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Glycyrrhizin attenuates hepatic ischemia-reperfusion injury by suppressing HMGB1-dependent GSDMD-mediated kupffer cells pyroptosis

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ABSTRACT

Gasdermin D (GSDMD), a genetic substrate for inflammatory caspases, plays a central role in pyroptosis of macrophages and release of interleukin-1β (IL-1β), but was mainly referred to microbial infection. High mobility group box-1 (HMGB1), served as an alarm molecule during various pathological process, has been widely recognized to be involved in liver ischemia-reperfusion (I/R). Glycyrrhizin, a natural anti-inflammatory and antiviral triterpene in clinical use, was recently referred to have ability to prevent I/R induced liver injury by inhibiting HMGB1 expression and activity. However, the mechanisms responsible for damage amelioration subsequently to HMGB1 inhibition during liver I/R remain enigmatic. This study was designed to explore the functional role and molecular mechanism of glycyrrhizin in the regulation of I/R induced liver injury. We found that liver I/R promotes GSDMD-mediated pyroptotic cell death of Kupffer cells, which was inhibited by glycyrrhizin. Interestingly, endogenous HMGB1, not exogenous one, was involved in hypoxia-reoxygenation (H/R) induced pyroptosis. Moreover, GSDMD knockdown protects kupffer cells against H/R induced pyroptosis in vitro. Here, we report, for the first time, that glycyrrhizin attenuated tissue damage and kupffer cells pyroptosis during liver ischemia-reperfusion injury (LIRI) and identify a previously unrecognized HMGB1- dependent GSDMD- mediated signaling pathway in the mechanism of kupffer cells pyroptosis induced by H/R. Our findings provide the first demonstration of GSDMD-determined pyroptotic cell death responsible for I/R induced release of IL-1β and this would provide a mandate to better understand the unconventional mechanisms of cytokine release in the sterile innate immune system.

1. Introduction

Ischemia-reperfusion injury in the liver is a major cause of morbidity and mortality from hepatic resectional surgery, liver transplantation and hemorrhagic shock with fluid resuscitation. Compared with ischemia injury, innate-immune-dominated tissue inflammatory response initiated by Kupffer cells after reperfusion involves more cytotoxic mechanisms [1,2]. The production of interleukin-1beta (IL-1β) by Kupffer cells has long been thought to be one of the primary initiating events for propagation of the hepatic inflammatory response [3]. IL-1 receptor blockade with IL-1ra resulting in attenuation of tissue injury after I/R [4] may be explained by the important role of IL-1β in mediating neutrophil-endothelium interactions, which is confirmed to be critical in liver I/R injury by applying monoclonal antibody against the neutrophil adherence glycoprotein (CD18) [5]. Furthermore, it has been reported that activation of Kupffer cells plays an important role in the production of IL-1 and exposure of mononuclear phagocytes to hypoxia followed by reoxygenation lead to significant production of IL-1 in vitro [6,7]. A growing number of evidence has confirmed that secretion of IL-1β by inflammatory cells is largely dependent on inflammasome assembly and the subsequent caspase-1 activation [8–10]. Our previous study also suggested NLRP3 inflammasome to be involved in the production of IL-1β in a murine model of liver I/R [11]. However, the mechanisms responsible for the unconventional release process of IL-1β subsequently to inflammasome activation remain enigmatic.

Apoptosis and necrosis are the two major modes of cell death

Abbreviations used in this paper: LIRI, liver ischemia-reperfusion injury; I/R, ischemia-reperfusion; H/R, hypoxia-reoxygenation; GSDMD, gasdermin D; NLRP3, nucleotide-binding domain, leucine-rich repeat containing protein 3; ASC, apoptosis-associated speck-like protein containing a C-terminal caspase-activating recruiting domain; NPC, nonparenchymal cell; sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; DC, dendritic cell; HMGB1, high-mobility group box 1

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accounts for the severe injury observed during hepatic reperfusion, the molecular mechanisms of which have been extensively studied [12,13]. Until pyroptosis, a novel form of programmed cell death that differs from other types of cell death has been improved. Pyroptosis is dependent on caspase-1 activation, features destruction of the actin cytoskeleton, pore formation in the plasma membrane resulting in release of proinflammatory intracellular contents and positive for PI staining [14]. It has been widely discussed in various diseases such as stroke [15], alcoholic hepatitis [16] and renal ischemia-reperfusion-induced acute kidney injury (AKI) [17]. However, the role of pyroptosis in hepatic ischemia-reperfusion injury remains unclear. More recently, as a genetic substrate for inflammatory caspases, accumulating evidences suggest a central role of gasdermin D (GSDMD) in pyroptosis of macrophages and release of IL-1β [18,19]. Despite these important functions, the mechanism underlying how inflammasome trigger IL-1β secretion during hepatic ischemia-reperfusion injury is unknown.

High-mobility group box 1 (HMGB1), a highly conserved ubiquitous protein present in nearly all cell types, mediates inflammation during sterile and infectious injury and contributes importantly to disease pathogenesis. The important roles of HMGB1 in regulating cells apoptosis and necrosis as an exogenous alarmin during liver I/R injury have been widely published by interacting with multiple receptors [20]. In addition to its extracellular role, HMGB1 also functions as an cytosolic signaling molecule during inflammation [21,22], including recently reported to activate caspase-1 and contribute to the proinflammatory response in liver I/R [23]. It is generally accepted that Glycyrrhizin, a natural triterpene glycoconjugate binding directly with HMGB1 to inhibit its chemoattractant activity and mitogenic activity, reduces I/R-induced liver injury [23], but the mechanism still needs clarifying.

Here, we report, for the first time, that pyroptosis is a key event during hepatic ischemia-reperfusion injury and identify a previously unrecognized role of glycyrrhizin in the mechanism of suppressing liver injury caused by I/R.

2. Materials and methods

2.1. Animals

Male 8–10-wk-old wild-type C57BL/6J mice were purchased from the Institute of Experimental Animal, Chinese Academy of Medical Sciences (Beijing, China). The mice were housed in the specific pathogen-free (SPF) facility at the Tongji Medical College for at least one week before inclusion in experiments. All the experiments were performed in compliance with the Tongji Medical College Animal Care and Use Committee guidelines.

2.2. Hepatic ischemia-reperfusion injury model

Partial hepatic ischemia was induced as previously described [24,25]. Briefly, mice were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). A midline laparotomy was performed, and a microvascular clamp was used to occlude blood supply to the left and median lobes of the liver. The caudal lobes retained intact portal and arterial inflow and venous outflow, preventing mesenteric venous congestion. The abdomen was covered with a sterile operating gauze to minimize evaporative loss during the ischemic period. After 90 min of partial hepatic ischemia, the clip was removed and the abdomen was closed with continuous 4–0 silk, initiating hepatic reperfusion. The animals received 1 ml of sterile saline subcutaneously to recover. Sham control mice underwent the same protocol without vascular occlusion. Glycyrrhizin (Minophagen Pharmaceutical, Tokyo, Japan) was administered twice, right before ischemia and when reperfusion begins, intravenously in the tail vein in a dose of 50 mg/kg. Rectal temperature was measured and was maintained at 37 °C by using an electronic heating pad with a rectal thermistor probe (FHC, Bowdoin, Maine, USA). At the end of the observation period following reperfusion, the mice were anesthetized and were killed by exsanguination.

2.3. Liver sample preparation

Liver tissue was harvested at indicated time points following reperfusion and divided as follows: [1] A representative section was fixed in 4% formalin for 24 h and embedded in paraffin; [2] another representative section was snap frozen in liquid nitrogen and embedded in optimal cutting temperature compound; [3] approximately 50 μg of tissue were placed in 0.5 ml of RNAlater Solution (Life Technologies, Carlsbad, CA); and [4] the remainder of the liver tissue was stored at −80 °C.

2.4. Liver damage assessment

Blood was obtained from inferior vena cava after reperfusion for analysis of serum alanine aminotransferase (ALT) and aspartate transaminase (AST) as index of liver injury. Measurements of sALT and sAST were made using the Opera Clinical Chemistry System (Bayer Co.)

2.5. Liver histology and immunostaining

Tissue paraffin sections (5 μm) were prepared and routinely stained for hematoxylin and eosin. The severity of liver IRI was scored blindly with Suzuki's criteria on a scale from 0 to 4. No necrosis or congestion/centrilobular ballooning was given a score of 0, whereas severe congestion and > 60% lobular necrosis were given a value of 4 [23]. Tissue immunohistochemistry (IHC) staining for Ly6G (BD Biosciences), NLRP3 (Santa Cruz Biotechnology) and HMGB1 (kindly provided by J. Tang, Institute of Biophysics, Chinese Academy of Science, Beijing, China) were performed in paraffin sections according to the manufacturer's instruction. F4/80 (Abcam, Cambridge, MA) was visualized in frozen sections by immunofluorescence (IF) with DAPI nuclear counterstaining.

2.6. Myeloperoxidase (MPO) assay

Neutrophil accumulation in the liver tissue was assessed with the enzymatic activity of Myeloperoxidase (MPO) as previously described [26]. One unit of MPO activity (U/g) was defined as the amount of enzyme degrading 1 μmol peroxide per minute at 25 °C per gram of tissue.

2.7. Isolation of Kupffer cells

Kupffer cells were isolated as previously described [11]. In brief, after saline solution perfusion followed by 0.05% collagenase digestion of the liver, the cell suspension was centrifuged twice at 50*g* for 3 min to remove most of the parenchymal cells, followed by centrifuged the cell supernatant (1500 rpm, 5 min, 4 °C) to enrich the NPCs. The NPC-enriched fraction was collected, washed in PBS, and positively selected using PE- conjugated anti-F4/80 antibody (Biolegend, CA) and anti-PE-conjugated magnetic MicroBeads (Miltenyi Biotec) following the manufacturer's protocol. Cells isolated after liver I/R were incubated with serum-free DMEM for 12 h until the supernatants and cells were collected for detection. For in vitro H/R assays, cells were plated and treated according to the succeeding protocol.

2.8. Hypoxia-reoxygenation protocol

To obtain hypoxia, the culture plates were transferred to a 'hypoxia chamber' that had similarly been purged with 95% N_2/5% CO_2. The 'hypoxia chamber' was established using an air-tight jar with a barometer, a gas inlet and outlet ports positioned at opposite ends (Advanced Instruments, MA, USA) as previously described [27]. The gas ports were sealed with a valve, except during the flushing of the

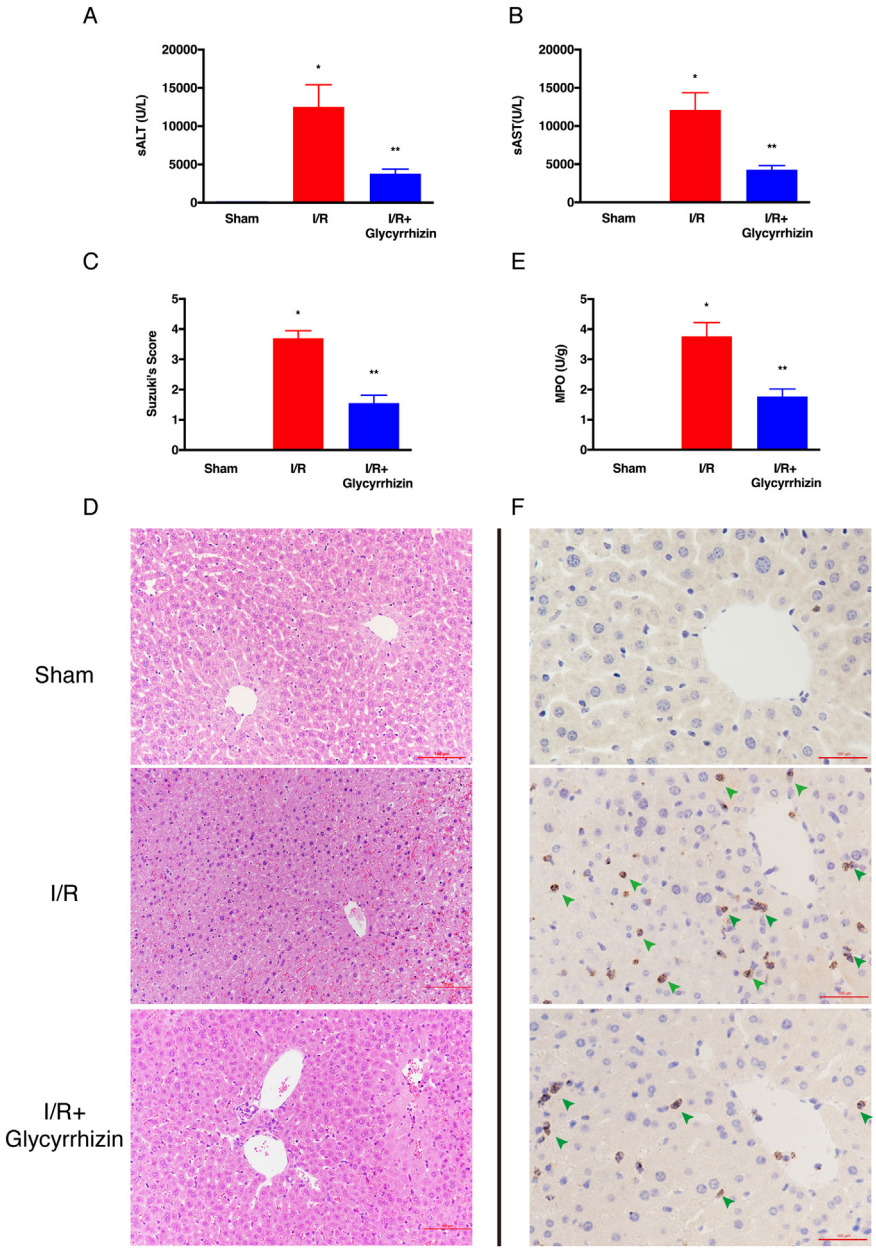

Fig. 1. Glycyrrhizin ameliorates I/R induced liver tissue damage and neutrophils infiltration. (A) sALT and (B) sAST levels (international units/liter), a measure of hepatocellular injury, are analyzed in sham mice and mice that underwent 1.5 h of ischemia followed by 6 h of reperfusion with glycyrrhizin pretreatment or not. Data represent Mean ± SD (n = 6 mice/group). *, P < 0.05 vs sham control. **, P < 0.05 vs I/R. Histological grading of liver I/R is determined by (C) Suzuki's score and (D) H&E staining (original magnification ×200). Data represent Mean ± SD (n = 6 mice/group). *, P < 0.05 vs sham control. **, P < 0.05 vs I/R. Images are representative liver sections from each group. Neutrophil infiltration is shown by (E) MPO activity and (F) Ly6G stained immunohistochemistry of liver tissues from sham and I/R mice. Data represent Mean ± SD (n = 6 mice/group). *, P < 0.05 vs sham control. **, P < 0.05 vs I/R. Images are representative liver sections from each group. Experiment is performed twice at different occasions.

chamber. To ensure a humidified atmosphere, the water trays were positioned at the bottom of the chamber. After the addition of the culture plates to the hypoxia chamber, it was flushed with 95% N_2/5% CO_2 for approximately 20 min. These conditions maintained an oxygen concentration of < 0.1% in the gas phase which was measured by an O_2 analyzer placed inside the chamber.

Cells were treated with 10 μg/ml LPS (Invivo Gen, San Diego, CA) for 3 h prior to stimulation with hypoxia-reoxygenation to activate NF-κB signaling. To start the H/R experiments, the cultured cells were washed twice in PBS buffer and covered with glucose-free/fetal bovine serum-free medium (Gibco, Carlsbad, CA). The hypoxia incubation was then achieved by flushing 95% N_2/5% CO_2 and maintained for 4 h. After hypoxia, the cells were reoxygenated by adding an equal volume of medium supplemented with fetal bovine serum (10%) and glucose (4500 mg/L) and placing under normoxia for 20 h. Glycyrrhizin (Tokyo Chemical Industry, Tokyo, Japan, at the concentration of 50 or 100 μM), HMGB1-neutralizing mAb (Anti-HMGB1 Ab; gift from Institute of Biophysics, Chinese Academy of Science, Beijing, China, at the concentration of 100 ng/ml) or recombinant HMGB1 (rHMGB1, Sigma- Aldrich, at the concentration of 1 μg/ml) were applied at the start of hypoxia and supplied when reoxygenation begins.

2.9. Cytokine ELISA

A mouse ELISA kit (eBioscience, Waltham, MA) was used to measure mature IL-1β levels in cell culture supernatants following the manufacturer's protocol.

2.10. Knockdown of GSDMD

The siRNA duplexes targeting mouse GSDMD and negative control (NC) siRNA were designed and synthesized by RiboBio (Guangzhou, China). The sequences used for knockdown of GSDMD were as follows: Gsdmd_001(GCCTCCATGAATGTGTGTA), Gsdmd_002(CCTTGAGTGTCTGGTGCTT), Gsdmd_003(CCTTGAGTGTCTGGTGCTT). Kupffer cells at a concentration of 2.0×10^4 were seeded in each well of a 48-well plate in 300 μl of culture medium to yield a confluence of 80%. Cells were allowed to adhere for 12 h. 2.5 pmol siRNA was diluted in 100 μl of

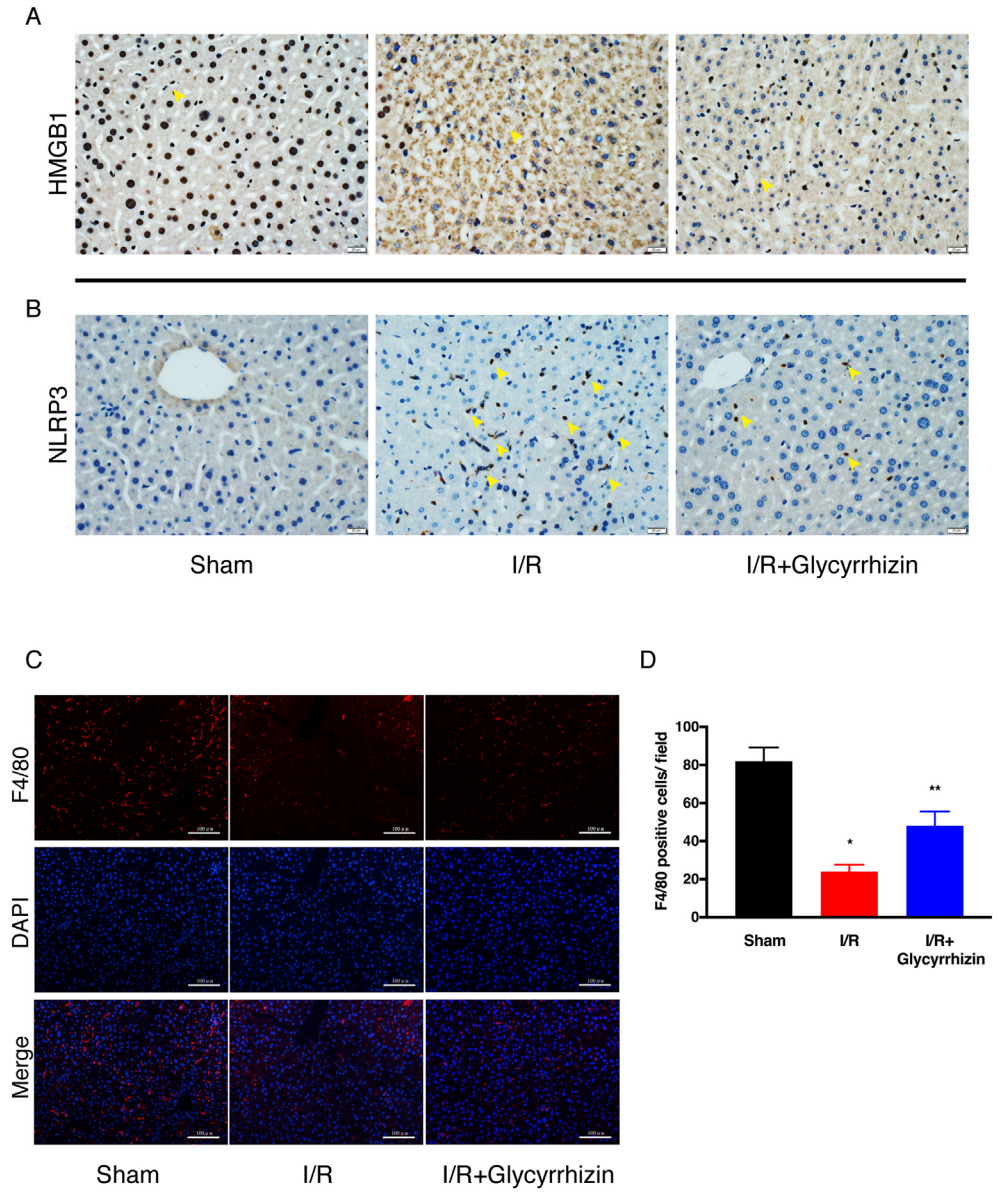

Fig. 2. Glycyrrhizin inhibits liver I/R induced HMGB1, NLRP3 activation and Kupffer cells death. Representative immunohistochemistry graphs show the location and expression of (A) HMGB1 (original magnification, ×400) and (B) NLRP3 (original magnification, ×400). Images are representative liver sections from six mice per group. (C) Immunofluorescent staining of F4/80 from sections of sham, I/R and I/R + Glycyrrhizin liver (original magnification ×200). Red, F4/80; blue, nuclei. Images representative of six experiments are shown and (D) F4/80 positive cells are quantified. *, $P < 0.05$ vs sham control. **, $P < 0.05$ vs I/R. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Opti-MEM (Gibco, Carlsbad, CA). 1 μl of Hiperfect transfection reagent (QIAGEN) was added to the diluted siRNA and mixed. The transfection mixture was incubated for 10 min at room temperature, then applied to each well and a final siRNA concentration of 100 nM. 6 h later, the transfection medium/mixture was replaced with normal medium containing 10% FBS. After being cultured for additional 24 h, the cells were ready for experiments. Transfection efficiency and specificity of RNAi was determined at 48 h by Western blot.

2.11. Caspase-1 inhibition assays

The pan-caspase inhibitor *Z*-VAD-FMK and caspase-1 inhibitor *Z*-YVAD-FMK were obtained from APExBio (Houston, TX). For in vitro application, Kupffer cells were plated on Petri dishes and stimulated with H/R with or without 20 μM Z-VAD-FMK or Z-YVAD-FMK.

2.12. Flow cytometry analysis of PI

Cells treated with various stimulations or grown under normal condition were gently scraped and transferred to flow cytometry tubes for subsequent analysis. Cells were washed twice with cold PBS and then re-suspended gently in 500 μl binding buffer. After stained with PI protected from light at room temperature for 15 min, the cells were then analyzed by flow cytometer (BD LRS II; BD Biosciences) immediately. Data analysis was performed using FlowJo (FlowJo, LLC) software. The rate of PI positive cell was calculated by the formula: (positive cells/total cells) × 100%. Background were determined by a control untreated-cell with PI staining.

2.13. Cell immunofluorescence microscopy

For caspase-1 activity determination, FAM-FLICA caspase-1 assay kit (ImmunoChemistry Technologie, Bloomington, MN) was used according to the instructions provided by the manufacturers. Briefly, pretreated Kupffer cells were incubated with caspase-1 FLICA at 37 °C protected from light for 1 h. After wash twice with PBS, the cells were analyzed by a Fluorescent Cell Imager (Bio-Rad) immediately in a dark place. For NF-κB P65 translocation detection, after all the indicated treatments, cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 3% bovine serum albumin in PBST (PBS with 0.2% Tween-20) for 2 h at room temperature to reduce non-specific staining. The cells were then

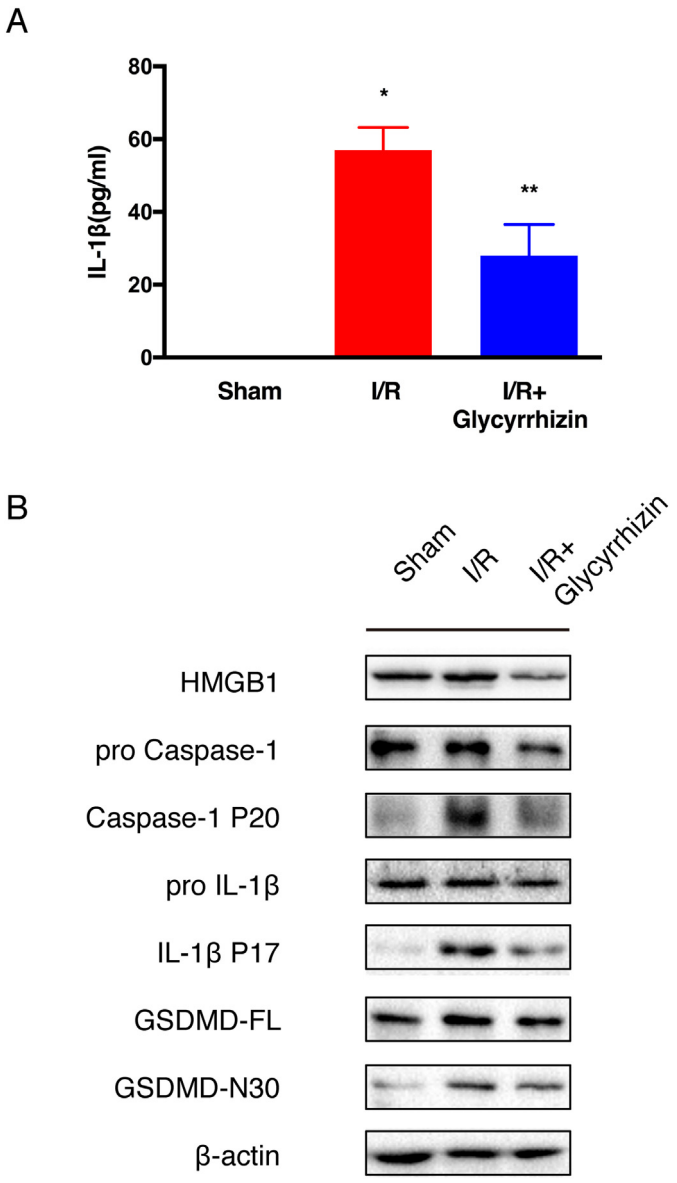

Fig. 3. Glycyrrhizin decreases liver I/R induced pyroptotic Kupffer cells death. (A) Release of IL-1β in serum-free culture supernatants of Kupffer cells obtained from mice after operation were measured by ELISA and compared within three groups. Data represent the Mean ± SD (n = 6 mice/group). *, P < 0.05 vs sham control. **, P < 0.05 vs I/R. (B) Western blot images showing the protein levels of HMGB1, caspase-1, IL-1β and GSDMD, in precursor and/or activated format, in kupffer cell lysates of sham, I/R and I/R+ Glycyrrhizin mice. The blots shown are representatives of at least three experiments with similar results.

incubated with mouse monoclonal NF-κB p65(sc-8008, Santa Cruz Biotechnology, CA) Antibody at 4 °C overnight, followed by staining with Alexa Fluor 488-conjugated goat-anti-mouse IgG (Cell Signaling Technology) for 1 h at room temperature. Subsequently, DAPI was used to stain nuclei for 3 min. Fluorescence images were captured with EVOS FL Colour Imaging System (Thermo Fisher Scientific, Waltham, MA).

2.14. Western blotting

Proteins (40 μg per sample) from liver tissues or whole cell lysates were subjected to 10%/12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a Polyvinylidene Fluoride membrane (Merck Millipore, Billerica, MA). The membrane was blocked and incubated with primary antibody overnight, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. Bands were visualized with the enhanced chemiluminescence reagent and digitized using a CCD camera (ChemiDoc, Bio-Rad, Hercules, CA). Expression intensity was quantified by ImageLab (Bio-Rad). Anti-Gasdermin D (GSDMD) Ab (dilution, 1:500), anti-NF-κB p65 (dilution, 1:500), anti-Casp1 (dilution, 1:1000), anti-IL-1β (dilution, 1:1000; both Abcam, Cambridge) and anti-HMGB1, anti-NLRP3 (dilution, 1:1000; ProteinTech Group Chicago) were used.

2.15. Statistical analysis

Analysis were performed with GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). Differences between experimental groups were analyzed with one-way ANOVA or two-tailed Student' *t*-test. Data were expressed as Mean ± SD. All differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Glycyrrhizin ameliorates I/R induced liver tissue damage and neutrophils infiltration

We analyzed the effects of Glycyrrhizin on mouse livers subjected to 90 min of warm ischemia followed by 6 h of reperfusion. Serum ALT (Fig. 1A) and AST (Fig. 1B) levels showed a remarkable decrease in glycyrrhizin administrated mice undergoing liver I/R and these data correlated with Suzuki's grading (Fig. 1C) of histological liver I/R damage. As shown in histologic observation (Fig. 1D), liver sections from mice undergoing I/R injury presented with significant features of severe centrilobular ballooning, congestion and lobular necrosis compared to those from sham-operated mice. In contrast, these pathological changes were mostly prevented in the glycyrrhizin-treated mice. The liver MPO activity, an index to quantify neutrophils infiltration, was suppressed in glycyrrhizin-treated mice versus liver I/R mice (Fig. 1E). Also, the number of infiltrating neutrophils was increased in I/R tissues, whereas the glycyrrhizin-treated group showed less neutrophil accumulation detected by Ly6G stained immunohistochemistry (Fig. 1F).

3.2. Glycyrrhizin inhibits liver I/R induced HMGB1, NLRP3 activation and Kupffer cells death

We found that serum IL-1β levels reached its highest level at 9 h after the initial of reperfusion and declined thereafter (data not shown) in accordance with others previous studies [25], which indicating a large amount death of kupffer cells. Thus, the following results are all acquired after 6 h reperfusion (unless indicated). As shown in Fig. 2A, HMGB1 translocated from nuclear to the cytoplasm in hepatocytes as well as in non-parenchymal liver cells (NPC) after liver I/R, which was inhibited by glycyrrhizin. Activation of NLRP3 located in Kupffer cells was confirmed by immunohistochemical staining and glycyrrhizin resulted in an obvious decrease in NLRP3 expression (Fig. 2B). Moreover, F4/80, a surface glycoprotein expressed by murine macrophages, was observed by immunofluorescent staining of liver sections after 9 h reperfusion, indicating the death of Kupffer cells which developed typical pyroptosis morphology with cell swelling and membrane rupture (Fig. 2C). And this was also inhibited by glycyrrhizin pretreatment (Fig. 2D).

3.3. Glycyrrhizin decreases liver I/R induced pyroptotic Kupffer cells death

To further clarify the way of Kupffer cells death, we analyzed the production of IL-1β, the mechanism by which the cytokine was released from cells could be pyroptosis, in Kupffer cells isolated from sham mice, mice subjected to liver I/R and I/R mice with glycyrrhizin pretreatment. After incubation under serum-free conditions for 12 h, IL-1β

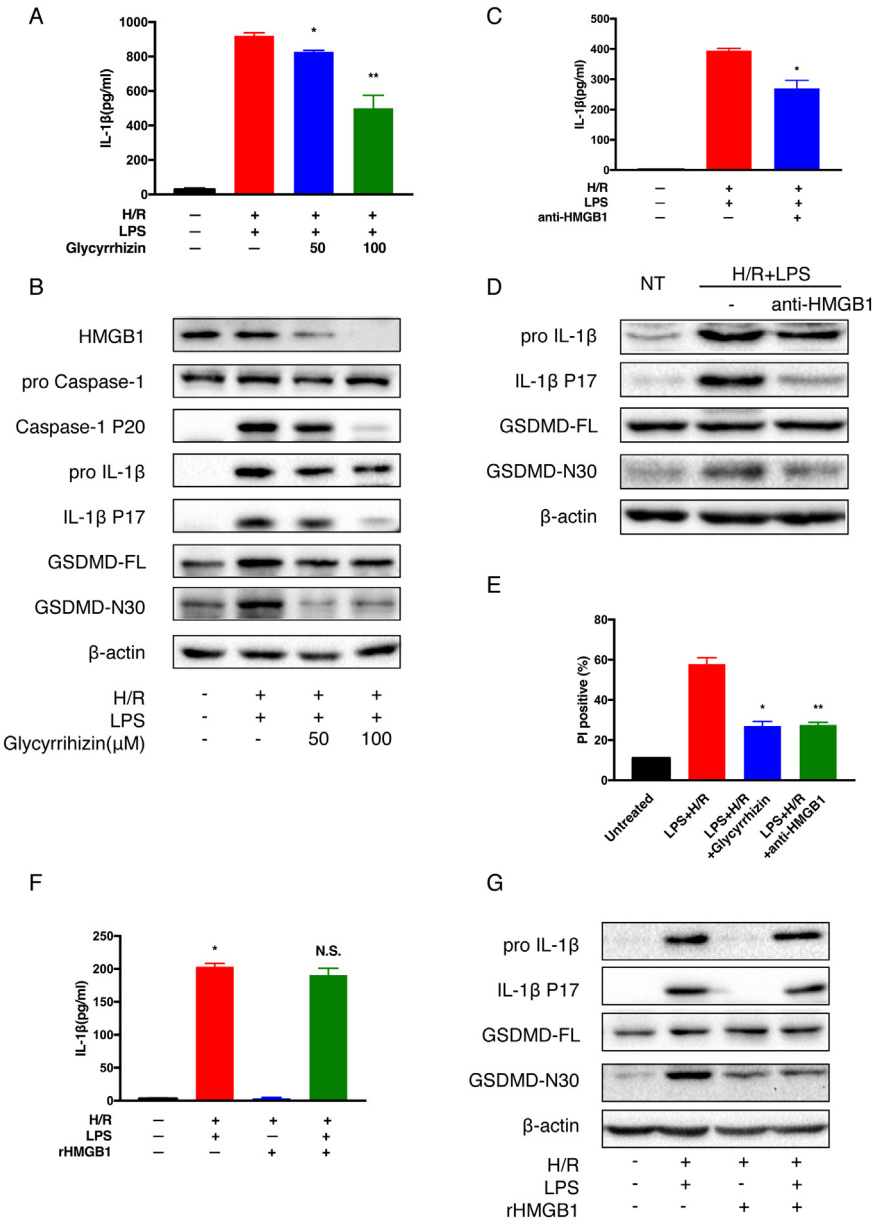

Fig. 4. Glycyrrhizin prevents H/R induced kupffer cells pyroptosis. Cells are treated with glycyrrhizin with LPS plus H/R stimulation. (A) IL-1β release is examined by ELISA in culture supernatants. Data represent Mean ± SD (n = 3/experiments). *, P < 0.05 vs LPS plus H/R stimulated cells. **, P < 0.05 vs 50 μM glycyrrhizin treated LPS plus H/R stimulated cells. (B) HMGB1, caspase-1, IL-1β and GSDMD expression are examined by Western blot in cell lysates. The blot shown is representative of three different experiments with similar results. (C) IL-1β release is examined by ELISA in culture supernatants. Data represent Mean ± SD (n = 3/experiments). *, P < 0.05 vs LPS plus H/R stimulated cells. (D) IL-1β and GSDMD expression are examined by Western blot in cell lysates. The blot shown is representative of three different experiments with similar results. (E) Cells are stained with propidium iodide (PI) and observed by flow cytometry. (Data shown are combined results from triplicate wells in a single experiment. *, P < 0.05 vs untreated cells. **, P < 0.05 vs LPS plus H/R cells.) And then cells are treated with recombinant HMGB1 with LPS and/or H/R stimulation. (F) IL-1β release is examined by ELISA in culture supernatants. Data represent Mean ± SD (n = 3/experiments). N.S., not significant vs LPS plus H/R stimulated cells. (G) IL-1β and GSDMD expression are examined by Western blot in cell lysates. The blot shown is representative of three different experiments with similar results.

released into the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) and its expression on protein levels in cells were determined by western bolt. As shown in Fig. 3A, supernatant IL-1β levels increased evidently followed by liver I/R and decreased with glycyrrhizin supplement. The protein-level expressing of activated IL-1β (IL-1β P17), which showed a noteworthy promotion in I/R mice versus sham-operated controls, was also inhibited by glycyrrhizin (Fig. 3B). Moreover, glycyrrhizin decreased expression of HMGB1 and activated caspase-1 (caspase-1 P20) in comparison with I/R group. In contrast to sham-operated controls, western blot–assisted expression of the N-terminal cleavage product of GSDMD (GSDMD-N30) was increased in I/R group, accompanied by markedly elevated caspase-1 activity, which demonstrated a remarkable GSDMD activation to drive pyroptosis. What's more, activation of GSDMD was also inhibited by glycyrrhizin.

3.4. Glycyrrhizin prevents H/R induced kupffer cells pyroptosis

To elucidate the molecular mechanisms of glycyrrhizin mediated inflammatory response in Kupffer cells pyroptosis after liver I/R, we used a hypoxia-reoxygenation (H/R)-stimulated cell culture system. As release of processed IL-1β required both precursor production and active secretion, herein, we applied widely used LPS to activate NF-κB signal producing IL-1β precursor. Our results suggested that abundant of mature IL-1β could be detected only in those culture supernatants of cells which were primed with LPS and treated with H/R stimuli. Cells stimulated with LPS or H/R alone did not lead obvious mature IL-1β release (Fig. S1A). The translocation of NF-κB p65 protein from the cytoplasm to nucleus was stimulated by LPS as measured using immunofluorescence microscopy (Fig. S1B).

We first used glycyrrhizin to study the involvement of HMGB1 in in vitro H/R induced kupffer cells pyroptosis. In agreement with in vivo results, as shown in Fig. 4A, glycyrrhizin treatment reduced the release of IL-1β in a dose-dependent manner by preventing caspase-1-induced IL-1β cleavage and GSDMD activation in comparison with LPS + H/R Group (Fig. 4B). The similar results were also acquired when using HMGB1 neutralizing antibody (anti-HMGB1) to specifically inhibit HMGB1 (Fig. 4C and D). Notably, cells from H/R plus glycyrrhizin or anti-HMGB1 treatment showed the same decrease extent in the number of PI positive cells, compared to pure H/R incubation (Fig. 4E),

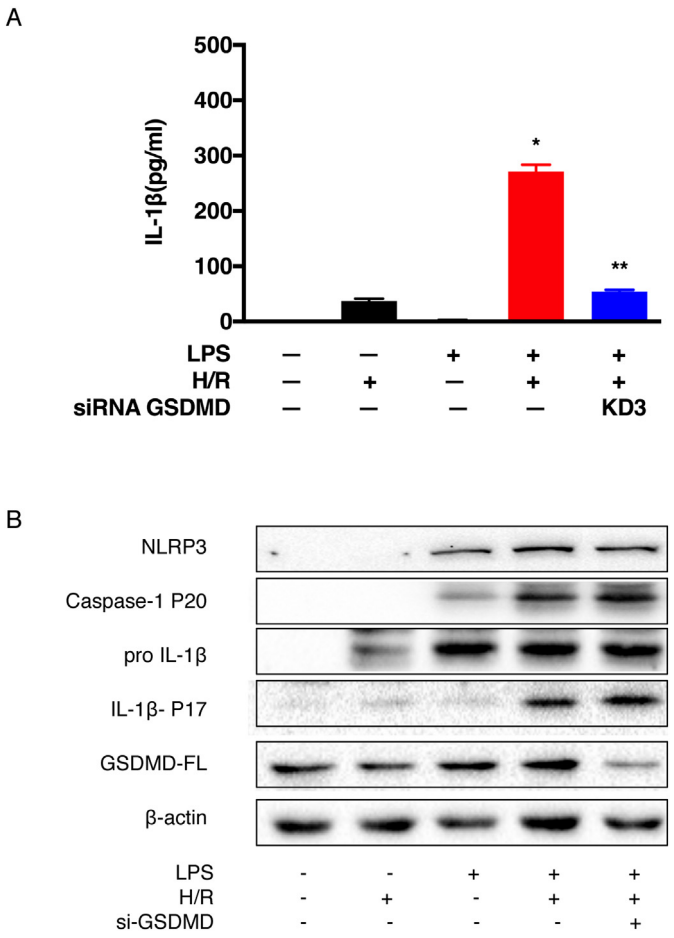

Fig. 5. GSDMD knockdown protects kupffer cells against H/R induced pyroptosis. (A) IL-1β release is examined by ELISA in culture supernatants of cells with indicated treatments. *, $P < 0.05$ vs LPS or H/R stimulated cells. **, $P < 0.05$ vs LPS plus H/R stimulated cells (red lane). Data represent Mean ± SD (n = 3/experiments). (B) Protein expression of NLPR3, activated caspase-1, IL-1β and GSDMD are examined by western blot in cell lysates with indicated treatments. The blot shown is representative of three different experiments with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggesting that glycyrrhizin suppressed kupffer cells pyroptosis resulted from H/R via inhibiting HMGB1. To further investigate the mechanism by which HMGB1 may exert in H/R induced kupffer cells pyroptosis, we next analyzed the release of IL-1β by applying recombinant HMGB1 (rHMGB1) while H/R incubation. However, different from what we expected, treatment with rHMGB1 had no effect on IL-1β secretion in culture medium (Fig. 4F). Moreover, it failed to promote caspase-1-induced IL-1β cleavage and GSDMD activation, as detected by western blotting assays (Fig. 4G). Previous studies emphasized HMGB1 role as transcriptional activator, which acted mainly through Toll-like receptor (TLR) 4 signaling in liver I/R injury [29] or through Receptors for Advanced Glycation End-products (RAGE) in I/R induced myocardial injury [28]. Recently, it was reported that glycyrrhizin have effects on stabilization of the cellular membrane structure by modulating the production of HMGB1 [31]. Endogenous HMGB1, not exogenous one, contribute to I/R induced myocardial apoptosis [21]. From the above, we speculated that the endogenous HMGB1 may exert an instinct role of the exogenous one on H/R induced kupffer cells pyroptosis.

3.5. GSDMD knockdown protects kupffer cells against H/R induced pyroptosis

The activation of NLRP3 inflammasome and caspase-1 (Caspase-1 P20) was also shown in cells exposed to H/R stimulation. Small interfering RNA (siRNA) technology was used to elucidate GSDMD involved in H/R induced kupffer cells pyroptosis and the specificity of the various GSDMD siRNAs were examined using Western blotting. KD3 showed the highest knockdown efficiency and was then used for the subsequent experiments (Fig. S1B). Kupffer cells were transfected with small interfering RNA (siRNA) before H/R stimulation. As shown in Fig. 5A and B, H/R-induced IL-1β release was inhibited after GSDMD knockdown without influencing IL-1β processing. In the meanwhile, knock down of GSDMD ameliorated H/R induced cells PI staining positive rate (Fig. 6A).

3.6. Activation of GSDMD is controlled by caspase-1 in H/R induced kupffer cells pyroptosis

Caspase-1 activation in our in vitro H/R model was further confirmed by western boltting (Fig. 6B) and FLICA fluorescent staining assay (Fig. 6C, D). Inflammatory caspases, limited to caspase-1, 4 and 11, were found capable of cleaving GSDMD after specific sequence, predicting that GSDMD could function downstream of the inflammatory caspases [18]. However, whether and how GSDMD interacts with caspase-1 during H/R induced kupffer cells pyroptosis is still unclear. The above results demonstrated that GSDMD contributes to the H/R induced inflammation response, we next investigated the role of caspase-1 on GSDMD by using a caspase-1 inhibitor *Z*-YVAD-FMK in our model. The disruption of caspase-1 signaling alleviated IL-1β release (Fig. 6E), as evidenced by not only diminished IL-1β activation but also reduced GSDMD cleavage (Fig. 6F). In agreement with these data, transfection with GSDMD siRNA resulted in a same significant reduction in PI-positive rate when compared to cells treated with Z-YVAD-FMK (Fig. 6A). On the contrary, GSDMD knocked down had no effect on caspase-1 activation (Fig. 6B and C).

4. Discussion

Macrophages can be activated and regulated by high-mobility group box 1 (HMGB1), as evidenced by a HMGB1-dependent inflammation response in the mechanism of liver IRI, in which rHMGB1 had ability to active caspase-1 in an TLR4-dependent way in murine Bone marrow–-derived macrophages (BMMs) [23]. However, our results in the current study showed little effect of rHMGB1 on IL-1β release resulted from caspase-1 activation either in LPS-primed or in LPS plus H/R-primed kupffer cells. Moreover, the expression of double-stranded RNA dependent protein kinase (PKR), a kinase which physically interacts with multiple inflammasome components and broadly regulates inflammasome activation [29], showed no obvious activation in our model (data not shown), suggesting rHMGB1 may not involve in activating caspase-1, as least in H/R stimulated kupffer cells. More recently, a study identified a novel pathway of HMGB1-induced pyroptosis. The authors showed that the endocytosis of HMGB1 initiated a cascade of molecular events which eventually induced cell pyroptosis [30]. Combining with the fact that HMGB1 mobilized from the nucleus to the cytoplasm in activated immune cells [31], we speculated a potential mechanism for endogenous HMGB1 in pyroptosome formation and caspase-1 activation. In agreement with this idea, our results showed a moderate reduction in pyroptosis induced IL-1β production when glycyrrhizin or anti-HMGB1 was applied during H/R incubation, indicating a distinct role of endogenous HMGB1 from exogenous ones in this H/R model.

Glycyrrhizin, a natural anti-inflammatory and antiviral glycoconjugated triterpene in clinical use, inhibits HMGB1 chemoattractant and mitogenic activities by binding directly to each of the two HMG boxes of HMGB1 [32]. It has been indicated that glycyrrhizin has a

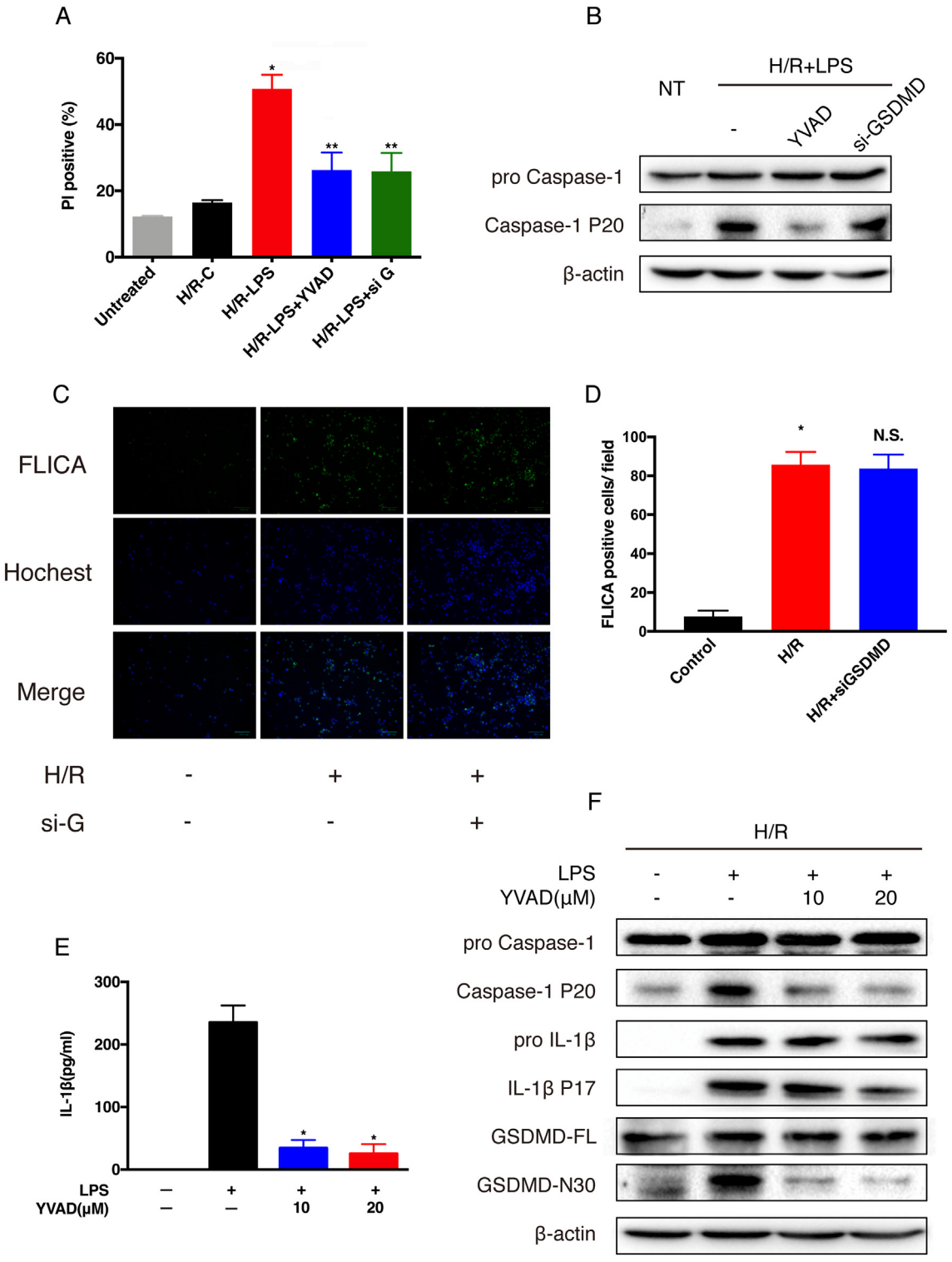

Fig. 6. Activation of GSDMD is controlled by caspase-1 in H/R induced kupffer cells pyroptosis. Cells with indicated treatments are (A) stained with propidium iodide (PI) and observed by flow cytometry. (Data shown are combined results from triplicate wells in a single experiment. *, $P < 0.05$ vs H/R cells. **, $P < 0.05$ vs LPS plus H/R cells.) or (B) lysed for caspase-1 protein expression by western blot (Blots are representative of three independent experiments with similar results). (C) Immunofluorescent staining of FLICA (green) (original magnification was ×100) and nuclei (blue) in cells underwent indicated treatments are as shown. Images are representative from two different experiments with similar results. (D) FLICA positive cells are quantified. *, $P < 0.05$ vs control. **, N.S. vs LPS plus H/R cells. Cells underwent H/R are treated with or without LPS and *Z*-YVAD-FMK. (E) IL-1β release is examined by ELISA in culture supernatants. Data represent Mean ± SD (n = 3/experiments). *, $P < 0.05$ vs LPS plus YVAD stimulated cells. (F) protein expression of caspase-1, IL-1β and GSDMD are examined by Western blot in cell lysates. The blot shown is representative of three different experiments with similar results. Each experiment is repeated three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

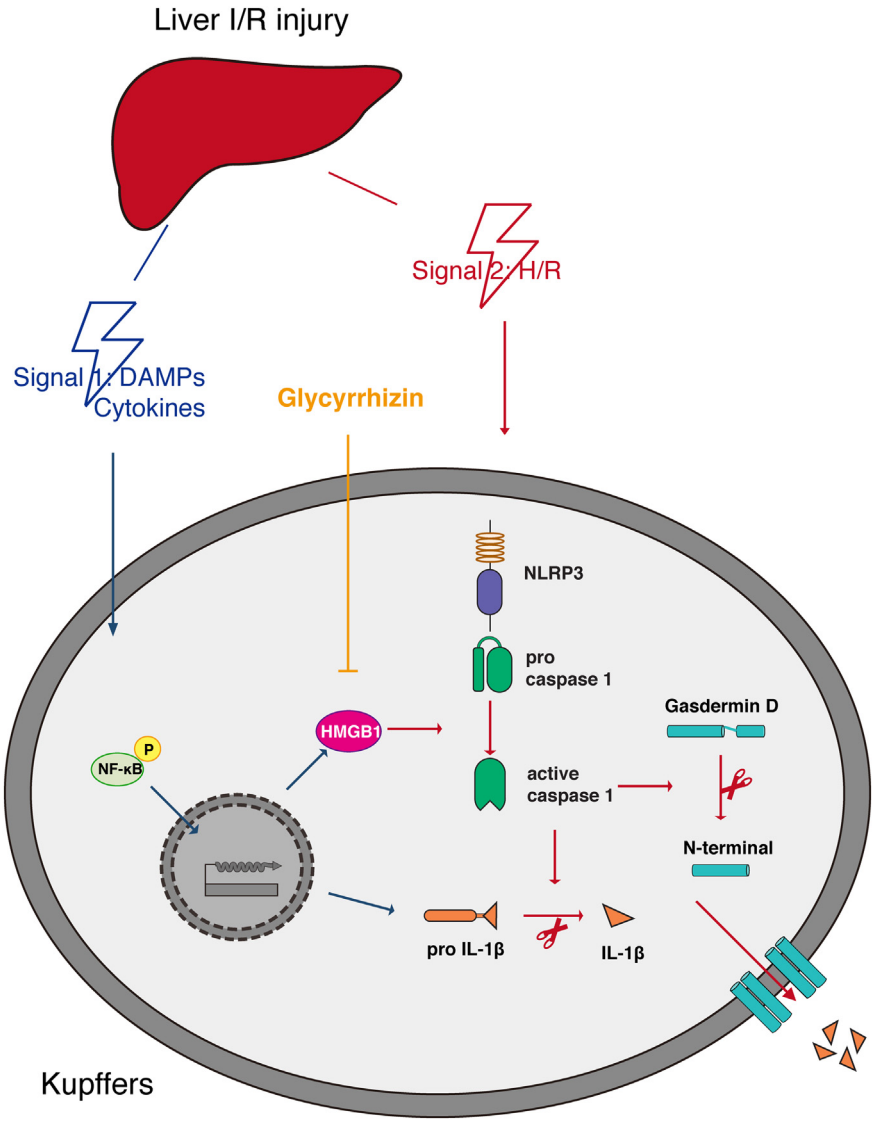

Fig. 7. Diagram representing the putative mechanism by which glycyrrhizin inhibits LIRI induced pyroptosis in kupffer cells. Glycyrrhizin blunts GSDMD activation and the subsequent kupffer cells pyroptosis accompanied with IL-1β release via inhibiting endogenous HMGB1 signal.

protective efficacy in I/R induced brain, spinal cord, renal and liver injury [23,33–37]. Remarkably, our own work has highlighted a possible role of glycyrrhizin in ameliorating experimental colitis [38]. The convergence of these pieces of information identified glycyrrhizin as a candidate inhibitor of the HMGB1-dependent inflammation. In support of these findings, we initially observed a significant decrease in I/R induced liver tissue injury and kupffer cells pyroptosis in mice pretreated with glycyrrhizin, suggesting that glycyrrhizin may alleviated liver I/R injury though inhibiting pyroptosis. An endogenous HMGB1-dependent caspase-1/GSDMD signaling pathway was further confirmed via in vitro H/R model, which may provide a new target for glycyrrhizin anti-inflammatory axis.

Pyroptosis is a form of programmed cell death distinct from apoptosis and necrosis and is defined by its dependence on the inflammatory caspase-1 and/or -11 in mice (caspase-1 and/or caspase-4/-5 in human) [39]. Although pyroptosis was first described in 1992 [40] and later identified in 2001 [41], how inflammasome-mediated inflammatory caspase activation causes a lytic form of cell death was a long-standing question till the identification of GSDMD as the executioner of pyroptosis [18]. In the absence of stimulation, full-length GSDMD remains intact with the N-terminal (GSDMD-N) and C-terminal (GSDMD-C) regions interacting with each other. This auto inhibitory conformation is released upon efficient cleavage at a conserved glutamic acid residue (Asp276 in mouse and Asp275 in human GSDMD) by inflammatory caspases, dividing GSDMD into GSDMD-N and GSDMD-C domains. The generation of N-terminal fragment allows for its oligomerization and only the oligomerized form of GSDMD-N is able to translocate to the plasma membrane and exhibit membrane-disrupting cytotoxicity in mammalian cells [42]. This lytic form of cell death is mostly observed in professional phagocytes, such as macrophages, monocytes, and DCs, but emerging evidence shows that it can be induced in other cell types [43]. Inflammasomes are cytosolic sensors that activate caspase-1 [44]. Different from the others, NLRP3 inflammasome formation was triggered by the cytosolic perturbation, such as potassium efflux [45], mitochondrial ROS production [46], and changes in cell volume [47], instead of any direct signals. To date, however, there is little evidence to indicate that pyroptosis acts in vivo downstream of NLRP3. Results of our laboratory have shown that NLRP3 silencing has a protective effect in murine liver I/R injury mainly through downregulation of caspase-1 activation and reduction of IL-1β, IL-18 and HMGB1 release [11], which indicated a potential role in pyroptosis. It was indicated that pyroptosis may accounts, at least in part, for the inflammasome-induced cell death in acute brain injury after stroke [15]. A study also revealed that pyroptosis was a key biological event in ischemia-

reperfusion injury (IRI)-induced renal tubule epithelial cell death via the CHOP- caspase-11 pathway triggered by overactivated ER stress [17]. Based on these facts, for the first time, we have shown that pyroptosis might play a critical role in Kupffer cells death during pathological process of liver I/R.

Nigericin, which has been recently confirmed as NLRP3 inflammasome activator and GSDMD-regulated pore formation stimulus, was known and widely used to promote the release of IL-1β from LPS primed macrophages [48]. However, several lines of evidence have led to the conclusion that hypoxia-reoxygenation (H/R) acts exclusively via GSDMD mediated pyroptosis to induce release of the IL-1β from activated macrophage. This has been directly demonstrated using kupffer cells, a finding we have repeated in the present study. Our findings suggest for the first time the involvement of GSDMD in mediating kupffer cells pyroptosis and IL-1β release in both in vivo I/R and in vitro H/R injury, which may provide a potential inflammatory mechanism in sterile innate inflammation.

We do, however, recognize the limitations of our assays, in that we have demonstrated the effects of glycyrrhizin on inhibiting GSDMD mediated kupffer cells pyroptosis in the development of liver I/R, but have not studied the effects on I/R induced liver damage resulting from inhibition of GSDMD in animals. Unimpeachable evidence supporting our conclusions awaits the development of assays that monitor pyrotosome formation, caspase-1 activation and IL-1β release simultaneously from single liver resident macrophages in vivo. Despite the caveat, the simplest interpretation of our data is that glycyrrhizin protects kupffer cells from I/R induced pyroptosis and GSDMD pores represent a mechanism of IL-1β secretion from H/R stimulated macrophages (Fig. 7). Validation of this hypothesis would provide a mandate to better understand the unconventional mechanisms of cytokine release in the sterile innate immune system, such as the hepatic ischemia-reperfusion injury.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.002.

Disclosures

The authors declare no conflict of interest.

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