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The novel hepatoprotective mechanisms of silibinin-phospholipid complex against D-GalN/LPS-induced acute liver injury

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ABSTRACT

Background & Aims: Silibinin-phospholipid complex (SPC) has been utilized to treat acute liver injury clinically. Nevertheless, the hepatoprotective mechanism of SPC remains to be further dissected in response to new insights into the pathogenesis of acute liver injury. Very recently, we have documented, for the first time, that M2-like macrophages exert the hepatoprotection against acute insult through inhibiting necroptosis-S100A9-necroinflammation. In the present work, we integrated this new finding into the mechanism of action of SPC, and attempted to dissect the hepatoprotective mechanism of SPC from this new perspective.

Methods: SPC and corresponding controls were administered intragastrically into control mice subjected to D-GalN/LPS challenge. The hepatic damage was assessed, and the expression of necroptosis-S100A9-necroinflammation signaling molecules was detected. The correlation between SPC and macrophage activation was investigated. The expression of miR-223-3p and its regulation on macrophage activation were analyzed. The targeted inhibitory effects of miR-223-3p on necroptosis and necroinflammation signaling molecules were confirmed.

Results: SPC alleviated remarkably the hepatic damage triggered by D-GalN/LPS. The administration of SPC inhibited the expression of necroptosis-S100A9-necroinflammation signaling molecules. The levels of M2-like macrophage markers were increased significantly in SPC-treated mice or macrophages. miR-223-3p expression was enhanced in SPC-treated mice. miR-223-3p transfer led to up-regulated expression of M2-like macrophage markers. miR-223-3p directly targeted 3′ UTR of RIPK3 and NLRP3, and the expression of necroptosis and necroinflammation signaling molecules was inhibited in miR-223-3p-transferred hepatocytes and macrophages.

Conclusions: SPC alleviates acute liver injury through up-regulating the expression of miR-223-3p. MiR-223-3p further promotes M2-like macrophage activation and the targeted inhibition of necroptosis and necroinflammation. Our findings provide novel insight into the hepatoprotective mechanism of SPC against acute liver injury.

1. Introduction

Acute liver injury (ALI) is caused by acute insults such as viruses, alcohol, and drugs in the setting of normal liver function. The most severe form of ALI, namely, acute liver failure (ALF), is considered as a life-threatening syndrome accompanied with rapidly destroyed hepatic function and high mortality (nearly 80 %) [1,2]. Unfortunately, the pathogenesis of ALI still needs to be further explored although great

Abbreviations: ALI, acute liver injury; ALF, acute liver failure; TNF, tumor necrosis factor; RIPK, receptor-interacting serine-threonine kinase; MLKL, pseudokinase mixed lineage kinase domain like protein; DAMP, damage-associated molecular pattern; D-GalN, D- galactosamine; LPS, lipopolysaccharide; SPC, silibinin-phospholipid complex; SPF, specific pathogen free; CMC-Na, sodium carboxymethyl cellulose; PL, phospholipids; ALT, alanine transaminase; AST, aspartate aminotransferase; H&E, hematoxylin-eosin; RT-qPCR, reverse transcription and quantitative polymerase chain reaction; PVDF, polyvinylidene difluoride; DMEM, Dulbecco's Modified Eagle Medium; NC, negative control.

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progress has been made in the past decades.

Necroptosis is a novel mode of programmed cell death, which is independent of caspase. It can be triggered by the ligation of tumor necrosis factor (TNF) alpha and death receptors, which leads to the sequential phosphorylation and activation of kinases including receptor-interacting serine-threonine kinase (RIPK) 1 and 3. RIPK3 then activates the pseudokinase mixed lineage kinase domain like protein (MLKL) through phosphorylation, thereby causing substantial conformational changes of MLKL and ensuing membrane permeabilization. The rupture of necroptotic cells is accompanied by the release of damage-associated molecular pattern (DAMP) and the development of necroinflammation [3–5]. Emerging evidence supports the pivotal role of necroptosis in multiple liver diseases [6–8]. Lately, we documented, for the first time, that M2-like macrophages exert the hepatoprotective effects against D-GalN/LPS-induced acute liver injury through inhibiting necroptosis-S100A9-necroinflammation [9].

Silymarin, also known as Shui Fei Ji Su in China, is a natural flavonoid which is extracted from the fruits and seeds of Compositae Milk thistle (Silybum marianum). Silibinin accounts for 50–70 % of this extract, and is the major bioactive flavonolignan in silymarin. Silibinin is composed of the stereoisomers silybin A and silybin B in approximately equal portions [10,11]. In recent years, the potentially biologic effects of silibinin have been extensively explored, and their efficacy against multiple diseases including cancer, diabetes and Alzheimer's disease has been verified [12,13]. In terms of liver diseases, silibinin has been utilized to treat drug-induced liver injury, alcoholic and non-alcoholic fatty liver diseases, chronic hepatitis B, and so on [14–18]. Unfortunately, conventional silibinin suffers from poor solubility and low bioavailability, which limits greatly the administration of silibinin in clinical practice [10,11,22]. In view of this, silibinin-phospholipid complex (SPC) has been developed. This complex has been documented to possess high bioavailability and good tolerance [23,24]. Studies revealed that the administration of SPC alleviates acute liver injury through anti-inflammatory or anti-oxidant effects [17]. However, the hepatoprotective mechanism of SPC remains to be further dissected in response to new insights into the pathogenesis of acute liver injury.

In the present work, we integrated the inhibition of necroptosis-S100A9-necroinflammation mediated by M2-like macrophages into the mechanism of action of SPC, and hypothesized SPC protects mice against acute hepatic injury through promoting M2-like macrophage activation and further inhibiting necroptosis-S100A9-necroinflammation. To testify this hypothesis, firstly, we confirmed the beneficial hepatoprotection conferred by SPC against acute liver injury; secondly, the inhibition of necroptosis-S100A9-necroinflammation was demonstrated in mice receiving SPC and acute insult, compared to acutely injured mice; thirdly, the close association between SPC and M2-like macrophage activation was verified through *in vivo* and *in vitro* experiments; finally, we dissected the molecular mechanism by which SPC exerts a hepatoprotective effect, and focused on the inhibition of necroptosis and necroinflammation mediated by miR-223. Our findings will help provide novel and powerful evidence for the clinical application of SPC into the treatment of acute liver injury.

2. Materials and methods

2.1. Animals

BALB/c mice (male, 6-week-old) were ordered from Laboratory Animal Breeding Center of Beijing LongAn, Beijing, China. Mice were housed in a specific pathogen free (SPF) environment under controlled conditions (22 °C–24 °C, 12-hour light/dark cycle). Animals were fed standard laboratory chow with water ad libitum. All animal care and experimental procedures performed in this study complied with the Guide for the Care and Use of Laboratory Animals, and were approved by Institutional Animal Care and Use Committee of Beijing YouAn Hospital, Capital Medical University.

2.2. Animal protocol

The experiment protocol was as follows: (1) Control: BALB/c mice were given with PBS, 0.5 % sodium carboxymethyl cellulose (CMC-Na) or phospholipids (PL), as appropriate. (2) Pharmacological interventions: Control mice were administrated intragastricly with silibinin (25 mg/kg) and SPC (25 mg/kg) dissolved in 0.5 % CMC-Na, respectively, 24 h before and 2 h after acute insult. (3) Acute insult: Mice were challenged intraperitoneally with D-GalN (350 μg/g, Sigma) plus LPS (10 ng/g, Invivogen). Sera and liver tissues were harvested 6 h after acute challenge for analysis.

2.3. Evaluation of liver injury

Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were measured using an automatic chemical analyser (Hitachi 7600, Japan) according to an automated procedure. Liver tissues were fixed in 10 % neutral-buffered formalin and embedded in paraffin, then the sections (3 μm) were stained with hematoxylin-eosin (H&E) using a standard protocol. Histological pictures were captured using an Olympus Bx51 microscope (Olympus America, Melville, NY, USA) equipped with cellSens standard software (version 1.4.1). The histological severity of hepatic damage was assessed and scored blindly by experienced pathologists [25].

2.4. Reverse transcription and SYBR Green quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from liver tissues and cells with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). One μg RNA was reverse-transcripted into cDNA using AMV retrotranscriptase system (TakaRa, Dalian, Liaoning, China). qPCR reactions in triplicate were run in ABI StepOne Plus System (Thermo Fisher Scientific) using SYBR Green reaction mix (TakaRa). In a final reaction volume of 20 μl, the followings were added: 1 × SYBR Green, cDNA, 0.5 mM each primer, and ROX. The optimal reaction conditions were as follows: 95 °C (10 min), followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min). The primers were designed by Primer 3 (Version 0.4.0) and listed in Supplementary Table 1. The relative expression of gene transcripts was calculated and normalized to the expression of reference gene GAPDH.

2.5. Immunofluorescence analysis

Immunofluorescent staining was performed on frozen liver sections, as previously described [26]. Liver sections were stained with FITC Mouse anti-iNOS/NOS Type II (1:400; BD Transduction Laboratories™, San Jose, CA, USA) primary antibody. Nikon Inverted Fluorescence Microscope ECLIPSE Ti equipped with NIS-Elements F 3.0 Software (Nikon Corporation, Tokyo, Japan) were applied for image capture.

2.6. Western blot analysis

The whole protein was extracted from frozen liver tissues or cells using RIPA lysis buffer supplemented with Halt™ protease inhibitor cocktail (Thermo Fisher Scientific). Proteins were then subjected to 10 % SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific). The membranes were blocked with 5 % skim milk (BD Bioscience, USA) for 2 h at room temperature, and then probed with the following primary antibodies overnight at 4 °C: RIP3 (E7A7F) XP rabbit mAb (1:1000; Cell Signaling Technology), anti-MLKL antibody (1:2000; Abcam), phospho-MLKL (Ser345) (D6E3G) rabbit mAb (1:1000; Cell Signaling Technology), anti-NLRP3 antibody (1:2000; Abcam), ASC/TMS1 (D2W8U) rabbit mAb (1:2000; Cell Signaling Technology), cleaved caspase-1 (Asp297) (D57A2) rabbit mAb (1:1000; Cell Signaling Technology), and β-actin (1:5000; Immunoway). After washing, the membranes were incubated with HRP-conjugated

anti-rabbit IgG for 1 h at room temperature. Bands were visualized using Luminol ECL reagent (Thermo Fisher Scientific).

2.7. Isolation and culture of mouse bone marrow-derived macrophages

The tibia and femur of mice were flushed under sterile conditions using Dulbecco's Modified Eagle Medium (DMEM). Cells were collected by centrifugation, then were differentiated in DMEM containing M−CSF (50 ng/ml) for 5 days to give rise to mature macrophages. A monolayer of macrophages was gently scraped on Day 6, and cultured in DMEM supplemented with 10 % FBS in a humidified atmosphere at 37 °C with 5 %CO_2.

2.8. Macrophage polarization

Mouse bone marrow-derived macrophages were activated with 50 ng/mL of IFN-gamma (Novoprotein) or 50 ng/mL IL-4 (Novoprotein). The phenotype of activated macrophages was identified through qPCR analysis for gene signature of representative markers.

2.9. Cell transfection

Mouse liver cell line AML-12 and BMDMs cultured in DMEM supplemented with 10 % FBS were seeded in 6- or 24-well plates, then miR-223-3p mimics, inhibitors, and their negative controls (NC) were transfected using Lipofectamine RNAiMAX Reagent (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions.

2.10. Dual luciferase reporter assay

A dual luciferase reporter assay was used to verify the target relationship between miR-223-3p and RIPK3 or NLRP3. The plasmid encoding the luciferase reporter gene (psiCHECK-2) was purchased from Promega Corporation (Madison, WI, USA). The luciferase gene reporter vectors [psiCHECK-2-m-RIPK3-3′UTR-wt (wild type), psiCHECK-2-m-RIPK3-3′UTR-mu (mutation type), psiCHECK-2-m-NLRP3-3′UTR-wt, psiCHECK-2-m-NLRP3-3′UTR-mu] were transfected into 293 T cells together with mmu-miR-223-3p or their negative controls (Sangon Biotech, Beijing, China) using Lipofectamine RNAiMAX reagent. After

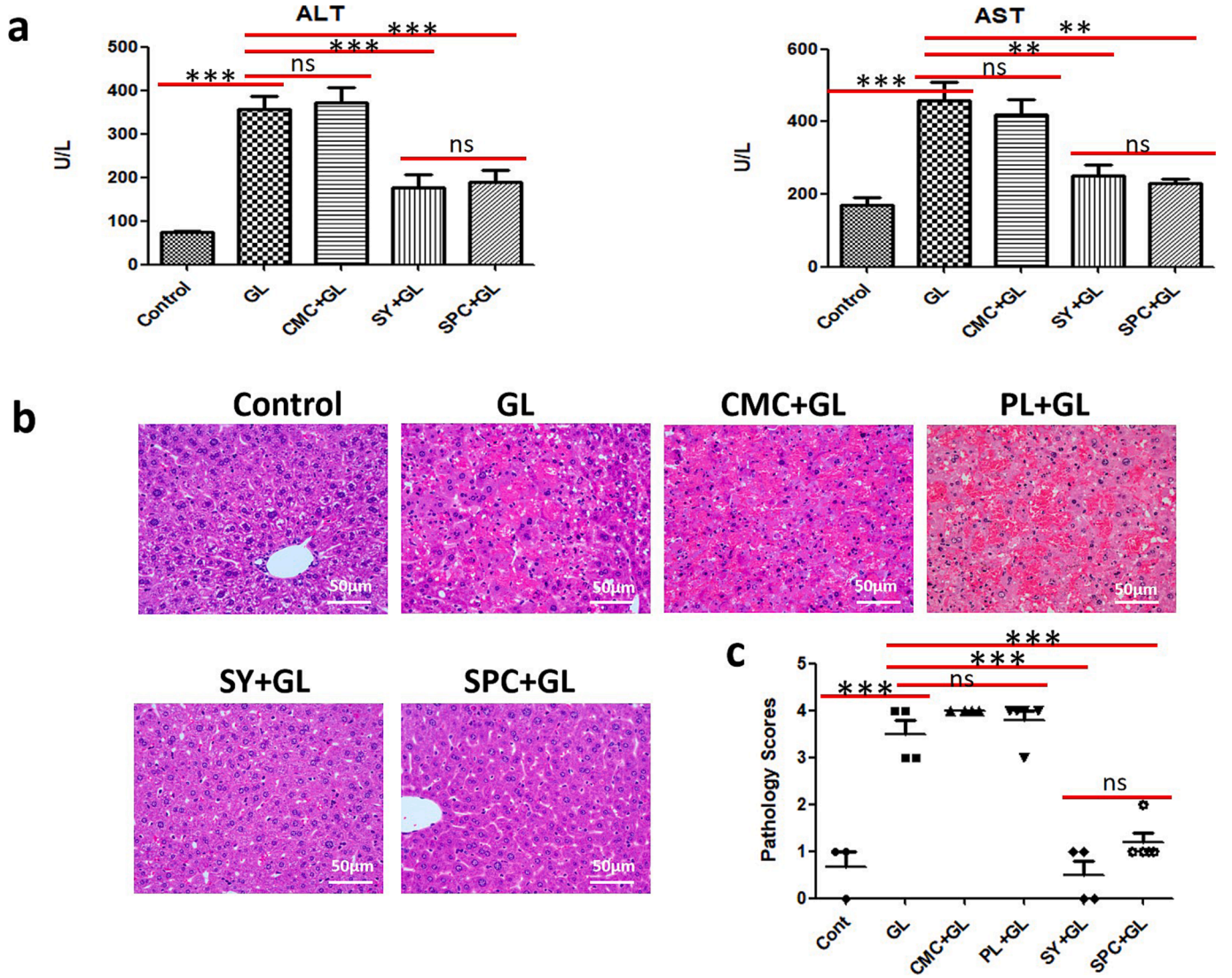

Fig. 1. SPC alleviates the acute hepatic damage induced by D-GalN/LPS. a. The hepatic damage assessed by serum ALT and AST levels in mice receiving SPC plus D-GalN/LPS compared to those in acutely injured mice. Group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test, **P < 0.01, ***P < 0.001 (n = 4–9). **b.** The hepatic damage assessed by histopathology with H&E staining in mice subjected to D-GalN/LPS with or without SPC treatment. **c.** The pathology scores of hepatic damage in mice subjected to D-GalN/LPS with or without SPC treatment (n = 3–5). Group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test, ***P < 0.001. GL, D-GalN/LPS; CMC, sodium carboxymethyl cellulose; PL, phospholipid; SY, silibinin; SPC, silibinin-phospholipid complex.

48 h of incubation, the luciferase activity was analyzed by Dual-Luciferase® Reporter Assay System (Promega).

2.11. Statistical analysis

Results were expressed as mean ± standard error of the mean or median (Min, Max). Group comparisons were performed using Student's *t* test, Mann-Whitney *U* test, or One-way ANOVA followed by Tukey's multiple comparison test, as appropriate. Statistics and graphs were generated using Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. SPC alleviates the acute hepatic damage induced by D-GalN/LPS

Firstly, we wanted to confirm whether SPC exerts a hepatoprotective effect on control mice subjected to a sub-lethal dose of D-GalN/LPS challenge. For this purpose, SPC was applied into control mice 24 h before and 2 h after acute insult. As expected, serum ALT and AST levels were remarkably reduced in mice receiving SPC compared to acutely injured mice (Fig. 1a). Moreover, the hepatic damage assessed by histopathology analysis was greatly improved in SPC-treated mice (Fig. 1b). And the pathology scores performed by well-experienced experts provided further support for the hepatoprotection conferred by SPC administration (Fig. 1b). Thus, SPC alleviates D-GalN/LPS-induced acute hepatic damage.

3.2. SPC inhibits necroptosis-S100A9-necroinflammation triggered by D-GalN/LPS

Our latest data showed that the administration of D-GalN/LPS triggered the remarkable up-regulation of necroptosis signaling, S100A9 expression and necroinflammation [9]. Herein, we tended to testify whether SPC exerts hepatoprotection against D-GalN/LPS insult through inhibiting necroptosis-S100A9-necroinflammation. To this end, the mRNA and protein expression of signaling molecules related to necroptosis-S100A9-necroinflammation was compared in mice subjected to acute insult with or without SPC treatment. At the protein level, the expression of RIPK3, MLKL, p-MLKL, NLRP3, ASC, and cleaved caspase-1 was markedly inhibited in mice receiving SPC treatment plus D-GalN/LPS insult as compared to that in acutely injured mice (Fig. 2a). Real-time PCR analysis revealed that the mRNA levels of RIPK3, MLKL, S100A9, NLRP3, and IL-1β were significantly down-regulated in acutely injured mice receiving SPC treatment (Fig. 2b). In addition, S100A9 secretion assessed by ELISA was also obviously suppressed in serum from mice receiving SPC treatment, compared to acutely injured mice (Fig. 2c). Therefore, the administration of SPC brings about the

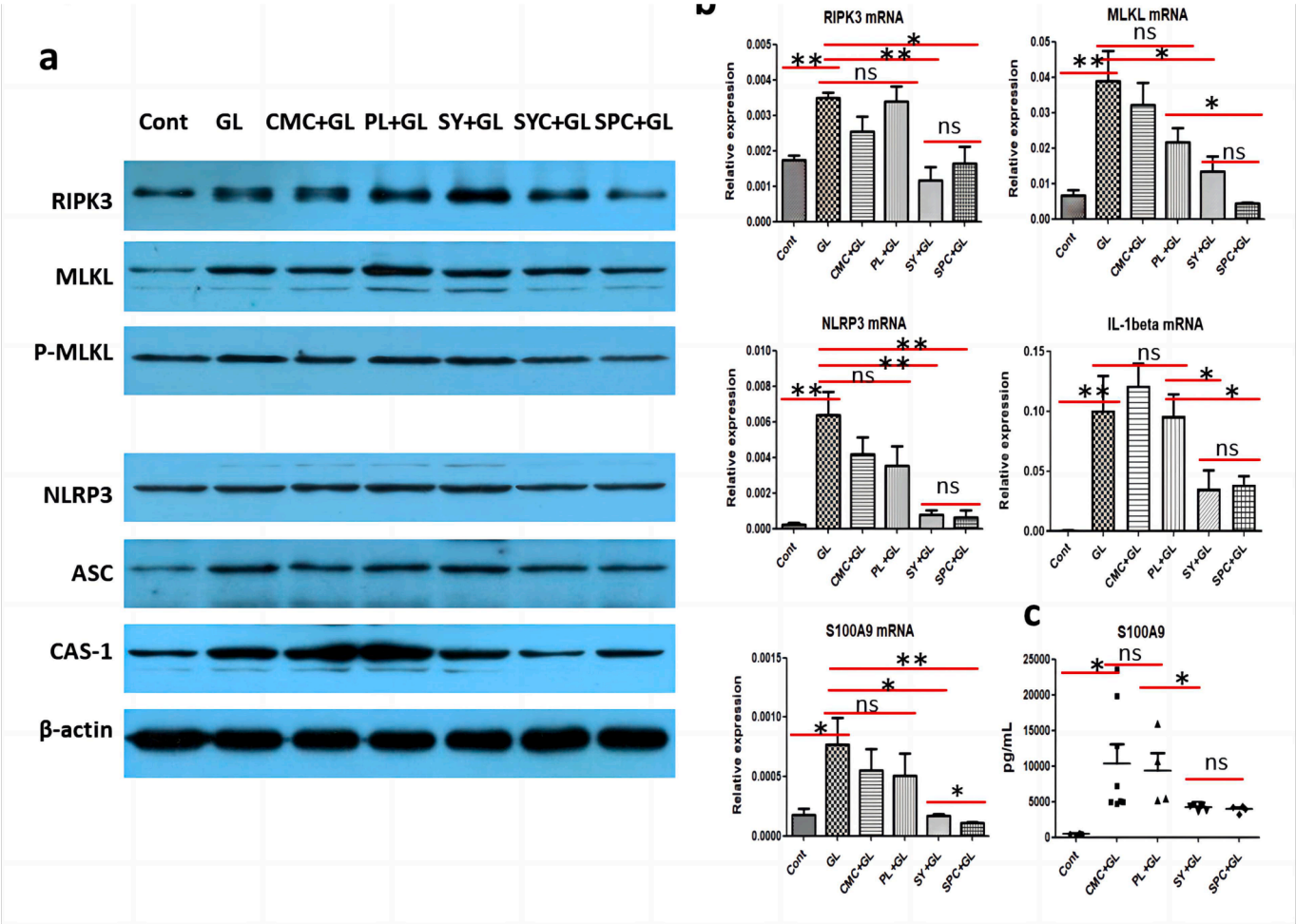

Fig. 2. SPC inhibits necroptosis-S100A9-necroinflammation triggered by D-GalN/LPS. a. The expression of RIPK3, MLKL, p-MLKL, NLRP3, ASC, and caspase-1 in acutely injured mice with or without SPC treatment. **b.** The mRNA levels of necroptosis markers (RIPK3 and MLKL) and necroinflammation mediators (S100A9, NLRP3 and IL-1beta) in acutely injured mice with or without SPC treatment (n = 3–5). **c.** Serum S100A9 levels in acutely injured mice with or without SPC treatment (n = 4–8). Group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test, *P < 0.05, **P < 0.01, ***P < 0.001. GL, D-GalN/LPS; CMC, sodium carboxymethyl cellulose; PL, phospholipid; SY, silibinin; SYC, silibinin capsule; SPC, silibinin-phospholipid complex.

remarkable inhibition of necroptosis-S100A9-necroinflammation in acutely injured mice.

3.3. The hepatoprotective effects exerted by SPC is closely related to M2-like activation of macrophages

Lately, we have demonstrated that M2-like macrophages exert beneficial hepatoprotection against D-GalN/LPS challenge through inhibiting necroptosis-S100A9-necroinflammation [9]. Herein, we hypothesized the alleviation of hepatic damage and the inhibition of necroptosis-S100A9-necroinflammation signaling in SPC-treated mice can also be ascribed to M2-like macrophages. To testify this hypothesis, we analyzed and compared the activation phenotype of macrophages in the livers of acutely injured mice with or without SPC treatment. Quantitative PCR analysis showed that the mRNA levels of M2 macrophage markers including Arg-1 and TGF-beta were obviously up-regulated in SPC-treated mice, compared to those in acutely injured mice. Meanwhile, the expression of M1 markers such as TNF-alpha and CD86 displayed remarkable reduction (Fig. 3a). Specially, the M2/M1 ratios including Arg-1/iNOS, Ym-1/iNOS, Arg-1/CD86, and TGF-beta/CD86 exhibited remarkable elevation in SPC-treated mice (Fig. 3b). Therefore, the hepatoprotection mediated by SPC is bound up with M2-like macrophage activation.

3.4. SPC promotes M2-like activation of macrophages in vitro

To provide substantive evidence on the close relationship between SPC administration and M2-like macrophage activation, we conducted macrophage polarization experiment *in vitro*. Mouse macrophages were isolated, and then stimulated with recombinant mouse IFN-gamma, IL-4, silibinin, and SPC, respectively. As a result, SPC-treated macrophages manifested remarkable M2 induction, as shown by much higher gene expression of M2 markers including CD206 and TGF-beta (Fig. 4a). Especially, the M2/M1 ratio of TGF-beta/iNOS was greatly higher in SPC-treated macrophages, which was even comparable with IL-4 stimulation (Fig. 4b). At the protein levels, the expression of M1 marker iNOS was markedly reduced in macrophages treated with SPC compared with that in IFN gamma-treated macrophages (Fig. 4c). Hence, SPC is capable of promoting M2-like activation of macrophages.

3.5. SPC up-regulates the levels of miR-223-3p

We then dissected the molecular mechanism by which SPC promotes M2-like macrophage activation, and focused on miR-223-3p, an important regulator of macrophage polarization [27,28]. For this aim, the expression of miR-223-3p was detected and compared in acutely injured mice with or without SPC treatment. qPCR detection showed that miR-223-3p expression was obviously up-regulated in the liver

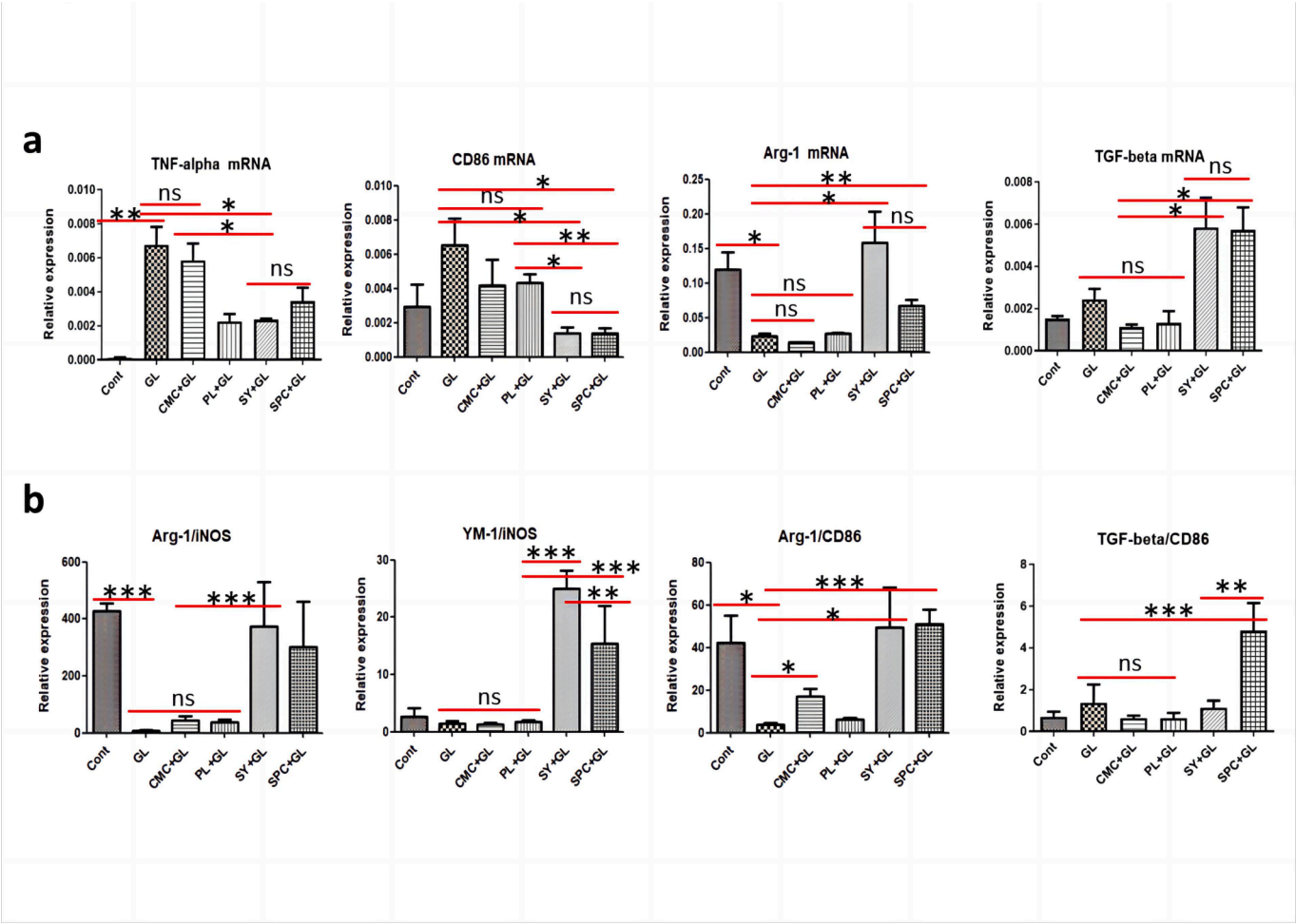

Fig. 3. The hepatoprotective effects exerted by SPC is closely related to M2-like activation of macrophages. a. The mRNA levels of M2 macrophage markers (Arg-1 and TGF-beta) and M1 markers (TNF-alpha and CD86) in acutely injured mice with or without SPC treatment (n = 3–4). **b.** The M2/M1 ratios including Arg-1/iNOS, Ym-1/iNOS, Arg-1/CD86 and TGF-beta/CD86 in acutely injured mice with or without SPC treatment (n = 3–4). Group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test, *P < 0.05, **P < 0.01, ***P < 0.001. GL, D-GalN/LPS; CMC, sodium carboxymethyl cellulose; PL, phospholipid; SY, silibinin; SPC, silibinin-phospholipid complex.

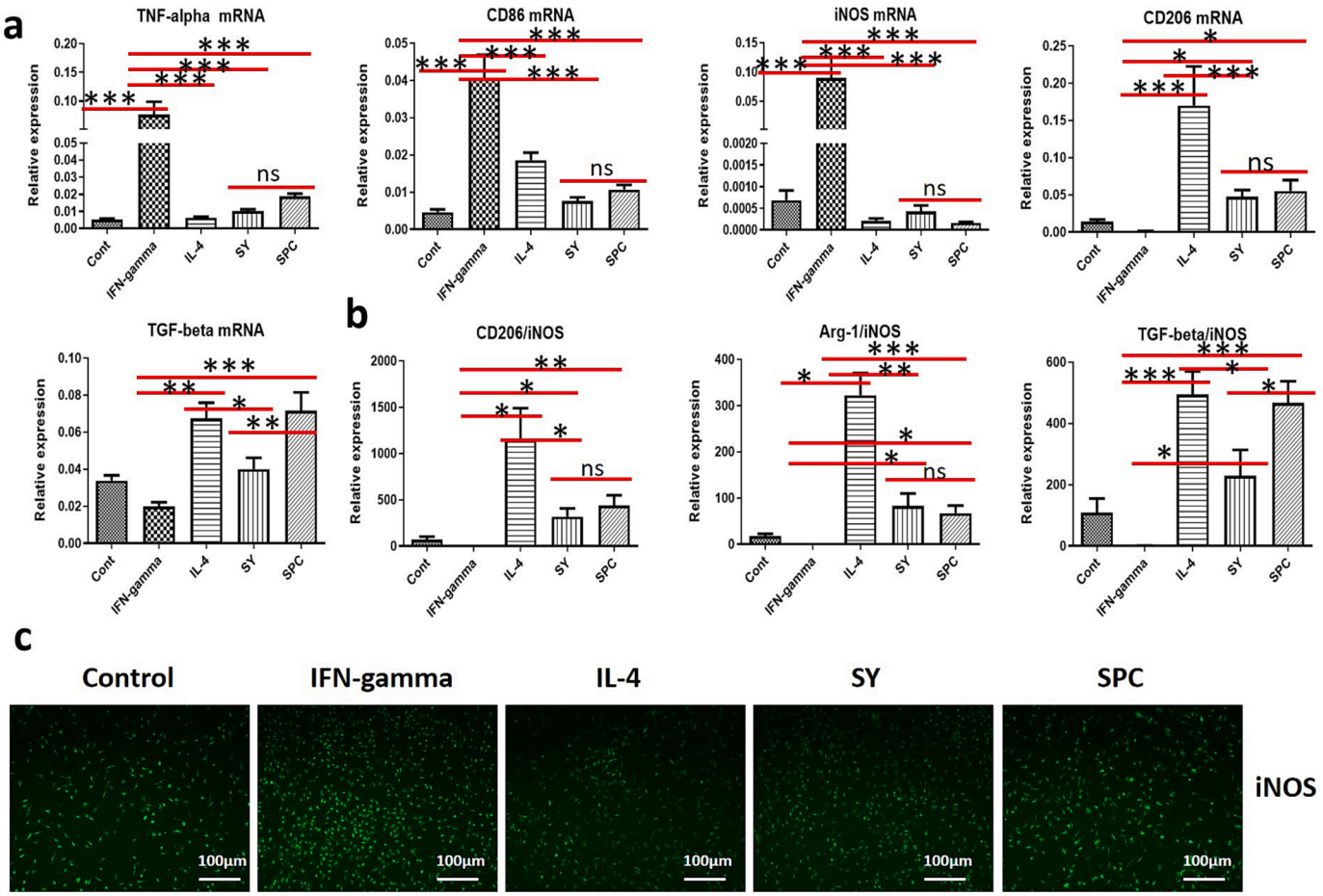

Fig. 4. SPC promotes M2-like activation of macrophages *in vitro*. a. The mRNA levels of M2 macrophage markers (CD206 and TGF-beta) in mouse macrophages stimulated with recombinant mouse IFN-gamma, IL-4, silibinin, and SPC (n = 4–7). **b.** The M2/M1 ratios including Arg-1/iNOS, CD206/iNOS and TGF-beta/ iNOS in mouse macrophages stimulated with recombinant mouse IFN-gamma, IL-4, silibinin, and SPC (n = 4–7). **c.** The protein expression of M1 marker iNOS in mouse macrophages stimulated with recombinant mouse IFN-gamma, IL-4, silibinin, and SPC. Group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test, *P < 0.05, **P < 0.01, ***P < 0.001. SY, silibinin; SPC, silibinin-phospholipid complex.

tissues of silibinin- or SPC-treated mice, as compared to that in acutely injured mice (Fig. 5). Therefore, SPC promotes the expression of miR-223-3p.

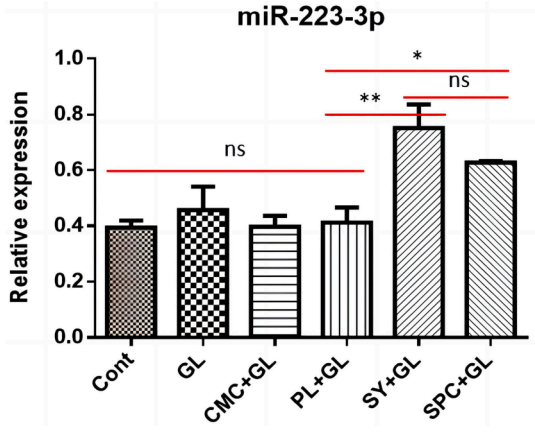

Fig. 5. SPC up-regulates the levels of miR-223-3p. miR-223-3p expression in the liver tissues from mice subjected to D-GalN/LPS with or without SPC treatment. Group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test, *P < 0.05, **P < 0.01. GL, D-GalN/LPS; CMC, sodium carboxymethyl cellulose; PL, phospholipid; SY, silibinin; SPC, silibinin-phospholipid complex (n = 3–4).

3.6. *miR-223-3p promotes M2-like activation of macrophages*

To clarify the regulation of miR-223-3p on macrophage activation, we conducted macrophage polarization experiment *in vitro*. Primary mouse macrophages were stimulated with recombinant mouse IFN-gamma, IL-4, or transfected with miR-223-3p, respectively. As a result, miR-223-3p-transfected and IL-4-stimulated macrophages manifested remarkable M2 induction, as shown by much higher gene expression of M2 markers including Arg-1, CD206 and TGF-beta but lower levels of M1 markers such as TNF-alpha, iNOS and IL-1beta (Fig. 6). Hence, miR-223-3p overexpression significantly promotes M2-like activation of macrophages.

3.7. *miR-223-3p negatively regulates necroptosis signaling*

Next, we attempted to evaluate whether necroptosis signaling molecules were subjected to the direct regulation of miR-223-3p. To this end, we first predicted the potential relationship between miR-223-3p and necroptosis signaling molecules by biological software Targetscan 8.0. As a result, the sequences between miR-223-3p and RIPK3 3′UTR exhibited a complementary relationship (Fig. 7a). To further verify whether RIPK3 is a direct target of miR-223-3p, we conducted the dual luciferase reporter assay. According to our data, miR-223-3p overexpression inhibited the luciferase reporter activity of RIPK3-3′UTR-wt, conversely, the administration of miR-223-3p mimics did not suppress the luciferase reporter activity of RIPK3-3′UTR-mu (Fig. 7b). We then

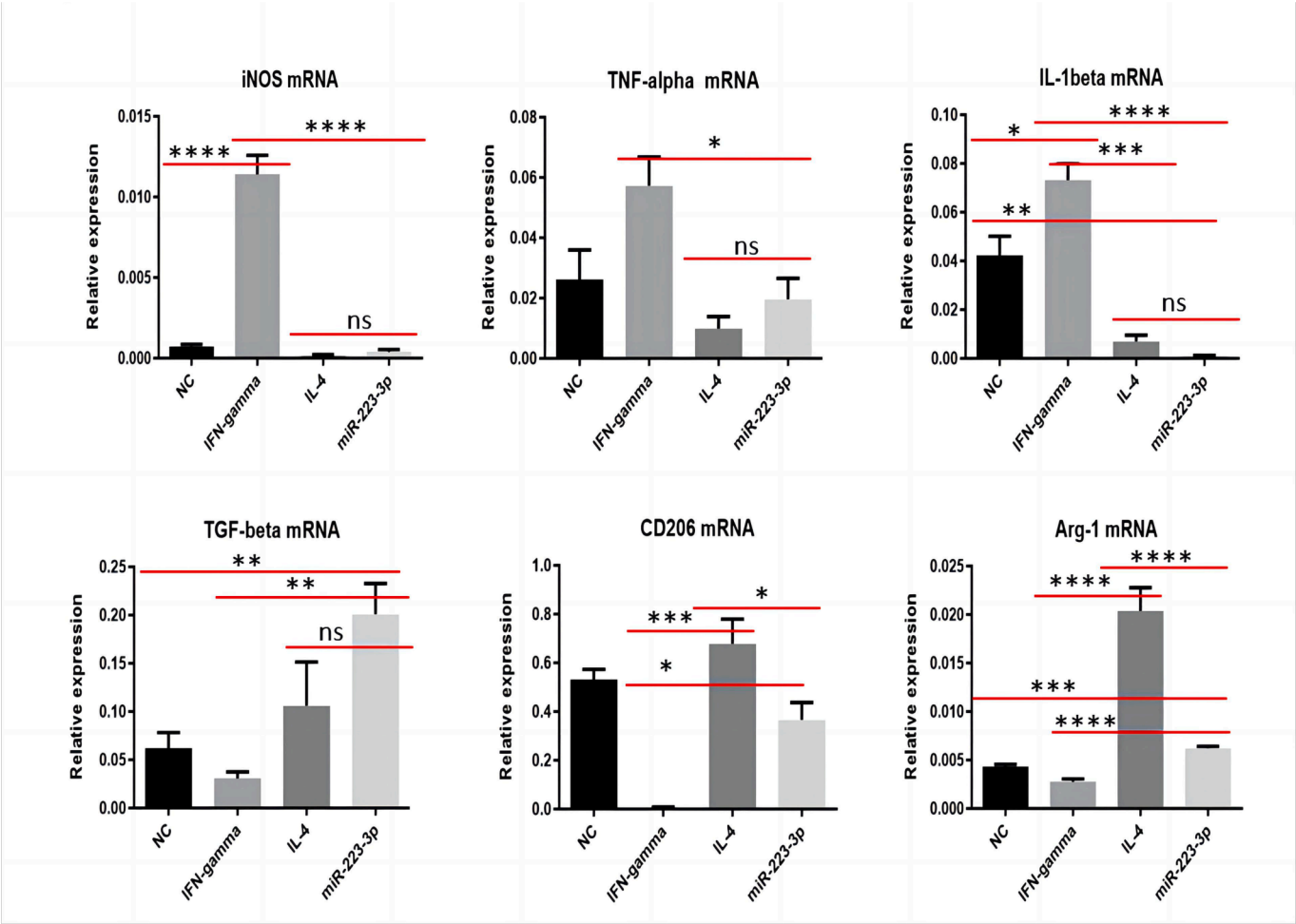

Fig. 6. miR-223-3p promotes M2-like activation of macrophages. The mRNA levels of M1 macrophage markers (iNOS, TNF-alpha and IL-1beta) and M2 markers (CD206, TGF-beta and Arg-1) in mouse macrophages stimulated with recombinant mouse IFN-gamma, IL-4, or transfected with miR-223-3p mimics (n = 3–4). Group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

analyzed the influence of miR-223-3p transfection on the severity of necroptosis in AML-12 cells with TNF-α/z-VAD treatment. As expected, the transfection of miR-223-3p mimics led to the obvious reduction in the levels of RIPK3 and MLKL mRNA, compared to NC mimics (Fig. 7c). Together, miR-223-3p suppresses necroptosis signaling by directly targeting the 3′ UTR of RIPK3.

3.8. miR-223-3p negatively regulates necroinflammation

Finally, we attempted to assess whether miR-223-3p regulates necroinflammation signaling molecules. For this reason, we first predicted the potential relationship between miR-223-3p and necroinflammation signaling molecules by Targetscan 8.0. As expected, the sequences between miR-223-3p and NLRP3 3′UTR exhibited a complementary relationship (Fig. 8a). To further determine whether miR-223-3p directly targets the 3′UTR of NLRP3, dual luciferase reporter assay was also performed. The results showed that miR-223-3p mimic significantly inhibited the luciferase reporter activity of NLRP3-3′UTR-wt but not NLRP3-3′UTR-mu (Fig. 8b). We then analyzed the influence of miR-223-3p transfection on the severity of necroinflammation in macrophages. Our data showed that the transfection of miR-223-3p mimics led to the obvious reduction in the levels of caspase-1 and S100A9 mRNA, compared to NC mimics (Fig. 8c). Together, miR-223-3p suppresses necroinflammation by directly targeting the 3′ UTR of NLRP3.

4. Discussion

In the present study, we documented that SPC protects against D-GalN/LPS-induced acute liver injury through up-regulating the levels of miR-223-3p, which promotes M2-like activation of macrophages and inhibits necroptosis-S100A9-necroinflammation. To the best of our knowledge, this is the first time to explain the potential molecular mechanisms of SPC-mediated hepatoprotection against ALI from this new perspective.

Silibinin has been used to cope with liver dysfunctions in humans, such as viral hepatitis, alcoholic liver disease, non-alcoholic liver disease, cirrhosis and acute liver injury [17]. However, it is well-known that the bioavailability of silibinin is rather low when it is administered orally [29]. Fortunately, the addition of phospholipid makes silibinin lipid-compatible, which further improves the bioavailability and enhances the efficacy of silibinin [23]. The identified pharmacologic actions of SPC include antioxidant, anti-inflammatory, anti-fibrotic effects and insulin resistance modulation [17,18]. Nevertheless, the mechanism of action of SPC is worthy of further investigation with the emerging reports on the novel form of cell death. Necroptosis is a newly-discovered, programmed cell death. Very recently, we have documented, for the first time, that necroptosis-S100A9-necroinflammation plays an important role in D-GalN/LPS-induced acute liver injury [9]. Therefore, we integrated necroptosis into the hepatoprotection mediated by SPC, and attempted to dissect the mechanism of action of SPC from this new viewpoint.

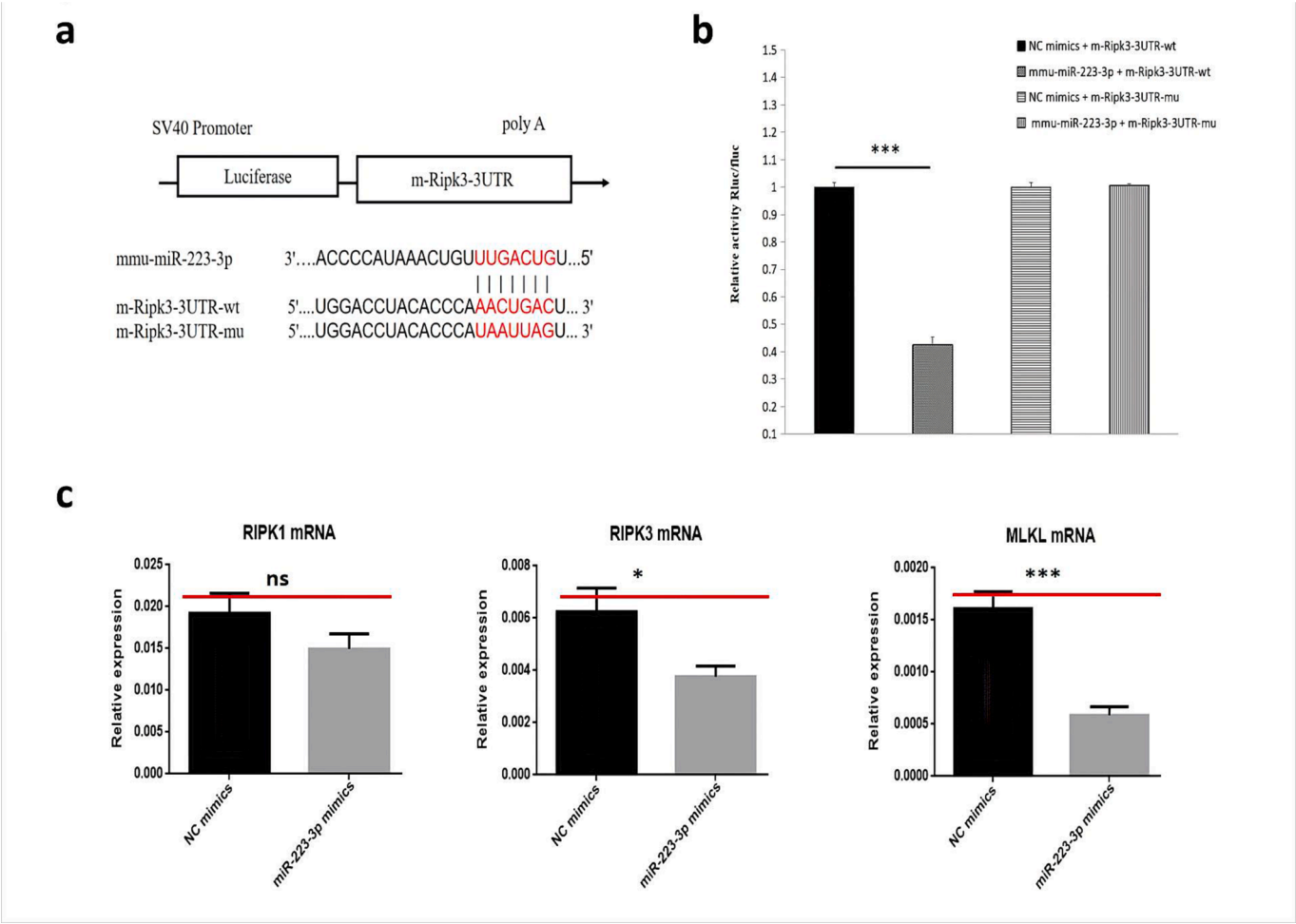

Fig. 7. miR-223-3p directly inhibits necroptosis. a. The speculated relationship between miR-223-3p and 3′UTR of RIPK3 by Targetscan 8.0; **b.** Dual luciferase reporter assay in order to validate the relationship between miR-223-3p and 3′UTR of RIPK3; **c.** The mRNA levels of necroptosis signaling molecules including RIPK1, RIPK3 and MLKL in mouse hepatocytes transfected with miR-223-3p mimics or NC (n = 3–4). Group comparisons were performed using Student's *t* test.

We first demonstrated that SPC remarkably alleviated the acute hepatic damage induced by D-GalN/LPS. This finding confirmed the hepatoprotection mediated by SPC, and provided evidence for the clinical use of SPC as a hepatic protectant. Notably, similar ALT/AST levels and histopathology were observed between SPC + GL group and SY + GL group in our study. Nevertheless, the hepatic damage should be less severe in SPC + GL group compared with SY + GL group theoretically considering that SPC has better bioavailability and tolerance than SY. We believe that the following four reasons can be used to explain the similar liver damage between these two groups. Firstly, we gavaged mice directly, which may not be the same as taking them orally. Secondly, the conclusion we have drawn in this manuscript is a comprehensive judgment. WB data in Fig. 2a showed that the expression of necroptotic and necroinflammatory biomarkers in SY + GL group was higher than that in SPC + GL group, suggesting that necroptosis and necroinflammation could be reduced more significantly by SPC compared with SY. Thirdly, the dose and course of SPC administration in this work may not be sufficient to bring about a significant improvement in liver histology in SPC + GL group compared with SY + GL group. Lastly, drugs may be absorbed and metabolized differently in human and mice.

We then analyzed the gene and protein expression of signaling molecules related to necroptosis-S100A9-necroinflammation in mice subjected to acute insult with or without SPC treatment. As a result, the levels of necroptosis signaling molecules (RIPK3, MLKL and/or p-MLKL), S100A9, and necroinflammation signaling molecules (NLRP3, ASC, CAS-1, IL-1beta) were obviously inhibited in SPC-treated mice. This data supported the crucial role of necroptosis-S100A9-necroinflammation in the hepatoprotection conferred by SPC. As far as our information goes, this is the first report demonstrating that SPC exerts a hepatoprotective effect through inhibiting necroptosis-S100A9-necroinflammation. The expression of necroptotic and necroinflammatory markers was higher in SY + GL group as compared with SPC + GL group according to WB data. It is reasonable considering that SPC has better bioavailability than SY. Although PCR data showed that the expression of necroptotic and necroinflammatory biomarkers was similar between SY + GL group and SPC + GL group, generally speaking, WB data can reflect the actual expression level of signaling molecules better than PCR results.

Next, we aimed to further dissect the mechanism by which SPC inhibits necroptosis-S100A9-necroinflammation. Our latest work has shown that the inhibition of necroptosis signaling and S100A9 expression is closely tied up with M2-like macrophage activation, and M2-like macrophages exert the hepatoprotection against D-GalN/LPS challenge through inhibiting necroptosis-S100A9-necroinflammation [9]. In view of the pivotal role of M2-like macrophages in the hepatoprotection against acute insult, we wanted to verify whether it also works when it comes to the hepatoprotective mechanism of SPC. To this end, we performed *in vivo* and *in vitro* experiment to analyze the activation phenotype of macrophages in response to SPC treatment. As expected, enhanced M2 markers including Arg-1, CD206 and TGF-beta but reduced M1 markers such as iNOS, CD86 and TNF-alpha were noticed in

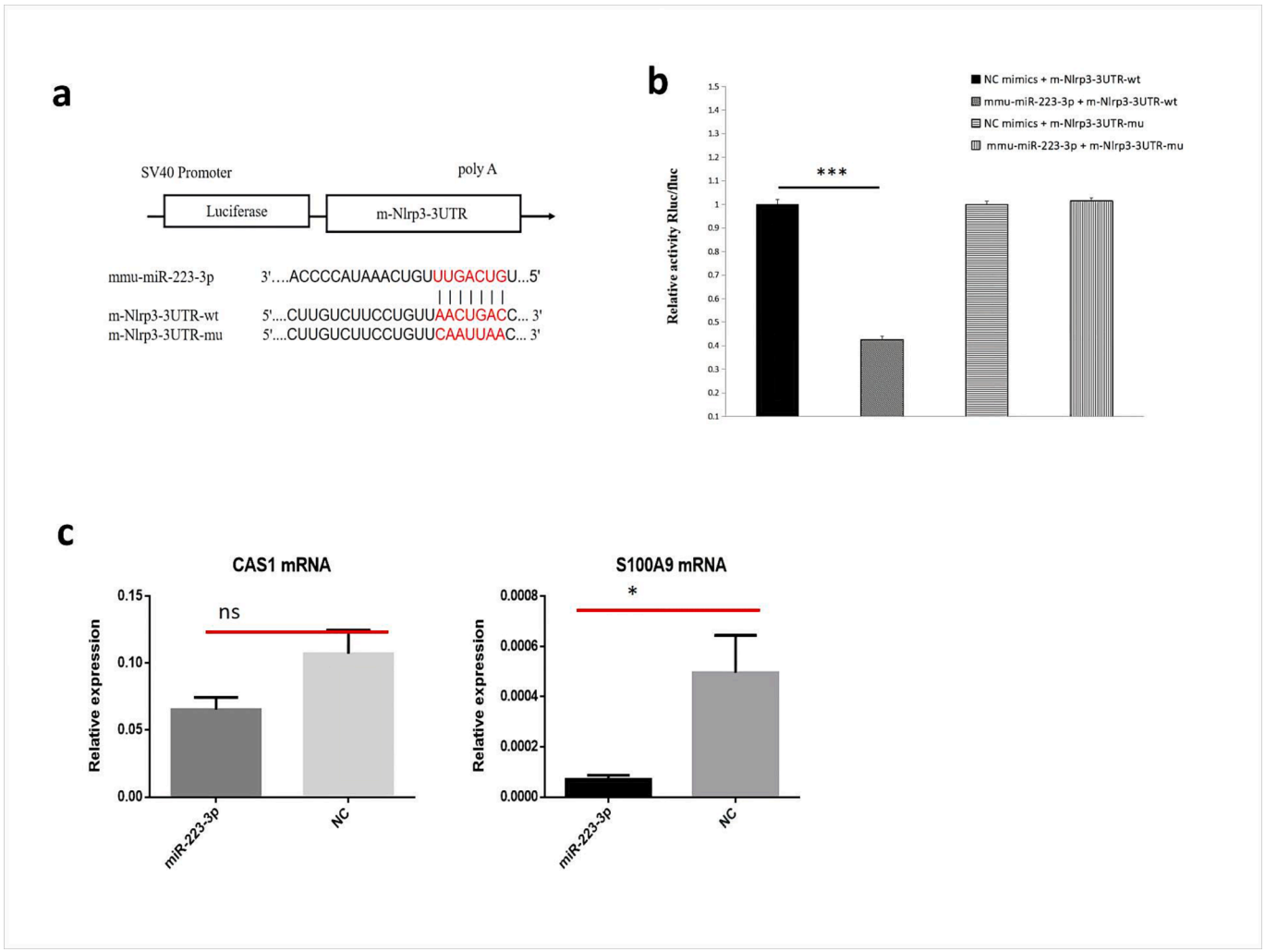

Fig. 8. miR-223-3p directly inhibits necroinflammation. a. The speculated relationship between miR-223-3p and 3′UTR of NLRP3 by Targetscan 8.0; **b.** Dual luciferase reporter assay in order to validate the relationship between miR-223-3p and 3′UTR of NLRP3; **c.** The mRNA levels of necroinflammation mediators including NLRP3 and S100A9 in mouse macrophages transfected with miR-223-3p mimics or NC (n = 3–4). Group comparisons were performed using Student's *t* test and Mann–Whitney *U* test, respectively.

SPC-treated mice or macrophages. Thus, the hepatoprotection exerted by SPC is closely bound up with M2-like macrophages. This finding is partially consistent with previous reports [30,31].

To further explore the mechanism by which SPC promotes M2-like macrophage activation, we analyzed the expression of miR-223-3p, an important regulator of macrophage polarization [28,32]. *In vivo* experiments indicated miR-223-3p was up-regulated in SPC-treated mice compared with acutely injured mice. As far as we know, this is the first to report the up-regulation of miR-223-3p expression by SPC. To confirm the promoting effects of miR-223-3p on M2-like activation of macrophages, we analyzed the activation phenotype of macrophages subjected to miR-223-3p treatment. Enhanced M2 markers including Arg-1, CD206 and TGF-beta but reduced M1 markers such as iNOS, IL-1beta and TNF-alpha were noticed in miR-223-3p-transferred macrophages. This finding is in line with the reports that consider miR-223-3p as a key regulator of the dynamic balance between M1/M2 macrophage activation [32,33]. Collectively, SPC up-regulates miR-223-3p and further promotes M2-like activation of macrophages.

As mentioned above, miR-223-3p promotes M2-like activation of macrophages, and M2-like macrophages inhibit necroptosis-S100A9-necroinflammation. In view of this, we speculated that miR-223-3p can target signaling molecules critically involving in necroptosis and ensuing necroinflammation. To testify this speculation, we performed dual luciferase reporter assay. The results validated miR-223-3p targeted directly 3′UTR of RIPK3 and NLRP3. We further documented the inhibitory effects of miR-223-3p on necroptosis and necroinflammation by transferring miR-223-3p into hepatocytes or macrophages. The mRNA levels of necroptosis signaling molecules (RIPK3 and MLKL) and critical molecules related to necroinflammation (caspase-1 and S100A9) were significantly reduced in miR-223-3p-transferred cells. Together, miR-223-3p inhibits necroptosis and ensuing necroinflammation. Our results are in accordance with the reports by Huang et al. who confirmed that miR-223-3p can inhibit RIPK3 expression by targeting the 3′ UTR of RIPK3 [34] and Jimenez et al. who identified that miR-223-3p negatively regulates the NLRP3 inflammasome activity in acute and chronic liver injury [35].

In brief, SPC exerts the hepatoprotection against D-GalN/LPS-induced acute liver injury through up-regulating miR-223-3p, which further promotes M2-like macrophage activation and inhibits necroptosis-S100A9-necroinflammation. This study manifests the immunopharmacol feature of SPC. Specially, our work illustrates the mechanism of action of SPC from a novel perspective, and provides an in-depth evidence for clinicians to treat acute liver injury with SPC in practice.

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Authors contributions

L.B. and Y.C. conceived the study and designed the experiments. L.B., Y.C., Z.P.D., and M.M.X. interpreted the results and generated the figures and tables. L.B. and S.T. wrote the manuscript. X.D.Z., S.T., M.M.X. and M.K. performed the experiments. S.J.Z., Z.P.D., and Y.C. provided feedback and supervised the study. Z.P.D., L.B. and Y.C. obtained funding and supervised the overall execution of this work.

Availability of data and materials

The dataset generated during the current study is available from the corresponding author upon reasonable request.

Author contribution

L.B. and Y.C.: conceptualization. L.B., Y.C., Z.P.D., and M.M.X.: Formal analysis. L.B. and S.T.: Wrotomg-original draft. X.D.Z., S.T., M. M.X. and M.K.: Data curation. S.J.Z., Z.P.D., and Y.C.: Writing-review & editing. Z.P.D., L.B. and Y.C.: Funding acquistion.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2023.109808.

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Further reading

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