Novel role of macrophage TXNIP-mediated CYLD-NRF2-OASL1 axis in stress-induced liver inflammation and cell death

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Supplementary materials and methods

Animals. The floxed TXNIP (TXNIP$^{FL/FL}$) mice (B6;129-*Txnip*^{tm1Rlee}/J) and the mice expressing Cre recombinase under the control of the Lysozyme 2 (Lyz2) promoter (LysM-Cre) were obtained from The Jackson Laboratory (Bar Harbor, ME). A targeting construct incorporating a loxP-exon1-FRT-PGKneobpA-FRT-loxP sequence was introduced to J1 129S4/SvJae-derived embryonic stem (ES) cells. Resultant mice were crossed with 129Sv background animals expressing *Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$ to excise the FRT-flanked neomycin cassette, leaving exon 1 flanked by loxP sites. This strain was maintained on a mixed 129 and C57BL/6 genetic background. To generate myeloid-specific TXNIP knockout (TXNIP$^{M\text{-}KO}$) mice, a homozygous loxP-flanked TXNIP mouse was mated with a homozygous Lyz2-Cre mouse to create the F1 mice that were heterozygous for a loxP-flanked TXNIP allele and heterozygous for the Lyz2-Cre. The F1 mice were then backcrossed to the homozygous loxP-flanked TXNIP mice, resulting in the generation of TXNIP$^{M\text{-}KO}$ (25% of the offspring), which were homozygous for the loxP-flanked TXNIP allele and heterozygous for the Lyz2-Cre allele (Fig. S1). Mouse genotyping was performed using a standard protocol with primers described in the JAX Genotyping protocols database. Male mice at 6-8 weeks of age were used in all experiments. This study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Animal protocols were approved by the Institutional Animal Care and Use Committee of The University of California at Los Angeles.

Mouse liver IRI model. We used an established mouse model of warm hepatic ischemia (90min) followed by reperfusion (6h) [5]. Mice were injected with heparin (100U/kg), and an atraumatic clip was used to interrupt the arterial/portal venous blood supply to the cephalad liver lobes. After 90min of ischemia, the clip was removed, and mice were sacrificed at 6h of reperfusion. Some animals were injected via tail vein with OASL1 siRNAs or non-specific (NS)

control siRNA (2.5 mg/kg) (Santa Cruz Biotechnology, Santa Cruz, CA) mixed with mannose-conjugated polymers (Polyplus transfection™, Illkirch, France) at a ratio according to the manufacturer's instructions 4h prior to ischemia as described [1, 5, 6]. Some animals were injected via tail vein with TBK1-expressing bone marrow-derived macrophages (BMMs) or control cells ($1x10^6$ cells in 0.1 ml of PBS/mouse) 24h before ischemia.

Hepatocellular function assay. Serum alanine aminotransferase (sALT) levels, an indicator of hepatocellular injury, were measured by ALT and AST kit (ThermoFisher, Waltham, MA) according to the manufacturer's instructions.

Histology, immunohistochemistry, and immunofluorescence staining. Liver sections (5-μm) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki's criteria [19]. Liver macrophages were detected using primary rat $CD11b^+$ monoclonal antibodies (mAb) (Abcam, Cambridge, MA) and secondary AlexFluor488-conjugated AffiniPure donkey anti-rat IgG (Jackson Immunoresearch, West Grove, PA) for immunofluorescence staining. DAPI was used for nuclear counterstaining. Liver neutrophils were detected by immunohistochemistry (IHC) staining using primary rat Ly6G mAb (ThermoFisher Scientific). The gene expression was detected in liver sections by IHC staining using primary rabbit p-TBK1, p-IRF3, p-P65, CYLD, NOX4, NRF2 (ThermoFisher Scientific), and RIPK3 (Santa Cruz Biotechnology) Abs. Immunofluorescence staining of p-STING and p-TBK1 in Kupffer cells was analyzed in the liver sections using primary rabbit p-STING and p-TBK1 mAbs (Cell Signaling Technology, Danvers, MA) and rat CD68 mAb (Bio-Rad, Hercules, CA). The primary mouse CYLD (Santa Cruz Biotechnology) and NOX4 (Novus Biologicals, Littleton, CO) Abs, and rabbit NRF2 (Cell Signaling Technology) and G3BP1 (Proteintech, Rosemont, IL) Abs, the secondary AlexFluor488-conjugated AffiniPure donkey anti-rabbit IgG Ab, Cy5-conjugated AffiniPure donkey anti-mouse IgG Ab, AlexFluor488-conjugated AffiniPure donkey anti-mouse IgG Ab and Cy5-conjugated AffiniPure donkey anti-rabbit IgG Ab (Jackson Immunoresearch) were used for

staining CYLD, NOX4, NRF2, or G3BP1 positive cells according to the manufacturer's instructions. Images for immunofluorescence staining were captured using a fluorescence microscope (Keyence BZ-X810, Osaka, Japan) and analyzed using Image-pro Plus software. Positive cells were counted blindly in 10 HPF/section (x200).

TUNEL assay. The DNA fragmentation characteristic of apoptotic cells in formalin-fixed paraffin-embedded liver sections was detected with a Klenow-FragEL DNA Fragmentation Detection Kit (EMD Chemicals, Gibbstown, NJ) according to the manufacturer's instructions. Apoptotic cell death in primary hepatocytes was measured by Cell Meter TUNEL Apoptosis Assay Kit (AAT Bioquest, Sunnyvale, CA). Briefly, treated hepatocytes were fixed in 4% paraformaldehyde for 30 min. After three wash with TBST, hepatocytes were incubated with Tunnelyte^{TM} Green for 60 min at 37˚C. Additional Hoechst staining was conducted for the nucleus identification. TUNEL-positive cells were visually identified with a fluorescence microscope by a FITC filter. Results were scored semi-quantitatively by averaging the number of apoptotic cells/microscopic field at 200× magnification. Ten fields were evaluated for each sample.

Quantitative RT-PCR analysis. Total RNA was purified from liver tissue or cell cultures using RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Reverse transcription to cDNA was performed by using SuperScript III First-Strand Synthesis System (ThermoFisher Scientific). Quantitative real-time PCR was carried out using the QuantStudio 3 (Applied Biosystems by ThermoFisher Scientific). In a final reaction volume of 25μl, the following were added: 1× SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen) cDNA and 10μM of each primer. Amplification conditions were: 50°C (2min), 95°C (5min), followed by 40 cycles of 95°C (15sec) and 60°C (30sec). The primer sequences that amplify IL-6, TNF-α, CXCL10, MCP-1, IFN-β, NQO1, GCLC, GCLM, OASL1, and HPRT were shown in Supplementary Table 1. The target gene expressions were calculated by their ratios to the housekeeping gene HPRT.

Western blot analysis. Protein was extracted from liver tissue or cell cultures with ice-cold protein lysis buffer (50mM Tris, 150mM NaCl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton-100). The buffer contains 1% proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Proteins (30 μg/sample) in SDS-loading buffer (50mM Tris, pH 7.6, 10% glycerol, 1% SDS) were subjected to 4-20% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% dry milk and 0.1% Tween 20 (USB, Cleveland, OH). The nuclear and cytosolic fractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific). The TXNIP, NRF2, p-STING, STING, p-TBK1, TBK1, p-IRF3, IRF3, p-IκBα, IκBα, p-P65, P65, p-Src (Tyr416), Src, Ubiquitin, Lamin B2, Apaf-1, cytochrome c, cleaved caspase-9, cleaved caspase-3, RIPK3, and β-actin (Cell Signaling Technology), CYLD (Santa Cruz Biotechnology), NOX4 (Novus Biologicals), OASL1 (Biorbyt Ltd., Cambridge, UK), and NOX2, p-ASK1, ASK1, or G3BP1 Ab (ThermoFisher Scientific) were used. The membranes were incubated with Abs and then added Western ECL substrate mixture (Bio-Rad) for imaging with the iBright FL1000 (ThermoFisher Scientific). Relative quantities of protein were determined by comparing the β-actin expression using iBright image analysis software (ThermoFisher Scientific).

Isolation of primary hepatocytes, Kupffer cells, and bone marrow-derived macrophages. Primary hepatocytes, Kupffer cells, and BMMs from the $TXNIP^{FL/FL}$, $TXNIP^{M\text{-}KO}$, or wild-type (WT) mice were isolated as described [5]. In brief, livers were perfused in situ with warmed (37°C) HBSS solution, followed by a collagenase buffer (collagenase type IV, Sigma-Aldrich). The Perfused livers were dissected and teased through 70-μm nylon mesh cell strainers (BD Biosciences, San Jose, CA). The nonparenchymal cells (NPCs) were separated from hepatocytes by centrifuging at 50 × *g* 2min three times. The NPCs were then suspended in HBSS and layered onto a 50%/25% two-step Percoll gradient (Sigma) in a 50-ml conical centrifuge tube and centrifuged at 1800 × *g* at 4°C for 15min. The Kupffer cells in the middle layer were collected

and plated to cell culture dishes in DMEM with 10% FBS, 10mM HEPES, 2mM GlutaMax, 100 U/ml penicillin, and 100 µg/ml streptomycin for 15min at 37°C. Murine bone-derived macrophages (BMMs) were generated as described [5]. In brief, bone marrow cells were removed from the femurs and tibias of the $TXNIP^{FL/FL}$, $TXNIP^{M-KO}$, or WT mice and cultured in DMEM supplemented with 10% FCS and 15% L929-conditioned medium for seven days.

Co-culture of macrophages and primary hepatocytes. Primary hepatocytes were cultured in 6-well plates at a concentration of $4x10^5$ cells per well. After 24h, the 0.4µm-pore size transwell inserts (Corning) containing $1x10^6$ BMMs were placed into the 6-well plate with the initially seeded hepatocytes. The co-cultures were incubated for 12h with or without adding H_2O_2 (200 µM) in the lower chamber.

ELISA assay. Cell culture supernatants were harvested for cytokine analysis. ELISA kit (ThermoFisher Scientific) was used to measure the TNF-α level according to the manufacturer's instructions.

LDH activity assay. BMMs ($1x10^6$) were cultured with primary hepatocytes *(*$4x10^5$/well) for 12h with or without adding H_2O_2 (200 µM) in the lower chamber*.* The activity of lactate dehydrogenase (LDH) in the cell culture medium from the lower chamber was measured with a commercial LDH activity assay kit (Stanbio Laboratory, Boerne, TX) according to manufacturer's instructions.

Reactive oxygen species assay. ROS production in Kupffer cells was measured using the 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (Carboxy-H2DFFDA, ThermoFisher Scientific), as described [5]. In brief, Kupffer cells ($2x10^5$) were isolated from ischemic livers and cultured on collagen-coated cover slips without or with LPS (100ng/ml) for 2h at 37°C. After washing with PBS, cells were incubated with 10µM of Carboxy-H2DFFDA. The Carboxy-H2DFFDA was converted to a green-fluorescent form when hydrolyzed by intracellular

esterase and oxidized in the cells. Cells were fixed with 2% paraformaldehyde and stained with Hoechst dye. ROS produced by Kupffer cells were analyzed and quantified by fluorescence microscopy. Positive green fluorescent-labeled cells were counted blindly in 10 HPF/section (x200).

In vitro transfection. BMMs ($1x10^6$/well) were cultured for seven days and then and then transfected with CRISPR/Cas9-CYLD knockout (KO), CRISPR-CYLD activation, CRISPR/Cas9-NRF2 KO, CRISPR/Cas9-OASL1 KO, CRISPR-OASL1 activation, CRISPR/Cas9-G3BP1 KO, or control vector (Santa Cruz Biotechnology) by using Lipofectamine™ 3000 according to the manufacturer's instructions (ThermoFisher Scientific). After 24-48h, cells were supplemented with LPS (100 ng/ml) for an additional 6h. For lentivirus-mediated gene transfer, BMMs ($1x10^6$/well) were added with lentivirus-mediated TBK1 (Lv-TBK1) or Lv-control (at a multiplicity of infection 10) (Applied Biological Materials, Richmond, BC, Canada) and incubated at 37°C overnight. The medium was removed and replaced with fresh medium. After 48h, cells were harvested for *in vivo* adoptive transfer.

Immunoprecipitation analysis. BMMs after LPS stimulation were lysed in NP-40 lysis buffer (50mM Tris pH7.4, 10 mM EDTA, 150 mM NaCl, 1% NP-40, ThermoFisher Scientific) containing protease inhibitors. The lysates were incubated with CYLD (Santa Cruz Biotechnology), NOX4 (Novus Biologicals), or control IgG and protein A/G beads at 4 °C overnight. After immunoprecipitation, the immunocomplexes were washed with lysis buffer three times and analyzed by standard immunoblot procedures.

Chromatin immunoprecipitation (ChIP). The ChIP analysis was carried out using ChIP Assay Kit (Abcam). 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. After 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. The purified chromatin was analyzed on a 1.5 % agarose gel to analyze DNA fragment size, and the sheared chromatin was immunoprecipitated with NRF2 antibody (Cell Signaling Technology) overnight. The antibody/chromatin samples were then mixed with protein A sepharose beads. As a control, the normal IgG was used. The protein-DNA complexes were washed and eluted, followed by a reversal of crosslinking and DNA clean-up steps, and the purified DNA from the immunoprecipitation reaction was examined by PCR. The primer sequences for the NRF2-responsive region of OASL1 promoter: forward: 5'-ATTGCCACGCCTAATGACCC -3', reverse: 5'- CCACCGCACGTGGATTTTAG -3'.

ChIP-sequencing (ChIP-seq). The ChIP-DNA was amplified to generate a 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 the 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 annotation. 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 various group comparisons. All analyses were used by SAS/STAT software, version 9.4.

Supplementary Figures

Fig. S1

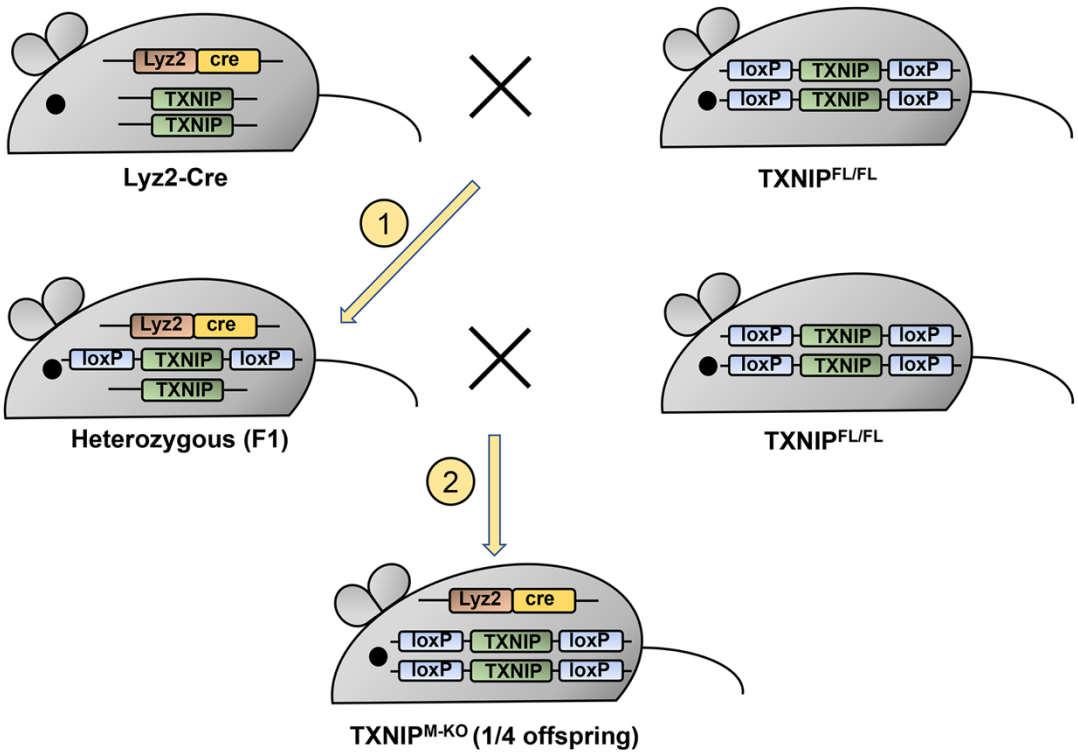

Fig. S1. Schematic illustration of generation of myeloid-specific TXNIP knockout mice. Two steps were used to generate myeloid-specific TXNIP KO mice. First, a homozygous loxP flanked TXNIP mouse is mated with a homozygous Lyz2-Cre mouse to generate the F1 mice that are heterozygous for a loxP-flanked TXNIP allele and heterozygous for the Lyz2-cre. Next, these F1 mice were backcrossed to the homozygous loxP-flanked TXNIP mice, resulting in the generation of myeloid-specific TXNIP KO mice ($TXNIP^{M\text{-}KO}$, 25% of the offspring), which were homozygous for the loxP-flanked TXNIP allele and heterozygous for the Lyz2-Cre allele.

Fig. S2

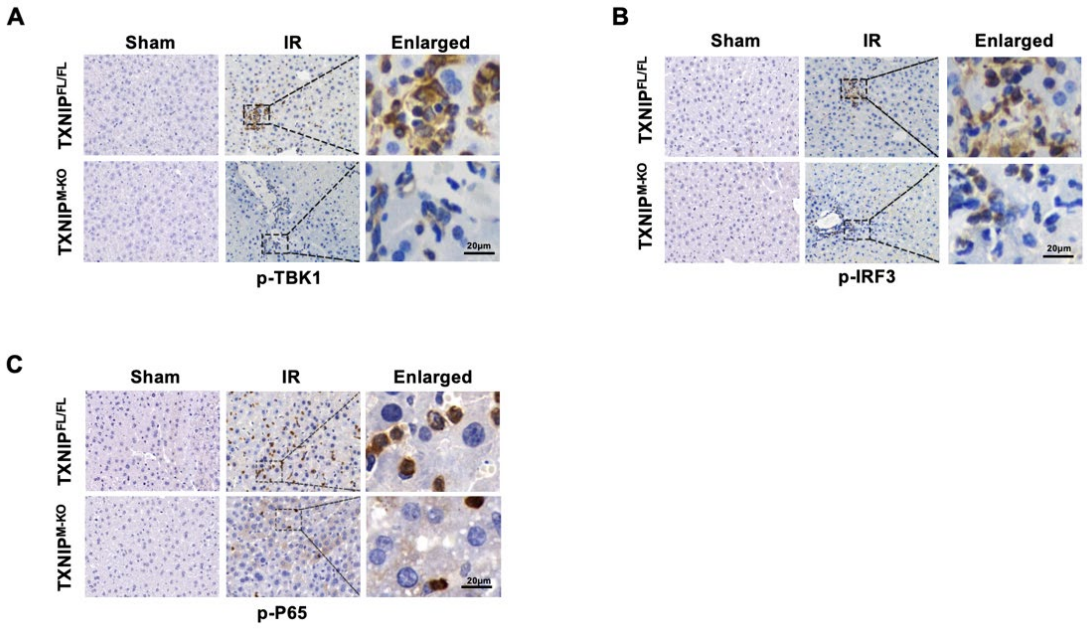

Fig. S2. Disruption of myeloid-specific TXNIP inhibits macrophage TBK1 and IRF3/NF-κB activation in IR-stressed liver. The $TXNIP^{FL/FL}$ and $TXNIP^{M\text{-}KO}$ mice were subjected to 90min of partial liver warm ischemia, followed by 6h of reperfusion. Immunohistochemistry staining of macrophage p-TBK1 (A), p-IRF3 (B), and p-P65 (C) expression in ischemic livers (n=6 mice/group). Scale bars, 20μm.

Fig. S3

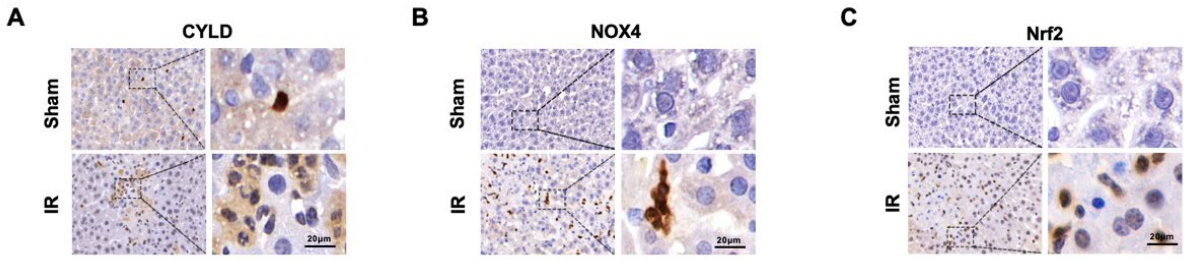

Fig. S3. **IR stress activates macrophage CYLD, NOX4, and NRF2 in ischemic livers.** The WT mice were subjected to 90min of partial liver warm ischemia, followed by 6h of reperfusion. Immunohistochemistry staining of macrophage CYLD (A), NOX4 (B), and Nrf2 (C) expression in ischemic livers (n=6 mice/group). Scale bars, 20μm.

Fig. S4

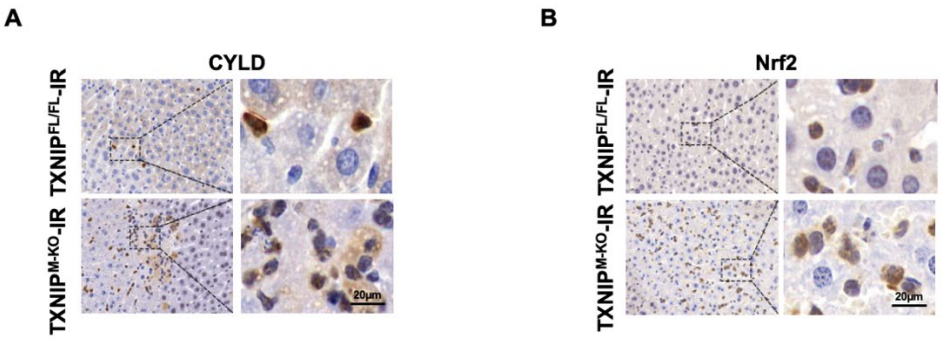

Fig. S4. Disruption of myeloid-specific TXNIP promotes macrophage CYLD and Nrf2 expression in IR-stressed liver. The TXNIP$^{FL/FL}$ and TXNIP$^{M\text{-}KO}$ mice were subjected to 90min of partial liver warm ischemia, followed by 6h of reperfusion. Immunohistochemistry staining of macrophage CYLD (A) and Nrf2 (B) expression in ischemic livers (n=6 mice/group). Scale bars, 20μm.

Fig. S5

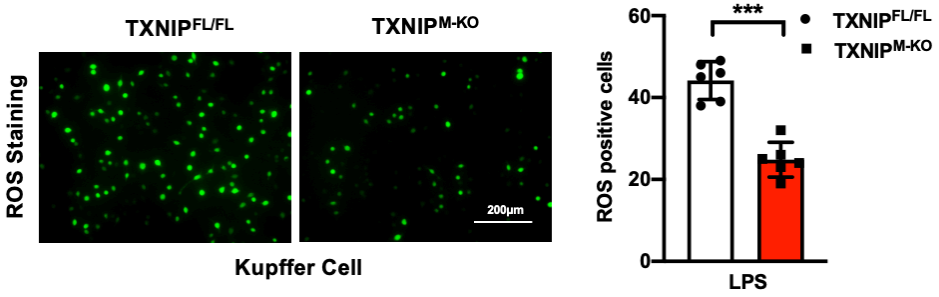

Fig. S5. Disruption of myeloid-specific TXNIP reduces ROS production in LPS-stimulated macrophages. The Kupffer cells ($2x10^5$) were isolated from ischemic livers and cultured with LPS (100ng/ml) for 2h. Detection of ROS production by Carboxy-H2DFFDA in Kupffer cells from the $TXNIP^{FL/FL}$ and $TXNIP^{M-KO}$ livers after IR stress. Quantification of ROS-producing Kupffer cells (green) (n=6 mice/group). Scale bars, 200μm. All data represent the mean±SD. Statistical analysis was performed using Permutation t-test. ***p<0.005.

Fig. S6

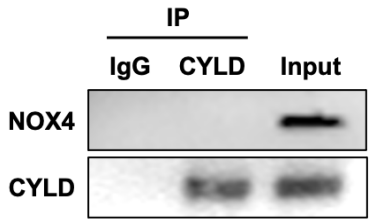

Fig. S6. CYLD could not bind to NOX4 in BMMs in the absence of LPS stimulation. Bone marrow-derived macrophages (BMMs, $1x10^6$) were cultured at 37°C for 6h without LPS stimulation. *Note:* Immunoprecipitation analysis revealed that CYLD could not bind to NOX4 in BMMs in the absence of LPS stimulation.

Fig. S7

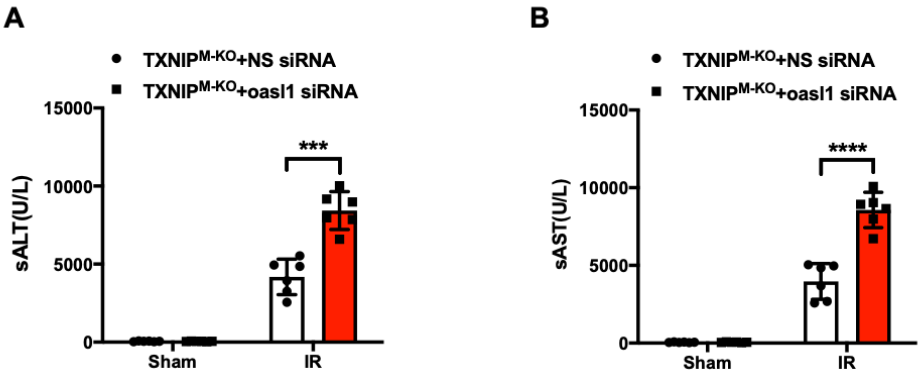

Fig. S7. Disruption of OASL1 in the TXNIP$^{M\text{-}KO}$ mice with the mannose-mediated siRNA treatment aggravated IR-induced liver injury. The TXNIP$^{M\text{-}KO}$ mice were injected via tail vein with OASL1 siRNA (2.5 mg/kg) or non-specific (NS) control siRNA mixed with mannose-conjugated polymers at 4h prior to ischemia. Liver function in serum samples was evaluated by serum ALT (A) and AST (B) levels (IU/L) (n=6 mice/group). All data represent the mean±SD. Statistical analysis was performed using Permutation t-test. ***p<0.005, ****p<0.001.

Fig. S8

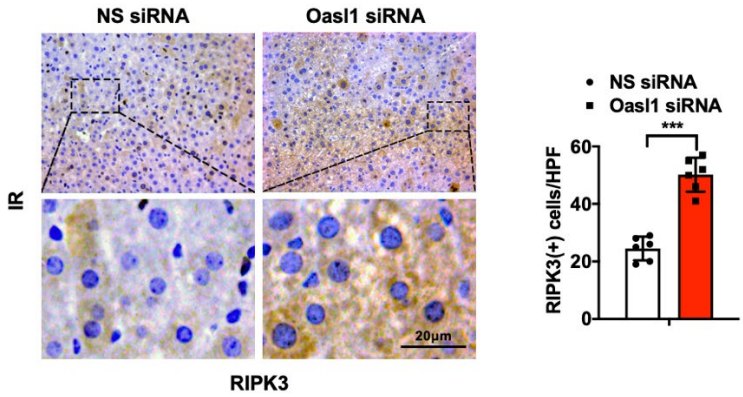

Fig. S8. OASL1 is essential for regulating RIPK3-mediated necroptosis in myeloid TXNIP-deficient livers in response to IR stress. The TXNIP$^{M\text{-}KO}$ mice were injected via tail vein with OASL1 siRNA (2.5 mg/kg) or non-specific (NS) control siRNA mixed with mannose-conjugated polymers at 4h prior to ischemia. Immunohistochemistry staining of RIPK3 expression in ischemic livers (n=6 mice/group). Scale bars, 100μm and 20μm. All data represent the mean±SD. Statistical analysis was performed using Permutation t-test. ***p<0.005.

Fig. S9

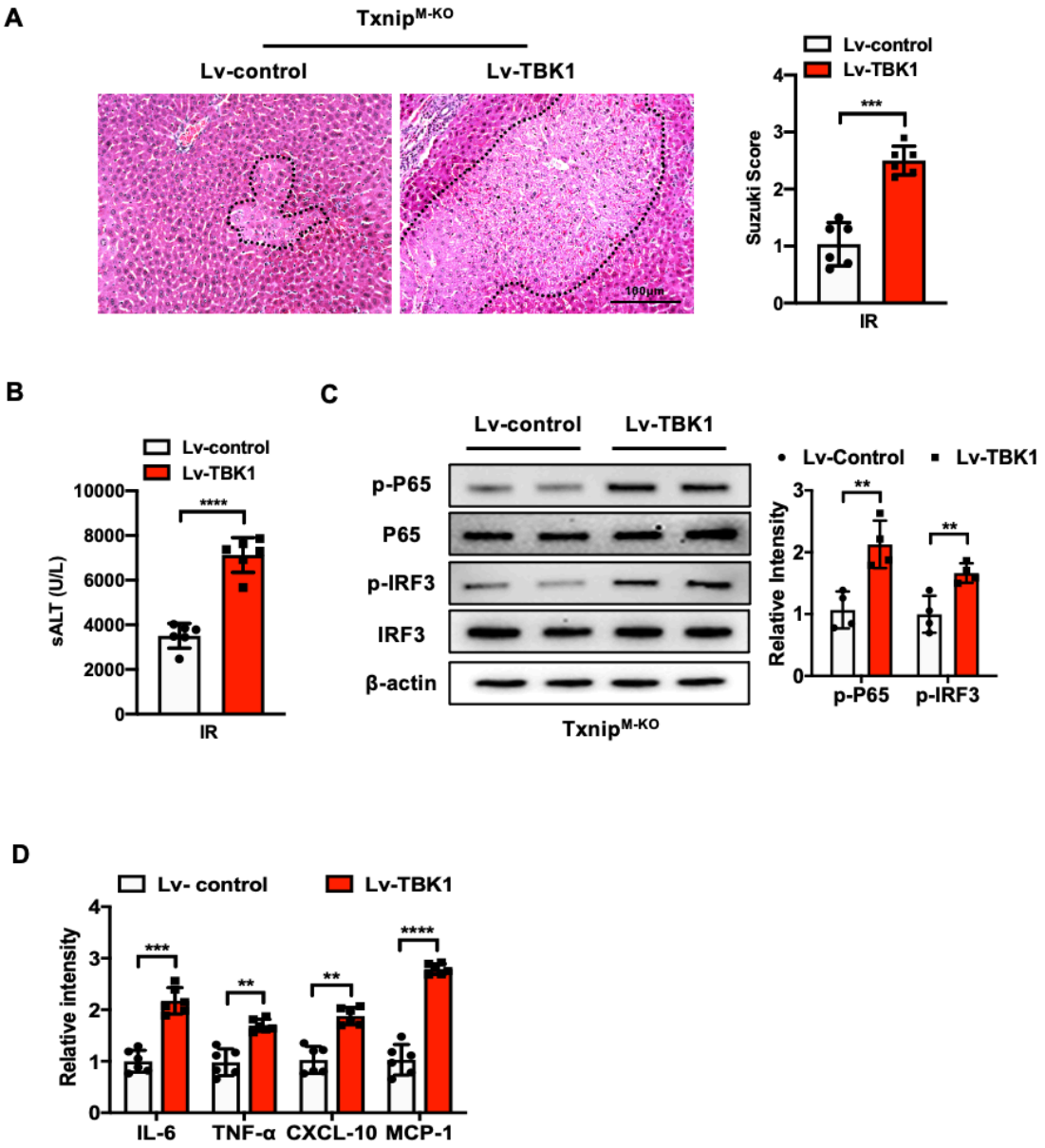

Fig. S9. Overexpression of TBK1 reverses the TXNIP$^{M\text{-}KO}$-mediated cytoprotection in IR-stressed livers and augments downstream immune and inflammatory pathway in IR-stressed livers. The TXNIP$^{M\text{-}KO}$ mice were injected via tail vein with bone marrow-derived macrophages (BMMs) transfected with lentivirus-mediated TBK1 (Lv-TBK1) or Lv-control ($1x10^6$ cells in PBS/mouse) 24h prior to ischemia. (A) Representative histological staining (H&E) of

ischemic liver tissue (n=6 mice/group) and Suzuki's histological score. Scale bars, 100μm. (B) Liver function in serum samples was evaluated by serum ALT levels (IU/L) (n=6 mice/group). (C) Western blot analysis and relative density ratio of p-P65 and p-IRF3. Representative of four experiments. (D) Quantitative RT-PCR analysis of IL-6, TNF-α, CXCL-10, and MCP-1 mRNA levels in ischemic livers (n=6 samples/group). All data represent the mean±SD. Statistical analysis was performed using Permutation t-test. **p<0.01. ***p<0.005, ****p<0.001.

Fig. S10

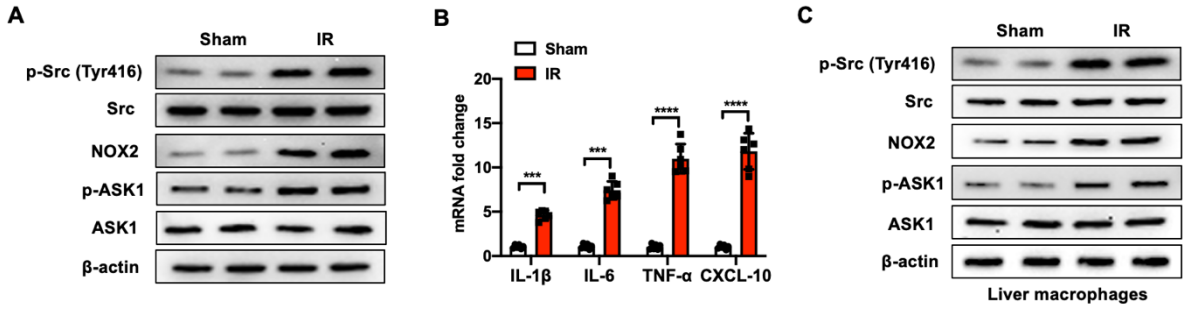

Fig. S10. IR stress activated Src tyrosine kinase signaling pathway and proinflammatory mediators in ischemic livers. The WT mice were subjected to 90min of partial liver warm ischemia, followed by 6h of reperfusion. (A) Western blot analysis and relative density ratio of p-Src (Tyr416), Src, NOX2, p-ASK1, ASK1 and β-actin in ischemic livers. Representative of four experiments. (B) Quantitative RT-PCR analysis of IL-1β, IL-6, TNF-α, and CXCL-10 mRNA levels in ischemic livers (n=6 samples/group). (C) Western blot analysis and relative density ratio of p-Src (Tyr416), Src, NOX2, p-ASK1, ASK1, and β-actin in liver macrophages from ischemic livers. All data represent the mean±SD. Statistical analysis was performed using Permutation t-test. ***p<0.005, ****p<0.001.

Table S1: Primers used in qRT-PCR studies.

Target genes	Forward primers	Reverse primers
HPRT	5'-TCAACGGGGGACATAAAAGT-3'	5'-TGCATTGTTTTACCAGTGTCAA-3'
TNF-α	5'- ACGGCATGGATCTCAAAGAC-3'	5'- AGATAGCAAATCGGCTGACG-3'
IL-6	5'- GCTACCAAACTGGATATAATCAGGA -3'	5'- CCAGGTAGCTATGGTACTCCAGAA -3'
IL-1β	5'-TGTAATGAAAGACGGCACACC-3'	5'-TCTTCTTTGGGTATTGCTTGG-3'
MCP-1	5'-GAAGGAATGGGTCCAGACAT-3'	5'-ACGGGTCAACTTCACATTCA-3'
CXCL-10	5'-GCTGCCGTCATTTTCTGC-3'	5'-TCTCACTGGCCCGTCATC-3'
Oasl1	5'- CCAGGAAGAAGCCAAGCACCATC -3'	5'- AGGTTACTGAGCCCAAGGTCCATC -3'
IFN-β	5'- CCACTTGAAGAGCTATTACTG -3'	5'- AATGATGAGAAAGTTCCTGAAG -3'
Nqo1	5'-AGCTGGAAGCTGCAGACCTG-3'	5'-CCTTTCAGAATGGCTGGCA-3'
Gclc	5'-ATCTGCAAAGGCGGCAAC-3'	5'-ACTCCTCTGCAGCTGGCTC-3'
Gclm	5'- TGGAGCAGCTGTATCAGTGG-3'	5'- AGAGCAGTTCTTTCGGGTCA-3'