Supplementary Materials

HIPPO SIGNALING CONTROLS NLRP3 ACTIVATION AND GOVERNS IMMUNOREGULATION OF MESENCHYMAL STEM CELLS IN MOUSE LIVER INJURY

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Experimental Procedures

Animals. The floxed YAP ($YAP^{FL/FL}$) mice (The Jackson Laboratory, Bar Harbor, ME) and the mice expressing Cre recombinase under the control of the Lysozyme 2 (Lyz2) promoter (LysM-Cre; The Jackson Laboratory) were used to generate myeloid-specific YAP knockout ($YAP^{M\text{-}KO}$) mice. Two steps were used to generate $YAP^{M\text{-}KO}$ mice. First, a homozygous loxP-flanked YAP mouse was mated with a homozygous Lyz2-Cre mouse to generate the F1 mice that were heterozygous for a loxP-flanked YAP allele and heterozygous for the Lyz2-Cre. Next, these F1 mice were backcrossed to the homozygous loxP-flanked YAP mice, resulting in generation of $YAP^{M\text{-}KO}$ (25% of the offspring), which were homozygous for the loxP-flanked YAP allele and heterozygous for the Lyz2-Cre allele (Supplementary Figure 1). Mouse genotyping was performed by using a standard protocol with primers described in the JAX Genotyping protocols database. The β-$catenin^{M\text{-}KO}$ was generated as described (14). Male C57BL/6 wild type (WT), $YAP^{FL/FL}$,

YAP$^{M\text{-}KO}$, β-catenin$^{FL/FL}$, and β-catenin$^{M\text{-}KO}$ mice at 6-8 weeks of age were used. Animals, housed in UCLA animal facility under specific pathogen-free conditions, received humane care according to the recommendations in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. The study protocols were approved by the Institutional Animal Care and Use Committee of University of California at Los Angeles.

Mouse liver IRI model and treatment. We used a well-established mouse model of warm hepatic ischemia followed by reperfusion, as described (13). Mice were injected with heparin (100 U/kg) and an atraumatic clip was used to interrupt the arterial/portal venous blood supply to the cephalad liver lobes. After 90 minutes of ischemia the clip was removed and mice were sacrificed at 6 hours of reperfusion. Some animals were injected via tail vein with bone marrow-derived MSCs ($1x10^6$cells in PBS/mouse) or pre-labeled with 5-chloromethylfluorescein diacetate (CMFDA) green fluorescent dye (Invitrogen) 24h prior to ischemia.

Hepatocellular function assay. Serum alanine aminotransferase (sALT) levels, an indicator of hepatocellular injury, were measured by IDEXX Laboratories (Westbrook, ME).

Histology and immunofluorescence staining. Liver sections were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki's histological criteria (18). CD68/arginase1 (Arg1) double positive macrophages were identified by immunofluorescence staining using goat anti-mouse CD68 (Santa Cruz Biotechnology) and rabbit anti-mouse Arg1 monoclonal antibodies (mAbs) (Abcam, MA) followed by incubating with secondary Cy3-conjugated AffiniPure donkey anti-goat IgG Ab and AlexFluor-conjugated AffiniPure donkey anti-rabbit IgG Ab (Jackson Immunoresearch, PA). The primary rabbit anti-mouse β-catenin or YAP mAb and the secondary Cy3-conjugated AffiniPure donkey anti-rabbit IgG Ab or AlexFluor-conjugated AffiniPure donkey anti-rabbit IgG Ab was used for staining β-catenin or YAP positive cells. The samples were covered with VECTASHIELD mounting medium containing DAPI (Vector, CA) and viewed with a fluorescence microscope (Leica).

Quantitative RT-PCR analysis. Quantitative real-time PCR was performed as described (14). In brief, total RNA was extracted from cells or liver tissues with Trizol reagent (Invitrogen), and 2μg of RNA was reverse transcribed into complementary first-strand cDNA using M-MLV reverse transcriptase (Invitrogen). The SYBR Green Mix (Applied Biosystems) was used for quantitative real-time PCR (qRT-PCR). Target gene expressions were calculated by their ratios to the housekeeping gene HPRT. Primer sequences used for the amplification of TNF-α, IL-1β, IL-10, TGF-β, and HPRT are shown in Supplementary Table 1.

Immunoblot analysis. Protein was extracted from liver tissue or cell cultures as described (14). In brief, protein was extracted from liver tissue or cell cultures with ice-cold protein lysis buffer (50 mMTris, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton-100). Proteins (30 μg/sample) were subjected to 4-20% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, CA). The nuclear and cytosolic fractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific). Rabbit anti-mouse β-catenin, p-β-catenin, p-MST1/2, MST1/2, p-LATS1, LATS1, p-YAP, YAP, Arg1, iNOS, XBP1s, NLRP3, cleaved caspase-1, p-Akt, Akt, Lamin B, and β-actin Abs (Cell Signaling Technology, MA) were used. Relative quantities of protein were determined and expressed in absorbance units comparing to β-actin or Lamin B using a densitometer (Kodak Digital Science 1D Analysis Software).

Immunoprecipitation analysis. BMMs from co-culture were lysed in NP-40 lysis buffer (50mM Tris pH7.4, 10 mM EDTA, 150 mM NaCl, 1% NP-40) containing protease inhibitors. The lysates were incubated with β-catenin antibody or control IgG and protein A/G beads at 4 ^{o}C overnight. After immunoprecipitation, the immunocomplexes were washed with lysis buffer three times and analyzed by standard immunoblot procedures.

Isolation of hepatic Kupffer cells, BMMs and bone marrow-derived MSCs. Primary liver macrophages (Kupffer cells) were isolated, as described (14). In brief, mouse livers were perfused in situ with warmed (37°C) HBSS solution, followed by a collagenase-buffer

(collagenase type IV, Sigma-Aldrich). Perfused livers were dissected and teased through 70-μm nylon mesh cell strainers (BD Biosciences). The nonparenchymal cells (NPCs) were separated from hepatocytes and layered onto a 50%/25% two-step Percoll gradient (Sigma-Aldrich). After centrifugation, KCs in the middle layer were collected and allowed to attach onto cell culture plates in DMEM with 10% FBS, 10 mM HEPES, 2 mM GlutaMax, 100 U/ml penicillin, and 100 μg/ml streptomycin for 15 min at 37°C. The purity of liver macrophages was 80% as assessed by immunofluorescence staining for $CD11b^+$ (data not shown). Murine bone marrow-derived macrophages (BMMs) were generated, as described (14). BMMs were cultured in DMEM supplemented with 10% FBS and 15% L929-conditioned medium. Bone marrow-derived MSCs were isolated, as described (19). In brief, the bone marrow cells were cultured with α-MEM (supplemented with 15% FBS, 0.1% 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin). Twenty-four hours later, culture medium was refreshed to remove non-adherent cells. MSCs were used in the experiments only after 2 to 3 expansion passages to ensure depletion of monocytes/macrophages.

In vitro transfection. BMMs ($1x10^6$/well) were cultured for 7 days and then transfected with CRISPR/Cas9 XBP1 knockout (KO), CRISPR YAP activation or CRISPR NLRP3 activation vector (Santa Cruz Biotechnology) by using Lipofectamine™ 3000 according to the manufacturer's instructions (Invitrogen). Control cells were treated with negative control CRISPR vector. After 24-48h, cells were supplemented with 100 ng/ml of LPS for additional 6h.

Co-culture of macrophages and MSCs. Macrophages were cultured in 6-well plates at a concentration of $1x10^6$ cells per well. After 24h, the 0.4μm-pore size Corning transwell inserts (Sigma-Aldrich) containing $2x10^5$ MSCs were placed into the 6-well plate with the macrophages that were initially seeded. Co-cultures were incubated for 24h with or without adding LPS (100 ng/ml).

ELISA assay. The cytokine TNF-α, IL-1β, IL-10, and TGF-β (eBioscience, CA), and PGE2 (R&D Systems, MN) levels were analyzed in mouse serum and co-culture supernatants by an ELISA assay according to the manufacturer's instructions.

Luciferase assays. The β-catenin promoter:luciferase reporter plasmid (β-catenin-luciferase) was constructed in pGL3 luciferase vector (Promega, WI) according to the manufacturer's instructions. BMMs were transfected with 1 μg of pGL3-β-catenin-luciferase vector. After transfection for 6h, the cells were washed and transfected with CRISPR YAP activation vector. 48h later, the cells were lysed with Passive Lysis Buffer, and the transcriptional activity was measured using a luciferase assay system (Promega).

Caspase-1 enzymatic activity assay. Caspase-1 enzymatic activity was determined by a colorimetric assay kit (R&D System), as described (11). Briefly, after co-cultured with MSC, A 50μl of cell lysate from BMMs was added to 50μl of caspase-1 reaction buffer in a 96-well flat bottom microplate. Each sample was then added to 200mM caspase-1 substrate, WEHD-pNA, followed by 2h of incubation at 37°C. The enzymatic activity of caspase-1 was measured on an ELISA reader at 405nm wavelength.

Chromatin immunoprecipitation (ChIP). The ChIP analysis was carried out using ChIP Assay Kit (Abcam, MA). Briefly, BMMs were treated with 1% formaldehyde for 10 min to cross-link proteins and chromatin. The reaction was stopped by adding 0.125M glycine for 5 min. Cells were washed with ice cold PBS and then resuspended with ChIP lysis buffer for 10 min. Cell lysates were centrifuged to pellet the nuclei. The cell nuclei were resuspended in nuclei lysis buffer and then subjected to sonication for 15 min. Purified chromatin was analysed on a 1.5 % agarose gel to analyze DNA fragment size. The sheared chromatin was immunoprecipitated with β-catenin or YAP antibody (Cell Signaling Technology) overnight. As a control, the normal IgG was used as a replacement for β-catenin or YAP antibody. The antibody/chromatin samples were mixed with protein A sepharose beads. Protein-DNA complexes were washed and eluted followed

by a cross-link reversal step, and the resulting DNA was purified. For sequential ChIP, sheared chromatin was first immunoprecipitated with β-catenin antibody, followed by elution with a second immunoprecipitation using YAP antibody. DNA from each immunoprecipitation reaction was examined by PCR. The primer for the β-catenin-responsive region of Xbp1 promoter: forward: 5'-ACTAATCCCTTACTACATGCTAGC-3', reverse: 5'-GTAGCAGGGTAGTACTCACTTG-3'.

ChIP-sequencing (ChIP-seq). The ChIP-DNA was then amplified to generate library for sequencing. The workflow consists of fragmentation of whole genome DNA, end repair to generate blunt ends, A-tailing, adaptor ligation and PCR amplification. Different adaptors were used for multiplexing samples in one lane. Sequencing was performed on Illumina HiSeq3000 (Illumina, San Diego, CA) for a single read 50 run at the Technology Center for Genomics & Bioinformatics (TCGB) at UCLA. Data quality check was done on Illumina SAV. Demultiplexing was performed with Illumina Bcl2fastq2 v 2.17 program. Reads were mapped to mouse mm10 genome using the Bowtie1 and MACS2 was used for the peak calling. ChIPseeker was used for the peak annonation. Genome browser representation files were generated by converting ChIP-seq data to bigWig format. This was done using genomeCoverageBed from bedtools v 2.17.0 to generate a bed file then UCSC bedGraphToBigWig to convert the bed to bigWig format.

Statistical analysis. Data are expressed as mean±SD and analyzed by Permutation *t* test and Pearson correlation. Per comparison two-sided *p* values less than 0.05 were considered statistically significant. Multiple group comparisons were made using one-way ANOVA followed by Bonferroni's post hoc test. When groups showed unequal variances, we applied Welch's ANOVA to make multiple group comparisons. All analyses were used by SAS/STAT software, version 9.4.

Supplementary Figure Legends

Supplementary Figure 1: Schematic illustration of generation of myeloid-specific Notch1 knockout mice. Two steps were used to generate myeloid specific YAP KO mice. First, a homozygous loxP flanked YAP mouse is mated with a homozygous Lyz2-Cre mouse to generate the F1 mice that are heterozygous for a loxP-flanked YAP allele and heterozygous for the Lyz2-cre. Next, these F1 mice were backcrossed to the homozygous loxP-flanked YAP mice, resulting in generation of myeloid specific YAP KO mice ($YAP^{M\text{-}KO}$, 25% of the offspring), which were homozygous for the loxP-flanked YAP allele and heterozygous for the Lyz2-Cre allele.

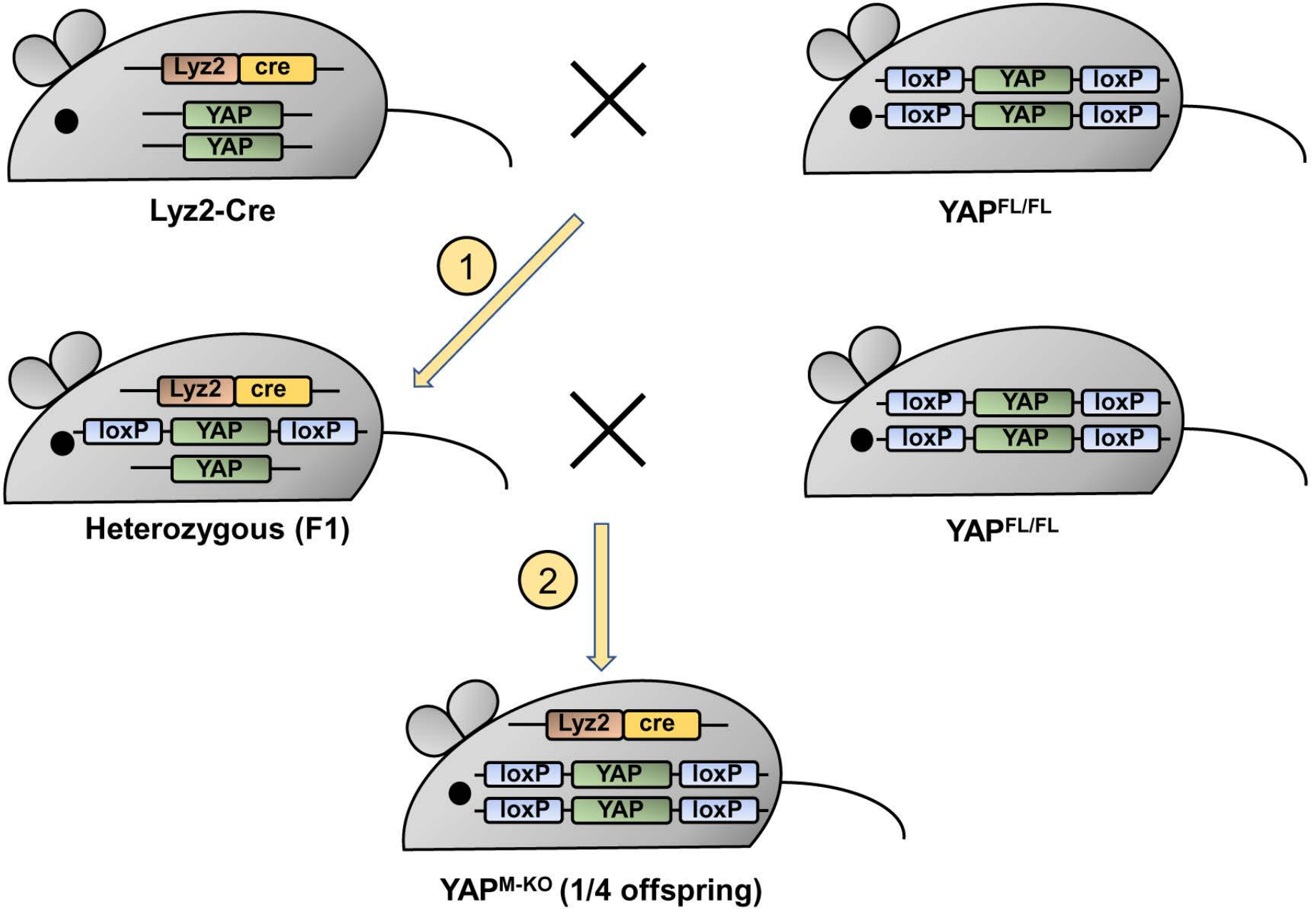
Supplementary Figure 2: Phenotype of macrophage deletion by clodronate liposomes dampened MSC-mediated immune regulation. Macrophage Depletion Kit (Encapsula NanoSciences, Brentwood, TN) was used to deplete macrophages according to the manufacturer's protocols. In brief, 200 µl of clodronate liposomes or control liposomes were injected via tail vein 24h prior to the onset of liver ischemia and 1h before the MSCs ($1x10^6$) administration. (A) Representative histological staining (H&E) of ischemic liver tissue. Scale bars, 100µm. Results representative of 4-6 mice/group. (B) Liver damage, evaluated by Suzuki's histological score. *$p<0.05$. (C) Hepatocellular function in serum samples was evaluated by sALT levels (IU/L). Results expressed as mean±SD (n=4-6 samples/group). *$p<0.05$.

Supplementary Table 1. Primers used in qRT-PCR studies.

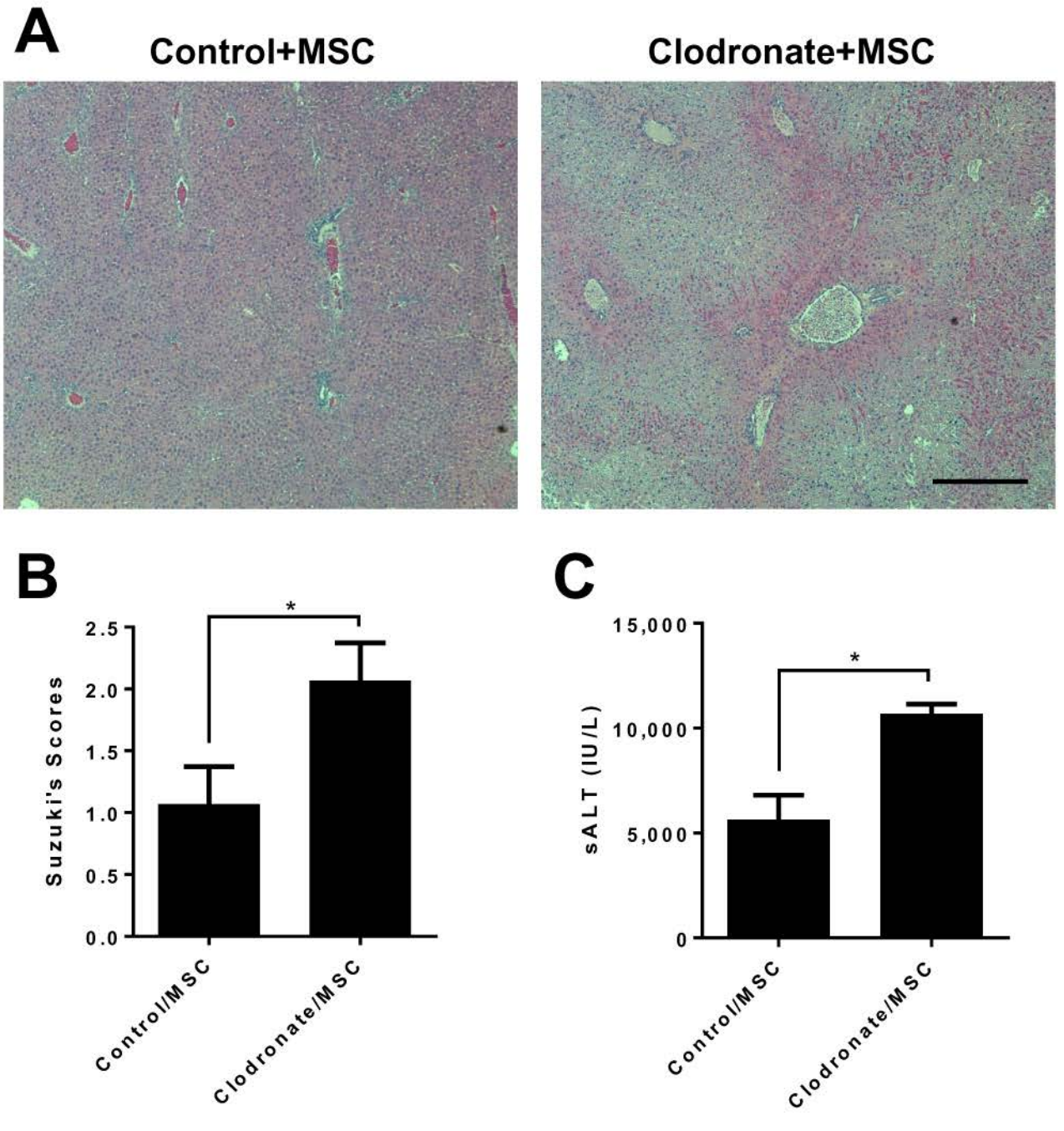
Supplementary Table 1: Primer sequences for the amplification

Target genes	Forward primers	Reverse primers
HPRT	5’-TCAACGGGGGACATAAAAGT-3’	5’-TGCATTGTTTTACCAGTGTCAA-3’
TNF-α	5’- ACGGCATGGATCTCAAAGAC-3’	5’- AGATAGCAAATCGGCTGACG-3’
IL-1β	5’-TGTAATGAAAGACGGCACACC-3’	5’-TCTTCTTTGGGTATTGCTTGG-3’
IL-10	5’-ACTGGCATGAGGATCAGCAG-3’	5’-CTCCTTGATTTCTGGGCCAT-3’
TGF-β	5´-TGCGCTTGCAGAGATTAAAA-3´	5´-CTGCCGTACAACTCCAGTGA-3´

Suppl. Fig. 1


Lyz2
cre
YAP
YAP
Lyz2-Cre
loxP
YAP
loxP
loxP
YAP
loxP
YAP^FL/FL
1
Lyz2
cre
loxP
YAP
loxP
YAP
Heterozygous (F1)
loxP
YAP
loxP
loxP
YAP
loxP
YAP^FL/FL
2
Lyz2
cre
loxP
YAP
loxP
loxP
YAP
loxP
YAP^M-KO (1/4 offspring)

Suppl. Fig. 2


A
Control+MSC
Clodronate+MSC
B
Suzuki's Scores
2.5
2.0
1.5
1.0
0.5
0.0
*
Control/MSC
Clodronate/MSC
C
sALT (IU/L)
15,000
10,000
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Control/MSC
Clodronate/MSC