from mice. CBC test was performed with Hemavet 950 FS Hematology Analyzer (Drew Scientific, Dallas, TX).

Histopathological and Immunohistochemistry analysis. Multiple organs were collected at various time points post-ILY (140 ng/g, i.v.) injection and samples were fixed in 10% formalin

and paraffin-embedded following standard procedure. Embedded adipose and liver tissues were cut to 4 μm thickness and subjected to staining with hematoxylin and eosin (H&E) or subjected to immune-histochemical staining for F4/80, myeloperoxidase (MPO) and TUNEL by using a pre-diluted rabbit anti-F4/80 (Bio-rad), anti-MPO polyclonal antibody (Biocare Medical, LLC, Concord, CA) and TUNEL assay kit (Milipore #S7100) and a rabbit ABC staining kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instruction. F4/80, MPO and TUNEL positive cells were quantified randomly from 10 fields at 100x magnification per mouse.

Cell culture. Cells were maintained in DMEM (MDA-MB-231, HELA and NIH3T3) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Western blot analysis. Samples were subjected to 4~12% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred from the gel to a polyvinylidene fluoride membrane. The membrane was probed with anti-hCD59 antibody (Sigma), phospho-HSL, HSL, ATGL, phospho-PKA (Cell signaling), alpha-SMA and β-actin antibodies (Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology).

Cell death assay. An assay of cell death was performed using a Trypan Blue dye exclusion assay after 2 h treatment with ILY. Trypan Blue positive cells were quantified randomly from 10 fields at 100x magnification per cells.

Flow cytometry analysis. Single-cell suspension was adjusted to 1×10^6 cell per 100 μl staining buffer and incubated with anti-F4/80 (clone BM8, eBioscience, San Diego, CA), anti-Gr-1 (clone RB6-8C5, eBioscience), anti-CD11b (clone M1/70, BD Biosciences), anti-iNOS (Cat: 12-5920-80) (Invitrogen) antibodies for 30 minutes at 4°C in the dark. For dead cell staining, cells with surface staining were stained with DAPI. Flow cytometry data were obtained from BD FACS Calibur and analyzed by FlowJo software (Tree Star Inc.).

Measurement of serum free fatty acids (FFAs). Serum 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.

Measurement of serum glycerols. Serum glycerols were measured with a commercial kit from BioVision (Catalog #K630-100; Milpitas, CA). Samples were prepared and measured according to the manufacturer's instruction.

Measurement of FFA concentration in cell medium

One-step direct transesterification developed by Lepage and Roy [(1)] was modified [(2)] and applied to measure the fatty acids in cell medium. Briefly, 50 µl of cell medium was added to 16 x 100 mm glass tubes containing 1.6 mL of methanol, 0.4 mL of hexane, and 200 µl of acetyl chloride. Docosatrienoic ethyl ester was used as internal standard. The test tubes were heated at 100C for 60 min and then neutralized by the addition of 5 mL of 6% Na2CO3. Two µl of hexane contained fatty acid methyl esters were injected into the Agilent 7890A gas chromatography flame ionization detector coupled with DB-FFAP capillary column (Agilent 15 m x 0.1 mm ID x 0.1 µm film thickness).

The fatty acid concentrations in the cell medium were calculated by comparing the integrated areas of each fatty acid peak with that of a known amount of ISTD in GC chromatogram.

Quantitative Real-time polymerase chain reaction (RT-PCR). Total RNA was isolated from tissues and cells using Trizol Reagent (Invitrogen) and 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-ΔΔCt). The primers used for real-time PCR are listed in Supporting Table 1.

Isolation of adipocytes, stromal vascular fraction (SVF), and adipogenesis. Adipocytes were isolated from white adipose tissue through a 100-µm mesh and cells were pelleted by

centrifugation at 250g for 5 min. The resulting pellet containing leukocytes was resuspended in 2 ml of ACK lysing buffer (BioWhittaker, Walkersville, MD). Cells were then resuspended with 10% FBS and counted using a hemocytometer. The floating adipocytes were collected for RNA or protein extraction. Pelleted SVF was plated at a density of 4×10^7 cells/15cm four plates. The SVF was also used for FACS. For adipocyte differentiation, the SVF was plated 1×10^5 cells/well. After confluence, cells were induced differentiation with 10% FBS + 5μg/ml insulin, 0.5 mM IBMX, 1μM Dex, T3 and Rosiglitazone. After 2 days, cells were changed to a medium containing 10% FBS, insulin, T3 and Rosiglitazone. After 4 days, cells were changed with the same medium again. Medium was repeatedly changed until 95% cell differentiation.

Measurement of caspase-3/7 activity. Caspase-3/7 activities in the liver samples were measured with a commercial kit from Promega (Catalog #G7791; Madison, WI, USA). Samples were prepared and measured according to the manufacturer's instruction.

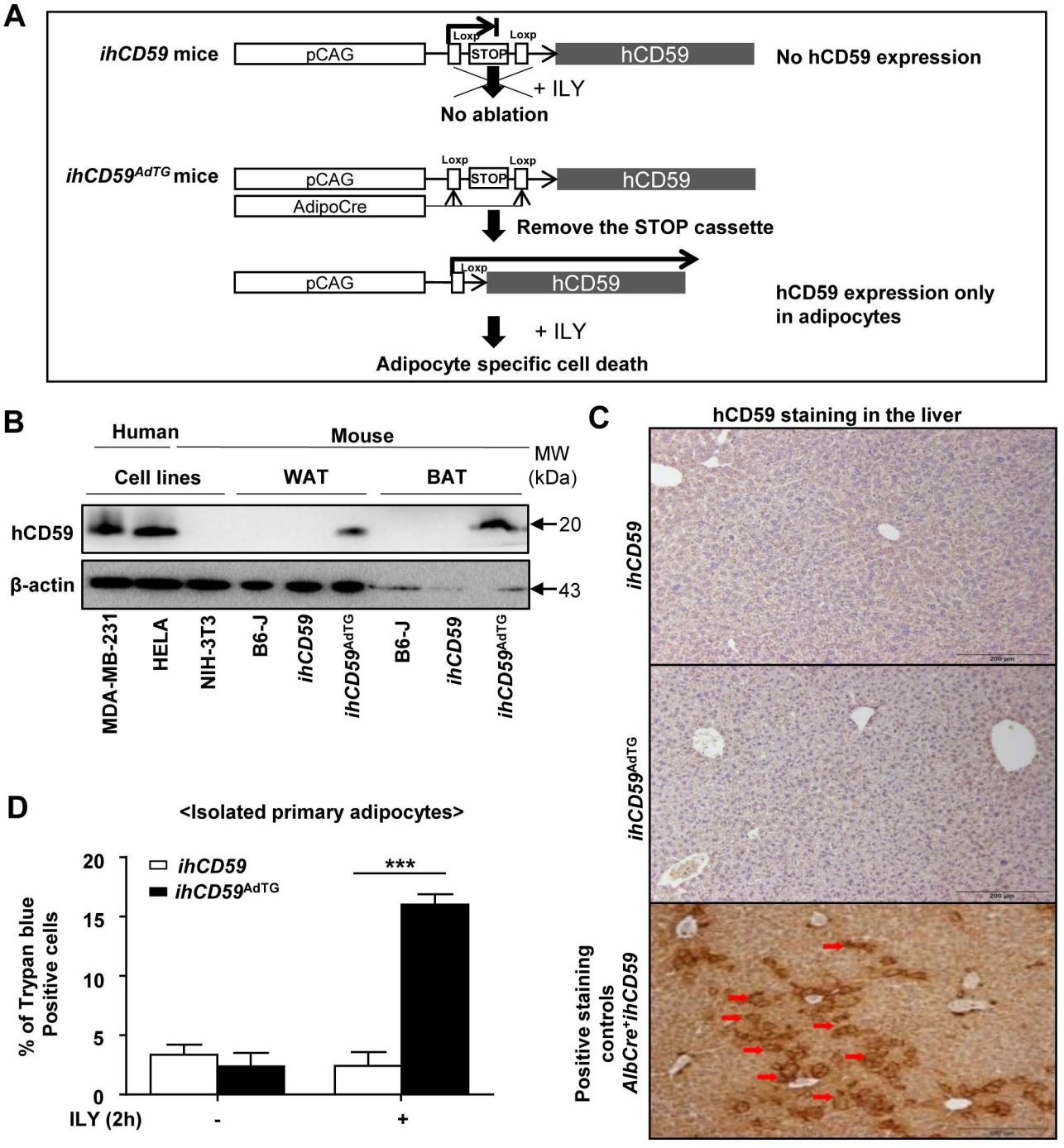
Immunohistochemistry analysis of cleaved caspase-3. Liver tissues were collected at 8-hour time point post-ILY (140 ng/g, i.v.) injection and samples were fixed in 10% formalin and paraffin-embedded following standard procedure. Embedded liver tissues were cut to 4 μm thickness and subjected to immune-histochemical staining for cleaved caspase-3 by using a prediluted rabbit anti-cleaved caspase-3 monoclonal antibody (Cell signaling, Catalog #9664) and a rabbit ABC staining kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instruction. Cleaved caspase-3 positive hepatocytes were quantified randomly from 10 fields at 400x magnification per mouse.

References:

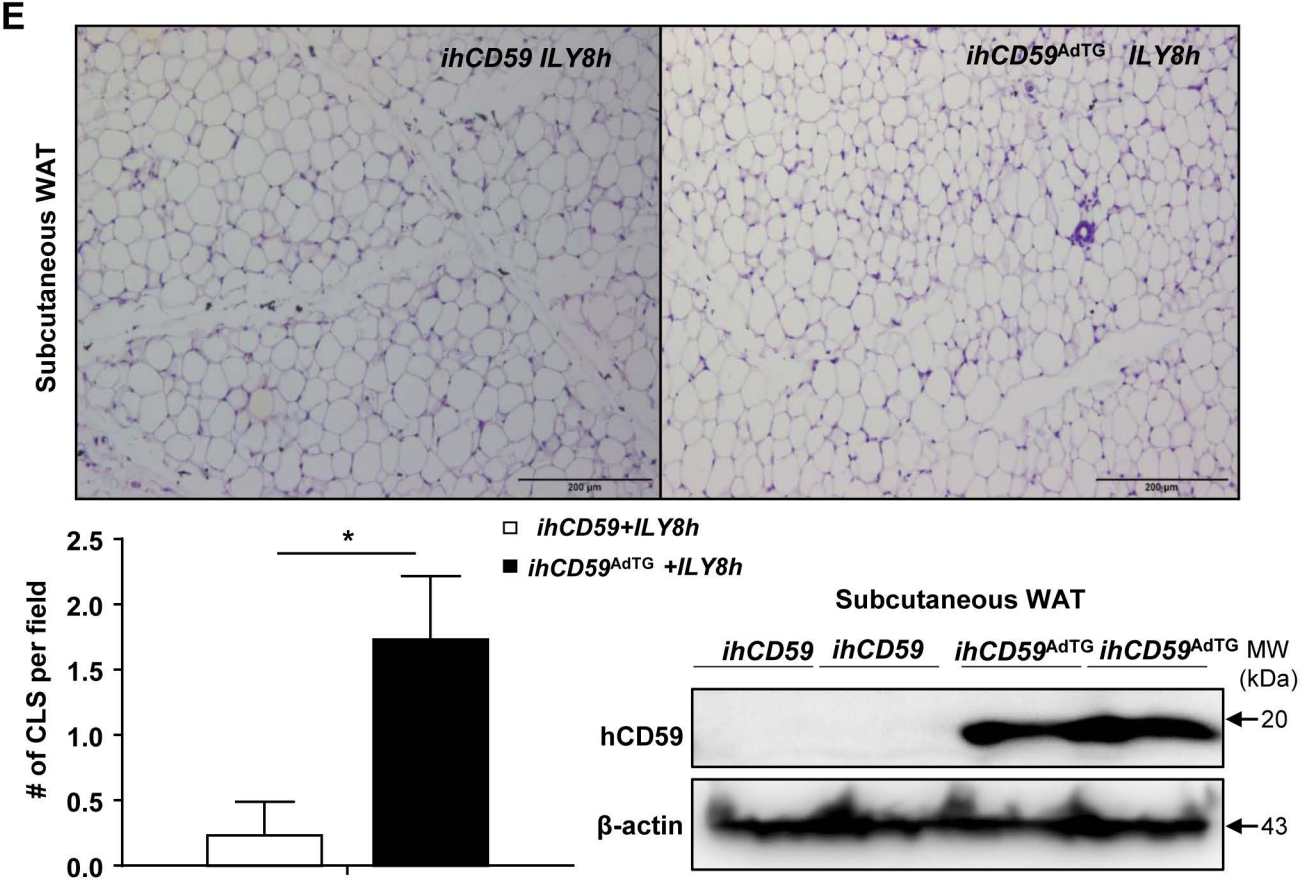
1. Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res 1986;27:114-120.

2. Lin YH, Salem N, Jr., Wells EM, Zhou W, Loewke JD, Brown JA, et al. Automated high-throughput fatty acid analysis of umbilical cord serum and application to an epidemiological study. Lipids 2012;47:527-539.

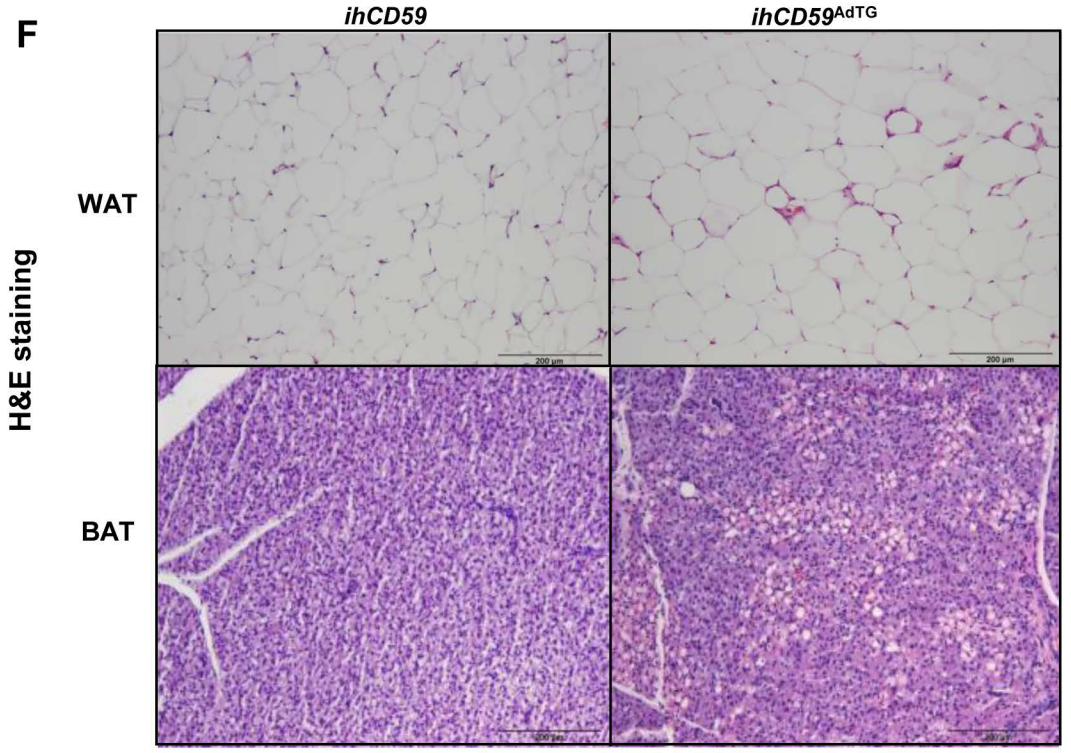
Supporting Fig. S1

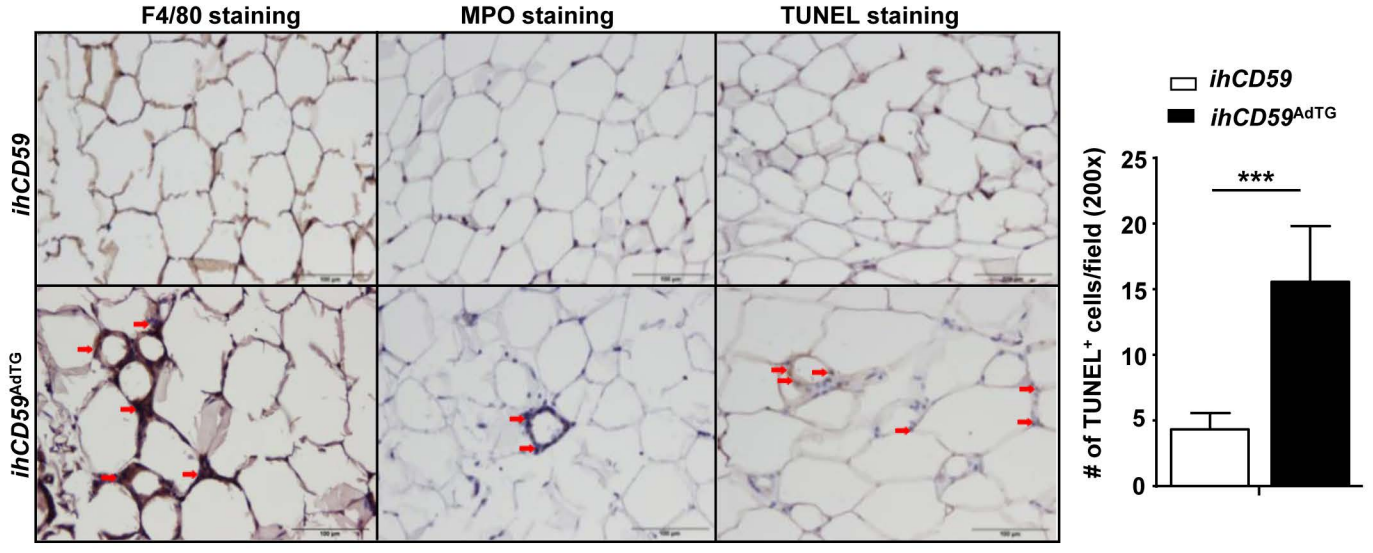


Supporting Fig. S1A-D. Characterization of *AdipoCre*$^{+}$*ihCD59 (ihCD59*^{AdTG}*)* mice. (A) Scheme for generation of the *ihCD59*^{AdTG} mice. *ihCD59*^{AdTG} mice was developed by crossing AdipoCre mice with *ihCD59* mice. (B) *ihCD59*^{AdTG} mice express hCD59 exclusively in adipocytes. Western blot analysis of hCD59 in human cell lines (MDA-MB-231, HELA), mouse cell line (NIH-3T3), white and brown adipose tissues (B6-J, WT *ihCD59* and *ihCD59*^{AdTG}). (C) hCD59 was not detected in the liver from *ihCD59*^{AdTG} mice. Positive control staining for hCD59 in liver tissue from *AlbCre*$^{+}$*hCD59* mice. Arrows indicate the hCD59 positive cells (red) in the liver tissue in *AlbCre*$^{+}$*hCD59* mice. (D) Primary adipocytes (10 days after adipocyte differentiation) from *ihCD59* and *ihCD59*^{AdTG} mice were incubated with ILY for 2 hours, cell death was determined by Trypan blue assay. ***P<0.001



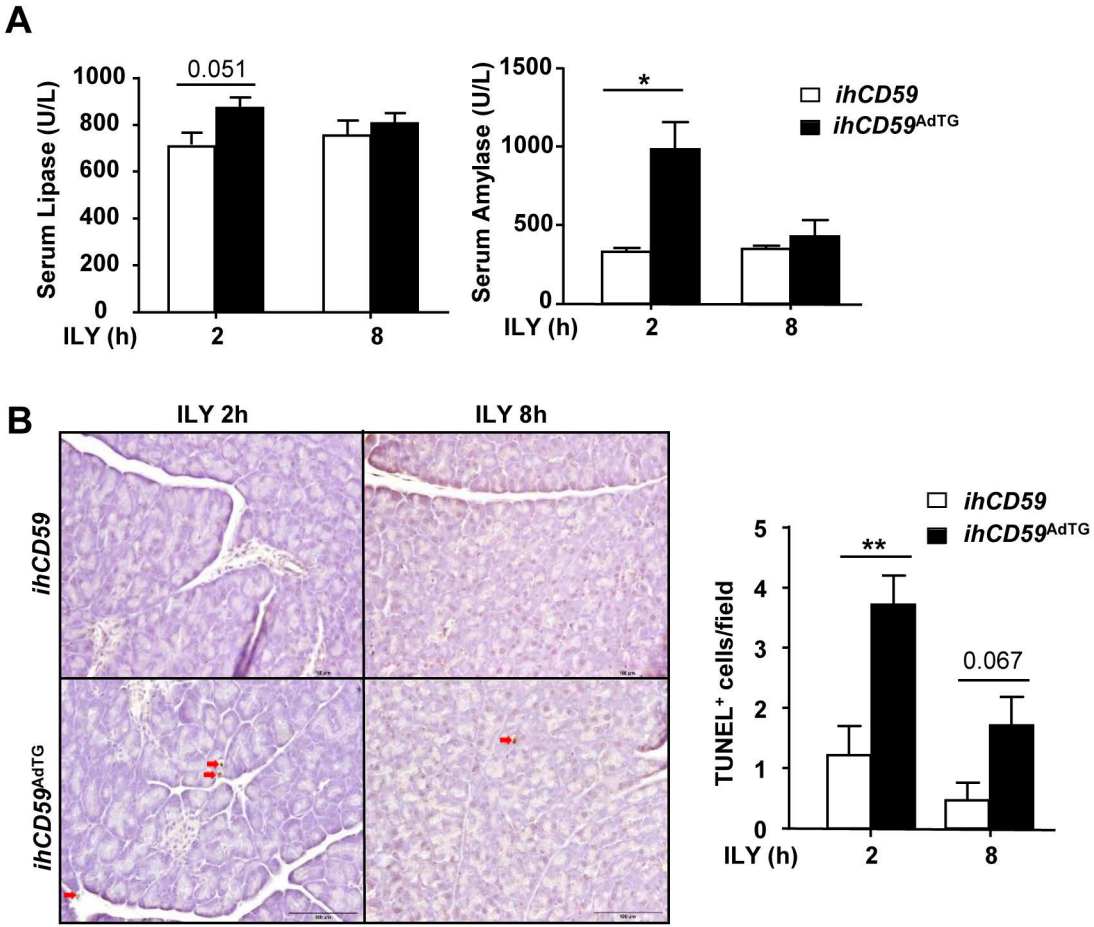
Supporting Fig. S1E. Characterization of *AdipoCre*$^{+}$*ihCD59 (ihCD59*^{AdTG}) mice.** (E) *ihCD59* and *ihCD59*^{AdTG} mice were treated with ILY for 8 hours. Subcutaneous adipose tissues were collected and subjected to H&E staining. Representative images are shown, and the number of CLS was quantified. Subcutaneous adipose tissues were also subjected to western blot analysis to confirm hCD59 expression (Right lower panel). *P<0.05





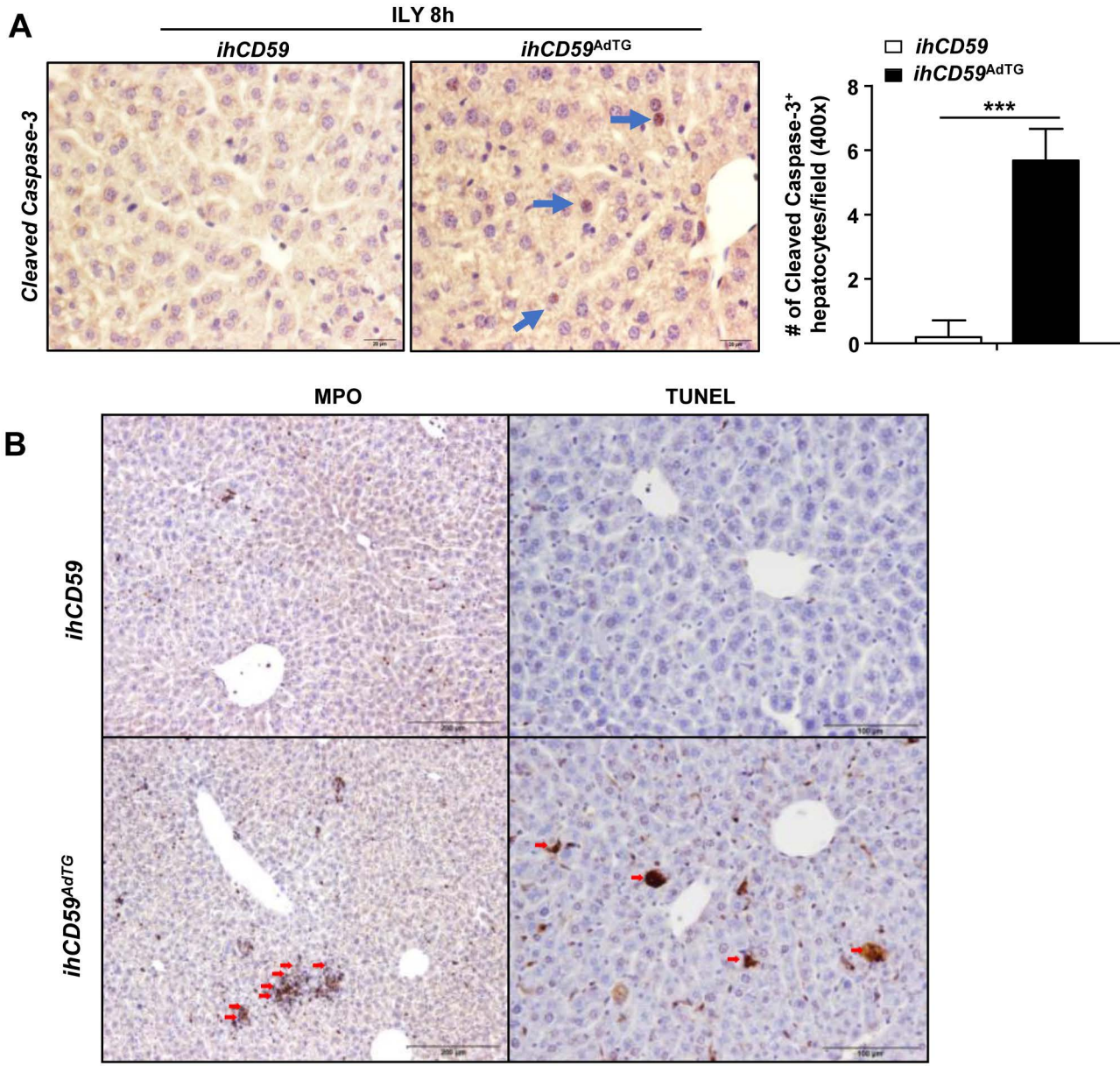
Supporting Fig. S1F, G. ILY injection induces adipocyte death and inflammation in *ihCD59*^{AdTG} mice. *ihCD59*^{AdTG} and their littermate control (*ihCD59*) mice were treated with ILY for 2 hours. Representative H&E staining of white adipose tissue and brown adipose tissue sections (upper panel). Representative immunostaining of F4/80 and MPO and TUNEL staining in white adipose tissues (lower panel). Arrows indicate F4/80^{+}, MPO^{+} and TUNEL^{+} cells (red) in the adipose tissue in *ihCD59*^{AdTG} mice. TUNEL^{+} cells are quantified and shown in the right. ***P<0.001

Supporting Fig. S2



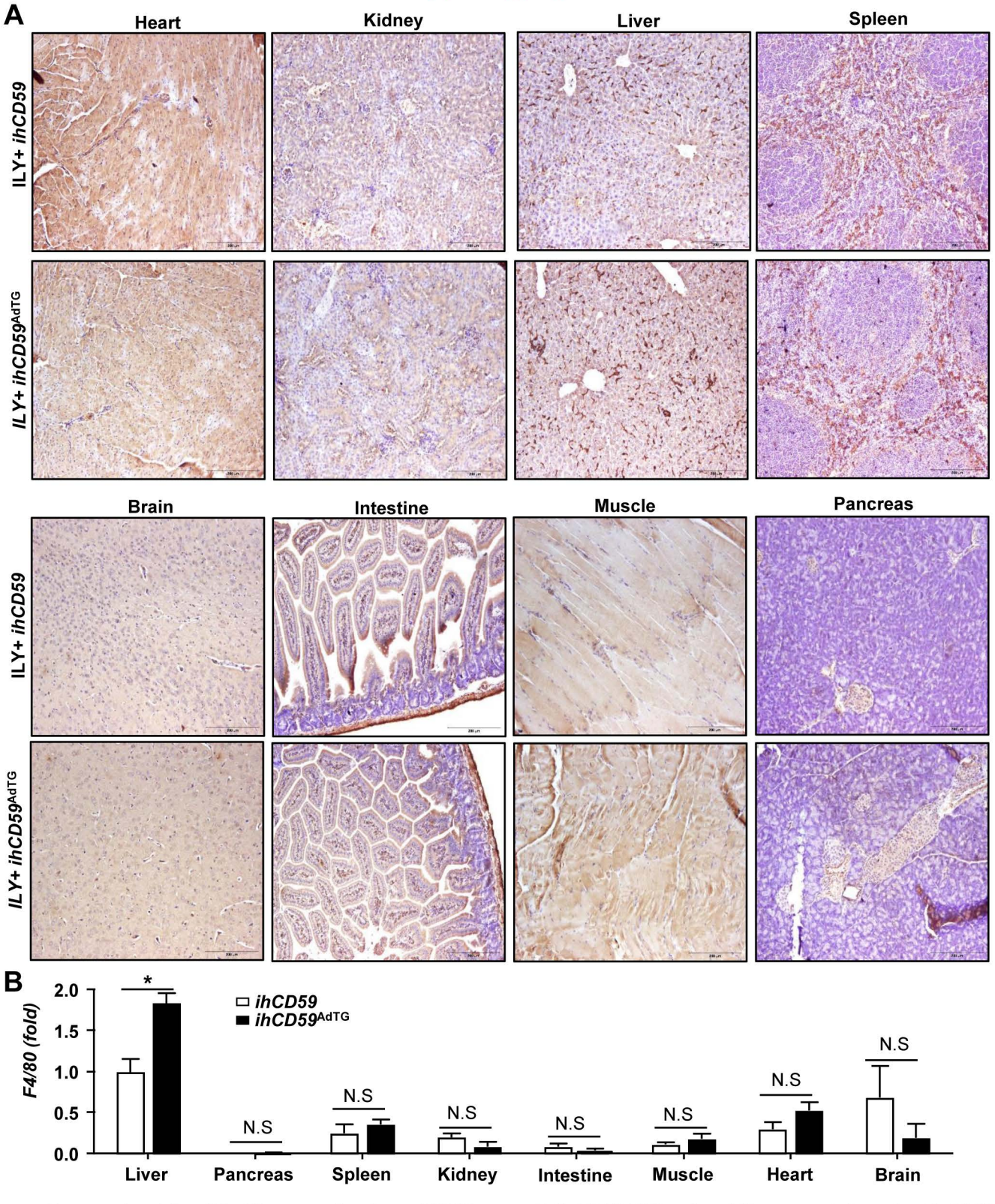
Supporting Fig. S2. Injection of ILY induces acute pancreatitis in *ihCD59*^{AdTG} mice. *ihCD59*^{AdTG} and their littermate WT (*ihCD59)* mice were treated with ILY for various time points. (A) Serum lipase and amylase levels. (B) Representative TUNEL staining is shown. Arrows indicate TUNEL^{+} cells. Quantitation is shown in the right. *P<0.05, **P<0.01

Supporting Fig. S3



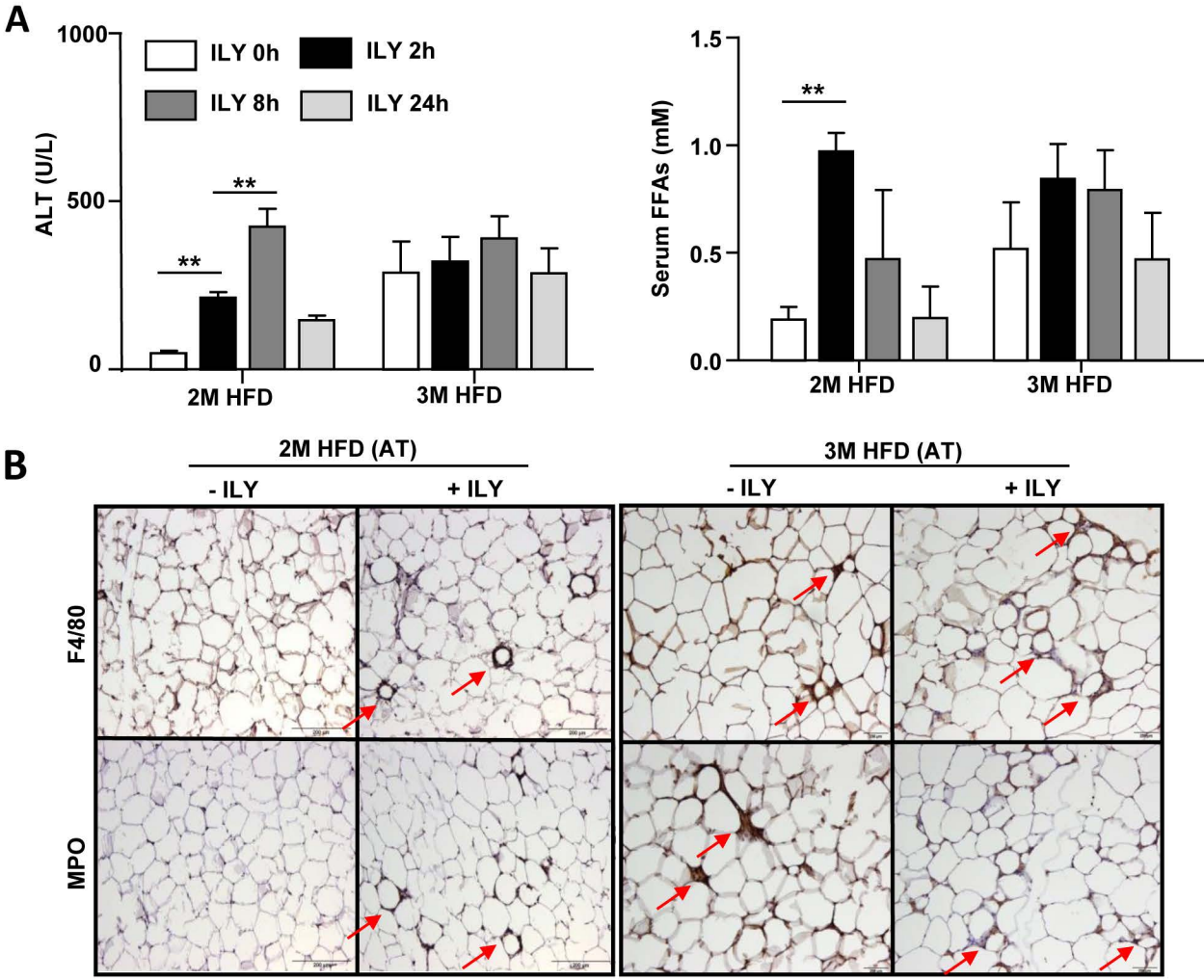
Supporting Fig. S3. Injection of ILY induces liver cell death in *ihCD59*^{AdTG} mice. *ihCD59*^{AdTG} and their littermate WT (*ihCD59)* mice were treated with ILY for 8 hours. (A) Liver tissues were subjected to immunohistochemistry analysis of cleaved caspase 3. Arrows indicate cleaved caspase 3^+ hepatocytes. Quantitation is shown in the right. ***$P<0.001$. (B) Liver tissues were subjected to MPO and TUNEL staining. Arrows indicate MPO^+ and $TUNEL^+$ cells.

Supporting Fig. S4



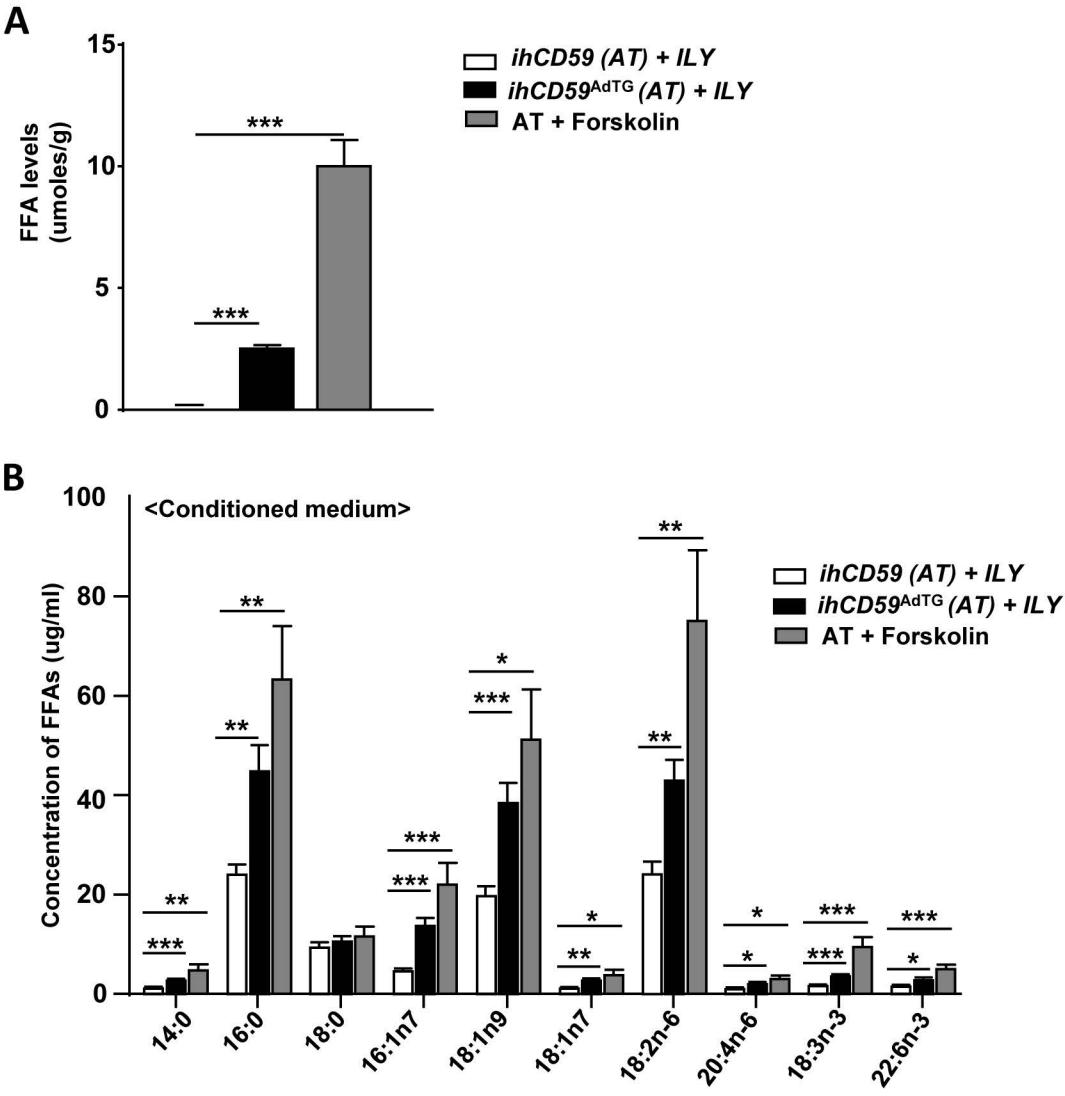
Supporting Fig. S4. Characterization of ILY treated *ihCD59*^{AdTG} mice. Mice were injected with ILY for 8 hours. Various organs were collected for analyses. (A) Representative F4/80 immunostaining for 4 mice in each group. (B) Various organs were subjected to qRT-PCR analyses of F4/80. Magnification in panel A x100. *P<0.05.

Supporting Fig. S5



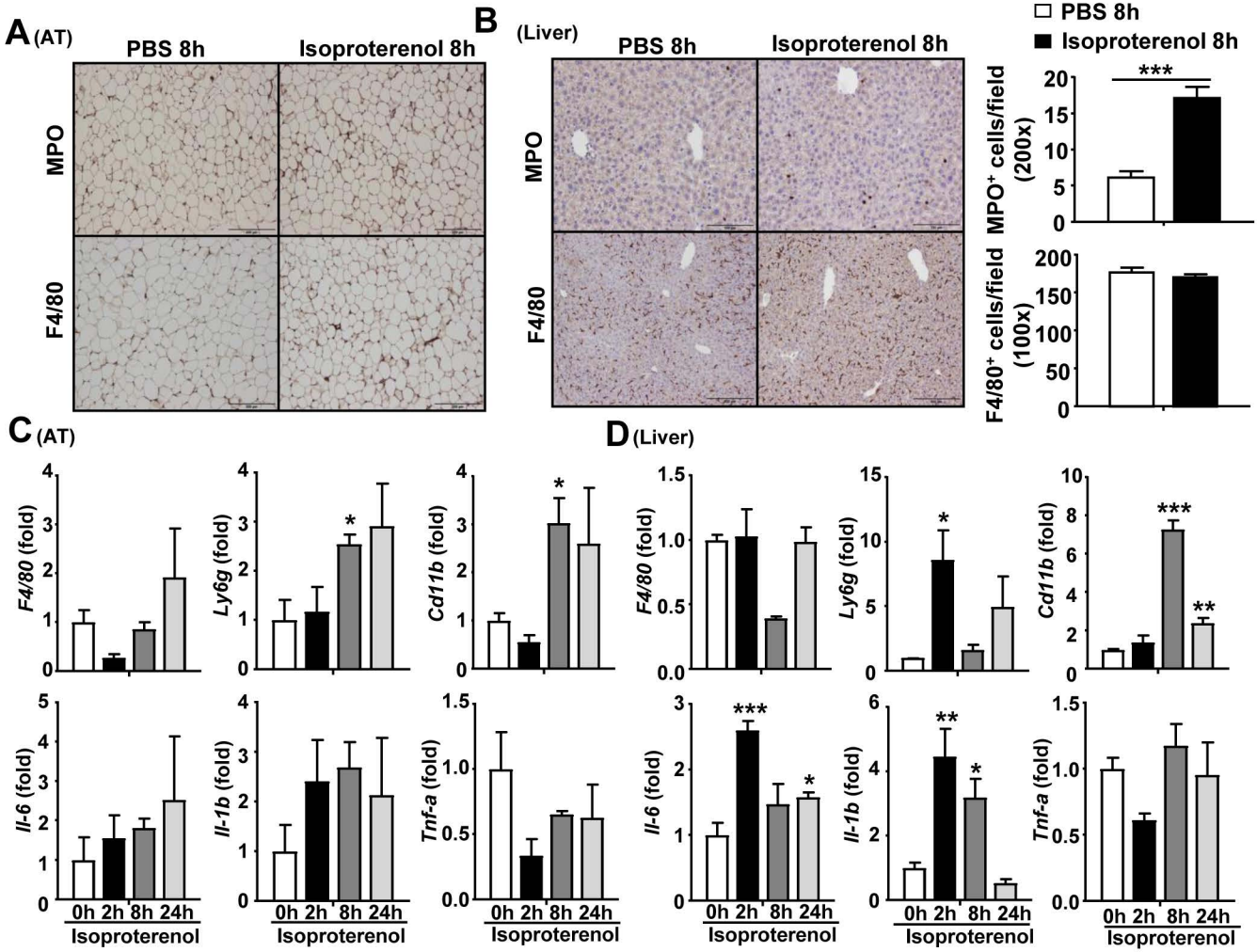
Supporting Fig. S5. Effects of ILY injection on 2-month and 3-month HFD-fed *ihCD59*^{AdTG} mice. *ihCD59*^{AdTG} were fed a HFD for 2 or 3 months, and subsequently treated with ILY for various time points. (A) Serum ALT and FFAs. (B) Representative immunostaining of white adipose tissue sections from 8-hour ILY-treated mice. Arrows indicate F4/80+ and MPO+ staining. $^{**}P<0.005$

Supporting Fig. S6



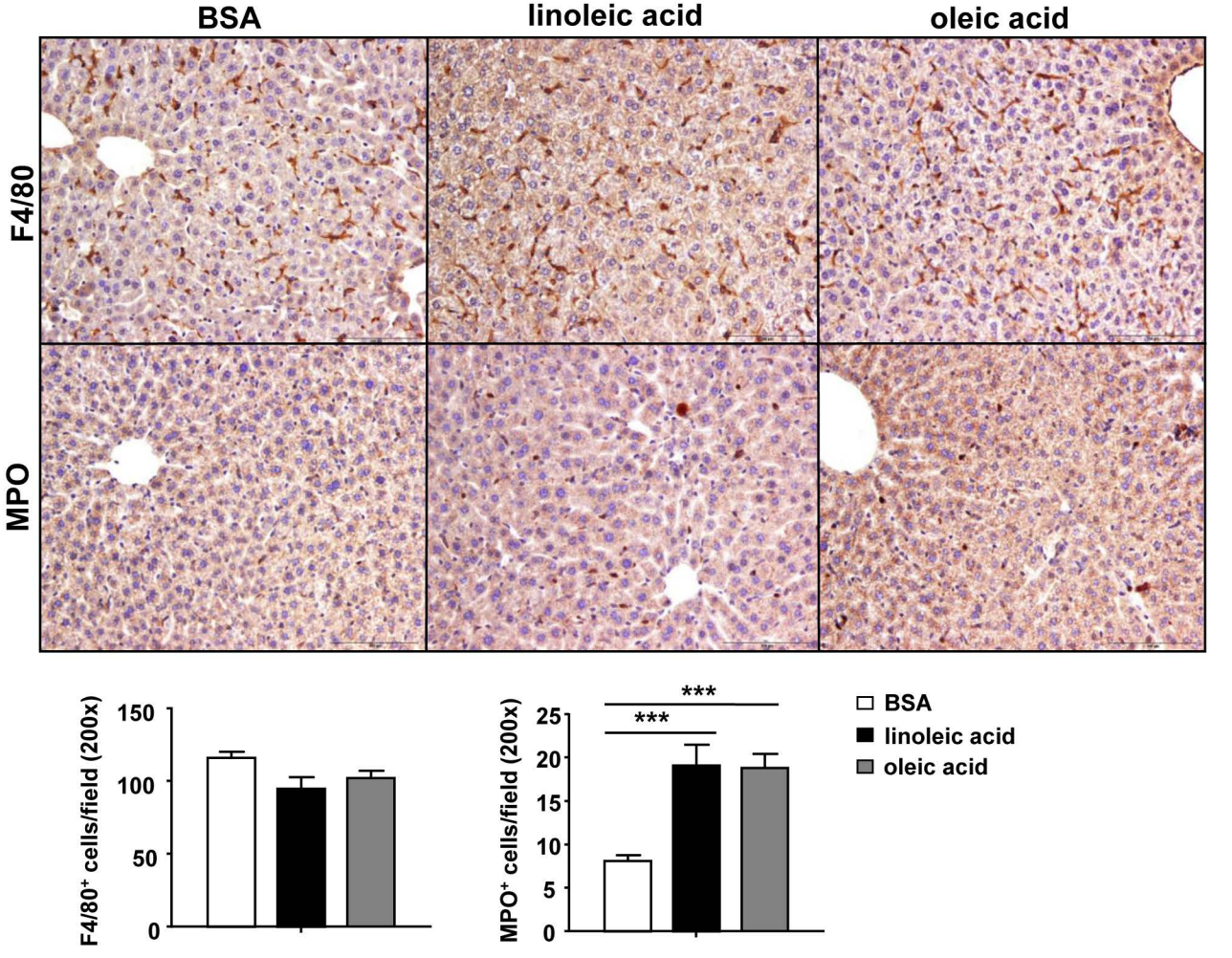
Supporting Fig. S6. Acute adipocyte death enhances lipolysis in adipocytes *ex vivo*. Isolated adipose tissues were incubated with ILY for 2 hours, and the release of FFA to media was determined and normalized to the fat tissue weight. (A) Released FFA from explants. (B) Concentration of fatty acids from explants (μg/mL medium). Values represent means ± S.E.M. (n=4). **P*<0.05, ***P*<0.01, ****P*<0.001. AT: adipose tissue; FFA: free fatty acid.

Supporting Fig. S7



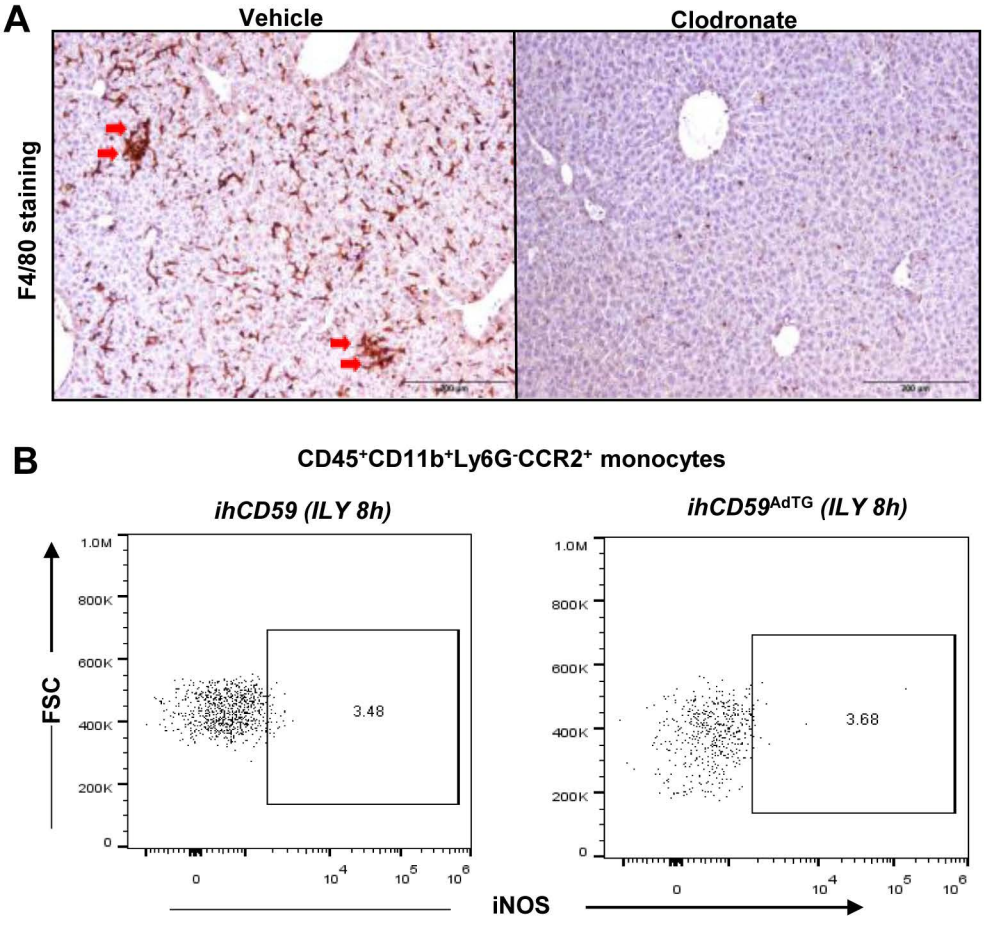
Supporting Fig. S7. Isoproterenol injection induces liver injury and inflammation. C57BL/6J mice were treated with isoproterenol for various time points. (A, B) Representative immunostaining of MPO and F4/80 in adipose tissue (AT) and liver tissue. Values represent means ± S.E.M. (n=5-6). ***P<0.001. (C, D) Pro-inflammatory cytokines in adipose and liver tissues. Values represent means ± S.E.M. (n=5-6). *P<0.05, **P<0.01, ***P<0.001 in comparison with 0h time point.

Supporting Fig. S8



Supporting Fig. S8. FFA injection induces hepatic neutrophil infiltration but not macrophage infiltration. C57BL/6 mice were injected with BSA, linoleic acid, or oleic acid for 6 hours. Liver tissues were collected and subjected to immunohistochemistry analyses with anti-F4/80 and anti-MPO antibodies. No $F4/80^+$ clusters were observed. The number of $F4/80^+$ and MPO^+ cells/per field was quantified. Values represent means ± SEM (n=7). ***P<0.001.

Supporting Fig. S9

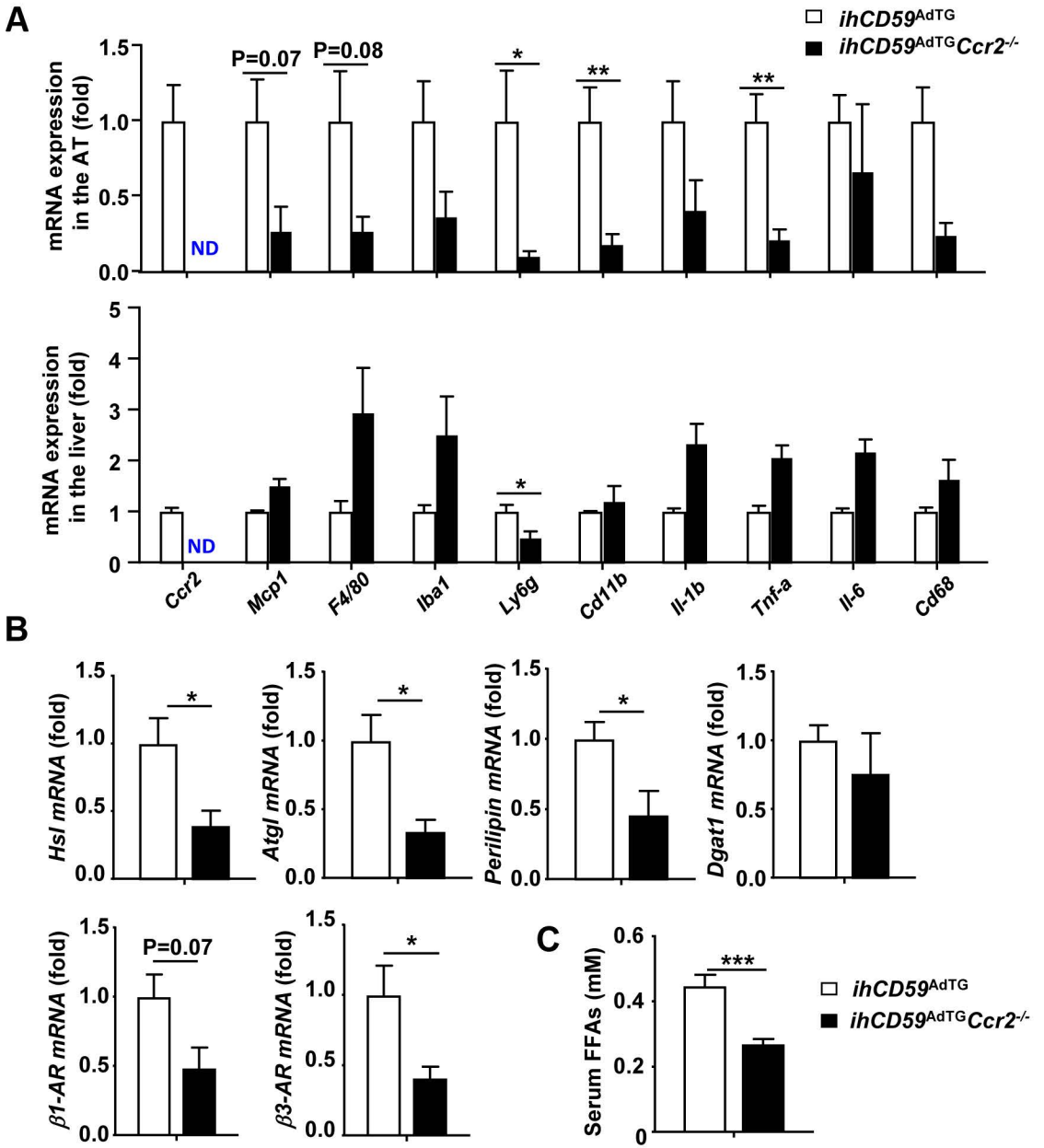


Supporting Fig. S9A-B.

A.. Immunostaining of F4/80 confirms macrophage depletion in liver tissues after clodronate injection. *ihCD59*^{AdTG} mice were injected with Liposomal Clodronate or vehicle control for 24 hours, and then injected with ILY. Mice were sacrificed 2 hours post ILY injection. Representative immunostaining of F4/80 in liver tissues.

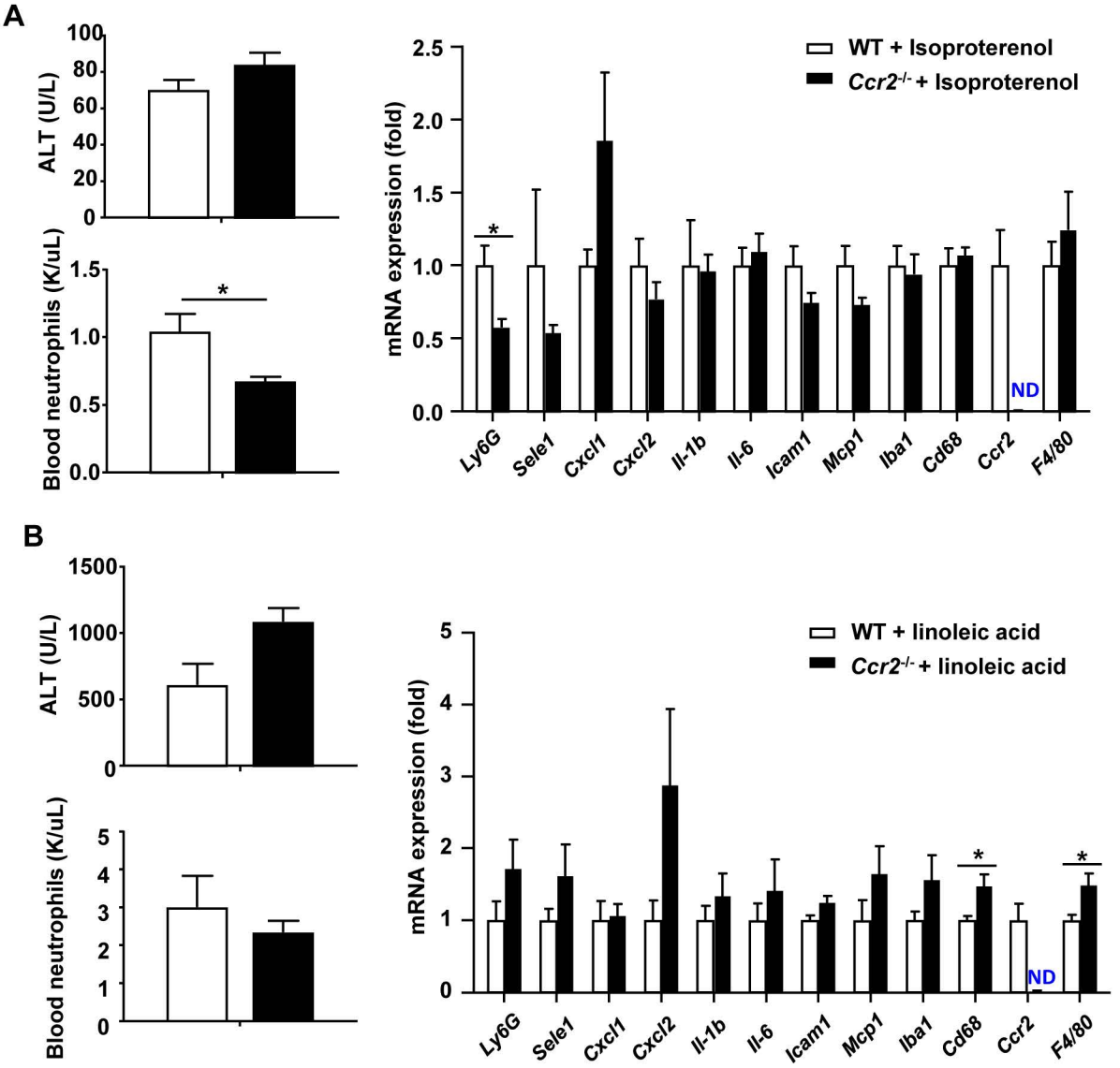
B. Expression of iNOS protein was not changed in CD45^{+}CD11b^{+}Ly6G^{-}CCR2^{+} monocytes from the peripheral blood in ILY-treated *ihCD59*^{AdTG} mice. *ihCD59* and *ihCD59*^{AdTG} mice were treated with ILY for 8 hours. Flow cytometric analysis of iNOS in circulating CD45^{+}CCR2^{+}CD11b^{+}Ly6G^{-} cells in the blood.

Supporting Fig. S10



Supporting Fig. S10. Expression of pro-inflammatory and lipolysis-related genes in adipose tissues (AT) in ILY-treated *ihCD59*^{AdTG} and *ihCD59*^{AdTG}*Ccr2*$^{-/-}$ mice. *ihCD59*^{AdTG} and *ihCD59*^{AdTG}*Ccr2*$^{-/-}$ mice were treated with ILY for 8 hours, adipose and liver tissues were subjected to RT-qPCR. (A) Pro-inflammatory gene expression in AT and liver tissues. (B) Lipolysis-related gene and β-adrenergic receptor (AR) gene expression in AT. (C) Serum FFA levels. Values represent means ± S.E.M. (n=5). **P*<0.05, ***P*<0.01, ****P*<0.001. ND; not detectable. AT: adipose tissue.

Supporting Fig. S11



Supporting Fig. S11. Isoproterenol or FFA injection induces liver injury independent of Ccr2. (A) *Ccr2*$^{-/-}$ mice were injected with isoproterenol for 8 hours. Serum ALT level was measured, Number of peripheral neutrophils were counted (Left panel). Pro-inflammatory cytokine mRNA levels in the liver were measured by RT-qPCR (Right panel). (B) *Ccr2*$^{-/-}$ mice were injected with linoleic acid for 6 hours. Serum ALT level was measured, Number of peripheral neutrophils were counted (Left panel). Pro-inflammatory cytokine mRNA levels in the liver were measured by RT-qPCR (Right panel). Values represent means ± S.E.M. (n=4-5). *P<0.05, ND; not detectable.

Supporting Table 1

Supporting Table 1. Primer sequences for Real-time PCR

Genes (mouse)	Forward Primer (5?3?)	Reverse Primer (5?3?)
Tnf-a	AGGCTGCCCCGACTACGT	GACTTTCTCCTGGTATGAGATAGCAAA
Il-6	TCCATCCAGTTGCCTTCTTG	TTCCACGATTTCCCAGAGAAC
Il-1b	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
Mcp-1	TCTGGACCCATTCCTTCTTGG	TCAGCCAGATGCAGTTAACGC
Ifn-g	TAGCCAAGACTGTGATTGCGG	AGACATCTCCTCCCATCAGCAG
Cd11b	ATGGACGCTGATGGCAATACC	TCCCCATTCACGTCTCCCA
Cd11c	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTCA
Hsl	CCTGCAAGAGTATGTCACGC	GGAGAGAGTCTGCAGGAACG
Atgl	CCACTCACATCTACGGAGCC	TAATGTTGGCACCTGCTTCA
Perilipin	TGAAGCAGGGCCACTCTC	GACACCACCTGCATGGCT
Dgat1	GACGGCTACTGGGATCTGA	TCACCACACACCAATTCAGG
Cd68	CCATCCTTCACGATGACACCT	GGCAGGGTTATGAGTGACAGTT
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Sele1	AGCAGAGTTTCACGTTGCAGG	TGGCGCAGATAAGGCTTCA
Cxcl1	TCTCCGTTACTTGGGGAC	CCACACTCAAGAATGGTCGC
Cxcl2	TCCAGGTCAGTTAGCCTTGC	CGGTCAAAAAGTTTGCCTTG
Mip1a	TGAGAGTCTTGGAGGCAGCGA	TGTGGCTACTTGGCAGCAAACA
Icam1	CAATTTCTCATGCCGCACAG	AGCTGGAAGATCGAAAGTCCG
Iba1	CAGACTGCCAGCCTAAGACA	AGGAATTGCTTGTTGATCCC
Il-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
Ly6g	TGCGTTGCTCTGGAGATAGA	CAGAGTAGTGGGGCAGATGG
β3-AR	CCTTCCCAGCCAGCCCTGTT	TGCTAGATCTCCATGGTCCTTCA
β1-AR	TCTACGTGCCCCTGTGCAT	GCTGTCGATCTTCTTTACCTGTTT
Inos	CCAAGCCCTCACCTACTTCC	CTCTGAGGGCTGACACAAGG
18s	ACGGAAGGGCACCACCAGGA	CACCACCACCCACGGAATCG

Supporting Table 2

Supporting Table 2. Serum biochemistry profiles of fatty acid injected B6J mice

Variables	Control group	Linoleic acid	Oleic acid	p Value* (Cont vs linoleic acid)	p Value* (Cont vs oleic acid)
Alkaline Phosphatase (U/L)	104.00±8.19	60.33±7.22	87.00±3.46	0.016	0.13
ALT (U/L)	55.00±7.81	661.00±177.10	794.00±109.00	0.03	0.003
AST (U/L)	139.00±23.07	1641.67±421.30	1354.00±24.88	0.02	3.63E-06
Albumin (g/dL)	2.80±0.10	1.93±0.24	2.90±0.20	0.03	0.68
Cholesterol (mg/dL)	84.00±1.73	51.67±5.67	86.00±5.29	0.006	0.74
Triglyceride (mg/dL)	105.00±12.12	72.33±7.88	153.00±7.94	0.087	0.03
Sodium (mmol/L)	479.00±1.00	374.67±52.67	489.00±1.73	0.12	0.008
Potassium (mmol/L)	7.10±0.10	6.27±1.07	8.00±0.10	0.48	0.003
Chloride (mmol/L)	415.00±1.00	326.00±47.00	420.00	0.13	0.008
Calcium (mmol/L)	2.20±0.03	1.64±0.23	2.16±0.02	0.07	0.28
Magnesium (mmol/L)	1.00±0.04	1.28±0.17	1.75±0.07	0.19	0.0007
Phosphorus (mg/dL)	33.30±0.17	28.73±4.28	37.10±0.20	0.35	0.0001
Amylase (U/L)	2671.00±87.71	10737.00±4161	17978.00±710.50	0.13	2.8E-05
BUN (mg/dL)	24.00±1.73	31.33±2.91	42.00±1.73	0.10	0.002
Creatinine (mg/dL)	0.1	0.1	0.1	1	1
CK (CKA5) (U/L)	775.00±181.70	5494.00±1866.00	9446.00±2495.00	0.07	0.03
Glucose (mg/dL)	301.00±13.11	162.00±12.00	312.00±48.59	0.001	0.84
Lactate Dehydrogenase (U/L)	484.00±117.80	5252.67±1214.00	3902.00±383.00	0.017	0.001
Protein (g/dL)	4.30±0.10	3.27±0.47	4.60±0.10	0.10	0.10
Uric Acid (mg/dL)	1.90±0.10	1.67±0.13	3.20±0.10	0.23	0.0008
Bilirubin (mg/dL)	<0.2	<0.2	<0.2	-	-

B6J mice were injected with fatty acids for 6 hours. Serum was collected for measurement of various parameters. Values represent means ± S.E.M. p Value* in this column was obtained from t-test analysis between two groups of vehicle control and linoleic acid or oleic acid.