MicroRNA-23a-3p targeting of HMGB1 inhibits LPS-induced inflammation in murine macrophages *in vitro*

QI SUN*, BING WANG* and MENGQIU LI

Department of Critical Care Medicine, The Jingzhou Central Hospital of Hubei, Jingzhou, Hubei 434000, P.R. China

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Abstract. Inflammatory cytokines, including high mobility group box 1 (HMGB1), play a key role in sepsis via various mechanisms, some of which remain unknown. Sepsis is a common cause of death in patients admitted to the intensive care unit. MicroRNAs (miRs) serve an important role in the inflammatory response. The present study aimed to investigate the role of miR-23a-3p in macrophage inflammation and the targeted regulation of HMGB1 expression. The murine macrophage cell line RAW264.7 was subjected to lipopolysaccharide (LPS) treatment to mimic the inflammation involved in sepsis *in vitro*. Reverse transcription-quantitative PCR was performed to measure miR-23a-3p expression and mRNA expression. Protein levels were determined using ELISA and western blotting. The target binding relationship between miR-23a-3p and the HMGB1 3'untranslated region was predicted and validated with a dual luciferase reporter assay. HMGB1 expression was increased and miR-23a-3p expression significantly reduced in patients with sepsis and in LPS-treated RAW264.7 cells in comparison with controls. Overexpression of miR-23a-3p reduced interleukin (IL)-6 and tumor necrosis factor (TNF)-α expression in RAW264.7 cells under LPS stimulation, while silencing of miR-23a-3p elevated the expression of IL-6 and TNF-α in comparison with controls. The inhibitory effect of miR-23a-3p on LPS-induced inflammation could be abolished by HMGB1 upregulation in RAW264.7 cells. HMGB1 was targeted by miR-23a-3p. miR-23a-3p is expressed at reduced levels during inflammation in sepsis, and overexpression of miR-23a-3p inhibits LPS-induced inflammation in murine macrophages *in vitro* by directly downregulating HMGB1. The results of the present study provided a novel insight into the molecular mechanism underlying HMGB1 expression at the post-transcriptional level in sepsis.

Correspondence to: Dr Mengqiu Li, Department of Critical Care Medicine, The Jingzhou Central Hospital of Hubei, 1 Renmin Road, Jingzhou, Hubei 434000, P.R. China
E-mail: nms75386412@sina.com

*Contributed equally

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Introduction

Sepsis is a systemic inflammatory response that is driven by a variety of pathogenic microorganisms including bacteria and their derived products such as endotoxins (1). Sepsis-mediated inflammation may lead to organ dysfunction or circulatory disorders (2). Globally, it's estimated that there are 31.5 million sepsis patients and potentially 5.3 million mortalities every year (3); 51.1% of sepsis patients receive intensive care and ~10% of all intensive care unit patients have severe sepsis in the United States (4,5). Several biomarkers of sepsis have been identified, including phase proteins and inflammatory cytokines such as interleukin (IL)-1, 6 and 10, as well as tumor necrosis factor (TNF)-α (6). Unfortunately, the outcome of patients with sepsis following treatment is often unsatisfactory, due to inflammatory cytokine secretion during the progression of sepsis (7,8). Therefore, it is essential to figure out novel biomarkers for an early diagnosis of sepsis, and thus to develop pharmacological therapy for sepsis by blocking the inflammatory cascade.

High-mobility group box 1 (HMGB1) is a highly conserved non-histone DNA-binding protein (9). It has been documented that HMGB, an extracellularly released mediator, can regulate the inflammatory response (10). Typically, the production of pro-inflammatory cytokines such as TNF-α and IL-1β is induced immediately once inflammation spreads into the blood stream, which then triggers HMGB1 expression (11). HMGB1 is known as a late mediator of inflammation (11). Existing evidence indicates that extracellular HMGB1 may exhibit pro-inflammatory activity in the pathogenesis of various inflammatory diseases (12). HMGB1 is secreted by injured cells and innate immune cells including macrophages (10,13). Moreover, in response to LPS stimulation, HMGB1 can translocate from the nuclei to the cytoplasm in macrophages (14). In the clinic, plasma HMGB1 levels are suggested to be positively correlated with organ dysfunction and death in patients with sepsis (15). Therefore, HMGB1 has been regarded as a promising therapeutic target in inflammatory diseases, including sepsis (16). However, the precise mechanism of HMGB1 involvement in sepsis remains unknown.

MicroRNAs (miRNAs/miRs) are endogenous non-coding transcripts of ~22 nucleotides in length that hinder protein

translation through direct base pairing to a broad range of biological systems in animal cells (17). miRNAs serve as essential regulators in inflammatory cytokine release and immune responses (18). In sepsis, it has been highlighted that numerous miRNAs are differentially expressed (19). Moreover, several miRNAs have been identified to be diagnostic or prognostic biomarkers in sepsis, including miR-25 and miR-150 (20-22). miR-23a-3p has been reported to contribute to pathology in cancer, cardiac hypertrophy and muscular atrophy (23). miR-23a-3p is observed to be upregulated in brain tissue, leukocytes and blood plasma during focal cerebral ischemia (24). Additionally, it has been reported that the downregulation of miR-23a-3p in inflammation is associated with TNF-α-induced insulin resistance, LPS-induced immune activation of rat testis and sepsis-induced acute kidney injury (25-27). Furthermore, circulating plasma miRNAs including miR-23a-3p can differentiate human sepsis and systemic inflammatory response syndrome (SIRS) (1). Macrophage activation is involved in the host immune response and inflammatory response to sepsis (28), and, thus, the role of miR-23a-3p in macrophage inflammation needs to be elucidated.

Although the role of HMGB1 in sepsis is well-known, the underlying regulatory mechanism has yet to be fully uncovered (29). The present study aimed to provide a novel insight into the targeted regulation of HMGB1 expression in sepsis by studying the role of miR-23a-3p in LPS-activated RAW264.7 macrophage cells and investigating the regulatory relationship between miR-23a-3p and HMGB1.

Materials and methods

Clinical specimens. A total of 20 patients (male:female, 12:8; age, 37-60 years) with severe sepsis were recruited from Jingzhou Central Hospital of Hubei Province (Jingzhou, China) during July 2018-December 2018 in the present study, together with 12 healthy controls (male:female, 7:5; age, 33-60 years). Peripheral venous blood (4 ml) was collected from all participants. Sepsis was defined according to the Sepsis-3 criteria (30). The excluding criteria for healthy controls: Pregnant women, bone marrow or organ transplant recipients, patients with cancer, individuals suffering with neutropenia, leukopenia or acquired immune deficiency syndrome. The present study was approved by the Ethics Committee of Jingzhou Central Hospital of Hubei, and informed written consent was obtained from all participants prior to study commencement.

Isolation of serum and peripheral blood mononuclear cells (PBMCs). Blood freshly collected in sodium citrate was used for isolation. For serum preparation, 2 ml blood was kept at -4˚C overnight, then centrifuged at 800 x g for 10 min at 4˚C. The supernatant was collected as serum samples and stored at -80˚C until further use. For PBMC isolation, 2 ml blood was centrifuged at 400 x g for 30 min at 18˚C on Ficoll-Paque PREMIUM (GE Healthcare; Cytiva) according to the manufacturer's instructions. The PBMC layer was collected and washed, and the cell pellet was re-suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Invitrogen; Thermo Fisher Scientific, Inc.).

Cell culture and lipopolysaccharide (LPS) treatment. The murine macrophage cell line RAW264.7 was acquired from the American Type Culture Collection and cultured in DMEM supplemented with 10% FBS at 37˚C in 5% CO_2. To induce inflammation *in vitro*, LPS (cat. no. L4516) was purchased from Sigma-Aldrich (Merck KGaA). RAW264.7 cells were exposed to 100 ng/ml LPS at 37˚C for 48 h prior to collection of cells and supernatants and RAW264.7 cells without LPS treatment served as the control.

Cell transfection. RAW264.7 cells were transferred to a six-well plate (Corning, Inc.) and incubated overnight. The pcDNA3.1$^+$ (Invitrogen; Thermo Fisher Scientific, Inc.) was used to overexpress HMGB1 via molecular cloning technology. Oligonucleotides (30 nM) and pcDNA-HMGB1 plasmids (2 μg) were transfected into RAW264.7 cells using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. GMR-miR™ miR-23a-3p mimic (miR-23a-3p; 5'-AUCACAUUGCCAGGG AUUU-3'), miR-23a-3p inhibitor (in-miR-23a-3p; 5'-AAAUCC CUGGCAAUGUGAU-3') and the indicated negative controls (miR-NC; 5'-CAGUACUUUUGUGUAGUACAA-3') and in-miR-NC (5'-UUGUACUACACAAAAGUACUG-3') were acquired from Shanghai GenePharma Co., Ltd. Transfected cells were cultured for an additional 48 h prior to further studies, among which transfected cells were treated LPS (100 ng/ml at 37˚C for 48 h).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). For examination of the mRNAs (including IL-6, TNF-α and HMGB1), first-strand cDNA was synthesized using All-In-One 5X RT MasterMix (Abcam), and the reaction thermal profile was 37˚C for 15 min, 60˚C for 10 min and 95˚C for 3 min. For examination of the miRs, a miScript Reverse Transcription kit (Qiagen GmbH) was used, and the reaction thermal profile was 37˚C for 60 min, and 95˚C for 5 min. To determine the levels of mRNAs and mature miR-23a-3p, qPCR was performed using SYBR Green qPCR Mix (Abcam) and miScript SYBR Green PCR Kit (Qiagen GmbH), respectively on an ABI PRISM 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were 95˚C for 15 min, and 35 cycles of 94˚C for 15 sec, 55˚C for 30 sec and 70˚C for 30 sec. GAPDH and U6 small nuclear RNA were used as internal controls for mRNA and miRNA, respectively. We chose $2^{-\Delta\Delta Cq}$ method for evaluation of data (31). The reactions were performed in triplicate for each sample. The primers involved were listed as follows: IL-6 forward (F), 5'-ACGGCC TTCCCTACTTC-3', and reverse (R), 5'-GCTGGACTGTTT CTAATGC-3'; TNF-α F, 5'-GGGTGTTCATCCATTCTC-3', and R, 5'-GGAAAGCCCATTTGAGT-3'; HMGB1 F, 5'-GGA GTGGCTTTTGTCCCTCAT-3', and R, 5'-TGCCTCTCGGCT TTTTAGGA-3'; GAPDH F, 5'-GGTTGTCTCCTGCGACTT CA-3', and R, 5'-GGTGGTCCAGGGTTTCTTACT-3'; U6 F, 5'-CTCGCTTCGGCAGCACA-3', and R, 5'-AACGCTTCA

CGAATTTGCGT-3'. These primer sequences were supported by a previous literature (32). Primers for hsa-miR-23a-3p and mmu-miR-23a-3p were purchased from Exiqon A/S (Qiagen AB).

Western blotting. Total protein was extracted in RIPA lysis buffer (Beyotime Institute of Biotechnology). After determining the protein concentration using a Bradford protein assay (Bio-Rad Laboratories, Inc.), 20 μg samples were loaded for the standard procedures of western blotting. In brief, 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and polyvinylidene fluoride membrane (MilliporeSigma) were used, and the blocking was performed in 5% skim milk for 1 h at 25˚C. β-actin served as a loading control to normalize protein levels. Primary antibodies including HMGB1 (cat. no. ab92310; 1:2,000; Abcam) and β-actin (cat. no. ab179467; 1:10,000; Abcam) were incubated overnight at 4˚C, and secondary antibody goat anti-rabbit IgG H&L (HRP-conjugated; cat. no. ab6721; 1:20,000; Abcam) was incubated at 25˚C for 1 h. Then, protein blot signals were detected by enhanced chemiluminescence (MilliporeSigma) and quantified using Image Lab™ v3.0 Software (Bio-Rad Laboratories, Inc.).

ELISA. The protein levels of IL-6 and TNF-α in the culture supernatants or serum were measured using mouse IL-6 ELISA kit (cat. no. EM004-48; Genetimes Technology, Inc.) and mouse TNF-α ELISA kit (cat. no. EM008-96; Genetimes Technology, Inc.) according to the instructions of the manufacturer. The serum supernatant was obtained from whole bloods after clot formation and culture supernatant was collected from cell culture media after LPS treatment by centrifuging at 800 x g for 10 min at 4˚C. The reactions were repeated for three times for each sample.

Dual luciferase reporter assay. DianaTools (Diana Lab, University of Thessaly, Thessaly, Greece) with microT-CDS algorithm (33) determined that there was a potential target binding site for miR-23a-3p in the 3'untranslated region (3'UTR) of HMGB1. For determination, the fragment of the HMGB1 3'UTR containing the putative sequence (positions 649-669) was mutated by replacing the AAU…GC… UGUGAU of the complementary sequence. Then, the wild type and mutant of HMGB1 3'UTR (HMGB1 WT/MUT) were cloned into a pGL3 vector (Invitrogen; Thermo Fisher Scientific, Inc.). RAW264.7 cells were co-transfected with HMGB1 WT/MUT (2 μg) and miR-23a-3p/NC mimic (30 nM), or co-transfected with HMGB1 WT/MUT (2 μg) and in-miR-23a-3p/NC (30 nM) using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). pRL-TK plasmids were used as an internal control and co-transfected with those at a dose of 50 ng. Every transfection group was carried out in triplicate. After 48 h, transfected cells were collected to measure *Firefly* and *Renilla* luciferase activity using a dual-luciferase reporter assay system (Promega Corporation), and relative luciferase activity was the ratio of *Firefly* and *Renilla*.

Statistical analysis. Data are presented as the mean ± standard error of mean. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.). Differences between groups were evaluated using one-way analysis of variance followed by Tukey's post hoc test, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of HMGB1 is increased and miR-23a-3p is downregulated in patients with sepsis. Whole blood from healthy volunteers (control; n=12) and patients with sepsis (sepsis; n=20) was collected, and serum and PBMCs were subsequently isolated. Expression levels of miR-23a-3p and HMGB1 were measured, as were the levels of pro-inflammatory cytokines. RT-qPCR analysis demonstrated that mRNA expression of two critical pro-inflammatory cytokines, IL-6 and TNF-α, was significantly elevated in sepsis group PBMCs compared with the control group (Fig. 1A and B). In addition, ELISA data showed that the secretion of IL-6 and TNF-α was significantly higher in serum and PBMCs from the sepsis group compared with the control group (Fig. 1C and D). In patients with sepsis, expression of miR-23a-3p was significantly downregulated (~0.22-fold) and the level of HMGB1 protein was significantly upregulated (~2.15-fold) in the serum and PBMCs (Fig. 1E and F) compared with levels in controls. These results suggested an inhibition of miR-23a-3p, and a promotion of HMGB1, IL-6 and TNF-α expression in patients with sepsis, indicating a potential role for miR-23a-3p in the inflammatory response during sepsis.

miR-23a-3p and HMGB1 are differentially expressed following LPS treatment in murine macrophage cells. To identify the biological function of miR-23a-3p and HMGB1 in innate immunity, a cell model of sepsis was constructed using RAW264.7 cells stimulated by LPS. The levels of pro-inflammatory cytokines released during LPS treatment were determined compared with control cells without LPS treatment. RT-qPCR data showed that LPS induced the expression of IL-6 and TNF-α at the mRNA level compared with the control (Fig. 2A and B), accompanied with increased secretion of these cytokines into the culture supernatants (Fig. 2C and D). These findings indicated the successful construction of a LPS-induced inflammation model in murine macrophages *in vitro*. RT-qPCR and western blotting determined that the expression of miR-23a-3p was significantly reduced, and that of HMGB1 protein was increased, in LPS-stimulated RAW264.7 cells compared with control cells without LPS treatment (Fig. 2E and F). These results suggested that LPS stimulation induced an inflammatory response comparable to sepsis in RAW264.7 cells, during which miR-23a-3p was downregulated and HMGB1 was upregulated.

miR-23a-3p modulates LPS-induced inflammation in murine macrophage cells in vitro. RAW264.7 cells were transfected with miR-23a-3p mimic to overexpress miR-23a-3p, and they were transfected with in-miR-23a-3p to silence miR-23a-3p. Efficiency of transfection was evaluated via RT-qPCR. miR-23a-3p expression was significantly increased following treatment with miR-23a-3p mimic, whereas it was inhibited by transfection of in-miR-23a-3p, compared with the corresponding NCs (Fig. 3A). RT-qPCR and ELISA were used to analyze LPS-induced TNF-α and IL-6 expression. As indicated

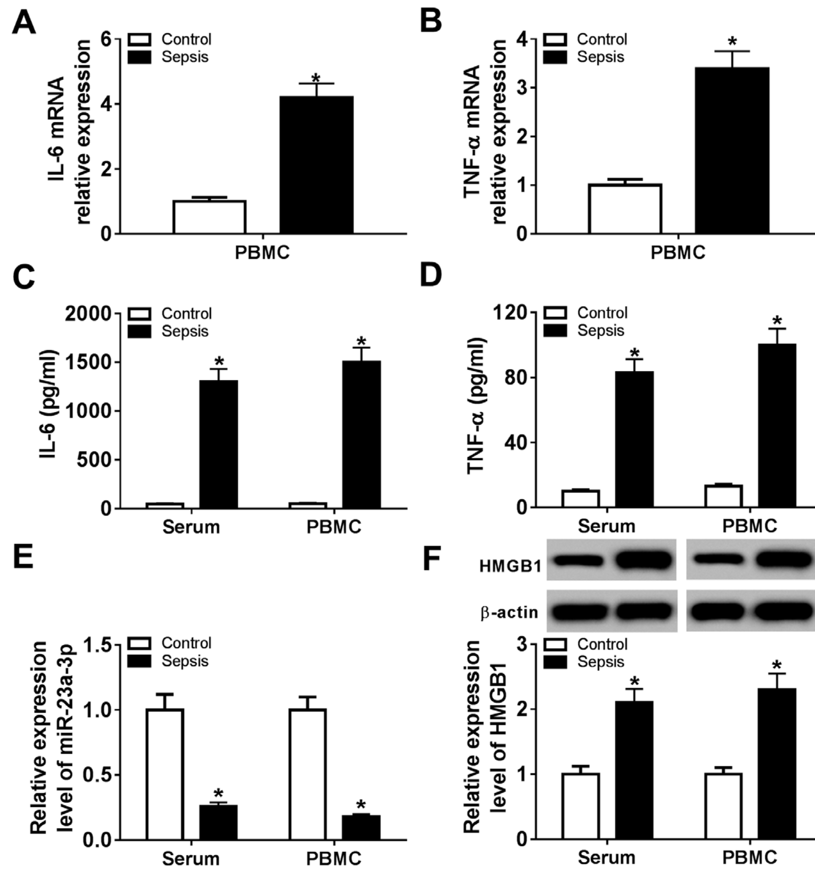

Figure 1. HMGB1 is upregulated and miR-23a-3p is downregulated in patients with sepsis. Serum and PBMCs were derived from the blood of healthy volunteers (n=12) and patients with sepsis (n=20). RT-qPCR analysis of (A) IL-6 and (B) TNF-α mRNA expression levels in PBMCs. ELISA analysis of (C) IL-6 and (D) TNF-α levels in serum and PBMCs. (E) RT-qPCR analysis of miR-23a-3p expression in serum and PBMCs. (F) Western blot analysis of HMGB1 protein expression in serum and PBMCs. *P<0.05 vs. Control. HMGB1, high mobility group box 1; IL, interleukin; miR-23a-3p, microRNA-23a-3p; PBMC, peripheral blood mononuclear cell; RT-qPCR, reverse transcription-quantitative PCR; TNF, tumor necrosis factor.

by Fig. 3B and C, overexpression of miR-23a-3p reduced IL-6 and TNF-α mRNA expression levels in RAW264.7 cells under LPS stimulation compared with control cells, whereas silencing of miR-23a-3p had the opposite effect. Moreover, the release of IL-6 and TNF-α into culture supernatants was decreased by miR-23a-3p overexpression, but promoted by miR-23a-3p inhibition (Fig. 3D and E). These data suggested miR-23a-3p may inhibit LPS-induced inflammation in murine macrophages *in vitro*.

miR-23a-3p physically targets HMGB1 via complementary binding. DianaTools *in silico* data predicted that there were complementary binding sites of miR-23a-3p in HMGB1 3'UTR at position 649-669. As presented in Fig. 4A, a HMGB1 MUT was constructed. A dual luciferase reporter assay showed that miR-23a-3p mimic transfection induced a significant decrease in the luciferase activity of HMGB1 WT in RAW264.7 cells, whereas in-miR-23a-3p transfection caused a significant increase (Fig. 4B and C). These results suggested a direct targeting relationship between HMGB1 and miR-23a-3p. RT-qPCR and western blotting data demonstrated that expression of HMGB1 at both the mRNA and the protein level was significantly inhibited in RAW264.7 cells transfected with miR-23a-3p, but it was promoted following in-miR-23a-3p transfection (Fig. 4D and E). These findings indicated that miR-23a-3p modulated LPS-induced inflammation in murine macrophages by targeting HMGB1.

Overexpression of HMGB1 attenuates the effects of miR-23a-3p overexpression in LPS-induced inflammation in vitro. RAW264.7 cells were divided into four transfection groups: miR-NC, miR-23a-3p, miR-23a-3p + pcDNA and miR-23a-3p + pcDNA-HMGB1. The expression of HMGB1 in RAW264.7 cells was evaluated via western blotting, and HMGB1 protein expression was significantly increased following pcDNA-HMGB1 transfection compared with the empty vector control (Fig. 5A). Furthermore, miR-23a-3p transfection inhibited HMGB1 in RAW264.7 cells, and this inhibition was attenuated following pcDNA-HMGB1 transfection (Fig. 5B). As presented in Fig. 5C and D, overexpression of miR-23a-3p reduced IL-6 and TNF-α mRNA expression levels in RAW264.7 cells under LPS stimulation compared with the NC, but overexpression of HMGB1 reversed this effect. Moreover, the release of IL-6 and TNF-α into the culture supernatant was suppressed by miR-23a-3p, which was subsequently reversed by HMGB1 restoration (Fig. 5E and F). These results suggested that the suppressive effect of miR-23a-3p overexpression on LPS-induced inflammation in murine macrophages was partially mediated via downregulation of HMGB1.

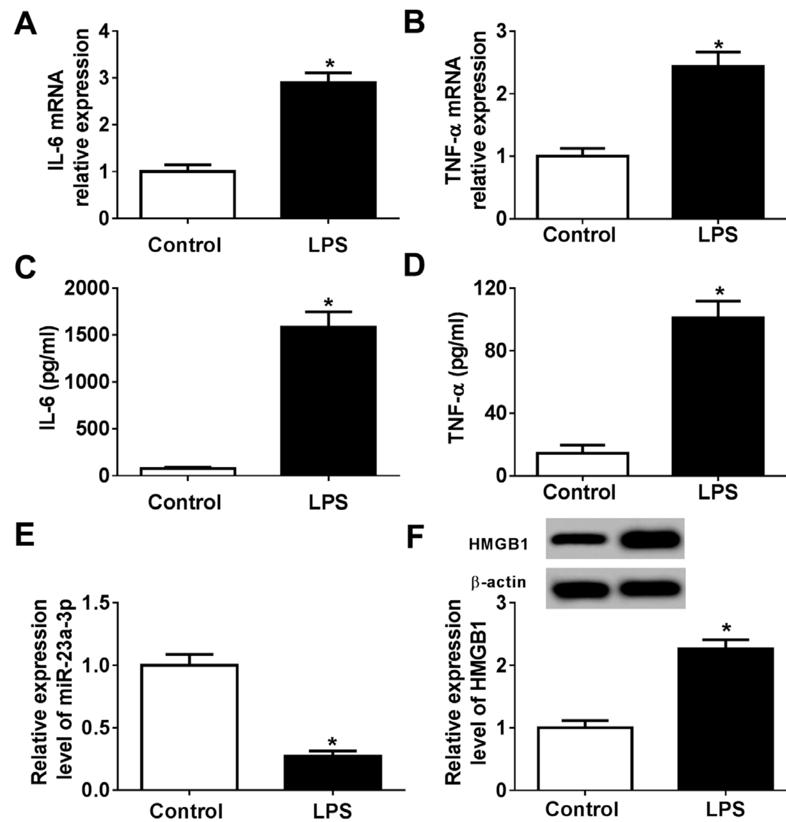

Figure 2. HMGB1 is upregulated and miR-23a-3p is downregulated following LPS stimulation of murine macrophage cells. RAW264.7 cells were exposed to 100 ng/ml LPS for 48 h to model inflammation. RT-qPCR analysis of (A) IL-6 and (B) TNF-α mRNA expression levels in RAW264.7 cells treated with LPS and control cells without LPS treatment. ELISA analysis of (C) IL-6 and (D) TNF-α levels in the culture supernatant of LPS-treated RAW264.7 cells. (E) RT-qPCR analysis of miR-23a-3p expression in RAW264.7 cells. (F) Western blot analysis of HMGB1 protein expression in LPS-treated RAW264.7 cells. *P<0.05 vs. Control. HMGB1, high mobility group box 1; IL, interleukin; LPS, lipopolysaccharide; miR-23a-3p, microRNA-23a-3p; RT-qPCR, reverse transcription-quantitative PCR; TNF, tumor necrosis factor.

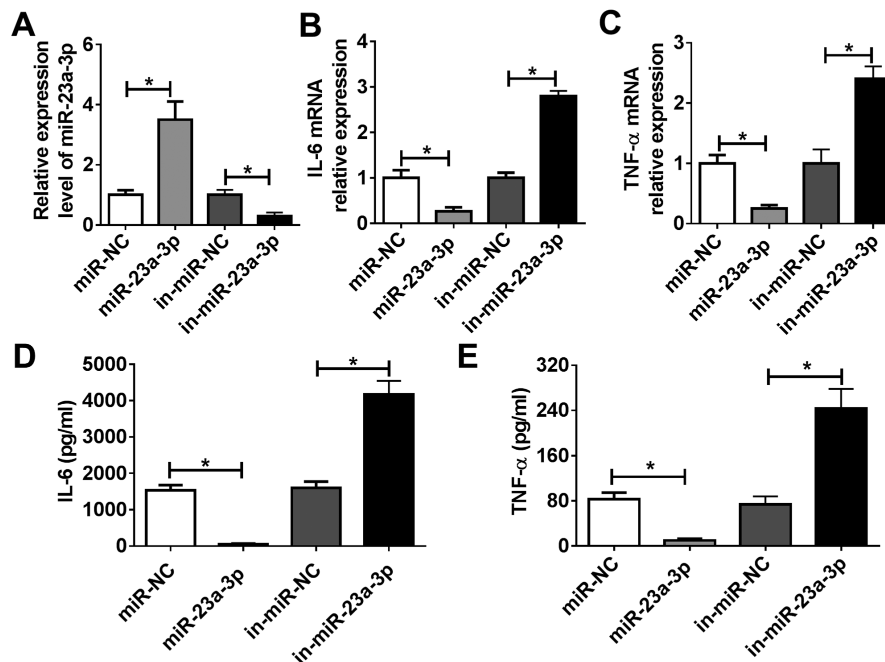

Figure 3. Role of miR-23a-3p in LPS-stimulated murine macrophage cells *in vitro*. RAW264.7 cells were transfected with miR-23a-3p mimic or in-miR-23a-3p, followed by LPS stimulation (100 ng/ml for 48 h). (A) RT-qPCR analysis of miR-23a-3p expression in RAW264.7 cells transfected with miR-23a-3p mimic or in-miR-23a-3p. RT-qPCR analysis of (B) IL-6 and (C) TNF-α mRNA levels in LPS-treated RAW264.7 cells transfected with miR-23a-3p mimic or in-miR-23a-3p. ELISA analysis of (D) IL-6 and (E) TNF-α in the culture supernatant of LPS-treated RAW264.7 cells transfected with miR-23a-3p mimic or in-miR-23a-3p. *P<0.05. IL, interleukin; in-miR, miR inhibitor; LPS, lipopolysaccharide; miR-23a-3p, microRNA-23a-3p; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; TNF, tumor necrosis factor.

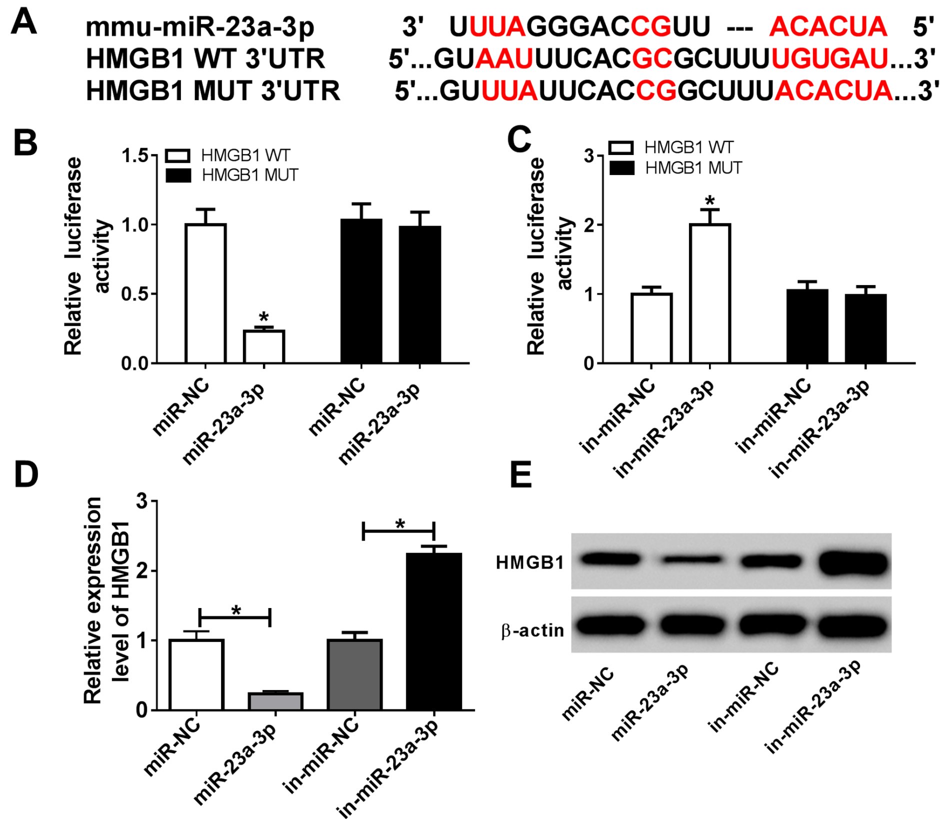

Figure 4. miR-23a-3p targets the 3'UTR of HMGB1 in murine macrophage cells. (A) DianaTools revealed ae binding site between miR-23a-3p and HMGB1 WT 3'UTR. Dual-luciferase reporter assays investigating the luciferase activity of vectors carrying HMGB1 WT 3'UTR or HMGB1 MUT 3'UTR in RAW264.7 cells transfected with (B) miR-23a-3p or (C) in-miR-23a-3p. (D) Reverse transcription-quantitative PCR and (E) western blot analyses of HMGB1 expression in RAW264.7 cells transfected with miR-23a-3p, miR-NC, in-miR-23a-3p or in-miR-NC. *$P<0.05$ vs. miR-NC, in-miR-NC or as indicated. 3'UTR, 3'untranslated region; HMGB1, high mobility group box 1; in-miR, miR inhibitor; miR-23a-3p, microRNA-23a-3p; MUT, mutant; NC, negative control; WT, wild type.

Discussion

Several preclinical studies in lethal sepsis have suggested that HMGB1 may be a promising target for improving therapeutic outcomes (34,35). In the present study, an increase of HMGB1 in patients with sepsis and LPS-challenged macrophages was observed alongside decreased expression of miR-23a-3p. In addition, overexpression of miR-23a-3p reduced, whereas silencing of miR-23a-3p elevated, pro-inflammatory cytokine expression (IL-6 and TNF-α) in RAW264.7 cells under LPS stimulation, and the inhibitory effect of miR-23a-3p overexpression on LPS-induced inflammation was attenuated following HMGB1 upregulation. Of note, HMGB1 was targeted by miR-23a-3p. These findings indicated a novel mechanism of HMGB1 in sepsis regulated by miR-23a-3p.

HMGB1 is a member of the HMGB family, which contributes to the regulation of gene expression (14,32). However, in response to LPS stimulation, HMGB1 is translocated from the nuclei to the cytoplasm of macrophages (14). Moreover, superfluous HMGB1 has been demonstrated to be secreted by macrophages and monocytes, thus participating in the occurrence of sepsis (36,37). In sepsis studies, Wang *et al* (9) indicated that HMGB1 and LPS in harmful concentrations were synergistically toxic or lethal. Additionally, it was reported that HMGB1 has a role in LPS-induced cytotoxicity for reasons that have not been fully elucidated (16,38). Deng *et al* (39) discovered that hepatocyte-released HMGB1 could bind and target LPS into the lysosomes of macrophages and endothelial cells. Therefore, HMGB1 enabled LPS to reach cytosolic caspase-11, thus forming multiple critical inflammatory mediators (40,41). In the present study, miR-23a-3p mimic-mediated upregulation of HMGB1 attenuated the expression of IL-6 and TNF-α in LPS-stimulated murine macrophages. Overexpression of HMGB1 promoted the production of IL-6 and TNF-α. Collectively, HMGB1 could be important for LPS to express its cytotoxicity.

A growing number of studies have indicated that multiple miRNAs are involved in the inflammatory response of macrophages by regulating HMGB1 expression. For example, miR-212-3p has been claimed to inhibit inflammatory responses in LPS-treated RAW264.7 cells by targeting HMGB1 (32). Zhou *et al* (29) demonstrated that HMGB1 was regulated by a handful of miRNAs, and that miR-205-5b expression was negatively associated with HMGB1 expression. Peroxisome proliferator-activated receptor γ suppresses inflammatory gene expression and pro-inflammatory transcription-factor signaling pathways in various cell types, and its agonist troglitazone mediates HMGB1 inhibition, which is associated with the upregulation of miR-142-3p in inflammatory responses *in vitro* and *in vivo* (42). In the present study, it was observed that miR-23a-3p was downregulated and inversely expressed with HMGB1 in LPS-induced RAW264.7 cells; overexpression of miR-23a-3p reduced the expression of IL-6, TNF-α and HMGB1 both at the mRNA and protein level by

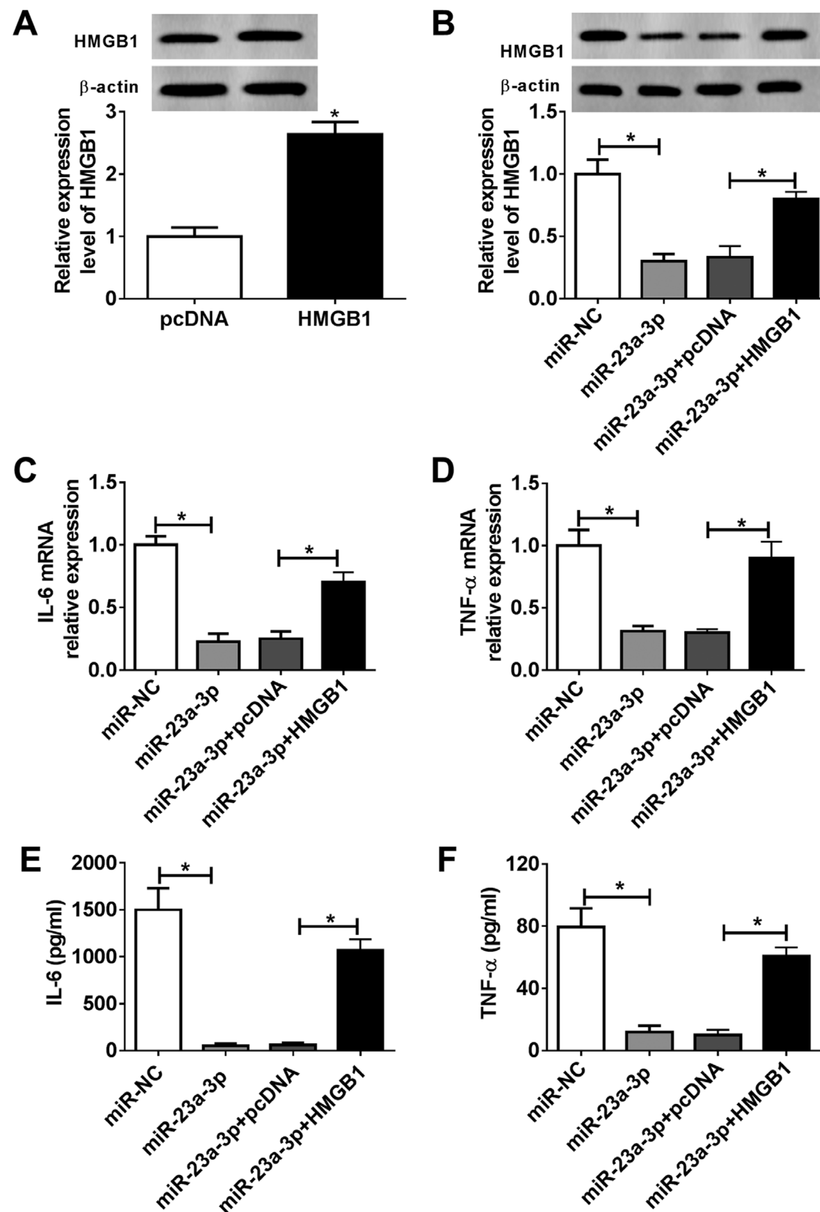

Figure 5. Role of HMGB1 in the effects of miR-23a-3p overexpression on LPS-stimulated murine macrophage cells. (A) Western blot analysis of HMGB1 protein expression in RAW264.7 cells treated with pcDNA-HMGB1 or pcDNA. RAW264.7 cells were transfected with miR-NC, miR-23a-3p, miR-23a-3p + pcDNA or miR-23a-3p + pcDNA-HMGB1 prior to LPS stimulation (100 ng/ml for 48 h). (B) Expression of HMGB1 as determined via western blotting. Reverse transcription-quantitative PCR analysis of (C) IL-6 and (D) TNF-α mRNA expression in LPS-treated RAW264.7 cells. ELISA analysis of (E) IL-6 and (F) TNF-α levels in the culture supernatant of LPS-treated RAW264.7 cells. *P<0.05 vs. pcDNA or as indicated. HMGB1, high mobility group box 1; IL, interleukin; LPS, lipopolysaccharide; miR-23a-3p, microRNA-23a-3p; NC, negative control; TNF, tumor necrosis factor.

targeting HMGB1. These data indicated the protective effect of miR-23a-3p in sepsis, and suggested that miR-23a-3p may act as a novel negative regulator of macrophage inflammation. Unfortunately, the role of miR-23a-3p in inflammation-related signal pathways, such as the MAPK (32), JAK/STAT1 (43) and NF-κB (44) pathways, was not further investigated in the present study. Animal experiments with miR-23a-3p should be performed for further investigation of the expression of IL-6, TNF-α and HMGB1 in serum and organs, including the liver, lung and kidney (29). In addition, miR-23a-3p together miRNAs were significantly decreased in both sepsis-induced acute kidney injury (AKI) and other AKI groups, and potential target genes of miR-23a-3p were predicted, including IL-6 (27). In the present study, it was suggested that IL-6 could be directly and indirectly regulated by miR-23a-3p in sepsis cell models. However, the target relationship between miR-23a-3p and IL-6 in LPS-induced RAW264.7 cells still requires further investigation.

miR-23a-3p functions are extensive. Accumulating evidence indicates that miR-23a-3p is involved in multiple diseases, including cancer, ischemia injury and inflammation. For example, in renal cell carcinoma (RCC), miR-23a-3p targeted proline-rich nuclear receptor coactivator 2 to act as an oncogene by enhancing cell proliferation and cell mobility, and inhibiting apoptosis in ACHN and 786-O cells, thus being a potential prognostic biomarker for RCC (45). miR-23a-3p, together with 8 other miRNAs, was observed to exhibit increased expression in brain tissue, leukocytes and

blood plasma 48 h after onset of photochemically-induced focal cerebral ischemia (24). Moreover, it was also reported that oxidative stress injury was alleviated in a mouse model of focal cerebral ischemia-reperfusion (46). Several studies indicated the downregulation of miR-23a-3p under inflammation. For example, inflammation response affected the miRNA profile of the male reproductive tract; five miRNAs, including miR-23a-3p, let-7f-5p, miR-200c-3p, miR-23b-3p and miR-98-5p, exhibited >2-fold downregulation after intraperitoneal injection of LPS in rats for 3 h (26). Serum miR-23a-3p was lower in sepsis-induced human AKI, as well as miR-4456, miR-142-5p, miR-22-3p and miR-191-5p (27). Furthermore, 20 circulating inflammation-associated miRNAs were downregulated in sepsis compared with SIRS, and miR-23a-3p was one of the top 6 most differentially expressed miRNAs in severe sepsis (1). However, there remained a lack of detailed information upon the dysregulation of miR-23a-3p and its molecular regulatory mechanism. Therefore, the expression of miR-23a-3p in sepsis was investigated. The results showed miR-23a-3p was downregulated to ~0.22-fold in patients with sepsis and an LPS-induced cell model of sepsis. Functionally, upregulation of miR-23a-3p resulted in the inhibitory influence on inflammation with decreased expression of IL-6 and TNF-α via targeting HMGB1.

The present study provided novel insight into regulation of HMGB1 by miR-23a-3p, and the miR-23a-3p/HMGB1 axis may represent a clinically relevant potential pharmacological target for effective therapeutic intervention in sepsis. Nevertheless, it should also be noted that targeted therapies, including those involving monoclonal antibodies or antagonists, could be limited, due to redundancy in the inflammatory response (47).

Collectively, it was demonstrated that miR-23a-3p negatively regulated LPS-induced inflammatory cytokine secretion in murine macrophages *in vitro*. Additionally, a novel mechanism for HMGB1 in sepsis was uncovered that was mediated by miR-23a-3p. Functional experiments suggested an inhibitory effect of miR-23a-3p on inflammatory cytokine expression via direct downregulation of HMGB1.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QS and BW analyzed and interpreted the patient data, performed all experiments and wrote the first draft. ML designed the experiments, agreed to be accountable for all aspects of the work, revised this manuscript critically and gave the final approval of the version to be published. All authors read and approved the final manuscript. QS and BW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Ethical approval was granted by the Ethics Committee of Jingzhou Central Hospital of Hubei. Participants provided their written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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