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Regular paper

MiR-135a is highly expressed and aggravates inflammatory response in sepsis by targeting MYOM1

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Background: Our research attempted to explore the effect of miR-135a on lipopolysaccharide (LPS)-induced THP-1cells damage and its potential mechanism. Methods: LPS (1 μg/mL) was selected to mimic the injury of sepsis *in vitro*. qRT-PCR was used to detect miR-135a expression. The association between miR-135a and Myomesin 1 (MYOM1) was speculated bythe predication website and confirmed by the dual-luciferase assay. MYOM1 expression was observed by qRT-PCR and western blotting assays. The biological properties of THP-1cells were analyzed by cell counting kit-8 and flow cytometry assays. The concentration of TNF-α, IL-6 and IL-8 in cell supernatant was calculated by enzyme-linked immunosorbent assay. Results: A marked augmentation of miR-135a was observed in LPS-induced THP-1 cells. Moreover, depletion of miR-135a alleviated the LPS-induced THP-1 cells injury from the perspective of improving cell viability and reducing cell apoptosis. Importantly, MYOM1, which was under expressed in LPS-induced THP-1 cells, was identified as a target of miR-135a and negatively regulated by miR-135a. Additionally, the mitigative impact of miR-135a inhibitor on THP-1cells damage triggered by LPS was suppressed by MYOM1 depletion. Conclusions: Our study suggested that the protective effect of miR-135a inhibitor on LPS-induced THP-1 cells injury was realized by regulation of MYOM1, which might afford a pair of novel molecules for sepsis clinical diagnosis.

Keywords: inflammation, LPS, miR-135a, MYOM1, sepsis

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Abbreviations: FBS, fetal bovine serum; ICU, intensive care unit; LPS, lipopolysaccharide; miRNAs, microRNAs; MYOM1, myomesin 1; NC, negative control; WT, wild type

INTRODUCTION

Sepsis is a severe disease, which originated from an infection and a systemic inflammatory reaction (Melvan *et al.*, 2010). Sepsis, one of the primary reasons of death in intensive care unit (ICU) patients, is characterized by the dysfunction of an organ and systemic inflammation reaction syndrome (Zhou *et al.*, 2016). Despite the presence of 170 different biomarkers, the complex clinical syndrome of sepsis and associated mortality have elevated morbidity (Silman, 2013). Therefore, more effective biomarkers need to be explored.

MicroRNAs (miRNAs), as non-coding RNA molecules with 17-22 nucleotides, can block mRNA expression and suppress the translation by directly modulating the specific mRNA (Mohr & Mott, 2015; Simonson & Das, 2015). Importantly, miRNAs have been reported to be implicated in multiple biological advances (Iorio & Croce, 2012; Catela Ivkovic *et al.*, 2017; Zendjabil *et al.*, 2017). Some reports claimed that miRNAs were tightly connected with the development of sepsis and they might serve as an important therapeutic target for sepsis (Benz *et al.*, 2016; Kingsley & Bhat, 2017). For example, Zhou and others (Zhou *et al.*, 2017) discovered that miR-155 suppressed the cardiac dysfunction caused by sepsis through targeting JNK. Additionally, miR-205-5b promoted the development of sepsis triggered by LPS through targeting HMGB1 (Zhou *et al.*, 2016). Moreover, miR-199a-5p has been reported to promote the intestinal barrier dysregulation in sepsis *via* targeting SP-D and activating the NF-κB pathway (Du *et al.*, 2020).

MiR-135a has been found to participate in a variety of diseases (Zhang *et al.*, 2019). For example, exhaustion of miR-135a alleviated the injury of acute pancreatitis via targeting FAM129A (Zhang *et al.*, 2019). Additionally, miR-135a has been noted for its pivotal role in various cancers. MiR-135a inhibited the malignant proliferation and diffusion of non-small cell lung cancer cells by reducing ROCK1 protein. Besides, miR-135a suppressed the invasion and migration of esophageal cancer stem cells *via* targeting Smo. Importantly, miR-135a was reported to be highly expressed in the serum of sepsis patients and aggravated the myocardial depression in sepsis (Zheng *et al.*, 2017). However, the effect of miR-135a on the biological behaviors of cells in sepsis and its underlying mechanism is not revealed.

In this study, we observed that miR-135a expression was increased in LPS-induced THP-1cells. Depletion of miR-135a suppressed the damage of THP-cells triggered by LPS. Moreover, we identified Myomesin 1 (MYOM1) as a target of miR-135a and down-regulated in LPS-induced THP-1cells. Importantly, we also discovered that knockdown of MYOM1 inhibited the alleviation effect of miR-135a inhibitor on THP-1 cells damage triggered by LPS. Collectively, our findings insinuated that miR-135a contributed to the progression of sepsis by targeting MYOM1, which afford the foundation for the targeted therapy of sepsis.

MATERIALS AND METHODS

Cell culture and transfection

The THP-1 cells were obtained from American Type Culture Collection (ATCC, Manassas, UAS). The cells

were cultured in DMEM medium including 10% fetal bovine serum (FBS) and antibiotic in a humidified atmosphere with 5% CO_2, at a 37°C incubator. The model was established by continuous stimulation with lipopolysaccharide (LPS) for 12 h.

THP-1 cells were transfected with miR-135a inhibitor (MH12315, Invitrogen, USA), negative control, pGL3-MYOM1, and pGL3-MYOM1 mut to regulate miR-135a and MYOM1 expression with the help of Lipofectamine™ 3000 (Invitrogen, USA), respectively.

RNA extraction and real-time PCR

Trizol reagent obtained from Invitrogen was applied to extract total RNA from the harvested cells according to the supplier's specification. The obtained total RNA was reverse transcribed with the support of PrimeScript® RT Master Mix (Takara, Japan). Real-time PCR was implemented to evaluate mRNA expression of MYOM1 and miR-135a. The relative expression of miR-135a and MYOM1 was measured by the $2^{-\Delta\Delta CT}$ method. U6 and GAPDH were deemed as internal controls for miR-135a and MYOM1 detection, respectively. The primers were listed below:

MiR-135a forward: 5'-GCGCTATGGCTTTTTATTCCT-3',
MiR-135a reverse: 5'-TGCAGGGTCCGAGGTAT-3';
U6 forward: 5'-CTCGCTTCGGCAGCACA-3',
U6 reverse: 5'-AACGCTTCACGAATTTGCGT-3';
MYOM1 forward: 5'-TCACTACCAGCGGGAGAAGA-3',
MYOM1 reverse: 5'-TGACATGCTTTTGACGTCCTG-3';
GAPDH forward: 5'-GCACCGTCAAGGCTGAGAAC-3',
GAPDH reverse: 5'-TGGTGAAGACGCCAGTGGA-3'.

Western blotting

The treated cells were lysed in lysis buffer. The total protein concentration was measured by the bicinchoninic acid (BCA) method. Afterwards, the same amount of protein (20 μg) was isolated by 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with non-fat milk and incubated overnight at 4°C against the following specific primary antibodies: MYOM1 (Abcam, Ab201228, 1:1000) and GAPDH (Abcam, Ab181602, 1:5000). This was followed by incubation with secondary antibodies. Finally, the blots were detected by enhanced chemiluminescence and analyzed by Image J software.

Determination of cell viability

Cell counting kit-8 (CCK-8) agent was implemented to assess cell viability. First, 1×10^3 cells were seeded into a 96-well plate and incubated overnight. At per time point (0 h, 24 h, 48 h, 72 h), 15 μL CCK-8 (Beyotime, Shanghai, China) reagent was put into per well and cultured for another 1.5 h at 37°C. Absorbance at 450 nm was detected by a microplate reader.

Enzyme-linked immunosorbent assay (ELISA)

The amount of TNF-α, IL-6 and IL-8 secreted into the culture supernatants was detected by using ELISA kit (Beyotime, Shanghai, China) based on the supplier's guidance.

Cell apoptosis detection

Flow cytometry was adopted to measure cell apoptosis with the support of an Annexin V/Propidium Iodide (PI) Apoptosis Detection Kit (Bytotime, Shanghai, China). First, THP-1 cells were collected, washed, resuspended in Annexin V binding buffer, and then stained with Annexin V and PI reagent. After gently vortex and cultivation away from the light at 37°C for 12 min, the cell apoptosis was detected by flow cytometry.

Plasmid Construction

pGL3-Basic vectors were obtained from Promega. Mutations of the miR-135a binding site of wild-type pGL3-MYOM1 plasmids were carried out using a Mut Express II Fast Mutagenesis kit following the manufacturer's instructions (Vazyme Biotech, Nanjing, China, C214-01). All constructs were confirmed by sequencing.

Dual-luciferase reporter assay

The association between miR-135a and MYOM1 was verified by dual-luciferase reporter assay. First, the wild type (wt) or mutant type (mut) of MYOM1was cloned into the pGL3 vector. Then, plasmid DNA (wt-MYOM1 or mut-MYOM1) and miR-135a inhibitor or negative control (NC) were co-transfected into cells. The luciferase activity was measured by using a double-luciferase reporter assay kit (Promega, Madison, USA) based on the supplier's instruction, which was normalized to firefly luciferase activity.

Statistical analysis

All experiment values were presented as mean ± standard deviation (S.D.) of at least three independent experiments. All statistical analyses were implemented by SPSS 22.0 and GraphPad Prism 5.0. The student's *t*-test was used to perform the difference between the two groups. Comparisons between two groups were implemented by one-way ANOVA followed by Dunnett's post hoc test. $p<0.05$ was deemed as statistically significant.

RESULTS

A marked augmentation of miR-135a was presented in LPS-induced THP-1 cells

To detect the impact of miR-135a in sepsis, an *in vitro* model was established. By analysing the viability of THP-1cells with different concentration of LPS stimulation, 1 μg/mL LPS was selected to mimic the injury of sepsis *in vitro* (Fig. 1A). Next, the data from qRT-PCR showed that the expression level of miR-135a is dose-dependently upregulated in response to LPS in THP-1 cells (Fig. 1B). To further investigate the impact of miR-135a in THP-1cells, we used the miR-135a inhibitor to reduce its expression. The data from Fig. 1C–D illustrated that miR-135a expression was significantly reduced after miR-135a inhibitor transfection, which provided a foundation for the following experiments.

Knockdown of miR-135a alleviated the damage of THP-1 cells induced by LPS

Thereafter, a series of biological experiments were adopted to evaluate the effect of miR-135a on LPS-induced THP-1cells. First, we observed that the reduced THP-1 cells viability caused by LPS stimulation was suppressed by miR-135a treatment (Fig. 2A). Moreo-

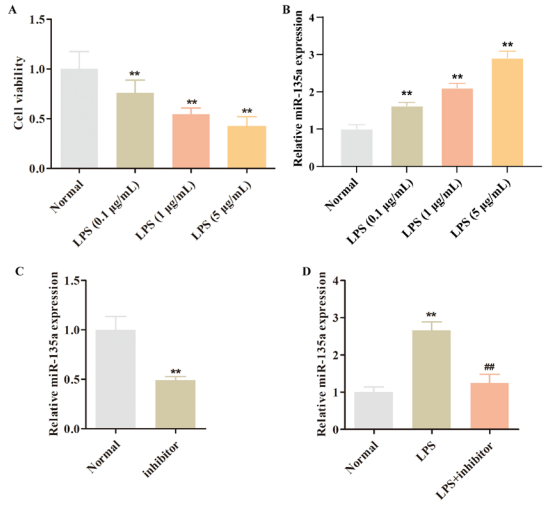

Figure 1. miR-135a expression was obviously strengthened in THP-1 cells treated by LPS.
(**A**) The decreased viability of THP-1cells caused by different concentrations of LPS was detected by the CCK-8 assay. (**B**) A marked augmentation of miR-135a in different concentrations of LPS-induced THP-1 cells was measured by qRT-PCR. (**C**) Data from qRT-PCR showed that miR-135a expression was obviously decreased after miR-135a inhibitor transfection. (**D**) In the LPS model, the qRT-PCR assay showed that miR-135a expression was significantly suppressed by miR-135a inhibitor stimulation.

ver, the strengthened apoptosis ability of THP-1cells caused by LPS was impaired through miR-135a inhibitor stimulation (Fig. 2B–C). Furthermore, the observation from ELISA kit showed that the increasing concentration of TNF-α, IL-6 and IL-8 in LPS-induced THP-1cells was reduced after miR-135a inhibitor treatment (Fig. 2D–F). All these findings suggested that depletion of miR-135a alleviated the damage of THP-1cells induced by LPS.

MYOM1 was confirmed as a target of miR-135a and down-regulated in LPS-induced THP-1 cells

To further explore the mechanism of miR-135a on LPS-induced THP-1 cells injury, the downstream target of miR-135a was excavated. By bioinformatics analysis, MYOM1 was predicted as a target of miR-135a. The targeting sites were exhibited (Fig. 3A). Then, the dual -uciferase assay exhibited that miR-135a inhibitor treatment elevated the luciferase activity of the wt MYOM1 group. But the change of luciferase activity in mut MYOM1 group was no statistical significance (Fig. 3B). In addition, we observed that MYOM1 was under expressed in THP-1 cells triggered by LPS. Furthermore, MYOM1 expression was obviously increased in LPS-induced THP-1 cells after miR-135a inhibitor stimulation (Fig. 3C–E). The whole findings indicated that a negative association existed between miR-135a and MYOM1.

The alleviation effect of miR-135a inhibitor on THP-1 cells injury can be inhibited by MYOM1 depletion

Based on the above observations, we performed biological experiments to investigate the function of miR-135a and MYOM1 on LPS-induced THP-1 cells damage. Firstly, we performed a qRT-PCR assay and western blot assay to investigate the expression of MYOM1 after being transfected with si-MYOM1. The data showed that the expression level of MYOM1 is

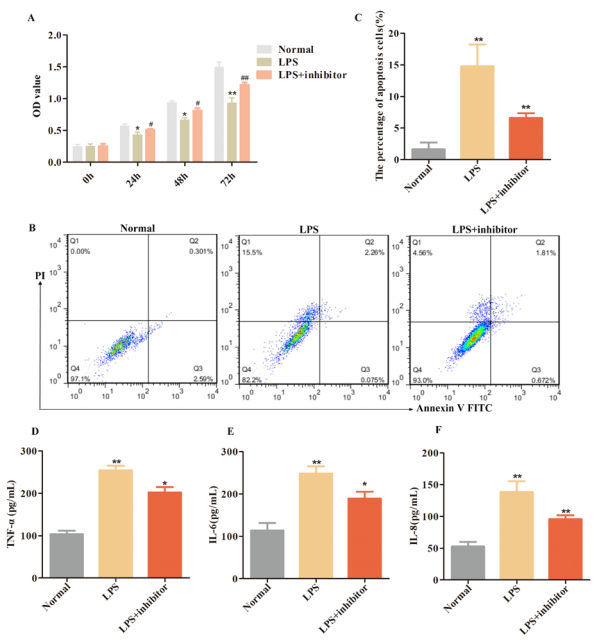

Figure 2. Exhausting miR-135a attenuated the injury of THP-1 cells triggered by LPS.
(**A**) The data from the CCK-8 assay showed that the decreased viability of THP-1 cells caused by LPS can be suppressed by the miR-135a inhibitor. (**B–C**) The result from flow cytometry presented that the increased apoptosis of THP-1 cells caused by LPS was reduced by the miR-135a inhibitor. ELISA kit exhibited that the increased concentrations of TNF-α (**D**), IL-6 (**E**) and IL-8 (**F**) in LPS-induced THP-1 cells were suppressed by miR-135a inhibitor stimulation. $^{*}p<0.05$, $^{**}p<0.01$ *vs*. Normal, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs*. LPS.

significantly downregulated both in mRNA level and protein level after being transfected with si-MYOM1 (Fig. 4A and 4B). We discovered that the elevated viability of THP-1 cells caused by miR-135a inhibitor in LPS condition was suppressed by MYOM1 knock-

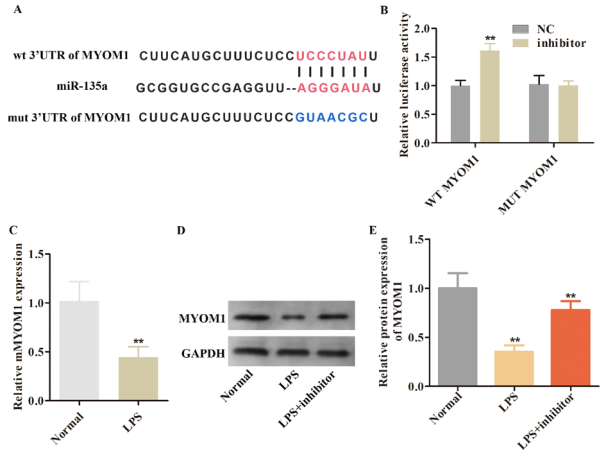

Figure 3. MYOM1 was identified as a target of miR-135a and lower expressed in LPS-treated THP-1 cells.
(**A**) The sequences of wt 3′UTR of MYOM1, miR-135a, and mut 3′UTR of MYOM1 were exhibited. (**B**) Dual-luciferase assay showed that miR-135a inhibitor transfection increased the luciferase activity of the wt MYOM1 group. (**C**) The qRT-PCR experiment exhibited that MYOM1 was lower expressed in THP-1 cells induced by LPS. (**D–E**) The western blotting assay showed that MYOM1 was down-regulated in LPS-induced THP-1 cells, but miR-135a inhibitor transfection elevated MYOM1 expression. $^{**}p<0.01$ *vs*. Normal, $^{\#\#}p<0.01$ *vs*. LPS.

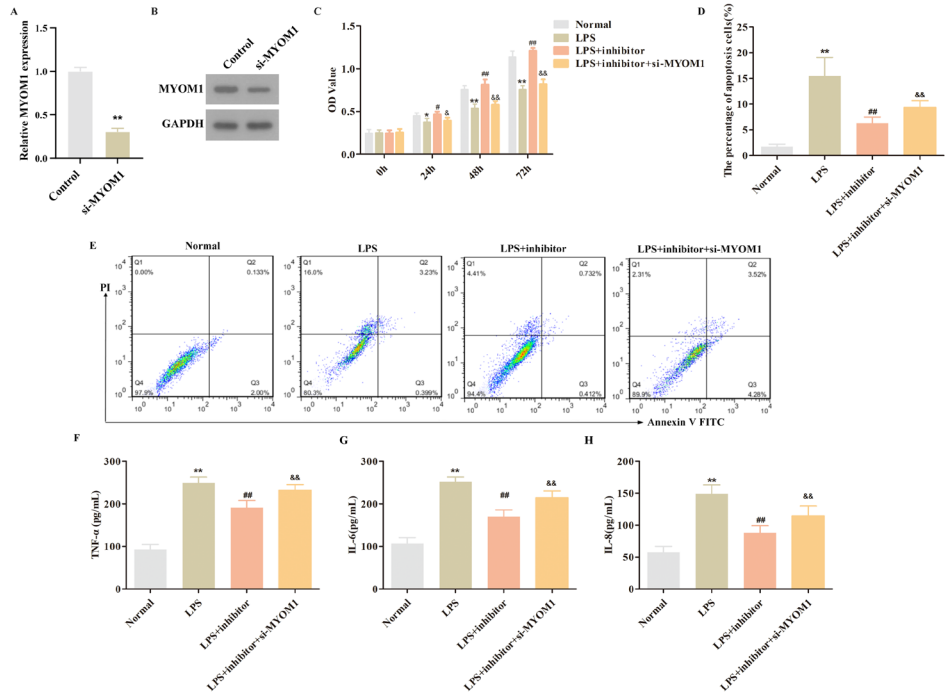

Figure 4. Depletion of MYOM1 suppressed the mitigative effect of miR-135a inhibitor on THP-1 cells damage caused by LPS.
(**A**) The qRT-PCR experiment exhibited that MYOM1 was lower expressed in THP-1 cells after being transfected with si-MYOM1. (**B**) The western blotting assay showed that MYOM1 was down-regulated in THP-1 cells after being transfected with si-MYOM1. (**C**) Data from CCK-8 showed that knockdown of MYOM1 reduced the increasing trend of miR-135a inhibitor on THP-1 cells viability. (**D–E**) The flow cytometry assay exhibited that MYOM1 depletion attenuated the inhibitory effect of miR-135a inhibitor on THP-1 cells apoptosis. ELISA assays presented that the inhibitory effect of miR-135a on TNF-α (**F**), IL-6 (**G**) and IL-8 (H) concentration can be reversed by MYOM1 depletion. $^{*}p<0.05$, $^{**}p<0.01$ *vs*. Normal, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs*. LPS, $^{\&}p<0.05$, $^{\&\&}p<0.01$ *vs*. LPS + inhibitor.

down (Fig. 4C). Then, the reduced apoptotic ability of THP-1cells created by miR-135a inhibitor was inhibited by MYOM1 depletion in LPS treatment (Fig. 4D–E). Additionally, we also observed that the decreased trend of TNF-α, IL-6 and IL-8 concentration in THP-1cells caused by miR-135a inhibitor was reversed by MYOM1 exhaustion (Fig. 4F–H). These results suggested that the function of miR-135a on LPS-induced THP-1cells was realized by targeting MYOM1.

DISCUSSION

Sepsis is a fearful clinical disease due to the host's inflammatory response to the infection (Xu *et al.*, 2020). Herein, we reported that miR-135a expression was obviously increased in LPS-induced THP-1cells. Exhausting miR-135a increased the proliferative ability of THP-1 cells, as well as alleviated the apoptosis ability and inflammatory response. Moreover, the downstream effector of miR-135a was identified as MYOM1, which presented a lower expression in LPS-induced THP-1 cells. The alleviation effect of miR-135a inhibitor on THP-1 cells damage triggered by LPS was suppressed by depletion of MYOM1. The observations suggested that the function of miR-135a on THP-1 cells was achieved by targeting MYOM1.

MiR-135a has been illustrated to be abnormally up-regulated and therefore could be a meritorious prognostic biomarker in numerous human diseases such as glioma, primary nasopharyngeal carcinoma and Parkinson's disease (Tang *et al.*, 2014; Liu *et al.*, 2016; Cheng *et al.*, 2017). Additionally, a marked augmentation of miR-135a was presented in the serum of a patient with sepsis (Zheng *et al.*, 2017). Interestingly, we also discovered that miR-135a expression was obviously increased in THP-1 cells with LPS stimulation. Furthermore, we illustrated that knockdown of miR-135a attenuated the injury of THP-1 cells triggered by LPS from the perspective of augmentation of cells proliferation, as well as reduction of cells apoptosis and inflammatory response. Moreover, we identified the downstream target of miR-135a in this model. Therefore, we not only revealed a marked augmentation of miR-135a presented in the *in vitro* model of sepsis, but also discovered the influence of miR-135a on the biological behaviors of THP-1 cells and the potential downstream mechanism.

It is well documented that miRNAs exert functions in the development and progression of various cells through modulating the expression of specific targets (Vangoor *et al.*, 2019). Thus, we investigated the underlying target gene, which mediates the role of miR-135a in LPS-induced THP-1 cells. Dual-luciferase assay illustrated that miR-135a inhibitor elevated the luciferase activity of cells cloned with wt MYOM1 3'UTR. MYOM1 encodes myomesin, a major binding partner of another sarcomeric protein Titin (Shamseldin *et al.*, 2015). MYOM1, which was discovered to be down-regulated in various cancer, was related to the worse clinical outcomes in tumors (Li *et al.*, 2017). In addition, MYOM1 has been identified as one of the lethal genes in human embryos (Shamseldin *et al.*, 2015). MYOM2, as an important paralog of MYOM1, has been reported to function in localized aggressive periodontitis by regulation of inflammatory processes (Sørensen *et al.*, 2008). However, little research has been done on the role of MYOM1 in inflammation. In our study, we creatively discovered that MYOM1

was lower expressed in THP-1 cells triggered by LPS. Moreover, knockdown of MYOM1 suppressed the alleviation impact of miR-135a inhibitor on THP-1 cells injury triggered by LPS. The whole observation underscored the important role of MYOM1 in the *in vitro* model of sepsis.

The original model of sepsis was the immune response to endotoxin, an LSP discovered in the cell walls of Gram-negative bacteria (Ulevitch & Tobias, 1999). The occurrence of sepsis stimulated macrophages to produce TNF and ILs (Faix, 2013). These pro-inflammatory cytokines produced a systemic inflammatory response which was characteristic of early sepsis (Faix, 2013). Additionally, several reports have suggested that levels of IL-6 and IL-8 were all higher in patients who died from sepsis (Andaluz-Ojeda *et al.*, 2012; Gouel-Chéron *et al.*, 2012). In our study, we observed that the levels of TNF-α, IL-6 and IL-8 were raised in THP-1 cells with LPS treatment, which was in step with the previous report (Faix, 2013). However, depletion of miR-135a suppressed the levels of TNF-α, IL-6 and IL-8 in THP-1 cells. While knockdown of MYOM1 reversed the above results triggered by the miR-135a inhibitor, which indicates that function of miR-135a/MYOM1 on THP-1 cells was achieved by regulating the inflammatory response.

Despite several findings that have been made in our study, limitations in this research need to be revealed. Firstly, we only found these results in LPS-induced THP-1 cells, more results should be verified *in vivo*. Second, we just found the downstream target of miR-135a, the upstream regulator of miR-135a should be explored in future.

CONCLUSIONS

Depletion of miR-135a alleviated the LPS-induced THP-1 cells injury from the perspective of improving cell viability and reducing cell apoptosis. The mitigative impact of miR-135a inhibitor on THP-1cells damage triggered by LPS was suppressed by MYOM1 depletion. In conclusion, we proposed that the therapeutic exploitation of miR-135a might afford intriguing perspectives to prevent the progression of sepsis.

Declarations

Ethics approval and consent to participate. Not applicable.

Consent for publication. Not Applicable.

Competing interests. The authors declare that they have no competing interests.

Acknowledgements. Not Applicable.

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

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Contributions. Sheng Chen provided the experimental design; Sheng Chen, Han Zhang, Haoming Li performed the experiments; Sheng Chen analyzed the data; Han Zhang prepared all figures, Sheng Chen wrote the draft of the manuscript; Sheng Chen, Han Zhang, Haoming Li wrote, reviewed and edited the manuscript. All authors reviewed the manuscript. The author (s) read and approved the final manuscript.

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