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ER stress promotes mitochondrial calcium overload and activates the ROS/NLRP3 axis to mediate fatty liver ischemic injury

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Abstract

Background: Fatty livers are widely accepted as marginal donors for liver transplantation but are more susceptible to liver ischemia and reperfusion (IR) injury. Increased macrophage-related inflammation plays an important role in the aggravation of fatty liver IR injury. Here, we investigate the precise mechanism by which endoplasmic reticulum (ER) stress activates macrophage NOD-like receptor thermal protein domain–associated protein 3 (NLRP3) signaling by regulating mitochondrial calcium overload in fatty liver IR.

Methods: Control- and high-fat diet-fed mice were subjected to a partial liver IR model. The ER stress, mitochondrial calcium levels, and NLRP3 signaling pathway in macrophages were analyzed.

Results: Liver steatosis exacerbated liver inflammation and IR injury and enhanced NLRP3 activation in macrophages. Myeloid NLRP3 deficiency attenuated intrahepatic inflammation and fatty liver injury following IR. Mechanistically, increased ER stress and mitochondrial calcium overload were observed in macrophages obtained from mouse fatty livers after IR. Suppression of ER stress by tauroursodeoxycholic acid effectively downregulated mitochondrial calcium accumulation and suppressed NLRP3 activation in macrophages, leading to decreased inflammatory IR injury in fatty livers. Moreover, Xestospongin-C–mediated inhibition of mitochondrial calcium influx decreased reactive oxygen species (ROS) expression in macrophages after IR. Scavenging of mitochondrial ROS by mito-TEMPO suppressed macrophage NLRP3 activation and IR injury in fatty livers, indicating that excessive mitochondrial ROS production was responsible for

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ER, endoplasmic reticulum; HFD, high-fat diet; IR, ischemia and reperfusion; MAMs, mitochondria-associated ER membranes; mROS, mitochondrial reactive oxygen species; NLRP3, NOD-like receptor thermal protein domain–associated protein 3; ROS, reactive oxygen species; TUDCA, tauroursodeoxycholic acid; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; WT, wild type.

Fei Li, Zhu Guan, and Yiyun Gao contributed equally to this paper.

macrophage NLRP3 activation induced by mitochondrial calcium overload. Patients with fatty liver also exhibited upregulated activation of NLRP3 and the ER stress signaling pathway after IR.

Conclusions: Our findings suggest that ER stress promotes mitochondrial calcium overload to activate ROS/NLRP3 signaling pathways within macrophages during IR-stimulated inflammatory responses associated with fatty livers.

INTRODUCTION

Liver ischemia and reperfusion (IR) injury is a frequent complication of various major liver surgeries, including partial hepatectomy and liver transplantation, and can affect the postoperative liver function, morbidity, and overall patient outcomes.[1] The pathogenesis of liver IR injury involves various mechanisms; including ATP depletion, oxidative stress dysregulation, and proinflammatory immune responses.[2]

NAFLD-induced end-stage liver disease is one of the most common indications for liver transplantation.[3] Moreover, owing to the shortage of liver donors, fatty livers are often accepted as marginal grafts.[4] However, emerging evidence has shown that liver steatosis aggravates liver IR injury and increases the risk of adverse outcomes. Impaired microcirculation, dysregulated mitochondrial function, and increased inflammation have been implicated in susceptibility of fatty liver to IR injury.[5]

Macrophages sense various damage-associated molecular patterns released from damaged hepatic cells to initiate an inflammatory cascade by secreting chemokines and cytokines that eventually contribute to liver IR injury.[6] Liver macrophages can be divided into proinflammatory-M1 and anti-inflammatory M2 macrophages, which regulate the progress of inflammation. We previously found that modulation of the macrophage innate immune response is a promising strategy for alleviating liver IR injury.[7–9] Recent studies have implicated the important role of macrophages in NAFLD and its progression to NASH.[10–12] Although macrophage innate immune activation has been reported to regulate IR injury in fatty liver, the precise regulatory mechanism remains obscure.[13]

The proper functioning of the endoplasmic reticulum (ER) and mitochondria is critical for maintaining cellular homeostasis. Mitochondria play an essential role in the metabolism of eukaryotes, which was found to be severely impaired by liver IR.[14] Excessive and prolonged generation of reactive oxygen species (ROS) by the mitochondria results in oxidative stress and cell injury. Calcium functions in many cellular processes and is maintained at appropriate concentrations in various cellular organelles and structures. Cells have adapted buffering strategies by compartmentalizing calcium into the mitochondria and ER. The physical and functional interactions between the ER and mitochondria can regulate each other's functions. Mitochondria-associated ER membranes (MAMs) are conserved structures and sites of physical communication between the ER and mitochondria that determine cell survival and death through the transfer of Ca^{2+} and other metabolites.[15] Increased MAM formation drives higher calcium accumulation in the mitochondria, leading to increased oxidative stress and ROS generation in obesity.[16] However, the function or dysfunction of mitochondria, regulated by the ER, in macrophage innate immune activation and IR injury in fatty liver has not been studied.

Here, we showed that liver IR induces ER stress in macrophages, which results in increased calcium flux from the ER to the mitochondria. Mitochondrial calcium overload promotes mitochondrial ROS production and subsequent NOD-like receptor thermal protein domain–associated protein 3 (NLRP3) activation in macrophages, leading to increased inflammatory tissue injury in the fatty liver after IR. The suppression of ER stress or scavenging of mitochondrial ROS improved intrahepatic inflammation and IR injury in fatty livers, suggesting a new therapeutic target for fatty liver IR injury.

METHODS

Animals

Wild-type (WT) male mice (8 wk) and NLRP3 myeloid knockout (NLRP3−/−) male mice (8 wk) were bred at Gempharmatech Co., Ltd. Mice were fed normal diet (CON) or high-fat diet (HFD) diet for 20 weeks and divided into 4 groups (WT-CON, $NLRP3^{-/-}$CON, WT-HFD, and $NLRP3^{-/-}$HFD). The mice were subjected to a 12-hour light/dark cycle in a standard pathogen-free environment. All animals received humane care and all animal procedures were in accordance with the relevant legal and ethical requirements approved by the NMU Institutional Animal Care and Use Committee (NMU08-092).

Mouse liver IR model

As mentioned previously,[7,17] a stable mouse model was established using 70% partial hepatic heat IR. Mice were anesthetized and a laparotomy was performed to expose the liver. The left and middle branches of the intrahepatic portal vein were clamped to block the blood supply. The clips were released for reperfusion after 90 minutes of ischemia. The sham mice underwent the same procedure but without clamping the blood vessels. The mice were sacrificed 6 hours after reperfusion, and liver and blood samples were collected for analysis.

To study the effects of ER stress, Control and HFD-fed mice were injected with tauroursodeoxycholic acid (TUDCA) i.p. (400 mg/kg) or PBS (Control) for 3 days, and then the model of liver IR was established.

To determine the role of mitochondrial oxidation, Control and HFD-fed mice were pretreated with the mitochondria-targeted antioxidant Mito-TEMPO (5 mg/kg, MedChemExpress, HY-112879) twice at 17 hours and 1 hour before surgery or PBS.

Examination of liver function

To assess the degree of liver injury in mice, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) concentrations were measured using an AU680 clinical chemistry analyzer (Beckman Coulter).

Histological examination

Liver specimens were fixed in 4% paraformaldehyde and embedded in paraffin for hematoxylin-eosin (H&E) staining. The Suzuki score was used to grade liver IR injury. The score was 0 for tissue with no necrosis, congestion, or lobular central globular change, and 4 for tissue with severe congestion and/or lobular necrosis $>60\%$. For Oil Red staining, frozen liver sections (10 μm) were rinsed with 60% isopropyl alcohol, then stained with Sigma-Aldrich oil red O solution (Sigma-Aldrich) for 15 minutes, then rinsed again with 60% isopropyl alcohol, and stained with hematoxylin before microscopic examination. The production of 4-HNE (4-hydroxynonenal) by lipid peroxidation was examined using immunohistochemistry with the anti-4-HNE antibody (ab46545; Abcam). Immunostaining images were analyzed using ImageJ.

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assay

The paraffin-embedded sections of the liver tissues were processed in toluene and dehydrated in a series of ethanol solutions. In addition, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining of the liver tissues was performed using a fluorescence detection kit according to the manufacturer's instructions.

Real-time quantitative PCR

Total RNA was extracted from tissues and cells, using the RNA Extraction kit (Beyotime Biotechnology). Then, it was used to synthesize complementary DNA with PrimeScript RT reagent kit with gDNA Eraser (TaKaRa), following the manufacturer's instructions. A real-time quantitative PCR was then performed. mRNA expression levels were normalized by Gapdh.

Immunofluorescence staining

Immunofluorescence staining was used to analyze the expression profiles of specific proteins in liver tissues. Liver tissue sections were incubated with primary antibodies of F4/80 and NLRP3 (Cell Signaling Technology) at 4°C overnight, and subsequent incubation with goat anti-mouse IgG (Sigma) for 1 hour. Then, 4′,6-diamidino-2-phenylindole was used to visualize the nucleus. Slides were rinsed twice with PBS and observed with a confocal microscope (Zeiss) according to the manufacturer's instructions.

Western blotting

Total proteins of liver tissues and cells were extracted with RIPA lysis buffer (Beyotime Biotechnology). Then, after being mixed with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were electrophoresized and transferred to a polyvinylidene fluoride nitrocellulose membrane. Antibodies against glyceraldehyde 3-phosphate dehydrogenase, NLRP3, Pro-caspase-1, C-caspase-1, IL-18, IL-1β, p-PERK, p-Eif2α, IRE1α, ATF6, GRP78, C/EBP homologous protein, and XBP1s (Cell Signaling Technology) were incubated with the membranes at 4°C overnight. Finally, membranes were incubated with secondary goat anti-rabbit IgG (Cell Signaling Technology) at 4°C for 2 hours. Glyceraldehyde 3-phosphate dehydrogenase served as an internal control.

Transmission electron microscopy

The macrophages were examined by transmission electron microscopy according to the manufacturer's instructions. Sections were stained with lead citrate and images were captured at 2K/6K magnification.

Detection of calcium

To detect calcium in cytoplasm and mitochondria, macrophages in glass bottom dishes were incubated with 1 μM Fluo4 AM and 200 nM Mitotracker (Yeasen Biotechnology) at 37°C for 30 minutes in the dark, respectively, and then observed with confocal microscopy.

Determination of malondialdehyde, superoxide dismutase, and the reduced glutathione/oxidized glutathione disulfide ratio

The levels of malondialdehyde, ROS, reduced glutathione, and oxidized glutathione disulfide were measured by the corresponding kit. The cells were cleaved with lysis buffer and centrifuged to remove debris and retain the supernatant. Finally, the contents of malondialdehyde, superoxide dismutase, reduced glutathione, and oxidized glutathione disulfide in the supernatant were determined by enzyme labeling.

Measurement of ROS levels

To detect ROS in cultured cells, cells were inoculated in 6-well plates and incubated with 10 μM 2,7-dichlorodihydrofluorescein diacetate (Yeasen Biotechnology) at 37°C for 30 minutes in the dark. Fluorescence intensity was measured by flow cytometry.

Cell culture

As described,[18] the liver was injected through the portal vein with 0.5% collagenase IV (Sigma) and isolated with a 70 μm nylon mesh cell filter (Biosharp). After resuspension, the cells divided into nonparenchymal cells and hepatocytes. Thirty minutes later, the medium of nonparenchymal cells was changed and the attached cells were considered to be macrophages.

Patients and specimens

Four patients diagnosed with NASH and an equal number of matched controls requiring partial hepatectomy in the First Affiliated Hospital of Nanjing Medical University were selected as the study subjects. Liver tissues adjacent to the lesion were collected at 0 and 30 minutes after partial hepatic portal occlusion. Peripheral blood was collected before operation and 1 day after resection. Informed consent was obtained from each patient. This research scheme was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (Institutional Review Board approval number: 2020-SRFA-219).

Statistical analysis

The data were shown as mean ± SD. One-way ANOVA was used to compare experiments between more than two groups. Statistical significance was assessed using the unpaired Student *t* test when comparisons were made between the two groups. Statistical analysis was performed using GraphPad Prism 9, and $p < 0.05$ is considered to be meaningful.

RESULTS

Increased inflammation and IR injury in fatty livers

Compared to the control group, mice fed with an HFD developed significant liver steatosis (Figure 1A). Liver IR injury was compared between the groups. Consistent with previous studies, mice fed with HFD showed increased liver IR injury as determined by serum ALT and AST levels and pathological analysis of liver sections using Suzuki's scores (Figures 1B–D). More TUNEL-positive cells were detected in fatty livers after IR (Figure 1E).

Intrahepatic inflammation was also compared between the control and fatty liver groups. As shown in Figure 1F, fatty livers exhibited much higher levels of proinflammatory gene induction of TNF-α, IL-6, IL-18, IL-1β, C-C motif chemokine ligand 2, and CXCchemokineligand-10. These findings suggest that liver steatosis exacerbates liver inflammation and IR.

Enhanced NLRP3 activation in macrophages aggravated inflammatory IR injury in fatty livers

We previously found that macrophage NLRP3 activation plays an important role in both liver IR injury and NASH.[11,19] In the present study, we evaluated the activation of NLRP3 signaling in macrophages. Interestingly, while comparable numbers of F4/80+ macrophages were found in the livers of control and HFD-fed mice (Figure 2A), enhanced NLRP3 activation in macrophages was detected in HFD-fed mice, as indicated by F4/80 and NLRP3 double staining (Figure 2B). Moreover, macrophages isolated from the livers of HFD-fed mice after IR showed increased protein levels of NLRP3, cleaved-caspase-1, IL-1β, and IL-18 (Figure 2C), indicating liver steatosis–mediated upregulation of NLRP3 activation in macrophages after IR.

Next, to confirm the crucial role of macrophage NLRP3 signaling in mediating IR injury in fatty liver, mice with myeloid NLRP3 deficiency or WT control were fed an HFD and then subjected to IR. Interestingly, myeloid NLRP3 deficiency protected fatty liver against IR injury, as

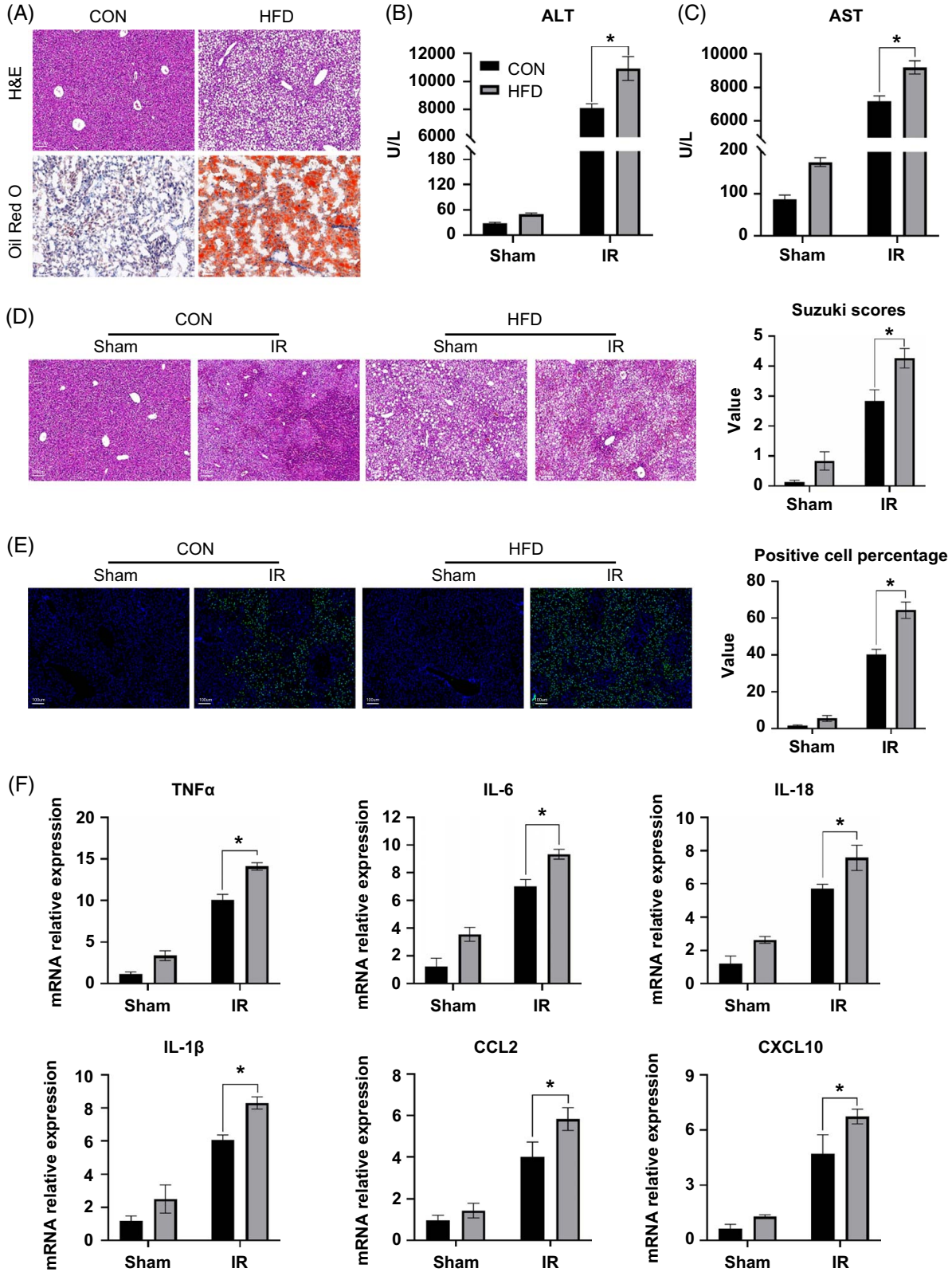

FIGURE 1 Fatty liver aggravated hepatic ischemia-reperfusion injury. Male C57BL/6 wild-type mice were fed with a normal chow or high-fat diet. Liver tissues were collected after 5 months of feeding. (A) H&E and Oil Red O staining of liver sections. Control and HFD-fed mice were treated with liver IR model or sham procedure. Liver tissues and blood samples were collected at 6 hours after reperfusion. (B, C) Serum ALT and AST levels. (D) H&E staining of liver sections; Suzuki scores based on H&E. (E) TUNEL staining of liver sections; percentage of positive cells were evaluated by ImageJ software. (F) Gene expression of TNF-α, IL-6, IL-18, IL-1β, CCL2, and CXCL10 in liver tissues measured by qRT-PCR. n = 4 mice/group. Values were presented as the mean ± SD. *$p < 0.05$. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCL2, C-C motif chemokine ligand 2; CXCL10, CXCchemokineligand-10; H&E, hematoxylin-eosin; HFD, high-fat diet; IR, ischemia and reperfusion; qRT, quantitative Real-Time; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

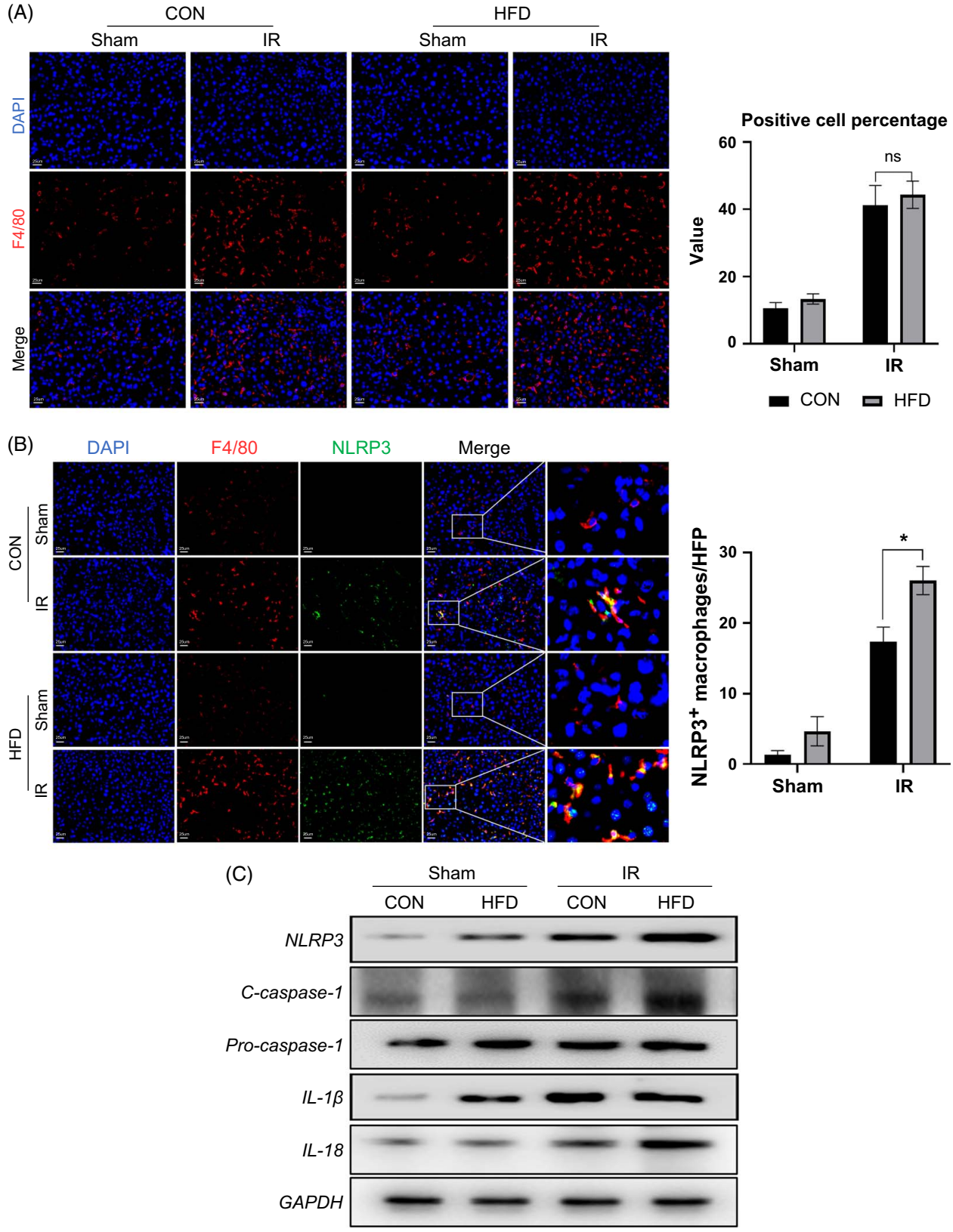

FIGURE 2 Fatty liver activated NLRP3 in intrahepatic macrophages. Control and HFD-fed mice were treated with liver IR model or sham procedure. Liver tissues and intrahepatic macrophages were collected at 6 hours after reperfusion. (A) Immunofluorescence staining of DAPI (blue) and F4/80 (red) in liver tissues; percentage of F4/80-positive cells was evaluated by ImageJ software. (B) Immunofluorescence staining of DAPI (blue), F4/80 (red), and NLRP3 (green) in liver tissues; percentage of F4/80 and NLRP3-positive cells was evaluated by ImageJ software. (C) Western blot analysis of NLRP3, C-caspase-1, Pro-caspase-1, IL-1β, IL-18, and GAPDH in isolated macrophages. n = 4 mice/group. Values were presented as the mean ± SD. *$p < 0.05$. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HFD, high-fat diet; IR, ischemia and reperfusion; NLRP3, NOD-like receptor thermal protein domain–associated protein 3.

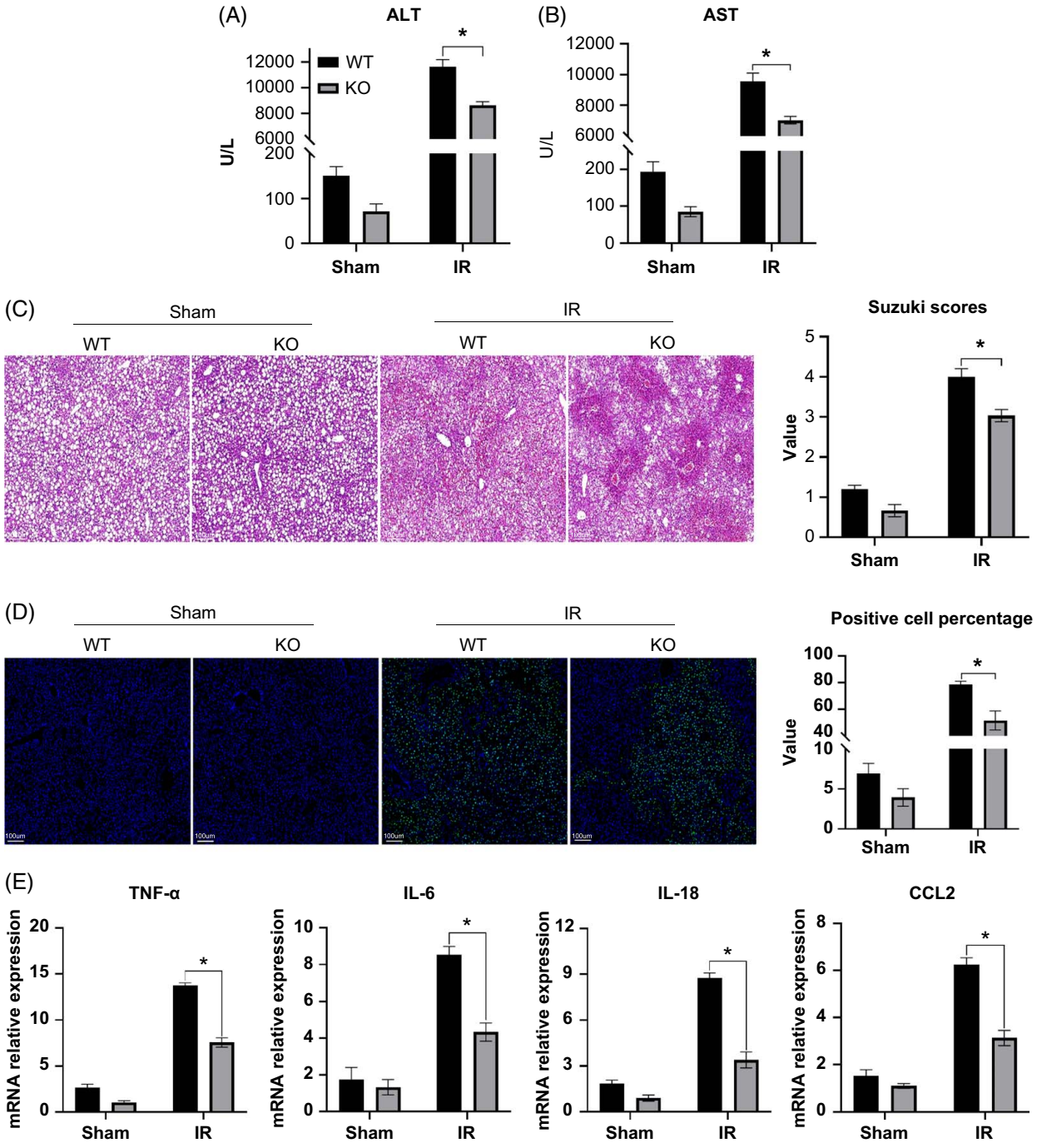

FIGURE 3 NLRP3 myeloid knockout alleviates IR injury in fatty liver. WT and NLRP3 MKO mice were fed with a high-fat diet, then subjected to liver IR model or sham procedure. Liver tissues and blood samples were collected at 6 hours after reperfusion. (A, B) Serum ALT and AST levels. (C) H&E staining of liver sections; Suzuki scores based on H&E. (D) TUNEL staining of liver sections; percentage of positive cells was evaluated by ImageJ software. (E) Gene expression of TNF-α, IL-6, IL-18, and CCL2 in liver tissues measured by qRT-PCR. n = 4 mice/group. Values were presented as the mean ± SD. *$p < 0.05$. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCL2, C-C motif chemokine ligand 2; H&E, hematoxylin-eosin; IR, ischemia and reperfusion; KO, knock out; NLRP3, NOD-like receptor thermal protein domain–associated protein 3; qRT, quantitative Real-Time; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; WT, wild-type.

evidenced by lower serum ALT and AST levels, better preserved liver architecture with lower Suzuki's scores, and fewer TUNEL-positive cells (Figures 3A–D). The suppression of intrahepatic inflammation was also observed in myeloid NLRP3-deficient mice after IR (Figure 3E). These results collectively suggest that enhanced macrophage NLRP3 activation contributed to increased inflammatory IR injury in fatty liver.

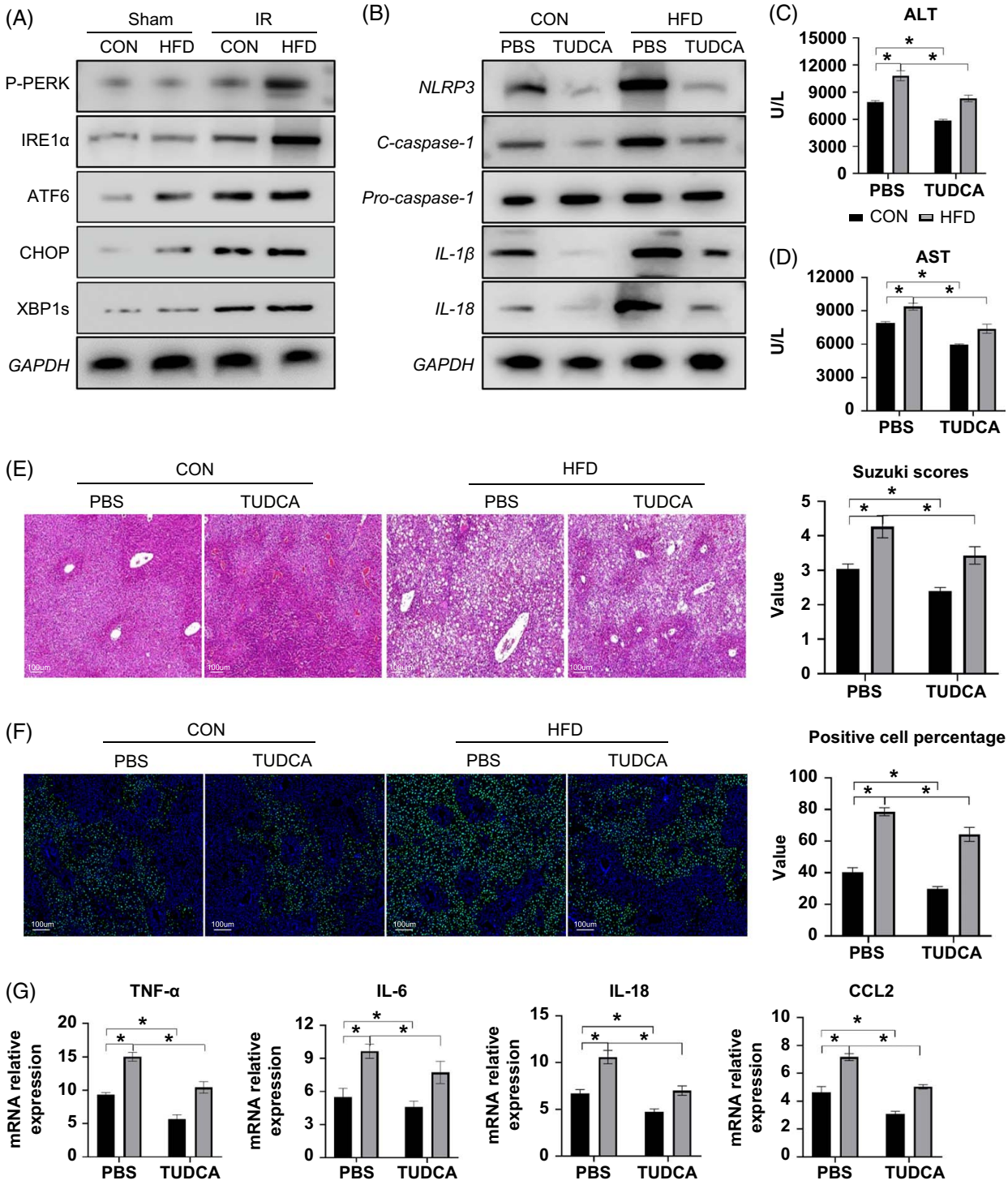

FIGURE 4 Fatty liver IR aggravates endoplasmic reticulum stress of macrophages in the liver and further activates NLRP3. Control and HFD-fed mice were treated with a liver IR model or sham procedure. Intrahepatic macrophages were collected at 6 hours after reperfusion. (A) Western blot analysis of P-PERK, IRE1a, ATF6, CHOP, XBP1s, and GAPDH in isolated macrophages. Control and HFD-fed mice were intraperitoneally injected with TUDCA (400 mg/kg) or PBS (Control), once per day for 3 days before surgery, and then the model of liver IR was established. Intrahepatic macrophages, liver tissues, and blood samples were collected at 6 hours after reperfusion. (B) Western blot analysis of NLRP3, C-caspase-1, Pro-caspase-1, IL-1β, IL-18, and GAPDH in isolated macrophages. (C, D) Serum ALT and AST levels. (E) H&E staining of liver sections. (F) TUNEL staining of liver sections. (G) Gene expression of TNF-α, IL-6, IL-18, and CCL2 in liver tissues measured by qRT-PCR. n = 4 mice/group. Values were presented as the mean ± SD. *$p < 0.05$. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCL2, C-C motif chemokine ligand 2; CHOP, C/EBP homologous protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin-eosin; HFD, high-fat diet; IR, ischemia and reperfusion; NLRP3, NOD-like receptor thermal protein domain–associated protein 3; qRT, quantitative Real-Time; TUDCA, tauroursodeoxycholic acid; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

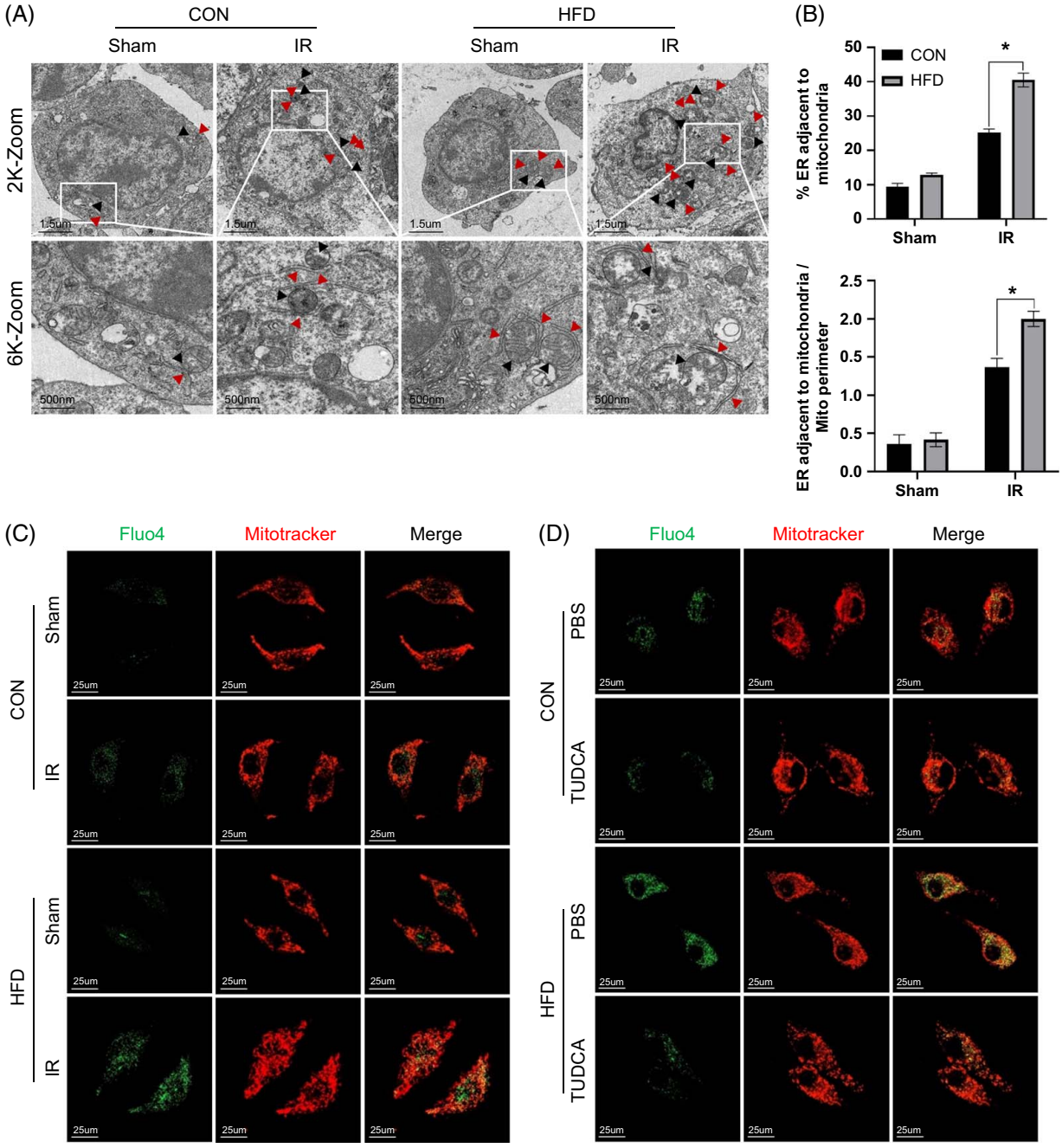

FIGURE 5 Fatty liver IR aggravates endoplasmic reticulum stress in macrophages and induces mitochondrial calcium overload. Control and HFD-fed mice were treated with a liver IR model or sham procedure. Intrahepatic macrophages were collected at 6 hours after reperfusion. (A) Transmission electron microscopy observe the physical interaction between mitochondria and endoplasmic reticulum in macrophages. (B) The length of the endoplasmic reticulum near mitochondria was measured quantitatively by the normalization of total endoplasmic reticulum length and mitochondrial circumference. (C) Representative fluorescent images of mitochondrial calcium level in macrophages showed green using meg-Fluo4, The mitochondria showed red using mitotracker Red. Control and HFD-fed mice were injected i.p. with TUDCA (400 mg/kg) or PBS (Control), once per day for 3 days before surgery, and then the model of liver IR was established. Intrahepatic macrophages were collected at 6 hours after reperfusion. (D) Representative fluorescent images of mitochondrial calcium level in macrophages showed green using meg-Fluo4. The mitochondria showed red using mitotracker Red. n = 4 mice/group. Values were presented as the mean ± SD. *$p < 0.05$. Abbreviations: HFD, high-fat diet; IR, ischemia and reperfusion; TUDCA, tauroursodeoxycholic acid.

ER stress induced by fatty liver IR injury promoted macrophage NLRP3 activation

Recent studies have shown that ER stress regulates macrophage proinflammatory responses during liver IR injury. To verify the involvement of ER stress in macrophage NLRP3 activation, we extracted macrophages from both control and fatty livers after IR and evaluated the ER stress signaling pathway. As shown in Figure 4A, liver IR triggered ER stress in macrophages,

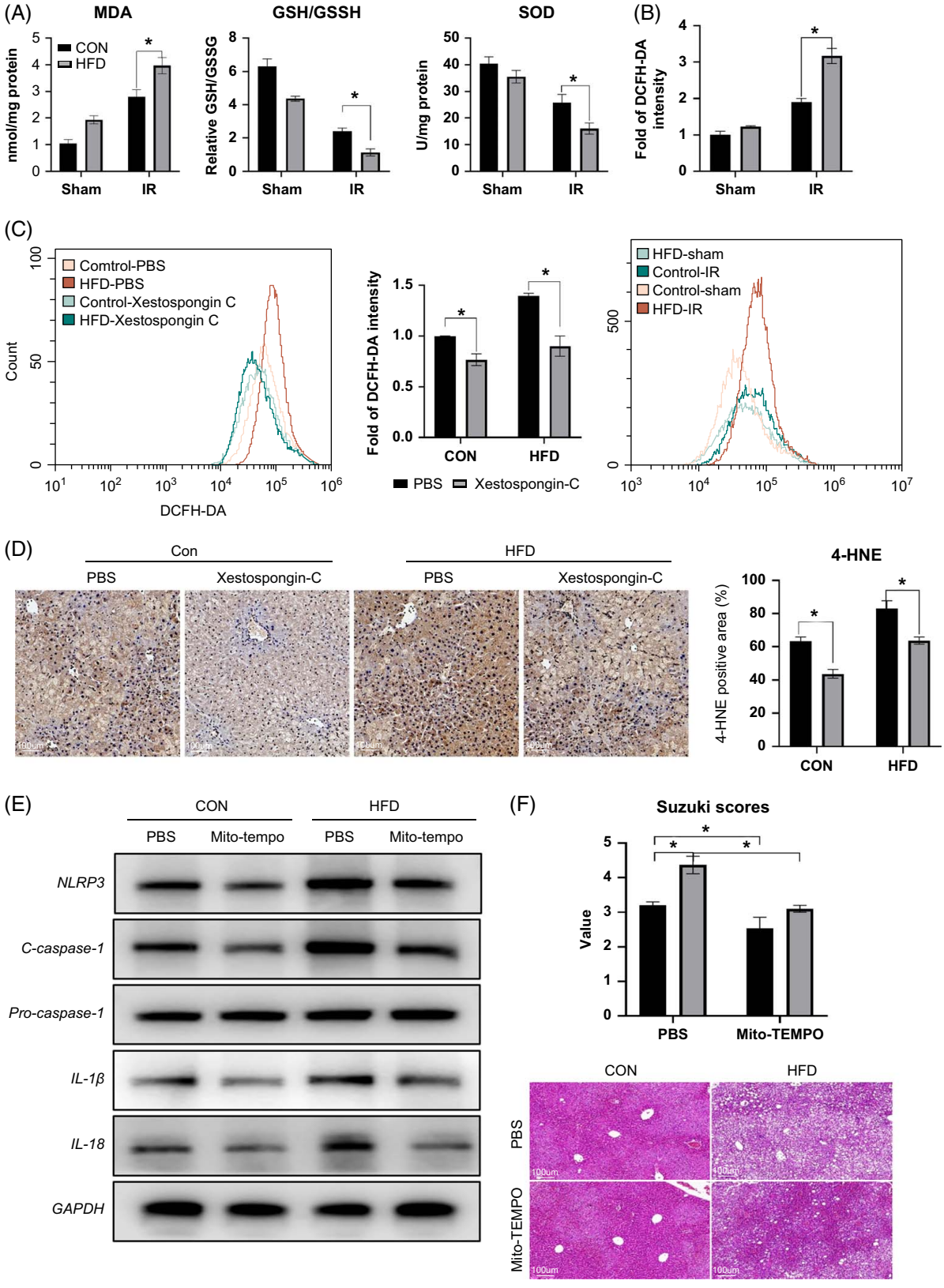

FIGURE 6 Mitochondrial calcium overload aggravates NLRP3 activation by oxidative stress in macrophages. Control and HFD-fed mice were treated with a liver IR model or sham procedure. Intrahepatic macrophages were collected at 6 hours after reperfusion. (A) MDA and GSH/GSSG levels and SOD activity in macrophages. (B) ROS levels were detected by DCFH-DA fluorescence. Control and HFD-fed mice were pretreated with Xestospongin-C (3 μM) or PBS (Control) before ischemia. Intrahepatic macrophages and the liver tissues were collected at 6 hours after reperfusion. (C, D) ROS levels were detected by DCFH-DA fluorescence and 4-HNE. Control and HFD-fed mice were pretreated with mito-TEMPO or PBS (Control) before ischemia. Intrahepatic macrophages were collected at 6 hours after reperfusion. (E) Western blot analysis of

NLRP3, C-caspase-1, Pro-caspase-1, IL-1β, IL-18, and GAPDH in isolated macrophages. (F) H&E staining of liver sections. n = 4 mice/group. Values were presented as the mean ± SD. *$p < 0.05$. Abbreviations: 4-HNE, 4-hydroxynonenal; DCFH-DA, 2,7-dichlorodi-hydrofluorescein diacetate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione disulfide; H&E, hematoxylin-eosin; HFD, high-fat diet; IR, ischemia and reperfusion; MDA, malondialdehyde; NLRP3, NOD-like receptor thermal protein domain–associated protein 3; ROS, reactive oxygen species; SOD, superoxide dismutase.

as indicated by increased protein levels of P-PERK, IRE1a, ATF6, C/EBP homologous protein, and XBP1s, which were further enhanced in macrophages from fatty livers after IR.

Next, an ER stress inhibitor, TUDCA, was used to determine the functional role of ER stress in regulating macrophage NLRP3 activation and IR-induced inflammatory tissue injury in fatty liver. TUDCA treatment significantly suppressed the activation of NLRP3 and its downstream signaling in macrophages from both control and fatty livers after IR (Figure 4B). Functionally, ER stress suppression by TUDCA effectively attenuated liver IR injury and the inflammatory response in fatty livers after IR (Figures 4C–G). These findings suggest that ER stress contributed to macrophage NLRP3 activation in response to IR injury in fatty liver.

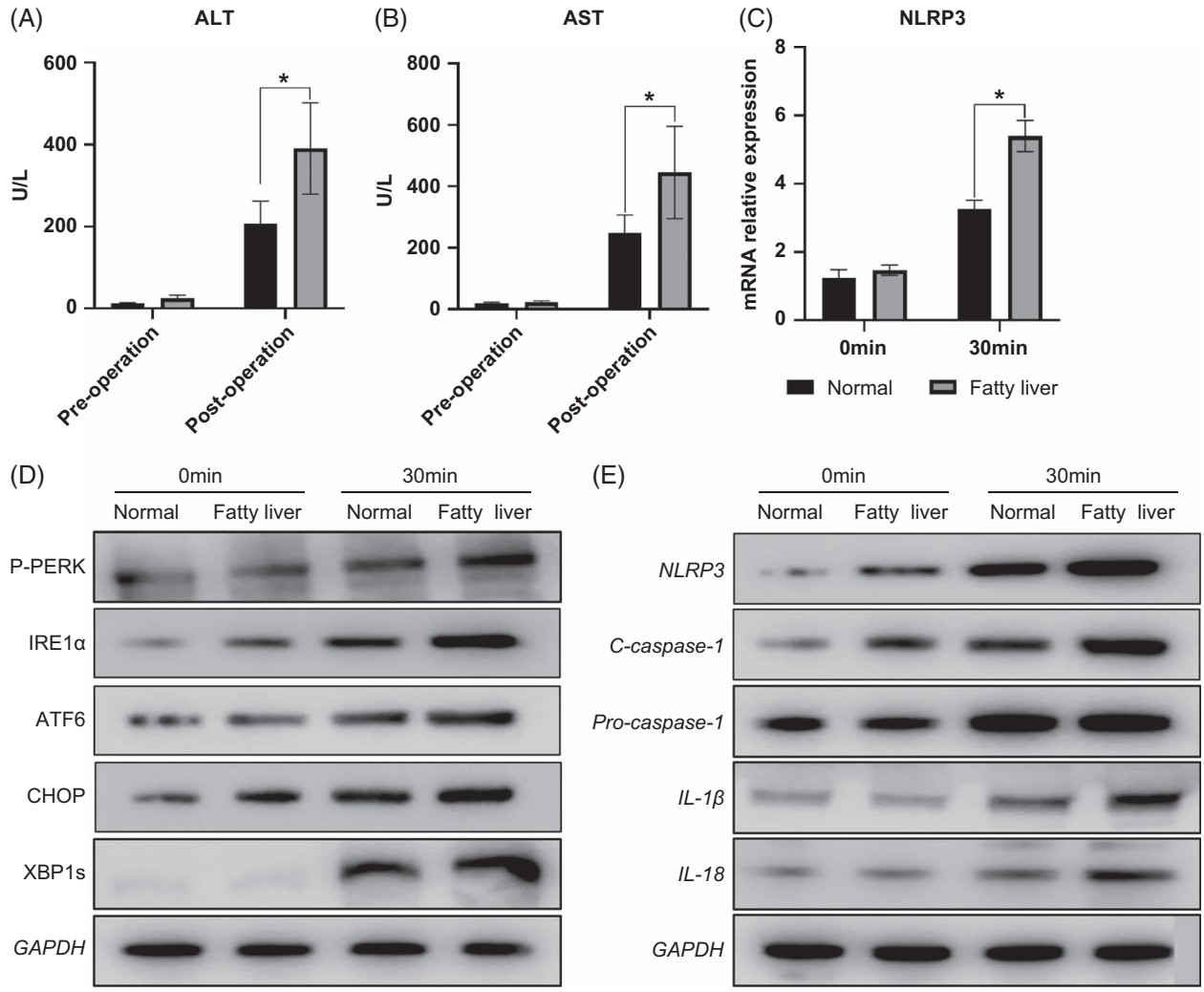

FIGURE 7 NLRP3 activation by endoplasmic reticulum stress of macrophages in patients with fatty liver exacerbates IR injury. Liver tissues and peripheral blood were collected respectively before transplantation and before the end of the surgery. (A, B) Serum ALT and AST levels. (C) Gene expression of NLRP3 in liver tissues measured by qRT-PCR. (D) Western blot analysis of P-PERK, IRE1a, ATF6, CHOP, XBP1s and GAPDH in liver tissues. (E) Western blot analysis of NLRP3, C-caspase-1, Pro-caspase-1, IL-1β, IL-18 and GAPDH in liver tissues. n = 4/group. Values were presented as the mean ± SD. *$p < 0.05$. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHOP, C/EBP homologous protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IR, ischemia and reperfusion; NLRP3, NOD-like receptor thermal protein domain–associated protein 3; qRT, quantitative Real-Time.

ER stress upregulated mitochondrial Ca^{2+} accumulation in macrophages from fatty livers after IR

To investigate the physical interaction between the ER and mitochondria in fatty livers after IR, we used transmission electron microscopy to examine macrophages collected from both control and HFD-fed mice after IR. As depicted in Figure 5A, we observed an increase in ER apposition to the mitochondria, and the proportion of ER in close contact with the mitochondria relative to the total ER content was significantly higher in fatty livers after IR (Figure 5B). In addition, macrophages showed upregulated mitochondrial Ca^{2+} accumulation in fatty livers after IR, as evidenced by the increased double-positive staining with Fluo4 and MitoTracker probes (Figure 5C). Moreover, the suppression of ER stress by TUDCA treatment significantly downregulated mitochondrial Ca^{2+} accumulation in macrophages from the livers of both control and HFD-fed mice after IR (Figure 5D), indicating a critical role of ER stress in promoting mitochondrial Ca^{2+} accumulation in IR-stressed macrophages.

ER stress-induced mitochondrial calcium overload activated macrophage NLRP3 through mitochondrial reactive oxygen species production in fatty livers after IR

As mitochondrial calcium overload induces oxidative stress, we tested whether the regulation of macrophage NLRP3 activation by ER stress-induced mitochondrial calcium overload was dependent on mitochondrial reactive oxygen species (mROS). As expected, increased oxidative stress was observed in macrophages obtained from livers of HFD-fed mice after IR, as indicated by the increased malondialdehyde, decreased levels of reduced glutathione/GSSH and superoxide dismutase (Figure 6A), and increased 2,7-dichlorodi-hydrofluorescein diacetate staining (Figure 6B). 4-HNE is a reactive lipid species that can induce oxidative modifications to other proteins and lipids and is an established marker of oxidative stress. As shown in Figures 6C, D, Xestospongin-C–mediated inhibition of mitochondrial calcium influx decreased ROS expression in the liver tissues and macrophages after IR. Interestingly, the scavenging of mROS by mito-TEMPO suppressed the activation of the NLPR3 signaling pathway in liver macrophages of HFD-fed mice after IR (Figure 6E), subsequently protecting fatty livers against IR injury (Figure 6F). Together, these results suggest that ER stress promoted mitochondrial calcium overload to activate NLRP3 signaling by inducing mROS expression in macrophages from fatty livers after IR.

Increased liver injury, NLRP3 activation, and ER stress in fatty livers after IR in patients

Finally, we compared liver function, the NLRP3 signaling pathway, and ER stress in normal and fatty livers after IR in humans. Consistent with the findings in mice, patients with fatty livers showed increased liver IR injury, as indicated by increased serum levels of ALT and AST (Figures 7A, B). Upregulated activation of the NLPR3 and ER stress signaling pathways was observed in human fatty livers after IR (Figures 7C–E).

DISCUSSION

Liver IR occurs in many clinical situations, including liver transplantation and partial hepatectomy, and results in impaired liver function and patient recovery. Fatty livers are more sensitive to IR insults and excessive inflammatory responses play an important role. In the present study, we found that mitochondrial calcium overload induced by ER stress promoted mROS production to activate NLRP3 in macrophages, leading to aggravated IR injury in fatty liver.

The mechanisms underlying liver IR are complex and unclear. The lack of nutrients and energy caused by ischemia leads to direct hepatocyte damage and the release of various types of damage-associated molecular patterns, which activate macrophages and other immune cells, thereby inducing severe inflammatory tissue injury. Both clinical and animal studies have implicated fatty liver as being more susceptible to IR injury.[5,20] However, effective and simple methods for attenuating fatty liver IR injury are currently unavailable. The critical role of macrophage-related inflammation in regulating IR injury has been revealed in both normal and fatty livers.[21,22] Fatty livers develop extensive hepatic necrosis and inflammation after IR, which can be suppressed by macrophage depletion.[23,24] A recent single-cell RNA sequencing analysis of immune phenotypes of liver grafts revealed that macrophages with a proinflammatory phenotype were enriched in transplanted steatotic livers.[25] Therefore, the suppression of macrophage proinflammatory activation may effectively alleviate IR injury in fatty liver.

Numerous studies have indicated the crucial role of ER stress in the pathogenesis of fatty liver disease and IR injury.[26,27] While unfolded protein response signaling pathways triggered by ER stress are essential for restoring ER homeostasis, defective unfolded protein response activation or prolonged ER stress can cause injury and disease.[28] Previously, we found that the ER stress signaling pathway not only regulates direct injury of liver parenchymal cells, but also modulates macrophage proinflammatory activation. XBP1 deficiency

promotes hepatocyte pyroptosis by impairing mitophagy during acute liver injury.[29] XBP1 was upregulated in liver tissues of patients with NASH. Hepatocyte-XBP1 depletion resulted in decreased lipid accumulation, whereas the genetic deletion of XBP1 in macrophages ameliorate atohepatitis and fibrosis in mice.[11,12] C/EBP homologous protein signaling has also been found to promote hepatocellular injury[9] and macrophage M1 polarization[30] after IR. In the present study, we observed increased ER stress in macrophages from fatty livers after IR, which further promoted macrophage activation and inflammatory liver injury.

Inflammasomes have recently been recognized as vital players in innate immune responses and proinflammatory signaling pathways. Among them, NLRP3 is the best-characterized inflammasome component that mediates caspase-1 activation and the secretion of proinflammatory cytokines in response to various dangerous stimuli.[31] NLPR3 is highly expressed in hepatic macrophages, and NLRP3 inflammasome activation plays an important role in promoting inflammatory liver IR injury.[32] We have previously found that aging aggravates liver IR injury by promoting macrophage NLRP3 activation.[8,19] A recent study also found that NLRP3 mediates the inflammatory response and pyroptosis in macrophages during fatty liver IR injury.[33] Pharmacological inhibition of the NLRP3 signaling pathway mitigates IR injury in the fatty liver.[34] Consistent with this, we found that myeloid NLRP3 deficiency protected the fatty liver against IR.

Emerging evidence indicates that ER stress plays a critical role in the regulation of ROS production and subsequent NLRP3 activation.[35,36] The ER stress–related PERK and IRE1a pathways induce cell death by activating NLRP3.[37,38] In addition, mROS was found to be critical for IRE1a in promoting NLRP3 activation.[39] Our group has previously found that XBP1 signaling plays an important role in regulating mROS by modulating mitophagy.[12,29] Liver IR stress activates Notch1 signaling and induces ROS-mediated NLRP3 activation to promote inflammatory liver injury.[40] Although the role of ROS in promoting inflammation and cell death during fatty liver IR has been well documented, the effect of macrophage NLRP3 signaling in regulating fatty liver IR remains unclear. In the present study, we demonstrated that ER stress promotes mROS production to activate NLRP3 signaling in macrophages during fatty liver IR.

Calcium ions are important regulatory factors for mitochondrial function. Dysfunction of the calcium balance system under stress causes its disordered distribution and overload, leading to functional abnormalities.[41] Mitochondrial calcium overload can promote mROS production, leading to the opening of the mitochondrial permeability transition pore, release of cytochrome C, and apoptosis.[42] Multiple studies have reported the important role of ER-mitochondria Ca2+ signals in regulating cell death in various animal models. Knockdown or pharmacological inhibition of Transient Receptor Potential Cation Channel Subfamily M Member 2 alleviated mitochondrial lipid peroxidation and ferroptosis by reducing mitochondrial calcium accumulation during liver IR injury.[43] In a mouse model of dibutyl phthalate–induced heart damage, ER stress suppression reduced mitochondrial calcium accumulation through the MAM, resulting in mtROS and NLRP3 inhibition and subsequent cardiomyocyte pyroptosis.[44] Silencing the MAM-associated protein FUNDC1 decreased ER-mitochondrial calcium exchange and suppressed mitochondrial calcium overload, leading to reduced cardiomyocyte necrosis caused by Sorafenib-induced myocardial toxicity.[45] The ER is an important intracellular calcium pool that regulates mitochondrial function by releasing calcium ions into the mitochondria. MAMs, also called ER mitochondrial contacts, are dynamic membrane structures that are involved in the regulation of lipid, Ca^{2+}, and ROS transmission between the ER and mitochondria. Recently, IRE1a expression was detected in MAMs, where it promoted the localized expression of IP3R and participated in the regulation of mitochondrial calcium influx, mitochondrial energy metabolism,[46] and apoptosis.[47] In summary, we found that fatty liver IR–induced ER stress activated macrophage ROS/NLRP3 signaling by promoting mitochondrial calcium overload.

In our previous study, we reported that patients undergoing partial hepatectomy demonstrated improved liver function with i.v. infusion of *N*-acetyl cysteine before surgery.[9] In addition, the utilization of TUDCA during liver transplantation resulted in a significant decrease in AST and cytolytic enzyme release, along with the amelioration of endothelial damage and reduction in mitochondria matrix and crystae damage.[48]

CONCLUSIONS

In the present study, we discovered a novel mechanism by which fatty liver exacerbates IR injury. This is the first study to demonstrate that ER stress promotes mitochondrial calcium overload to activate macrophage ROS/NLRP3 signaling during fatty liver IR injury. Our findings suggest a potential target to reduce IR injury in patients with fatty livers.

AUTHOR CONTRIBUTIONS

Zhuqing Rao developed the study concept. Haoming Zhou and Jian Xu directed experimental design and interpreted data. Fei Li, Zhu Guan, and Yiyun Gao performed experiments. Fei Li, Zhu Guan, Yiyun Gao, Yan Bai, Xinyu Zhan, and Xingyue Ji conducted data analysis. Zhuqing Rao and Haoming Zhou contributed reagents, analytic tools, and grant support. Fei Li, Haoming Zhou, and Jian Xu wrote the manuscript. All authors read, edited, and approved the manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This research scheme was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (Institutional Review Board approval number 2020-SRFA-219). All participants were provided with the subject information and gave informed consent to the research. All animals received humane care and all animal procedures were in accordance with the relevant legal and ethical requirements approved by the NMU Institutional Animal Care and Use Committee (NMU08-092).

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