

miR-23a downregulation modulates the inflammatory response by targeting ATG12-mediated autophagy

XIANG SI^{1*}, DAIYIN CAO^{2*}, JUAN CHEN¹, YAO NIE¹, ZHIYI JIANG¹,
MIN-YING CHEN¹, JIAN-FENG WU¹ and XIANG-DONG GUAN¹

¹Department of SICU, The First Affiliated Hospital of Sun Yat-sen University; ²Department of Critical Care Medicine, The Sixth Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510000, P.R. China

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Abstract. Autophagy, part of the innate immune defense mechanisms, is activated during the initial phase of septic insult. Previous studies indicated that micro (mi)RNAs are additionally involved in the host response to sepsis; however, the association between miRNAs and autophagy during this process is not fully understood. To study the role of miRNA (miR)-23a in autophagy initiated by sepsis, macrophages treated with lipopolysaccharides, in addition to blood samples from patients, were evaluated for miR-23a expression levels. Cell viability, inflammatory mediators and autophagic markers were investigated following overexpression or inhibition of miR-23a. The results suggested that miR-23a was suppressed subsequent to septic insult, promoting autophagy and suppressing a hyper inflammatory response, leading to enhanced cell viability. A luciferase assay and western blot analysis confirmed ubiquitin-like protein ATG12 to be the target of miR-23a. The present study revealed that the downregulation of miR-23a regulates an inflammatory response during septic insult via autophagy promotion.

Introduction

Sepsis is the third most common disease in the USA, causing ~300,000 cases of mortality annually (1). Despite the availability of various potent antibiotics, the mortality rates of sepsis have

failed to reduce (2), suggesting that a more comprehensive approach is required in its management. Genome wide expression studies indicated that pro- and anti-inflammatory cytokines encoding genes were upregulated during the host immune response (3), and the disruption of this balance may lead to hyper-inflammation (4) and immunosuppression (5).

Autophagy is an evolutionary conservative process critical for cell survival under stress (6). A microarray assay demonstrated that autophagy related gene lysosomal associated membrane protein 1 was upregulated in patients with sepsis compared with patients with non-infectious systemic inflammatory response syndrome (SIRS), which is defined as the SIRS associated with previously existing non-infectious diagnosis, while no sign of ongoing infection was observed (7). Polymorphisms of the immunity related GTPase M gene, which is autophagy-associated, was additionally associated with mortality due to sepsis, suggesting a protective role for autophagy (8). To investigate this hypothesis, various factors potentially regulating autophagic activities were studied, including microRNA (miRNA).

miRNAs are a group of short single-stranded RNA, ~21 nucleotides in length. miRNAs cause the degradation of target mRNA by selectively binding to its 3'untranslated region (UTR), fine-tuning gene expression post-transcriptionally (9). Previous studies demonstrated that the expression profile of microRNAs differs significantly between patients with sepsis and patients with non-infectious SIRS (10). A similar alteration was additionally confirmed in microarray studies focused on macrophages stimulated by lipopolysaccharide (LPS) (11). The two aforementioned studies demonstrated miR-23a downregulation in sepsis; however, the underlying mechanism for the involvement of miR-23a in sepsis response remains unclear.

In the present study, microRNA expression profiling data from previous studies were adopted and analyzed. Additionally, the downregulation of miR-23a during septic insult was confirmed with *in vivo* and *in vitro* experiments. Further study on the acting mechanism of miR-23a was conducted, aiming to better understand the molecular mechanism for sepsis response.

Materials and methods

Gene expression omnibus (GEO) data analysis. To identify a group of microRNAs that shared a similar expression pattern

Correspondence to: Dr Xiang-Dong Guan, Department of SICU, The First Affiliated Hospital of Sun Yat-sen University, 58 Zhongshan Second Road, Yuexiu, Guangzhou, Guangdong 510000, P.R. China
E-mail: guanxiangdong1962@163.com

*Contributed equally

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GEO, gene expression omnibus; IL, interleukin; LPS, lipopolysaccharide; miRNA/miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SIRS, systemic inflammatory response syndrome; TNF, tumor necrosis factor; UTR, untranslated region

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following LPS stimulation, clustering analysis on a dataset from the GEO database (12) was conducted. The dataset (accession no. GSE55414) contains expression profile from a previous study (11) was adopted in the present study. This dataset (accession no. GSE55414) evaluated miRNA levels at different time points following LPS stimulation. The initial screening of data was performed using Linear Models of Microarray Analysis 3.3 in R 3.4.2 (13), with a false discovery rate and minimal log fold change set as 0.05 and 1, respectively. Clustering analysis of the filtered microarray expression data was conducted with heatmap 1.0.8 (14) utilizing the Euclidean distance algorithm.

Cell culture. To conduct the *in vitro* study on macrophages, the RAW264.7 macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) at 37°C. The 3rd passage of cells were used for further experiments.

Cell transfection. Manipulation of the miRNA (miR)-23a expression level was achieved by oligonucleotide transfection. RAW264.7 cells were cultured in six-well plates at a concentration of 2×10^5 /well prior to transfection. miR-23a mimics (100 nM; 5'-AUCACAUGCCAGGGGAUUUCC-3') and miR-23a inhibitor (100 nM; 5'-GUGGUAUCCUGGC AAUGUGAU-3') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China), and a miRNA negative control was purchased from Exiqon, Inc. (10 nM, www.qiagen.com/us/shop; cat. no. 479903; Woburn, MA, USA). A Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) system was employed for microRNA transfection following the manufacturer's protocol. Following incubation at 37°C for 1 day, three groups of cells were generated, denoted as the control, miR-23a mimics and miR-23a inhibitor groups, respectively. The expression level of miR-23a was confirmed in all three groups of cells with a reverse transcription-quantitative polymerase chain reaction (RT-qPCR), the detailed protocol was described later in this article.

Immunofluorescence staining. To study the influence of the miR-23a expression level on autophagy activity, immunofluorescence imaging of autophagy markers was employed. RAW264.7 macrophages were fixed with 4% formaldehyde for 15 min at room temperature and incubated in 5% Tris buffered saline with Tween-20 (TBS-T; pH 8.3) diluted non-fat dry milk for 1 h. Immunofluorescence staining was performed using Microtubule-associated protein light chain 3 (LC-3) primary antibody (1:100; Abcam, Cambridge, UK; cat. no. ab62720) incubation overnight at 4°C and subsequent secondary antibody (1:200; Alexa Fluor 488 anti-rabbit IgG; Thermo Fisher Scientific, Inc.; cat. no. A10235) incubation at room temperature for 1 h. Cells were incubated using DAPI (100 ng/ml) for nuclear staining at room temperature for 30 min. Images were obtained from a confocal laser scanning microscope (LSM710; Zeiss, Oberkochen, Germany) at magnification, x600. LC3 puncta were

quantified with Image-J 1.51 (National Institutes of Health, Bethesda, MD, USA) (15).

Blood sampling and miRNA isolation. To further confirm the association between miR-23a levels and the host response to sepsis in the clinical setting, blood samples were collected from 27 patients with sepsis and 22 patients with non-infectious SIRS admitted to the SICU department of The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) during the period of June 2016 to February 2017. Patients under the age of 18 or for whom it was impossible to obtain informed consent within 6 h of admission were excluded. Patients were categorized as sepsis or non-infectious SIRS using standard criteria (16), and a blood microbial culture assay result was obtained from all patients as required by the standard criteria (17). Demographic data and Sequential Organ Failure Assessment scores are listed in Table I, revealing no statistically significant difference. miRNA isolation from plasma samples was conducted using a mirVana kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Informed consent was obtained from all individual participants included in the present study. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University.

ELISA. An ELISA was employed to evaluate the inflammatory response of macrophages subjected to LPS stimulation. RAW264.7 cells were seeded on 24-well plates at 4×10^4 /well. Following LPS (10 µg/ml, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) stimulation for 4 h in room temperature, the cell culture supernatant was collected. Subsequently, inflammatory cytokine levels of interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α were measured with ELISA kits (cat. nos. 550950 and 560478; BD Biosciences, Franklin Lakes, NJ, USA), following the manufacturer's protocol.

Western blot analysis. Protein expression levels were evaluated with western blot analysis. Cells were seeded on 6 well plates at a density of 1×10^6 per well. Western blotting was conducted following the protocol reported in a previous study (18). Total protein extracts were obtained with a radioimmunoprecipitation assay and transferred to nitrocellulose membranes. The membranes were incubated with blocking solution overnight, and primary antibodies were subsequently applied and incubated at room temperature for 2 h. Then the membranes were incubated with secondary antibody at room temperature for 1 h. Primary antibodies employed in the present study included LC3 (1:1,000; cat. no. ab48394; Abcam), Beclin-1 (1:1,000; cat. no. 3495; Cell Signaling Technology, Inc., Danvers, MA, USA), ATG12 (1:1,000; cat. no. ab155589) and Sequestosome-1 (p62; 1:1,000; cat. no. ab91526; both Abcam), and β-actin (1:1,000; cat. no. sc-130657; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used as a loading control. HRP-conjugated anti-rabbit (1:5,000; cat. no. ab205718) was used as secondary antibody. Image-J 1.51 was used for densitometry analysis.

RT-qPCR analysis. RNA expression levels were evaluated with RT-qPCR. Total RNA was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed to cDNA with the QuantiTect RT kit (Qiagen, Inc., Valencia, CA, USA). RT-qPCR was performed

Table I. Demographic data and SOFA scores of patients included in the present study.

Group	Age, years	Statistic	P-value	Male/female ratio	Statistic	P-value	SOFA score	Statistic	P-value
Sepsis, n=27	62.3±10.3	-1.768 ^a	0.107	13:14	0.025 ^b	0.874	8.1±2.3	1.299 ^a	0.215
Non-infectious SIRS, n=22	64.2±11.5			12:10			7.92±2.6		

^aIndependent t-test, ^b χ^2 test. SOFA, Sequential Organ Failure Assessment; SIRS, systemic inflammatory response syndrome.

Table II. Primers for reverse transcription-quantitative polymerase chain reaction analysis with the SYBR green system.

mRNA	Sense (5'-3')	Anti-sense (5'-3')
TNF- α	GTGAGGAGGACGAACATC	GAGCCAGAAGAGGTTGAG
IL-6	TGACCCAACCACAAATGC	TGACCAGAAGAAGGAATGC
GAPDH	TCATCCCTGCCTCTACTG	TGCTTCACCACCTTCTTG

TNF, tumor necrosis factor; IL, i nterleukin.

with a SYBR Mix (Qiagen, Inc) and the Bio-Rad Real-time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following a protocol used in a previous study (19), these cycling parameters were used: Initial denaturation at 95°C for 30 sec, followed by 35 cycles: Denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec and extension at 72°C for 30 sec. The expression level of mRNAs were first normalized to the GAPDH mRNA level, and the $2^{-\Delta\Delta C_q}$ method (20) was used for quantification. The relative expression level was compared between the three groups of cells. For microRNAs, the U6 level was used as the internal control. AlleleID 6 (Palo Alto, CA, USA, www.premierbiosoft.com/) was used for primer design and sequences are provided in Table II.

Luciferase reporter assays. To confirm the targeting association between miR-23a and ATG12 mRNA, luciferase reporter assays were conducted. The PsiCHECK-2 system (Promega Corporation, Madison, WI, USA) was used for the construction of the dual-luciferase assay plasmid. Bioinformatics analysis tool miRanda (microrna.org/) revealed a potential binding sequence for miR-23a in the 3'UTR of ATG12 mRNA. The predicted target sequence was cloned into the PsiCHECK-2 plasmid (Promega), generating an ATG12 wild-type (wt) dual-luciferase reporter plasmid. Subsequently, the mutated target sequence was generated with site-directed mutagenesis and was additionally cloned into the PsiCHECK-2 plasmid system, creating an ATG12 mutant (mut) reporter plasmid. RAW264.7 cells with miR-23a mimics, miR-23a inhibitor and negative controls were first seeded on 96-well plates at a concentration of 1×10^4 /well and transfected with ATG12 wt or ATG12 mut (200 ng/ml) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Luciferase activities were evaluated with the Modulus[™] dual-luciferase reporter assay system (Turner Designs, Sunnyvale, CA, USA) following 48 h incubation, normalized to *Renilla* activity, and all assays were performed in triplicate.

Cell viability assay. A cell viability assay was performed to study the influence of the miR-23a expression level on cell survival subsequent to LPS stimulation. All three groups of cells were seeded on 96-well plates at 5×10^3 cells per well. A Cell Counting kit-8 (Engreen Biosystem, Ltd., Auckland, New Zealand) was used for cell viability evaluation, and an absorbance value at 450 nm was used for the quantification of cell viability.

Statistical analysis. All experiments were repeated three times and data are expressed as the mean \pm standard deviation. Comparisons between groups were made using one-way analysis of variance, and Fisher's Least Significant Difference test was used for post hoc analysis. Baseline data for patients in sepsis or Non-infectious SIRS groups was compared, independent t-test was used for SOFA score and age comparison, while χ^2 test was used for sex ratio comparison. $P < 0.05$ was considered to indicate a statistically significant difference. R studio 1.1.383 (RStudio, Inc., Boston, MA, USA) was used for statistical analysis.

Results

miRNA expression levels in LPS-stimulated macrophages. A group of miRNAs that were consistently down regulated following LPS stimulation were identified (Fig. 1A) via clustering analysis on the GEO dataset (GSE55414). Expression levels of these miRNAs were further validated by RT-qPCR analysis in RAW264.7 cells (Fig. 1B), among which the down regulation of miR-23a was the most significant ($P < 0.01$). Consistently, RT-qPCR analysis of circulating miRNAs (Fig. 1C) additionally demonstrated that the miR-23a serum level was decreased in patients with sepsis (0.524; 95% confidence interval (CI), 0.036-1.012), compared with patients with non-infectious SIRS (1.557; 95% CI, 1.191-1.923) with a statistical significance ($P < 0.05$). The receiver operating characteristic curve analysis on the miR-23a level yielded an area under the curve value of 0.758 (Fig. 1D), and at the

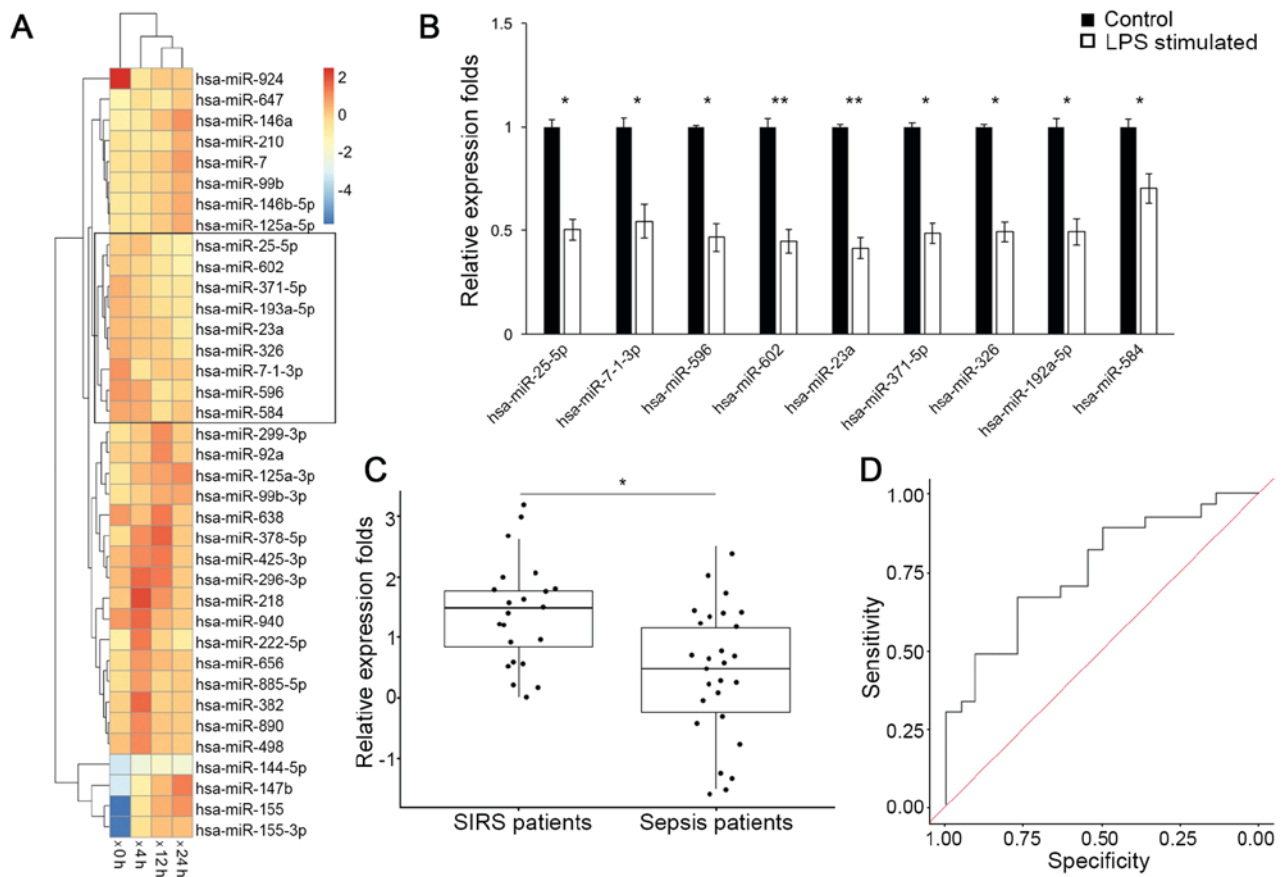


Figure 1. Expression analysis of miRNAs during the sepsis response. (A) Heat map for clustering analysis of miRNA expression data; 0, 4, 12 and 24 h are the times elapsed following LPS stimulation. (B) Relative expression fold changes of miRNAs following LPS stimulation. Expression levels prior to LPS administration were used as reference values. (C) Relative expression fold-changes of miR-23a in serum from patients with non-infectious SIRS or sepsis. (D) Receiver operating characteristic curve for the measurement of miR-23a in serum to differentiate between non-infectious SIRS and sepsis. *P<0.05, **P<0.01 vs. respective control. miRNA/miR, microRNA; LPS, lipopolysaccharide; SIRS, systemic inflammatory response syndrome.

threshold of 1.01 for miR-23a relative expression level, a sensitivity and specificity of 70.3 and 68.3%, respectively, was achieved for differentiating between patients with sepsis and patients with non-infectious SIRS. These results demonstrated that miR-23a is downregulated in response to sepsis insult.

Downregulation of miR-23a protects cells by modulating inflammatory mediators. Results of the RT-qPCR analysis on miR-23a expression levels suggested that oligonucleotide transfection was successfully conducted in all three groups of RAW264.7 cells (Fig. 2A and B). The Cell Counting kit-8 result suggested that 4 h following LPS stimulation, the cell viability of RAW264.7 cells decreased in all three groups of cells. Compared with the control group, the cell viability in the miR-23a mimics group was significantly decreased (P<0.05), while inhibition of miR-23a demonstrated a protective effect with an increased cell viability (P<0.05), suggesting that the downregulation of miR-23a is protective for cells following LPS stimulation (Fig. 2C).

Expression levels of the pro-inflammatory cytokines IL-6 and TNF- α were evaluated with RT-qPCR (Fig. 2D and E) and ELISA (Fig. 2F and G). A positive association was identified between miR-23a and IL-6 or TNF- α levels, suggesting a pro-inflammatory role for miR-23a. These results indicated that the protective effect of miR-23a downregulation following

LPS stimulation is associated with the regulation of inflammatory factors.

Effects of mir-23a on autophagic activity. The western blot analysis of the autophagy markers demonstrated an increase in the LC3 conversion (LC3-II/LC3-I ratio) (Fig. 3A) in the miR-23a inhibition group compared with the control group. Immunofluorescence imaging and LC3 puncta quantification further confirmed that miR-23a inhibited autophagic activity in macrophages following LPS stimulation (Fig. 3B and C). The result of western blot analysis also shown that other autophagy markers were influenced by miR-23a level after LPS stimulation (Fig. 3A). Compared with control group, beclin-1 was upregulated while p62 was downregulated in miR-23a inhibited cells. Such negative association between miR-23a level and autophagy markers was further confirmed with densitometry analysis (Fig. 3D-F; P<0.05), while no significant association was found between them in macrophages prior to LPS stimulation. These result is consistent with a previous study (21).

miR-23a targets ATG12 specifically to modulate autophagic activity. ATG12 serves an integral role in the autophagy pathway by forming a complex with Autophagy protein 5(ATG5) (22). Bioinformatics analysis tools miRanda (microrna.org/) suggested that miR-23a may bind to ATG12

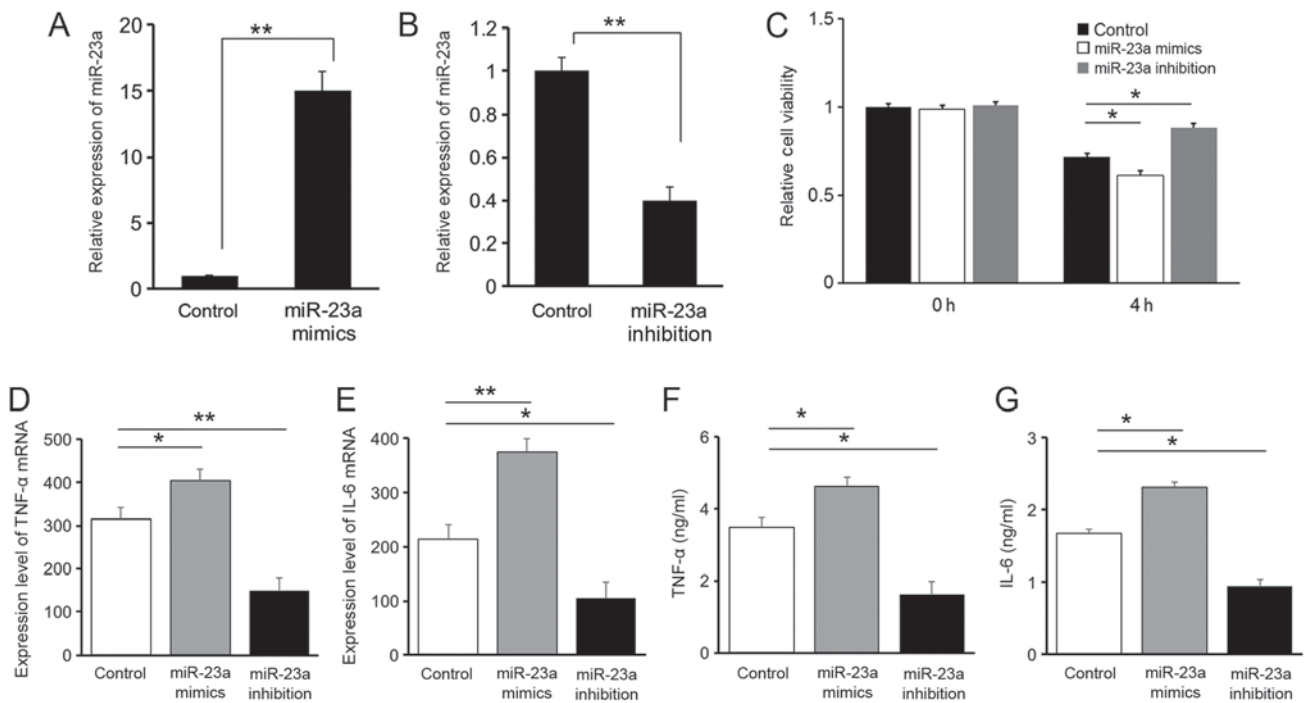


Figure 2. Influence of miR-23a on inflammatory mediators. The expression level of miR-23a in the negative control was compared with (A) the miR-23a mimics and (B) the miR-23a inhibition groups. miR-23a expression in the negative control group was used as reference. (C) Relative cell viability was assessed by Cell Counting kit-8 analysis. The expression levels of (D) TNF- α and (E) IL-6 were measured. ELISA was used to measure the concentrations of (F) TNF- α and (G) IL-6. * $P < 0.05$, ** $P < 0.01$. miR, microRNA; TNF, tumor necrosis factor; IL, interleukin.

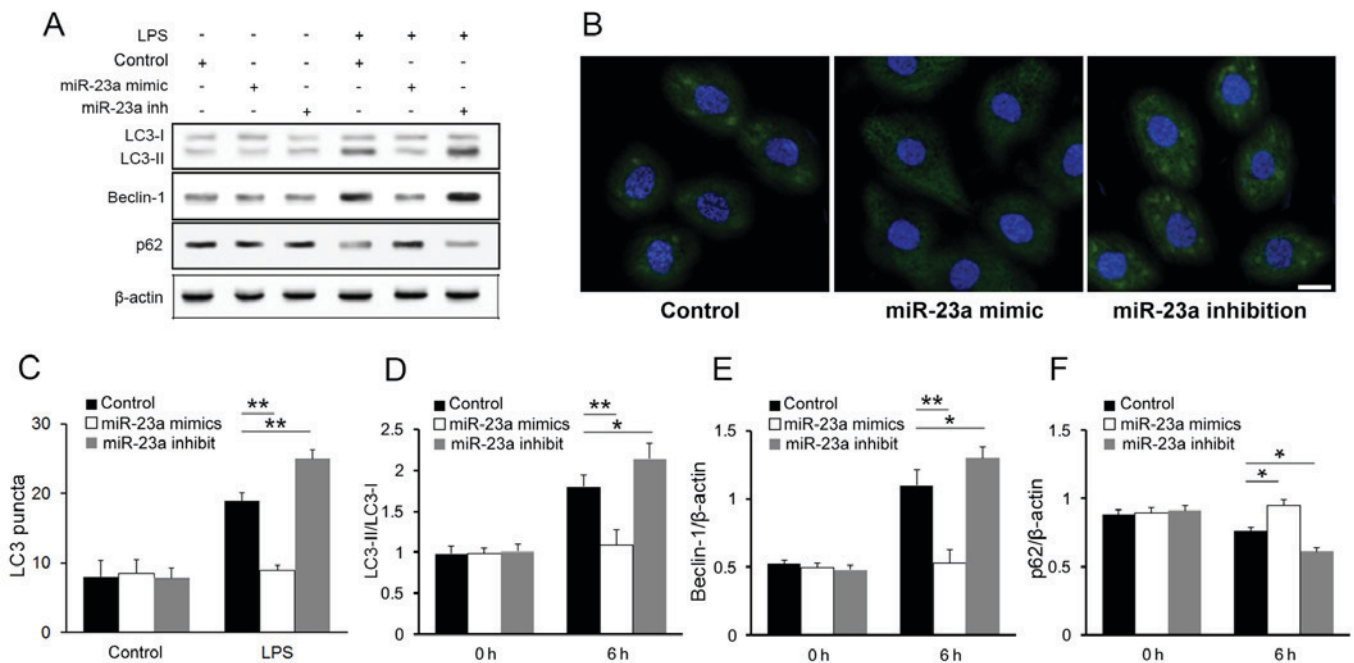


Figure 3. Modulation of autophagy by miR-23a. (A) Western blotting results for autophagy markers. (B) LC3 immunofluorescence staining results subsequent to LPS stimulation. Scale bar, 20 μ m. Densitometry analysis of the autophagy markers (C) LC3-II/LC3-I, (D) beclin-1 and (E) p62. (F) LC3 puncta quantification of immunofluorescence results. Blue staining represents nuclei; green staining represents LC3. * $P < 0.05$, ** $P < 0.01$. LPS, lipopolysaccharide; miR, microRNA; LC3-II/LC3-I, microtubule-associated protein light chain 3 conversion ratio.

selectively via a specific sequence in the 3'UTR of ATG12 mRNA (Fig. 4A). A dual-luciferase assay was conducted for confirmation and a negative association was identified between luciferase activities and miR-23a levels in cells transfected

with the ATG12-wt plasmid; however, no significant difference was observed between groups when ATG12-mut plasmids were applied ($P > 0.05$), suggesting that miR-23a targets ATG12 specifically (Fig. 4B). Downregulation of

ATG12 expression by miR-23a was further confirmed with western blot analysis (Fig. 4C).

Discussion

Sepsis is a principal challenge in the management of critically ill patients (1). The incomplete understanding of mechanisms underlying organ damage and immune defense during septic insult hinder the development of more comprehensive management (23). Accumulating evidence suggests that autophagic activity is increased during the initial phase of sepsis (24,25) and LPS, a key bacterial product, may initiate autophagy through toll-like receptor 2 and toll-like receptor 4 activation (26,27). Two studies indicated a protective role for such a response (24,28); however, the detailed underlying mechanism requires further investigation.

miRNA are a group of small non-coding RNAs that act as post-transcriptional regulators in various processes. The results of the present study demonstrated that multiple microRNAs inhibiting autophagy (29-32) were downregulated subsequent to LPS stimulation. Among these, the downregulation of miR-23a was the most significant. The *in vivo* study additionally demonstrated that the miR-23a serum level in patients with sepsis differed distinctly compared with patients with non-infectious SIRS, a result consistent with previous studies (33,34). These results collectively demonstrate that miR-23a is involved in the host response to sepsis.

The present results from the western blot analysis and immunofluorescence studies demonstrated for the first time, to the best of the authors' knowledge, that miR-23a is negatively associated with autophagic activities following septic insult. Downregulation of miR-23a mitigates the inhibition of autophagy, leading to the suppression of inflammatory mediators, a role proposed in a previous study for autophagy in sepsis response (35).

The present bioinformatics study and dual luciferase analysis indicated that miR-23a suppressed autophagy via ATG12. The targeting association was demonstrated with the dual-luciferase study, and a negative association was revealed between the miR-23a and ATG12 expression levels. The inhibitory effect that miR-23a exerts on ATG12 was further demonstrated by the LC3-II/LC3-I ratio and immunofluorescence imaging in the present study, since ATG12, along with autophagy protein 5 and autophagy-related protein 16-1 comprise the E3 ubiquitin ligase, which facilitates LC3 family conversion from LC3-I to LC3-II (22).

In conclusion, the present study demonstrated that miR-23a was downregulated during initial septic insult. It was demonstrated for the first time, to the best of the authors' knowledge, that miR-23a downregulation promoted the autophagic activity of macrophages in response to LPS stimulation, and this consequently suppressed inflammatory mediators, preventing an overwhelming inflammatory response. Finally, it was demonstrated that miR-23a may selectively bind to the 3'UTR of ATG12 mRNA, modulating the formation of the E3 ligase and the subsequent facilitation of LC3 conversion.

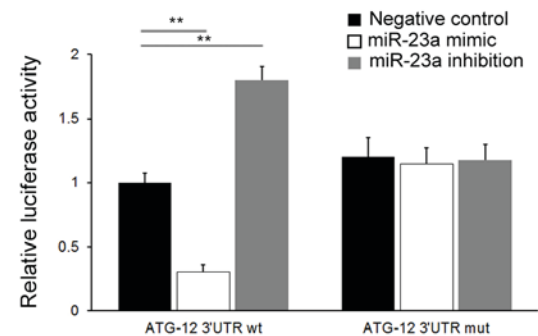
Acknowledgements

Not applicable.

A

ATG12 3' UTR WT 5' auggacucagaaaAAUGUGAu 3'
hsa-miR-23a 3' ccuuuagggaccgUUACACUa 5'
ATG12 3' UTR mut 5' auggacucagaaaAAacacAU 3'

B



C

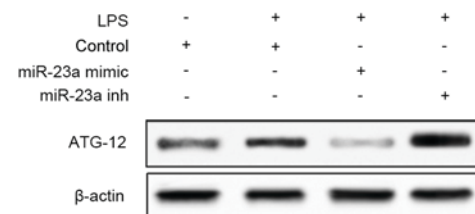


Figure 4. Luciferase and western blot analysis indicated ATG12 to be the target of miR-23a. (A) Predicted binding site for miR-23a in 3'UTR of ATG12 mRNA, and the mutated sequence. (B) Luciferase activity comparison between ATG12-wt and ATG12-mut plasmid. (C) Western blot analysis of ATG12 in the negative control, miR-23a mimics and miR-23a inhibition groups. ** $P < 0.01$. ATG12, ubiquitin-like protein ATG12; miR, miRNA; UTR, untranslated region; wt, wild-type; mut, mutant.

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Availability of data and materials

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

Authors' contributions

XS and DC carried out the statistical analysis and drafted this article. JC and YN were responsible for blood sampling and data analysis. ZJ, M-YC and J-FW carried out western blot and RT-qPCR analyses. X-DG conceived the idea and provided guidance.

Ethics approval and consent to participate

Informed consent was obtained from all individual participants included in the present study. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University [approval no. (2016)025].

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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