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Upregulation of miR-142-5p induced by lipopolysaccharide contributes to apoptosis of hepatocytes and hepatic failure

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Abstract. – OBJECTIVE: This study was probed to uncover the mechanism of miR-142-5p in septic liver injury.

MATERIALS AND METHODS: In this study, *in-vitro* and *in-vivo* models of sepsis were used. For *in-vitro* sepsis model, hepatocyte cell line (L02 cells) was treated with LPS (lipopolysaccharide). Whereas for in-vivo sepsis model, cecal ligation and puncture were performed in mice. Mice were assigned into three groups: control, CLP (Cecal Ligation Puncture), CLP + miR-142-5p inhibitor group. Liver injury was assessed via H&E staining. IL-6, TNF-α, and IL-1β expressions were assayed through ELISA kits. C-caspase-9, C-caspase-3, ERK, p65, and IκBα expressions were determined via western blot and RT-qPCR. Apoptosis in LPS-induced L02 cells was detected by TUNEL staining.

RESULTS: Our results show that miR-142-5p exhibited perspicuous upregulation in CLP mice tissues and LPS-induced L02 cells. On the other hand, inhibition of miR-142-5p could promote LPS-induced L02 cell activity and reduce apoptosis and inflammation. In terms of molecular mechanism, downregulation of miR-142-5p could abate sepsis-mediated acute hepatic injury by targeting SOCS1, through ERK and NF-κB pathway.

CONCLUSIONS: Overall our results demonstrate that miR-142-5p inhibitors can mitigate septic liver injury by downregulating the inflammation and apoptosis *via* targeting SOCS1. Thus, miR-142-5p can serve a potential therapeutic target for sepsis mediated acute hepatic injury.

Key Words:
MiR-142-5p, Septic liver injury, Hepatocytes, Hepatic failure.

Introduction

Sepsis, an invasive disease, triggers organ dysfunction[1]. Although medicinal treatments, molecular targeted therapies and immunotherapies have improved the pathological conditions of patients suffering with sepsis, still the incidence and death toll of sepsis are high[2,3]. At present, sepsis is a considered the main cause of death among ICU patients[4]. As a weighty organ of host defense dynamic balance, liver is frequently damaged in sepsis[5,6]. Sepsis-induced acute hepatic injury (AHI) is one of the complications of sepsis, which may further trigger multiple organ dysfunction syndrome (MODS) among advanced cases[7]. Therefore, it is necessary to dig into the pathogen-

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esis of septic AHI, as well as to explore efficient treatment.

MiRNA, an endogenous non-coding RNA, is widely distributed in human body. It is capable of modulating gene expression at the post-transcriptional level *via* binding with the 3'UTR of the target mRNA[8]. Many human diseases are related to the abnormal expression of miRNAs. By exploring these miRNAs, we may get novel clues for disease diagnosis and targeted treatment[9-11]. Some investigations[12,13] have displayed the alterations in miRNA expression in patients with sepsis that mediate the progression of sepsis through immune regulation and inflammatory regulation in the occurrence and development of sepsis. Recently, it has been demonstrated that miRNAs play a role in sepsis-mediated AHI. For example, miR-155 represses its target gene Nrf-2 to inhibit oxidative stress-induced endoplasmic reticulum stress, apoptosis, as well as mitochondrial dysfunction, and reduce septic AHI, suggesting that miR-155 may be a target for septic AHI[14]. Similarly, miR-30e inhibits hepatocyte apoptosis and induces hepatocyte proliferation through JAK/STAT signaling pathway, indicating that miR-30e may be a therapeutic target for septic AHI[15]. Likewise, miR-142-5p is engaged in progress of inflammation. For example, miR-142-5p contributes to ulcerative colitis progression *via* SOCS1. Thus, miR-142-5p exerts damaging roles in ulcerative colitis[16]. However, the influence of miR-142-5p in sepsis-mediated AHI remains unclear.

In view of the above research basis, we extrapolated miR-142-5p exerts influences in sepsis-mediated AHI. We intended to delve into the role of miR-142-5p in LPS-induced human liver epithelial cell (L02) model and CLP mouse model at molecular level. Our study revealed that miR-142-5p is upregulated in LPS induced L02 cells. Mechanistically, downregulated miR-142-5p could target suppressor of cytokine-signAHIng-1 (SOCS1) and block NF-κB and ERK pathway to allay sepsis-mediated AHI.

Materials and Methods

Cell Culture

Human liver epithelial cell line (L02) was procured from the Chinese Academy of Sciences. The DMEM (Hyclone, Shengke Bio, Cat: 12100046, Beijing, China) supplemented with 10% heat-devitalized FBS, 100 U/mL penicillin and 100 μg/mL streptomycin was used to cultivate the cells in the incubator containing 5%CO_2 at 37°C. 0.25% trypsin (Solarbio, Cat: P1400, Beijing, China) was used for subculture.

Cell Transfection

SiRNA negative control (si-NC), sh-SOCS1, miRNA NC (miR-NC), miR-142-5p mimics and inhibitor were synthesized by RiboBio (Beijing, China). Lipofectamine 3000 (Invitrogen, Cat: L3000008, Shanghai, China) was used to transfect the L02 cells with oligonucleotides. The transfection validity was detected by RT-PCR. The cells were cultured for 24 to 48 hours before the subsequent experiment.

MTT

After trypsin digestion, the transfected L02 cells were seeded into 96-wel plate (2.5×10^4 cells/well). MTT experiment was carried out in the incubator at 24 hours. Each well was cultured with 20 μL (5 g/L) MTT (Medchem Express, New Jersey, NJ, USA) at 37°C for 4 hours, and added with 150 μL DMSO. The absorbance ("A") value was measured at 492 nm with enzyme labeling instrument. The calculation was done as follows: inhibition rate of cell viability (%) = (average "A" of control group - average "A"of experimental group)/average "A" of experimental group × 100%.

TUNEL

TUNEL kits were bought from Invitrogen (Cat: C10245, Shanghai, China). Subsequently, cells were fixed in 4% POM solution for 30 min, incubated with blocking agent (0.3% H_2O_2 methanol solution) at room temperature for 30 min. to block endogenous peroxidase activity. Cell permeability was achieved using Triton X-100 for 2 minutes. Then, we added 50 μL TUNEL reaction mixed solution for labeling, and cultivated it in a wet box at 37°C for 1 h. At last, we added 50 μL of conversion agent, fostered it in a wet box at 37°C for 30 min, washed with PBS for 3 times, and 50-100 μL DAPI was used to stain the nucleus. Anti-fluorescence quenching agent was used to seal the cells. Fluorescence microscope was used we observe and store the images.

Western blot

After the cell treatment, total protein was collected using radio immunoprecipitation assay (RIPA) and quantified through bicinchoninic acid assay (BCA) assay. Then, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis

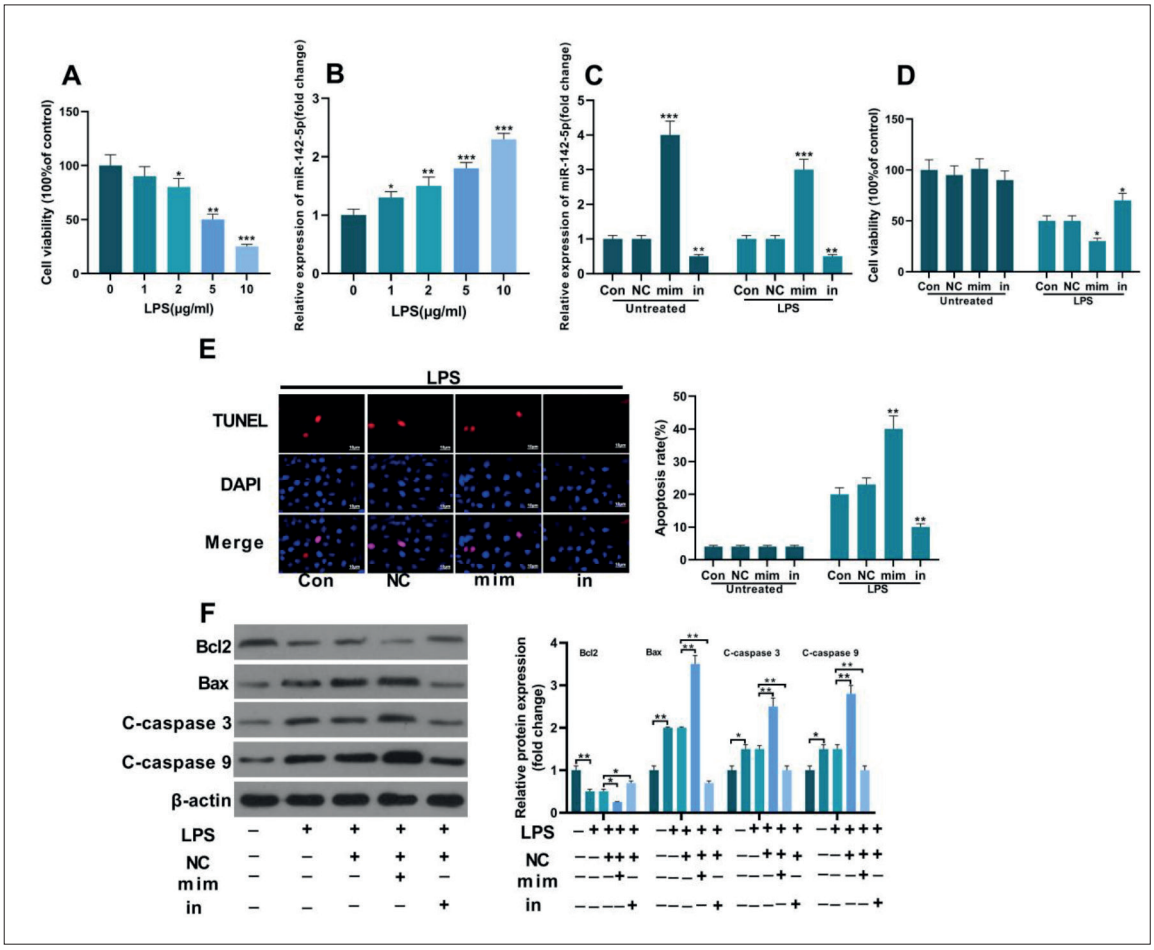

Figure 1. MiR-142-5p inhibitors inhibited LPS-induced apoptosis arrest in L02 cells. **A**, Incubating L02 cells with 0, 2, 5 and 10 μg/mL LPS resulted in a dose-dependent decrease in cell viability. **B**, In LPS, miR-142-5p level was up-regulated in dose-dependent fashions. **C-D**, We transfected miR-142-5p mimics and inhibitors into L02 cells. RT-PCR to assay miR-142-5p level in L02 cells. (**E**) TUNEL to test apoptosis (scale bar: 10 μm, 100x). (**F**) Western blot to monitor Bcl2, Bax, C-caspase-3 and C-caspase-9 protein levels in L02 cells. *p<0.05, **p<0.01, ***p<0.001.

(SDS-PAGE) electrophoresis was carried out and the proteins were transferred to polyvinylidene difluoride (PVDF) membrane. After transfer, the membrane was incubated in 5% skimmed milk for 2 h. The primary antibodies embracing SOCS1 (1:1000, CST, Shanghai, China), ERK (1:1000, CST, Shanghai, China), p65 (1:1000, CST, Shanghai, China), IκBα (1:1000, CST, Shanghai, China) and β-actin (1:2000, CST, Shanghai, China) were incubated nightlong at 4°C.Later, after washing membrane with TBST for 3 times, it was subsequently incubated with the corresponding HRP labeled second antibody at room temperature for 1 h. Finally, the membrane was washed with TBST for 3 times, ECL chemiluminescence Kit (Amersham pharmacy biotechnology, little Chalfont, UK) was used to expose the membrane. Using fusion software for quantitative analysis, the control group was standardized as "1". The experiment was repeated for three times.

ELISA

IL-6, TNF-α, and IL-1β levels were assayed via ELISA. Briefly, L02 cells were incubated with 2.5×10^5 cells in 24-well plates. Cells were lysed in RIPA lysate buffer (Beyotime, Shanghai, China), and centrifuged at 14000 × g for 5 min. The supernatant was collected for ELISA. After the supernatant of the culture was collected, the protein concentrations of IL-6, TNF-α and IL-1β were assayed by using rapid bio kit (R & D systems, Abingdon, UK).

Dual-Luciferase Reporter Gene Assay

The transfected L02 cells were trypsinized, counted and seeded into 24 well plates (5×10^4 /

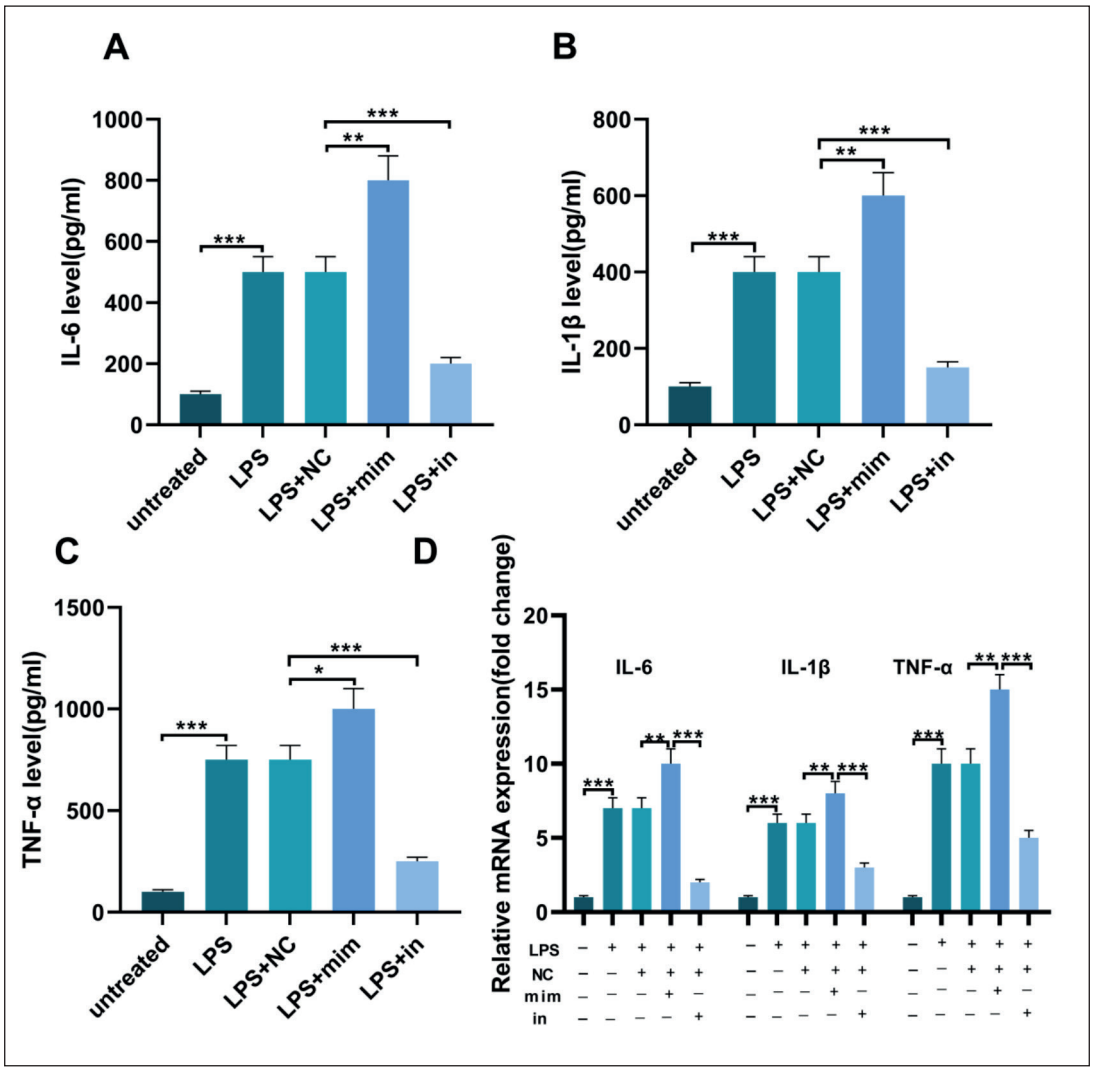

Figure 2. MiR-142-5p inhibitors abated inflammation of LPS on L02 cells. (**A-C**) ELISA to test TNF- α, IL-1 β and IL-6 levels in L02 cells. (**D**) RT-PCR to assay TNF- α, IL-1 β and IL-6 mRNA expression. *p<0.05, **p<0.01, ***p<0.001.

well). When the confluency reached at 70%, the cells are transfected. 200 ng top flash report plasmid and 10 ng FOP flash marine fluorescent enzyme control plasmid were co transfected into the cells. Cells were collected 48 hours later. The cells were resuspended by adding 1 × passive lysis buffer. Cells were cleaved at 4°C for 24 h. Centrifugation was implemented at 12000 r/min and 4°C for 10 min. Afterwards, we harvested the supernatant and measured the activity of luciferase.

Establishment of Cecal Ligation and Perforation Model

Our team acquired C57BL/6J male mice (weight 20 ± 2 g, aged 8-12 weeks) from Shanghai SLAC experimental animal Co., Ltd. Animal protection committee of Fudan University had ratified this animal program. The mice were randomized into control, CLP and miR-142-5p inhibitor groups. Fabrication of sepsis model was achieved via cecal ligation and perforation[16,17]. The specific steps were as follows: mice were placed in the isoflurane anesthesia room, gave isoflurane inhalation anesthesia, fixed the mice on the operating board after anesthesia, continued isoflurane inhalation, prepared skin in the middle area of abdomen and disinfected, took the median abdominal incision about 1.0-1.5 cm long, and incised the skin and subcutaneous tissue layer by layer. The rectus abdominis and peritoneum were cut at the white line

of abdomen, and the cecum and its adjacent intestine were palpated and exposed with non-invasive probe. We gently pulled out the cecum and used 3-0 silk thread to ligate the cecum and mesenteric vessels at the place about 1/3-1/2 of the length of the whole cecum. After that, 20 g needle was used to puncture the ligated part of the cecum. The puncture point was selected from the lack of blood vessels on the opposite side of the mesenteric vessels of the ligated part of the cecum. For the puncture through the cecum, a little stool was squeezed out slightly to ensure the unobstructed puncture well. All intestinal loops were incorporated into the abdominal cavity, and the wound was sutured with 4-0 silk thread layer by layer. After the operation, the mice were injected with

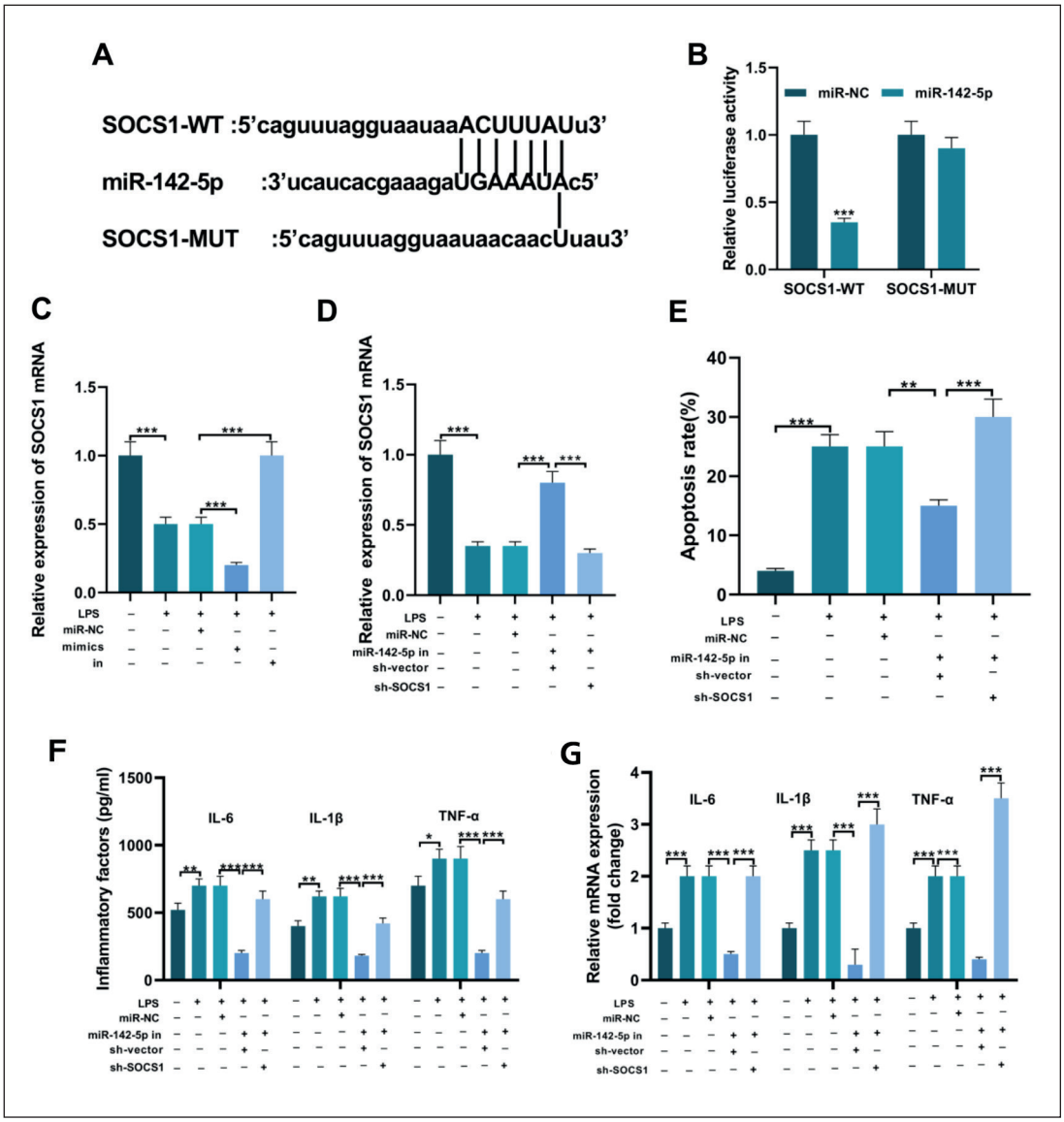

Figure 3. SOCS1 down regulation partially relieved miR-142-5p inhibitors induced repression of LPS-mediated L02 cell inflammation and apoptosis. (**A**) Through TargetScan database, we determined that the 3'UTR sequence of SOCS1 is complementary to miR-142-5p. (**B**) Luciferase reporter experiment to detect binding relation of SOCS1 with miR-142-5p. (**C**) Western blot assay was used to detect the level of SOCS1 mRNA in L02 cells transfected with miR-142-5p mimics and inhibitors. (**D**) Western blot assay was used to detect the level of SOCS1 mRNA in L02 cells transfected with miR-142-5p mimics and inhibitors. (**E**) The adenovirus vector with low SOCS1 expression was transfected into LPS-mediated L02 cells, and the apoptosis was assayed *via* TUNEL. (**F**) The adenovirus vector with low SOCS1 expression was transfected into LPS-mediated L02 cells, and the level of inflammatory cytokines was detected by ELISA. (**G**) The adenovirus vector with low SOCS1 expression was transfected into L02 cells induced by LPS, and the level of inflammatory cytokine mRNA was assayed *via* RT-PCR. *p<0.05, **p<0.01, ***p<0.001.

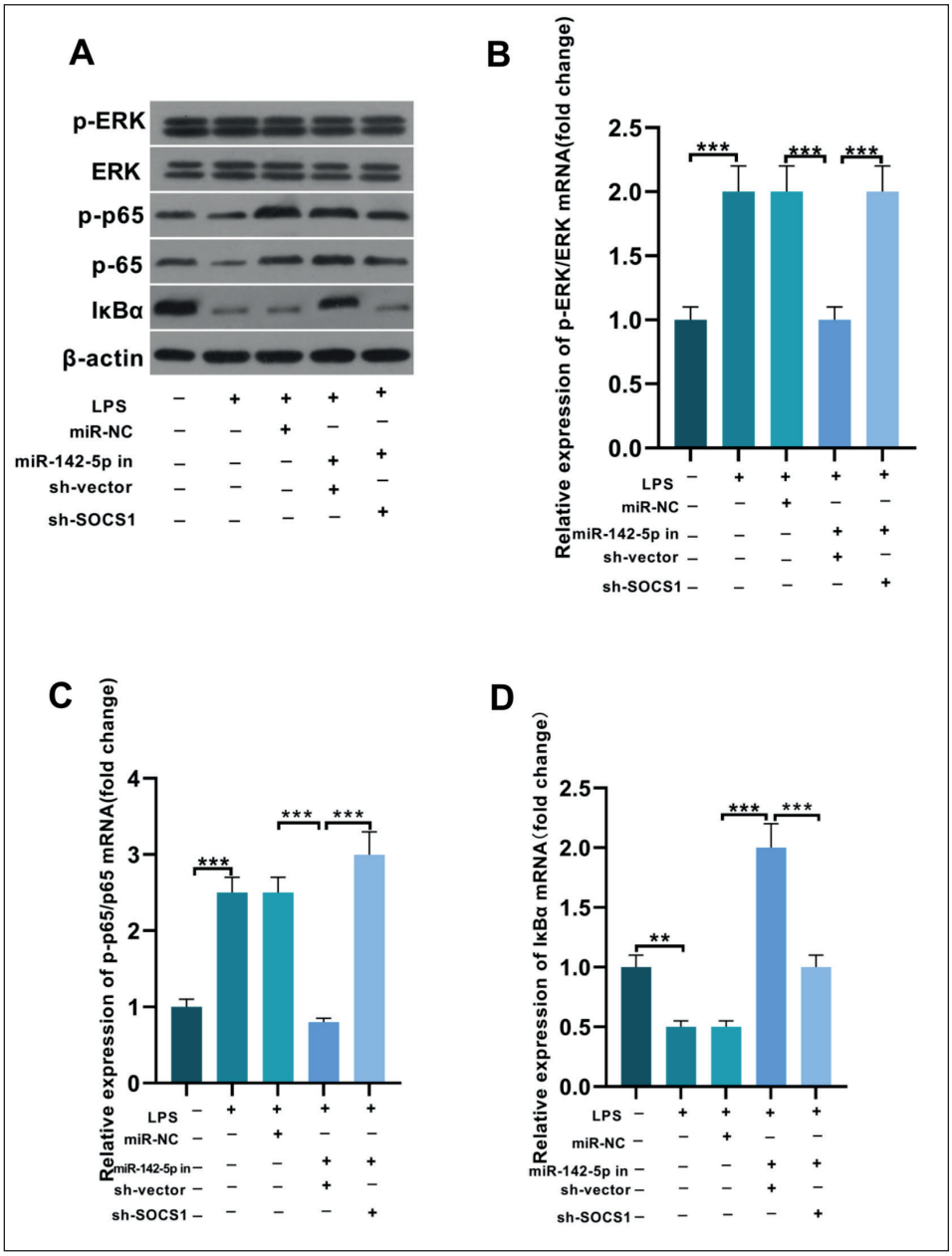

Figure 4. MiR-142-5p inhibitors restrained the activation of NF-κB and ERK signaling. After transfecting miR-142-5p inhibitor and SOCS1 low expression plasmid, p-p65/p65, p-ERK/ERK and IκBα protein **(A)** and mRNA **(B-D)** expression levels in LPS treated L02 cells were monitored via western blot and RT-PCR, separately. **p<0.01, ***p<0.001.

saline into the back subcutaneously, put back into the heated cage with warm air machine, and the mice were free to wake up. The mice were free to feed and drink. 100 μL adenovirus solution (1×10^{11}) was injected into tail vein to carry empty constitutional particles or miR-142-5p high expression plasmids (Hongtuo Biotechnology Co., Ltd, Hangzhou, China) to construct the model of miR-142-5p high expression CLP.

HE Staining

After the mice were sacrificed, their livers were separated and excised. Then we cut the appropriate size of liver tissue, washed it with 4°C normal saline and placed in fresh 4% paraformaldehyde for fixation. Later we embedded it in paraffin and prepared the 4 mm thick slices. The pathological changes of liver were observed after the HE staining.

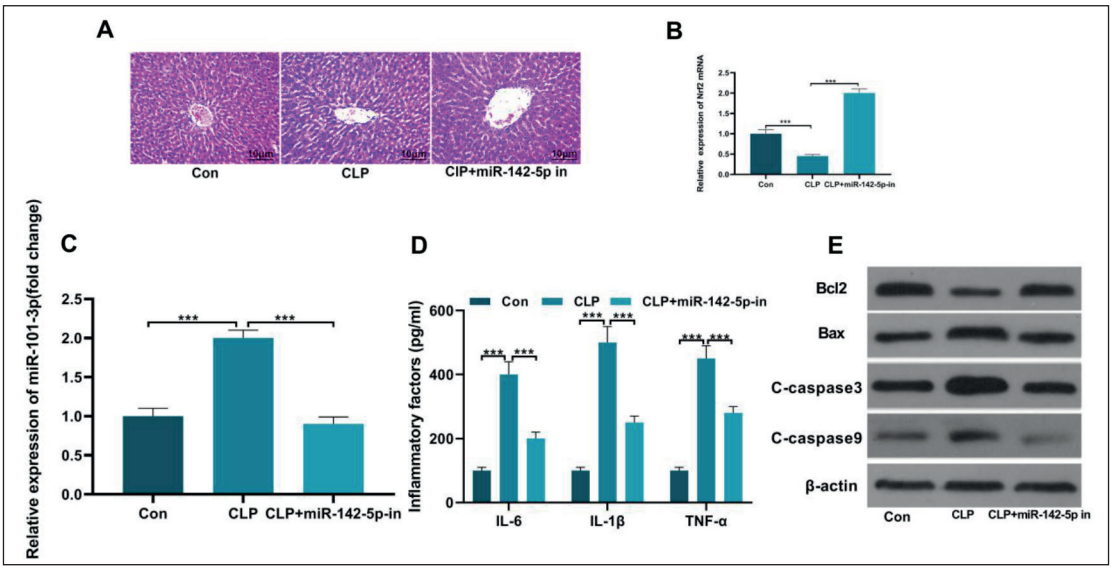

Figure 5. MiR-142-5p attenuated AHI-mediated inflammation and apoptosis by targeting SOCS1. **A**, HE staining to prospect the morphological changes of liver in septic mice after low miR-142-5p expression (scale bar: 10 μm, 100x). **B**, RT-PCR to test SOCS1 mRNA level in mouse tissue after low miR-142-5p expression. **C**, RT-PCR to determine miR-142-5p level in mouse tissue after low miR-142-5p expression. **D**, ELISA to assess the level of inflammatory cytokines after low miR-142-5p expression. **E**, Western blot to evaluate Bcl2, Bax, C-caspase-3 and C-caspase-9 levels. ***p<0.001.

Statistical Analysis

SPSS 22.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The analyzed data was displayed as mean ± standard deviation. *t*-test and ANOVA was used to compare the differences among groups.

Results

MiR-142-5p Inhibitors Inhibited LPS-Induced Apoptosis in L02 Cells

We tended to explore whether miR-142-5p was abnormally expressed in L02 cells stimulated by LPS. As shown in the figure, incubation of L02 cells with 0, 2, 5 and 10 μg/mL LPS resulted in a dose-dependent decrease in cell viability (p<0.05, Figure 1A). Meanwhile, miR-142-5p level exhibited up-regulation in a dose-dependent manner (p<0.05, Figure 1B). Hence, we probed that miR-142-5p might play critical role in LPS induced cell damage. Previous studies[18] have shown that apoptosis in hepatocyte is the most basic central link of liver injury and liver disease, which exerts functions in AHI progress[18]. Secondly, we inquired the miR-142-5p influence on LPS-mediated apoptosis. For this reason, we transfected miR-142-5p mimic and inhibitor into L02 cells, and monitored the efficiency (p<0.05, Figure 1C). We found that miR-142-5p repressed cell activity, whereas miR-142-5p inhibitors enhanced cell activity in LPS-treated L02 cells (p<0.05, Figure 1D). In addition, miR-142-5p could promote apoptosis (p<0.05, Figure 1E). Western blot undressed that LPS could reduce Bcl2 level in cells, but miR-142-5p inhibitors could up regulate it. Moreover, LPS could increase the level of C-caspase-9, Bax and C-caspase-3 protein. However, miR-142-5p mimics could enhance its level, and miR-142-5p inhibitors could inhibit its level (p<0.05, Figure 1F). Hereby, our results exhibited that miR-142-5p can promote LPS induced apoptosis of L02 cells and inhibit cell activity.

MiR-142-5p Inhibitors Attenuated Inflammation of LPS on L02 Cells

To dig out the anti-inflammatory effects of miR-142-5p inhibitors, we investigated the effects of miR-142-5p mimics and inhibitors on LPS induced secretion of inflammatory factors in L02 cells. ELISA evinced that IL-6, IL-1β, and TNF-α level was increased owing to the transfection of miR-142-5p mimics, while transfecting miR-142-5p inhibitors had opposite effect (Figure 2A-C). In addition, RT-qPCR unveiled that IL-6, IL-1β and TNF-α mRNA expression could be up-regulated by transfecting miR-142-5p mimics, while IL-6, TNF-α and IL-1β mRNA expression could

be enhanced for transfection of miR-142-5p inhibitors ($p<0.05$, Figure 2D). Thus, miR-142-5p inhibitors may attenuate LPS induced L02 cell damage.

SOCS1 Down Regulation Partially Relieved MiR-142-5p Inhibitors Based Inhibition of LPS-Mediated L02 Cell Inflammation and Apoptosis

Through TargetScan, we determined that 3'UTR sequence of SOCS1 had binding site for miR-142-5p (Figure 3A). To verify the binding of miR-142-5p with SOCS1, we evaluated the Luciferase activity in HEK293T cells. We found that miR-142-5p could significantly reduce the luciferase activity of SOCS1-wt, but not that of SOCS1-mut ($p<0.05$, Figure 3B). Western blot exhibited that miR-142-5p could remarkably reduce SOCS1 level in LPS induced L02 cells, while miR-142-5p inhibitors could up-regulate SOCS1 ($p<0.05$, Figure 3C). To further investigate the SOCS1 impact on miR-142-5p modulation of LPS-induced L02 cell inflammation and apoptosis, we transfected the silenced SOCS1 expression into LPS induced L02 cells. Thus, silencing SOCS1 expression could reverse the suppression of miR-142-5p inhibitors on cell inflammation and apoptosis ($p<0.05$, Figure 3D-G). Consequently, we resulted that miR-142-5p regulates LPS-meditated apoptosis and inflammation of L02 cells via SOCS1.

MiR-142-5p Inhibitors Blocked the Activation of NF ÐB and ERK

We further studied the miR-142-5p impact on downstream NF-κB and ERK signaling, and explored the LPS-induced injury's downstream pathways. Using western blot analysis, we found that miR-142-5p inhibitors perspicuously suppressed p-p65/p65 and p-ERK/ERK protein expression in LPS-mediated L02 cells, while the trend of IκBα was opposite (Figure 4A-E). However, SOCS1 silencing weakened this effect ($p<0.05$). This revealed that downregulation of miR-142-5p can block the NF-κB and ERK signaling pathways, which is achieved through the interaction with SOCS1.

MiR-142-5p Attenuated AHI Mediated Inflammation and Apoptosis by Targeting SOCS1

To further explore the role of miR-142-5p in reducing AHI mediated inflammation and apoptosis by targeting SOCS1. We constructed mir-142-5p inhibitors sepsis *in-vivo* model. The experimental data revealed that miR-142-5p inhibitors could significantly reduce liver tissue injury (Figure 5A) by down-regulating miR-142-5p, while inhibition of miR-142-5p up-regulates SOCS1 in septic mice ($p<0.05$, Figure 5B, C). Moreover, ELISA and western blot data exhibited that the levels of inflammatory cytokines and apoptosis-related proteins in serum were significantly down-regulated after down-regulation of miR-142-5p ($p<0.05$, Figure 5D, E). This suggests that miR-142-5p can attenuate AHI-mediated apoptosis as well as inflammation by targeting SOCS1.

Discussion

MiRNA plays a role in sepsis by regulating the inflammatory genes. For example, the suppression of miR-208a-5p attenuates myocardial injury via regulating NF-κB/HIF-1α pathway, implying that miR-208a-5p is likely a diagnostic marker and curative target for septic mice[13]. Similarly, miR-20a could exert anti-inflammatory role and anti-apoptotic impacts in LPS-mediated HK-2 by activating CXCL12/CXCR-4, NF-κB and ERK1/2 pathways[19]. In sepsis-mediated AHI, miR-155 inhibitors attenuate AHI via augmenting SOCS1 expression and devitalizing JAK/STAT signals[20]. Some studies have emphasized the significance of miR-142-5p in liver disease. For example, the up-regulation of miR-142-5p expression in NAFLD inhibits Nrf2 expression and facilitates lipid accumulation in hepatocytes. Likewise, miR-142-5p expression is augmented in non-alcoholic fatty liver, which inhibits Nrf2 expression but accelerates lipid accumulation in hepatocytes[21]. In addition, miR-142-5p overexpression exerts a defensive part in hepatocellular carcinoma by curbing cell growth and inducing apoptosis[22]. This study was designed to investigate the miR-142-5p function in sepsis-mediated AHI *in vivo* and *in vitro* model. Our results showed that the miR-142-5p expression in liver tissue from septic mice and LPS-induced hepatocytes was significantly up-regulated. This up-regulation of miR-142-5p could restrain the survival of hepatocytes by increasing the apoptosis and inflammation of hepatocytes. Thus, we hypothesized that down-regulation of miR-142-5p can attenuate sepsis-mediated AHI.

When sepsis-mediated AHI occurs, the liver is exposed to circulating antigens, endotoxins, and microbes, which circulate through the portal vein

from gastrointestinal tract to liver, or through arterial blood from systemic circulation to liver, posing the overmuch release of proinflammatory cytokines and inflammatory mediators[23]. Rigato et al[24] demonstrated that TNF-α is the most crucial pro-inflammatory factor in sepsis and considered as the "core factor" causing damage in multiple organs and death. Likewise, interleukin-1β exerts a synergistic effect. Furthermore, in sepsis-mediated AHI, the pathway of mitochondrial apoptosis is retarded by autophagy. The loss of mitochondrial potential leads to apoptosis, whereas the ROS produced by damaged mitochondria accelerates apoptosis[25-28]. We found that miR-142-5p can enhance expressions of pro-inflammatory cytokines and apoptosis-related proteins in hepatocytes induced by LPS and activate ERK and NF-κB pathway. These findings demonstrated miR-142-5p is involved in AHI induced by sepsis.

SOCS1, belongs to the SOCS family, is located at 16p12murp13.1 and consists of three domains: N region (amino terminal), the central SH2 region and the carboxyl terminal SOCS box[29]. The promoter of SOCS1 gene embraces binding sites of STAT1, STAT3 and STAT6, and regulates its expression by binding to the corresponding STAT[29]. SOCS1 regulates cytokines, but also functions monumentally in modulating the immune response of innate immunity and adaptive immunity[30,31]. Some studies[32,33] have emphasized the significance of SOCS1 in cancer. For example, in breast cancer, downregulation of SOCS1 can activate JAK/STAT signaling pathway to promote breast cancer progression. The downstream JAK-STAT signal pathway can also inhibit the therapeutic potential of SOCS1 in ovarian cancer. This demonstrated that SOCS1 can regulate tumor progression through JAK-STAT signal pathway in cancer. In this study, we proposed a new mechanism through which SOCS1 expression is down-regulated in sepsis-mediated AHI. Later, we unmasked that SOCS1 is a downstream target of miR-142-5p and harbors negatively modulatory relationship with it.

However, there are still some shortcomings in this study. In the mechanism study, other downstream targets of miR-142-5p need further screening which will further clarify the downstream mechanism of miR-142-5p. Moreover, miR-155 inhibitors can reduce AHI *via* enlarging SOCS1 expression and devitalizing JAK/STAT signals[20]. Therefore, it is necessary to probe the relation of miR-142-5p/SOCS1 axis with downstream JAK/STAT signal pathway in follow-up studies. Importantly, for further looking into the importance of miR-142-5p to AHI, clinical AHI samples need to be further analyzed. In short, miR-142-5p manifested up-regulation in CLP mice, as well as hepatocytes induced by LPS. Functional experiments showed miR-142-5p inhibition could reduce the apoptosis and inflammation in LPS-induced hepatocyte and enhance cell viability. Further studies revealed that miR-142-5p functions mainly by regulating the activation of ERK and NF-κB pathway by targeting SOCS1.

Conclusions

To conclude, these findings provide novel mechanism to attenuate CLP/LPS-induced sepsis by inhibiting pro-inflammatory microenvironment through miR-142-5p/SOCS1/NFκB signaling pathway. We suggest that miR-142-5p may be a new potential target for sepsis. Moreover, miR-142-5p inhibitors can mitigate septic liver injury by blocking inflammation and apoptosis via targeting SOCS1. Thus, miR-142-5p can emerge as a potential target for sepsis with acute hepatic injury.

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Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Statement

All experimental procedures conformed with institutional guidelines, the animal experiment research protocol was approved by the Second Affiliated Hospital of Kunming Medical University (NO. 20133) and performed in accordance with the "Guidelines for the care and use of experimental animals".

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