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MicroRNA-146 regulates the inflammatory cytokines expression in vascular endothelial cells during sepsis

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Aims: The purpose of this study was to investigate the functional role of microRNA (miR)-146 in sepsis, as well as the underlying mechanism. **Methods:** Human vascular endothelial cell line EA.hy926 cells were treated with lipopolysaccharide (LPS) and/or transfected with miR-146 mimics, inhibitor, and their corresponding controls. Expression of miR-146 was then analyzed after treatment and/or transfection, as well as the expression of inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, intercellular adhesion molecule (ICAM)-1, and E-selectin, and nuclear factor kappa B (NF-κB) binding activity. **Results:** The results showed that the expression of miR-146 was significantly downregulated by LPS stimulation compared to the control group ($P < 0.05$). Also, the expression of miR-146 was remarkably increased by miR-146 mimics but decreased by miR-146 inhibitor following stimulation with LPS ($P < 0.05$). In addition, the expression levels of TNF-α, IL-6, ICAM-1, and E-selectin were shown to increase following induction by LPS, and further markedly elevated by miR-146 inhibitor (all $P < 0.05$). However, the expression levels of these inflammatory cytokines were outstandingly decreased by miR-146 mimics (all $P < 0.05$). Moreover, we observed that the relative NF-κB activity was statistically upregulated by miR-146 inhibitor but downregulated by miR-146 mimics. **Conclusions:** MiR-146 may play an important role in the pathogenesis and development of sepsis by suppressing the expression of inflammatory cytokines.

1. Introduction

Sepsis is a frequent life-threatening medical condition characterized by systemic inflammatory reaction caused by infectious factors, which can progress in sepsis-associated immunosuppression (Angus and Van der Poll 2013; Dirkes 2013). It is one of the major and significant causes of mortality worldwide. Uncontrolled inflammation has emerged as a pathophysiologic basis for sepsis (Yao et al. 2015). The cumulative amount release of inflammatory mediators and the activation of inflammatory cascades ultimately lead to tissue injury and organ failure (Glauser 2000; Lewis et al. 2012). It has been well known that vascular endothelial cells are considered as a critical interface between the blood and tissues. These cells play an important role in the adhesion and aggregation of leukocyte in the presence of inflammatory response (Hernandez et al. 2007). Previous studies have confirmed that the vascular endothelial cells are significantly involved in systemic inflammation responses during sepsis, including the crosstalk between coagulation and inflammation (Aird 2001; Levi et al. 2012). In addition, it has been well demonstrated that the nuclear factor (NF)-κB signaling pathway is responsible for sepsis (Adamzik et al. 2013; Raspé et al. 2013). Inhibition of the endothelial NF-κB pathway might be beneficial in treating sepsis (Xu et al. 2010).

MicroRNAs (miRNAs) are a group of endogenous, small (18-25 nucleotides long), single-stranded and non-coding RNAs that have the ability to post-transcriptionally regulate the expression of target mRNA transcripts (Ying et al. 2008). Emerging evidence has suggested that miRNAs are involved in diverse physiological and pathological processes including cell proliferation, cell differentiation, development, apoptosis and metabolism (Bartel 2004). Recently, several studies have confirmed that miRNAs regulate inflammatory responses (O'Connell et al. 2012) and contribute to the pathogenesis of sepsis (Benz et al. 2016; Essandoh and Fan, 2014). Among miRNAs, miR-146 was previously found to be involved in regulating inflammatory reactions, which was responsible for sepsis (Cheng et al. 2013). Moreover, miR-146a has been confirmed to be an effective new biomarker for sepsis with high specificity and sensitivity (Wang et al. 2010). However, the exact mechanism by which miR-146 exerts anti-inflammatory function in sepsis is poorly understood.

Herein, we investigated the functional role of miR-146 in sepsis, as well as its underlying mechanism. Human vascular endothelial cell line EA.hy926 cells were incubated with lipopolysaccharide (LPS), and/or transfected with miR-146 mimic, inhibitor, or their corresponding controls. Expression of inflammatory cytokines and NF-κB binding activity were then analyzed. Our results revealed a potent anti-inflammatory function of miR-146 on sepsis, and the effects achieved might be related to an inhibition of NF-κB activation in endothelial cells.

2. Investigations and results

2.1. LPS downregulates miR-146 expression in EA.hy926 cells

To explore the functional role of miR-146 in sepsis, we first analyzed the expression of miR-146 in human vascular endothelial cell line EA.hy926 cells stimulated by LPS and/or transfected with miR-146 mimics, inhibitor, or their corresponding controls. As demonstrated in Fig. 1 A, compared to the control group, the relative expression levels of miR-146 were significantly decreased by LPS ($P < 0.05$), which indicated that vascular endothelial cell activation in sepsis was accompanied by the inhibition of miR-146 expression. Moreover, the results demonstrated that miR-146 expression was significantly increased in the cells transfected

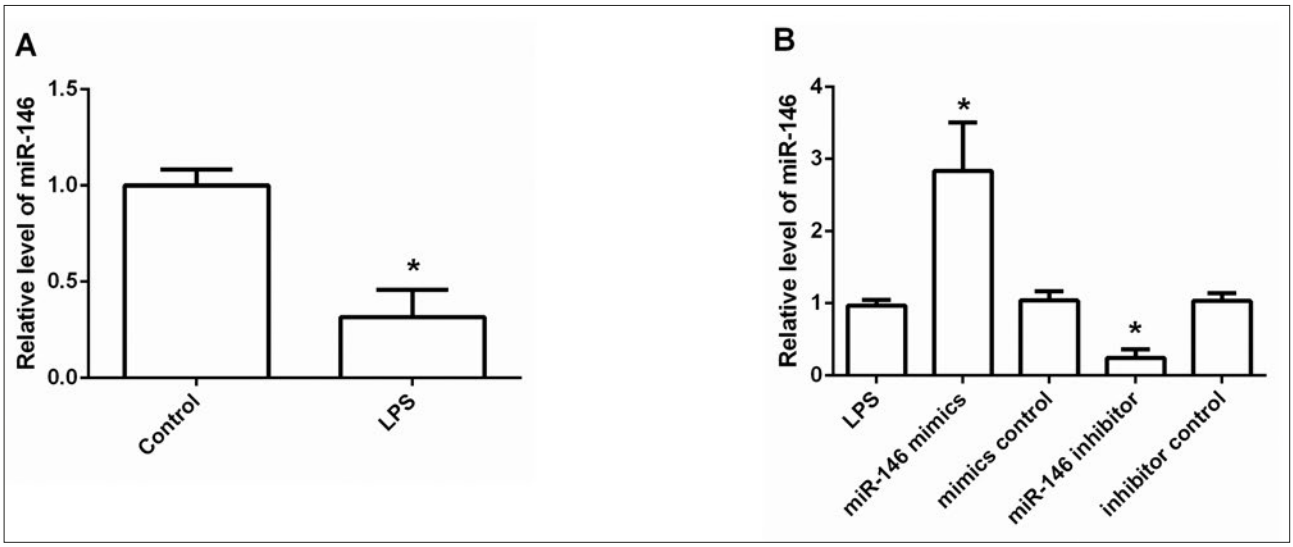

Fig. 1: LPS downregulates miR-146 expression in EA.hy926 cells. Human vascular endothelial cell line EA.hy926 cells were stimulated by LPS for 4 h and then transfected with miR-146 mimics, inhibitor, or their corresponding controls. The expression of miR-146 was determined. A, the relative expression levels of miR-146 in EA.hy926 cells after 4 h of stimulation with LPS; B, the relative expression levels of miR-146 in EA.hy926 cells after transfection with miR-146 mimics, inhibitor, or their corresponding controls following stimulation with LPS.(MiR, microRNA; LPS, lipopolysaccharide,$^{*}P < 0.05$ compared to the control group (A) or LPS group (B))

with the miR-146 mimics but was markedly decreased in the cells transfected with the miR-146 inhibitor after LPS stimulation when compared with LPS group ($P < 0.05$) (Fig. 1 B), confirming that the transfection was efficient.

2.2. LPS promotes inflammatory cytokines expression in EA.hy926 cells

After LPS stimulation, we determined the expression levels of inflammatory cytokines, including TNF-α, IL-6, ICAM-1, and E-selectin. As shown in Fig. 2, we observed that the expression levels of TNF-α, IL-6, ICAM-1, and E-selectin were all significantly increased after LPS stimulation in the human EA.hy926 cells compared to the cells did not undergo LPS stimulation (all $P < 0.05$). The results demonstrated that LPS stimulated the expression of inflammatory cytokines in human EA.hy926 cells, contributing to the inflammatory reaction in sepsis.

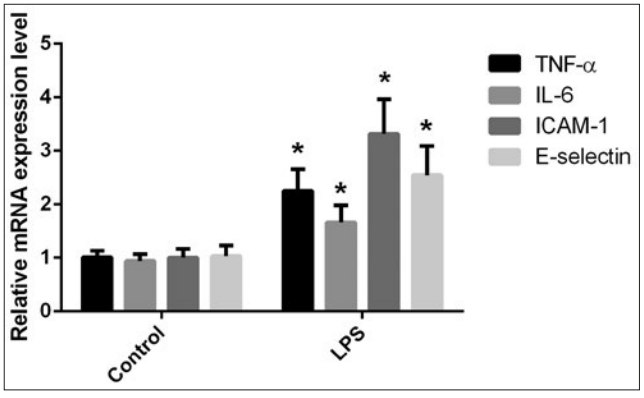

Fig. 2: LPS promotes inflammatory cytokine expression in EA.hy926 cells.The expression levels of inflammatory cytokine were determined after LPS stimulation. The expression levels of TNF-α, IL-6, ICAM-1, and E-selectin were all significantly increased after LPS stimulation in the human EA.hy926 cells. (MiR, microRNA; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; ICAM, intercellular adhesion molecule,$^{*}P < 0.05$ compared to the control group)

2.3. MiR-146 inhibitor stimulates the LPS-stimulated expression of inflammatory cytokines in EA.hy926 cells

Then, we investigated the effects of miR-146 suppression on the LPS-stimulated expression of inflammatory cytokines in EA.hy926 cells. The mRNA expression levels of TNF-α, IL-6, ICAM-1, and E-selectin were further significantly increased in EA.hy926 cells by miR-146 inhibitor compared to the inhibitor control group after LPS stimulation (all $P < 0.05$) (Fig. 3 A). In addition, the results of Western blot analysis also revealed that the protein expression levels of TNF-α, IL-6, ICAM-1 and E-selectin were all significantly higher in the miR-146 inhibitor group than the inhibitor control group after LPS stimulation (Fig. 3 B).

2.4. MiR-146 mimics inhibits the LPS-stimulated expression of inflammatory cytokines in EA.hy926 cells

Next, the effects of miR-146 overexpression on the LPS-stimulated expression of inflammatory cytokines in EA.hy926 cells were evaluated. As demonstrated in Fig. 4 A, we found that the mRNA expression levels of TNF-α, IL-6, ICAM-1, and E-selectin were all significantly downregulated in the cells transfected with miR-146 mimics when compared to the cells transfected with inhibitor control after LPS stimulation (all $P < 0.05$). Besides, as expected, the protein expression levels of TNF-α, IL-6, ICAM-1 and E-selectin were all significantly lower in the cells transfected with miR-146 mimics than the cells transfected with mimics control after LPS stimulation (Fig. 4 B).

2.5. MiR-146 negatively regulates NF-κB activation

To further investigate the underlying mechanism of the effects of miR-146 on the expression of inflammatory cytokines, we analyzed the regulation of miR-146 on the NF-κB signaling pathway by determining the NF-κB activity in the nucleus. The results revealed that the relative NF-κB activity was dramatically increased by LPS stimulation compared to the control group ($P < 0.05$). Moreover, we observed that the relative NF-κB activity was further elevated by the miR-146 inhibitor compared to the LPS group ($P < 0.05$). However, the relative NF-κB activity was observably reduced by miR-146 mimics compared to the LPS group ($P < 0.05$) (Fig. 5). The results indicated that the NF-κB activation was negatively regulated by miR-146.

3. Discussion

Sepsis is a devastating medical problem characterized by uncontrolled infection, which may lead to shock, multiple organ dysfunction syndrome (MODS) and mortality (Duran-Bedolla et al. 2014). However, the pathogenesis of sepsis is multifactorial and far more complex (Stearns-Kurosawa et al. 2011). Here, we demonstrated that miR-146 plays a significant role in the pathogenesis of sepsis.

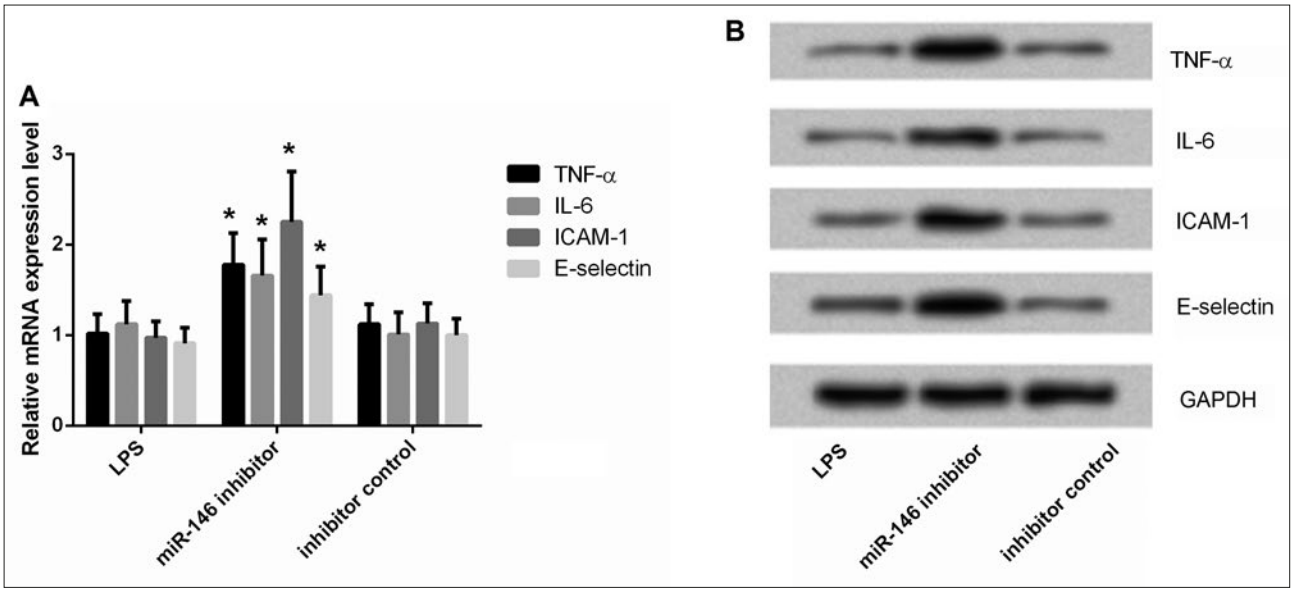

Fig. 3: MiR-146 inhibitor stimulates the LPS-stimulated expression of inflammatory cytokines in EA.hy926 cells. The effects of miR-146 suppression on the LPS-stimulated expression of inflammatory cytokines in EA.hy926 cells were determined. A, the mRNA expression levels of TNF-α, IL-6, ICAM-1, and E-selectin after transfection with miR-146 inhibitor; B, representative Western blot pictures of TNF-α, IL-6, ICAM-1 and E-selectin after transfection with miR-146 inhibitor.(MiR, microRNA; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; ICAM, intercellular adhesion molecule,$^{*}P < 0.05$ compared to the LPS group)

MiR-146 has shown lower levels in LPS-induced endothelial cells. Suppression of miR-146 significantly increased the expression levels of TNF-α, IL-6, ICAM-1, and E-selectin following induction by LPS, and while overexpression of miR-146 presented contrary results. Besides, the NF-κB activity was statistically promoted by suppression of miR-146 but inhibited by overexpression of miR-146.

Currently, an increasing number of studies have been concerned with the functional role of miRNA in sepsis. Sepsis is a systemic inflammatory response to infection, and upon inflammatory response, diverse miRNAs have been produced by host cells. Then the miRNAs promote or degrade the release of inflammatory mediators, causing immune hyperactivity or immunosuppression (Du and Ma 2009). Many miRNAs have been identified as diagnostic biomarkers of sepsis (Vasilescu et al. 2009; Wang et al. 2012, 2010), and could also predict the survival in patients with sepsis (Roderburg et al. 2013; Wang et al. 2012). Therefore, miRNAs play a critical role in regulating inflammatory responses in sepsis. Among miRNAs, miR-146 has been shown to inhibit inflammatory reactions; thus, it is critical in preventing inflammatory diseases (Bhaumik et al. 2009; Taganov et al. 2006). Actually, the functional role of miR-146 in sepsis has been studied. Wang et al. (2010) found that serum miR-146a levels were significantly decreased in septic patients, and the levels of miR-146a could be served as potential new biomarkers for sepsis. Besides, it has been suggested that the plasma levels of miR-146a were significantly reduced in sepsis patients compared to the non-sepsis-SIRS patients, demonstrating that measuring the levels of miR-146a might provide a potential method differentiating between sepsis and non-sepsis-SIRS (Wang et al. 2013). However, the exact mechanism underlying the anti-inflammatory function of miR-146 in sepsis still needs further investigations. Hence, in this paper, we

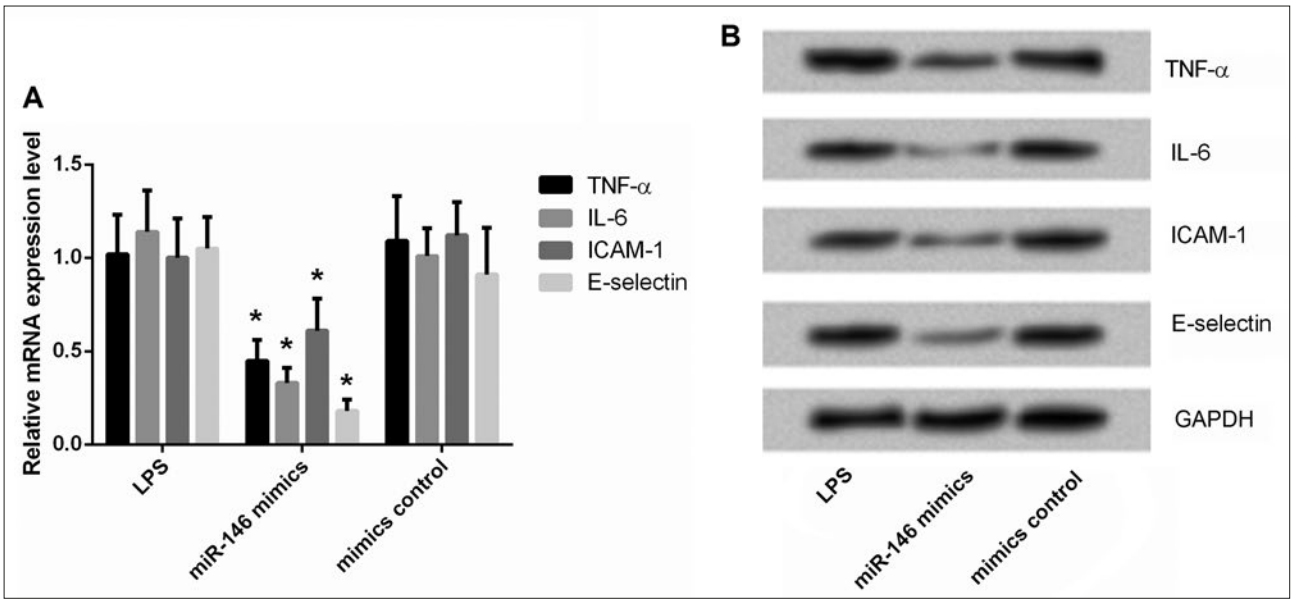

Fig. 4: MiR-146 mimics inhibits the LPS-stimulated expression of inflammatory cytokines in EA.hy926 cells. The effects of miR-146 overexpression on the LPS-stimulated expression of inflammatory cytokines in EA.hy926 cells were determined. A, the mRNA expression levels of TNF-α, IL-6, ICAM-1, and E-selectin after transfection with miR-146 mimics; B, representative Western blot pictures of TNF-α, IL-6, ICAM-1 and E-selectin after transfection with miR-146 mimics.(MiR, microRNA; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; ICAM, intercellular adhesion molecule ,$^{*}P < 0.05$ compared to the LPS group)

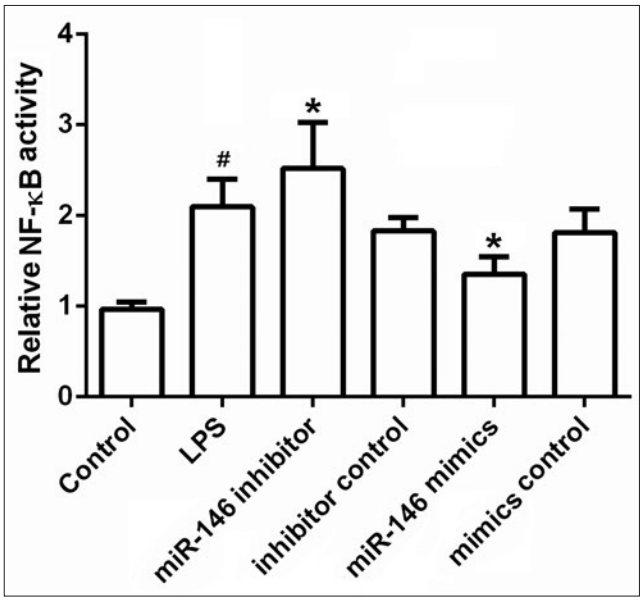

Fig. 5: MiR-146 negatively regulates NF-κB activation.The regulation of miR-146 on NF-κB signaling pathway was measured by determining the NF-κB activity in the nuclear. The relative NF-κB activity was elevated by miR-146 inhibitor but reduced by miR-146 mimics compared to the LPS group.MiR, microRNA; (LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B, $^{\#}P < 0.05$ compared to the control group,$^{*}P < 0.05$ compared to the LPS group)

focused on the functional role of miR-146 in sepsis, together with the potential underlying mechanism.

In the present study, firstly, we used LPS-stimulated human vascular endothelial cells to activate endothelial cells. LPS is a potent and pleiotropic stimulus of immune cells, promoting the release of inflammatory cytokines from circulating immune competent cells. Thus, LPS is a common stimulator to induce sepsis model (Anand et al. 2009). In addition, it has been well established that the endothelium plays an important role in the pathogenesis of many vascular inflammatory diseases, including sepsis (Aird 2003; Shapiro et al. 2010). Endothelial cells show diverse functions and are highly responsive to the extracellular environment (Aird 2005). Certain agonists, such as LPS and proinflammatory cytokines, could activate the endothelial cells (Shapiro et al. 2010). Furthermore, it has been reported that inhibition of endothelial activation was shown to reduce mortality in mouse models of sepsis (van Griensven et al. 2006). As expected, our results showed that LPS was able to activate the endothelial cells and thereby initiated a cascade of inflammatory reactions by producing various inflammatory factors, including TNF-α, IL-6, ICAM-1, and E-selectin. It was noteworthy that, we also found that miR-146 expression was significantly decreased in LPS-stimulated human endothelial cells. The results were in line with previous studies showing the lower levels of miR-146 in sepsis compared to the normal controls. In order to explore the regulatory mechanisms of miR-146 on inflammatory mediator expression in endothelial cells during sepsis, the expression of miR-146 in human endothelial cells was upregulated and downregulated by transfection with miR-146 mimics and inhibitor, respectively. Interestingly, we observed that the upregulation of miR-146 dramatically inhibited the expression of TNF-α, IL-6, ICAM-1, and E-selectin, while downregulation of miR-146 markedly promoted the expression of inflammatory mediators. These results suggested that the potential anti-inflammatory function of miR-146 in sepsis might be by regulating the expression of inflammatory mediators.

It is well known that the inflammatory mediators interact with each other and form a complex network, which is responsible for sepsis (Molloy et al. 1993; Walley et al. 1996). NF-κB signaling pathway has been shown its significant roles in sepsis by cooperatively stimulating endothelial activation and vascular inflammation (Liu and Malik 2006). A previous study has suggested that selective blockade of endothelial NF-κB pathway differentially affects systemic inflammation and MODS in septic mice (Xu et al. 2010). Also, it has been found that miR-146a/b was a negative regulator of constitutive NF-κB activity in breast cancer (Bhaumik et al. 2008) and sepsis (Cheng et al. 2013). To further investigate whether miR-146 modulates the expression of inflammatory mediators by regulating NF-κB signaling pathway, we analyzed the NF-κB activity after alternating the expression of miR-146. Our results revealed that the relative NF-κB activity was obviously increased by miR-146 inhibitor, while was reduced by miR-146 mimics compared to the LPS group. The present results were in consistent with previous studies that miR-146 negatively regulated the NF-κB signaling pathway.

In general, the present study reveals that miR-146 modulates sepsis by suppressing the expression of inflammatory mediators in endothelial cells. MiR-146 may be a potential therapeutic target for the treatment of sepsis.

4. Experimental

4.1. Cell culture

The human vascular endothelial cell line EA.hy926 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 0.1 mM nonessential amino acids (Life Technologies, Inc., Carlsbad, CA, USA), 1 mM sodium pyruvate (Life Technologies), 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES; Life Technologies) and 1% penicillin-streptomycin-neomycin antibiotics mixture at 37 °C in a 5% CO_2 humidified atmosphere.

4.2. Cell treatment and transfection

For cell treatment, 2×10^4 - 5×10^4 cells were inoculated into 12-well plates. The cells were maintained in DMEM containing 10% FBS (500 µl) and were grown to 70-90% confluency. Then LPS was added to each well at a final concentration of 1 µg/ml. The cells were cultured for another 4 h and the cells were subjected to cell transfection. For cell transfection, miR-146 mimics, inhibitor, and their corresponding controls were purchased from Sangon Biotech (Shanghai, China). Briefly, the cells (2×10^5) were seeded in 96-well plates after 4 h of administration or not with LPS. Then the cells were transiently transfected with miR-146 mimics (10 nM), inhibitor (30 nM), or their corresponding controls for 24 h according to the manufacturer's instructions. The miR-146 sequences were listed as follows: miR-146 inhibitor sequence, 5'- CCCAUGGAAUUCAGUUCUCAUU-3'; miR-146 inhibitor control sequence, 5'- CAGUACUUUUGUGUAGUACAA-3'; miR-146 mimics sequence, 5'-UGAGAACU GAAUUCCAUGGGUU-3'; miR-146 mimics control sequence, 5'-UUCUCCGA ACGUGUCACGUTT -3'. Cell transfection was carried out using the Lipofectamine 2000 according to the manufacture's protocol (Invitrogen). After 24 h of transfection, the cell suspension was collected for further analyses.

4.3. Quantitative real time PCR (qRT-PCR) analysis

After treatment or transfection, total RNA was extracted from the cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's introductions. First-strand complementary DNA (cDNA) was generated using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Schwerte, Germany). The reverse transcription reaction products were quantitatively analyzed using THUNDERBIRD SYBR qPCR Mix Kit (Toyobo Co., Ltd., Osaka, Japan) in a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression of miRNA was measured by TaqMan miRNA assays (ABI, Forest City, CA), and the expression of mRNA was measured by PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan). GAPDH and U6 snRNA were used as loading control for mRNA and miRNA, respectively. All primers were synthesized by Sangon Biotech. Co., Ltd. (Shanghai, China).

4.4. NF-κB binding activity assay

Nuclear was extracted from treated and/or transfected cells using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). The NF-κB activity in the nuclear was measured using an enzyme linked immunosorbent assay (ELISA)-based TransAM NF-κB kit (TransAM™ NF-κB p65 assay; Active Motif) according to the manufacturer's instructions and analyzed using Dual-Glo Luciferase assay (Promega, Madison, WI, USA).

4.5. Western blotting

After treatment and/or transfection, protein was extracted from the cells and the protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Illinois, USA). Equal amounts of protein were resolved in 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred electrophoretically onto a polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). The membranes were then blocked with 5% skimmed milk in Tris Buffered Saline with Tween (TBST) for 2 h at room temperature and incubated overnight at 4°C with the corresponding antibodies: anti-tumor necrosis factor (TNF)-α (1 : 1000; Abcam, Cambridge, MA), anti-interleukin (IL)-6 (1 : 1000; Abcam), anti-intercellular adhe-

sion molecule (ICAM)-1 (1 : 1000; Abcam), or anti-E-selectin (1 : 1000; Abcam). GAPDH was used as a loading control. Afterwards, the membranes were washed three times with phosphate buffer saline (PBS) buffer and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. The bands were visualized by using enhanced chemiluminescence (GE Healthcare UK Ltd, Little Chalfont, UK) according to the manufacturer's instructions.

4.6. Statistical analysis

Data are expressed as mean±standard deviation (SD). All collected data were firstly tested for the normal distribution using one-sample K-S test. Enumeration data were analyzed by chi-square test or rank-sum test. Student t-test (for two groups) and one way analysis of variance (ANOVA) with post hoc Tukey test (for more than two groups) were used to determine the significant differences in groups. A statistical significance was defined when $P < 0.05$.

Conflict of interests and financial disclosure: None declared.

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